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PROCEEDINGS OF THE TECHNICAL SEMINAR

4th International Exhibition & Seminar on Dairy, Aqua and Pet-2018

8-10th March 2018

Venue: Bangabandhu International Conference Center (BICC)
Sher-E-Bangla Nagar, Dhaka, Bangladesh.

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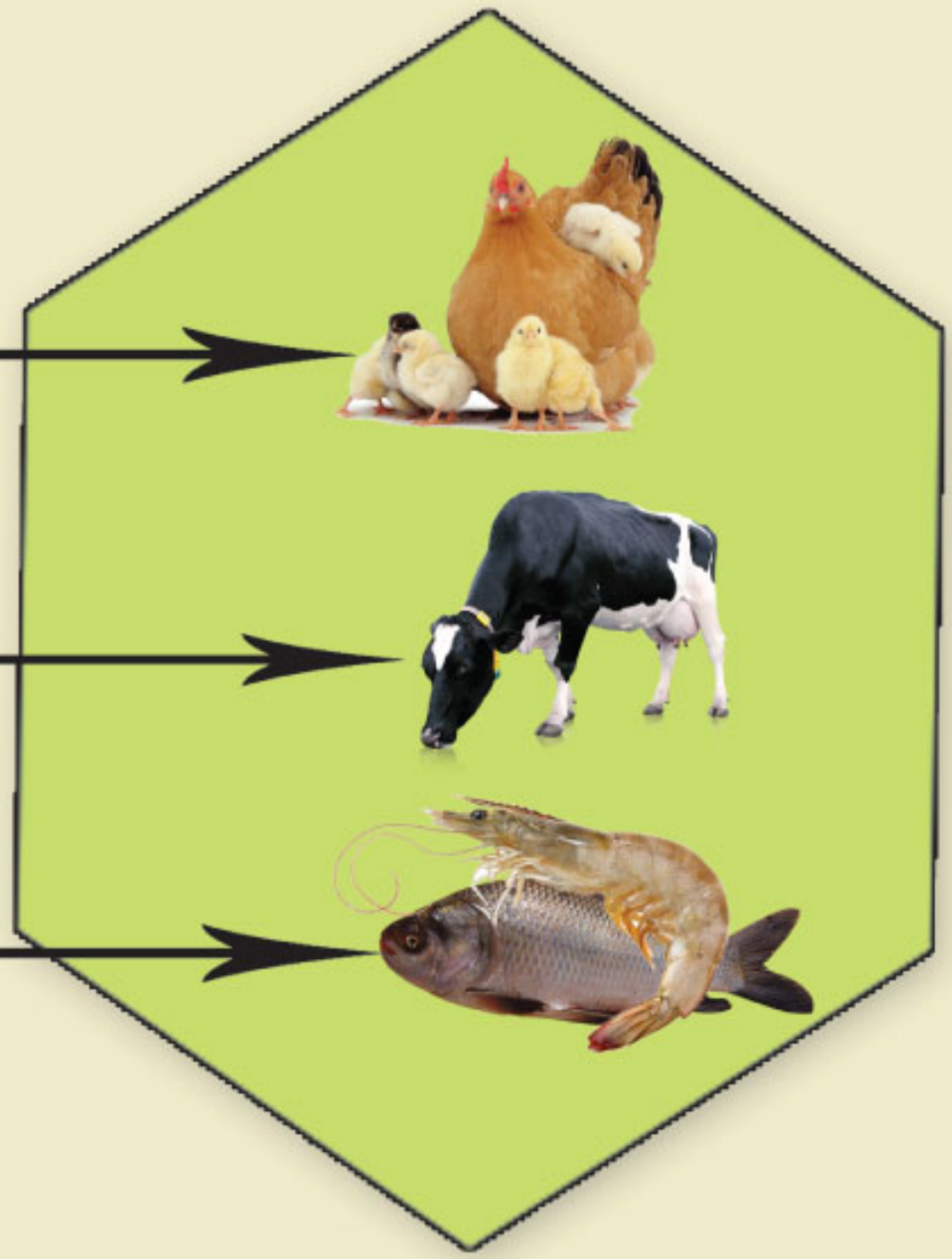

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Proceedings of the Technical Seminar

Date: 8-10 March 2018

Venue: BICC, Sher-E-Bangla Nagar, Dhaka, Bangladesh

Organized by:

AHCAB

Animal Health Companies Association of Bangladesh

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4th International Exhibition & Seminar on Dairy, Aqua and Pet-2018

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CEO, BIO-LAB, Dhaka

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Dr Md Kamruzzaman
Convener
Technical Sub-Committee
4th IEDAP 2018

4th International Exhibition
& Seminar on Dairy, Aqua & Pet-2018
8-10 March 2018

Editorial Notes

Animal Health Companies Association of Bangladesh (AHCAB) organizes 4th International Exhibition & Seminar on Dairy, Aqua & Pet-2018. It is a biennial programme. This is an important event for the scientist and specialists, practitioners and consultants, breeders and farmer's entrepreneurs and traders having interests in Dairy, Aqua and Pet. This magnificent event has two major components : Show and Seminar.

A total of 61 scientific papers from home and abroad will be presented in this 4th International Exhibition and Seminar on Dairy, Aqua and Pet-2018 during 8-10 March 2018. These papers have been distributed in eight oral and Poster presentation session such as Dairy Health, Aqua, Genetics, Disease, Feed & Nutritions, Zoonosis, Pet and Industrial. A Technical Committee thoroughly reviewed the papers. The full papers are published in this proceedings.

On behalf of the Technical Committee, I take this opportunity to thank the authors and the members of the Technical Committee for their excellent contribution. I also welcome the participants and wish them all a pleasant time and successful meeting.

Dr Md Kamruzzaman
Convener
Technical Sub-Committee
4th IEDAP 2018

4th International Exhibition
& Seminar on Dairy, Aqua & Pet-2018
8-10 March 2018

Few Words from Member Secretary

First of all, season's Greetings! Time flies without realizing it; I have been serving as the Member Secretary of the Technical Committee of 4th IEDAP. We have been working for last eight months for preparing the technical proceedings and finally it has been published with the special exertion of all members of Technical Sub- Committee.

International Exhibition on Dairy Aqua and Pet (IEDAP) is a biennial exhibition, Organized by Animal Health Companies Association of Bangladesh (AHCAB). The 4th International Exhibition & Seminar on Dairy, Aqua and Pet will be detained at Bangabandhu International Conference Centre (BICC) from 8th to 10th March 2018. In this episode, a technical proceeding is going to be published. In this era we have invited hundreds of scientist from home and abroad and we have received more than one hundred and eighty scientific credentials on Dairy, Aqua and for Pet.

A fully technical and competent team has worked and studied all the papers. But due to constrain only sixty-one is preferred for oral and poster presentation. We are very much apologetic for not to have room for all the papers and expectantly it may be possible for more in upcoming IEDAP. There are eight session for completing these technical proceedings. We have lot of efforts intended for accuracy of this proceeding; Pardon us for unforeseen slip-up.

Before I finish, I would like to convey my earnest thanks to our intact technical team members, colleagues, officials, patrons, well-wishers and publishers who are courageously gave their effort to make this booming periodical.

Dr. Md. Jasim Uddin
DVM, MS (BAU), MBA
Member Secretary, Technical Sub Committee
4th IEDAP 2018

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Technical Session - I : Pet

Chairperson : **Dr S M Nazrul Islam,** Curator, Bangladesh National Zoo.Dhaka

Co-Chairperson : **Md. Masudur Rahman, PhD,** Professor of Pathology, Faculty of Veterinary and Animal Science, Sylhet Agricultural University , Sylhet

Rapporteurs : **Dr Md Rashedul Islam,** Asst Professor & Chairman, Dept of Surgery and Theriogenology, Sher-E-Bangla Agriculture University, Dhaka

: **Dr Sajeda Sultana,** Asst Professor & Chairman, Department of Pathology Sher-E-Bangla Agricultural University, Dhaka

: **Mr Prince Rahman Pavel,** CEO, PDF Agro

Venue : Media Bazar, BICC, 8th March 2018, Time : 04:00pm-05:04pm

From	To	Title of Presentation
04:00pm	04:12pm	A Serological Survey of Newcastle Disease Virus in Pigeons at Chittagong Metropolitan Area, Bangladesh Elina K.C., Abdul Ahad, Nayem Akhter Palash, A.K.M. Mostafa Anower
04:13pm	04:25pm	Epidemiological investigation of clinical diseases and conditions of pet animals at Chittagong city area, Chittagong, Bangladesh. Dr. Md. Selim Ahmed
04:26pm	04:38pm	Present Status of Commercial Pigeon Farming in Selected Areas of Bangladesh Abu Nasar Md. Aminoor Rahman and Ziban Chandra Das
04:39pm	04:51pm	Fluid Therapy in Small Animal Practice Sagir Uddin Ahmed
04:52pm	05:04pm	Incidence of pigeon diseases in northern Bangladesh Md. Jalal Uddin Sarder, K.M. Mozaffor Hossain, Md. Hemayatul Islam, Md. Najmul Hassan Parvez, Most. Tahera Dil Akter, Md. Reazul Islam and Sharina Akhtar

Poster Presentation

A Comparative Study on a Canine Parvovirus Infection of Dogs in Bangladesh and India
Mohammad Mahbub Hasan and Md. Masuduzzaman

Surgical management of femoral fracture in cats by retrograde intramedullary pinning
Bhajan Chandra Das,Md.Afzal Hossain, Saraj Kumar Yadab, Bayezid Bostami and Bibek Chandra Sutradhar

Technical Session - II : Aqua

Chairperson : **Prof Dr Md Jahangir Alam,** Dean, Faculty of Fisheries, BSMRAU, Gazipur

Co-Chairperson : **Dr. Debangshu Bikash Bhowmik,** Director, Agata Feed Mills Ltd

Rapporteurs : **Dr. A. K. Shakur Ahmmad,** Associate Professor, Department of Fisheries Biology and Genetics, Bangladesh Agricultural University, Mymensingh

: **Dr Ibrahim Rashid,** Lecturer, Dept of Fisheries Biology & Aquatic Environment, BSMRAU, Gazipur

: **Mr Md. Sohel Iqbal,** Managing Director, Panacia Agro Limited

Venue : Media Bazar, BICC, 9th March 2018, Time : 09:15am-10:16am

From	To	Title of Presentation
09:00am	09:10am	Isolation, molecular identification and antibiotic susceptibility profile of Aeromonas hydrophila from diseased stinging catfish shing (Heteropneustes fossilis) Tanvir Ahammed, Shuvho Chakra Borty, Md. Shirajum Monir, Nazneen Begum, Md. Alimul Islam and S. M. Lutful Kabir
09:11am	09:21am	Genetic diversity and phylogenetics in Puntias sarana and Barbodes gonionotus fish populations of Bangladesh Shirin Sultana, Mohammad Shahdat Hossain, Mohammad Nazrul Islam, Md. Shahidul Islam Bhuiyan, Md. Salimullah and Jahangir Alam
09:22am	09:32am	Effect of red seaweed Hypneamusciformisas replacement of fish meal on growth and immune response of Tilapia *(Oreochromis niloticus)* Asaduzzaman Nur, Md. Nahid Hasan , Saida Zannat and S. M. Rafiquzzaman
09:33am	09:43am	Occurrence of Salmonella and Vibrio species in fresh fishes collected from different markets of Mymensingh, Gazipur and Sherpur districts and their characterization Amit Dutta and S. M. Lutful Kabir
09:44am	09:54am	Growth and Production of endangered Ompokpabda (Hamilton) in ponds based on stocking density Md. Abdus Samad, Kamrun Nahar, Mustafizur Rahman, EsmotJahan Alice
09:55am	10:05am	A review on commercially available methionine sources for aquaculture. Alexandros Samartzis, Cláudia Figueiredo-Silva andGirish Channarayapatna
10:06am	10:16am	Role of Micro Organism in Aquaculture M P Ananda, PR Daneb, KC Dash

Technical Session - III : Dairy Health

Chairperson : **Dr Priya Mohan Das,** Professor of Pathology, BAU, Mymensingh

Co-Chairperson : **Dr Md Abdul Halim,** DD, Dhaka Division, DLS, Dhaka

Rapporteurs : **Dr Mst Nelima Ibrahim,** Scientific Officer, LRI, Mohakhali, Dhaka

: **Dr Shukla Halder,** Former FAO Staff

: **Dr Md Mazharul Islam,** Asst Professor, BSMRAU, Gazipur

Venue : Media Bazar, BICC, 9th March 2018, Time : 10:30am-11:24am

From	To	Title of Presentation
10:30am	10:40am	In silico analysis of VP1 gene for prediction of peptide vaccine against three serotypes (O, A & Asia) of Foot & Mouth Disease Virus circulating in Bangladesh M. S. Mahmud, M. Giasuddin, M. A. M. A. Asari, E. Islam , M. A. Samad, M. R. Karim
10:41am	10:51am	Control of infectious diseases in dairy cattle through biosecurity and vaccination programmes Md. Ariful Islam
10:52am	11:02am	Advances of immunogenomics and targeted genome editing for disease resistance in livestock: application of CRISPR/Cas9 technology Md. Aminul Islam, M Jasim Uddin, M Ariful Islam, Sharmin Aqter Rony, M Munir Hossain, M Bozlur Rahman, Maren Julia Pröll, Christiane Neuhoff and Karl Schellander
11:03am	11:13am	Prevalence and Molecular detection of the causal agents of Sub-clinical Mastitis of dairy cows in Sirajganj and Pabna Districts Md. Humayun Kabir, Md. Ershaduzzaman, Md. Giasuddin, KHM Nazmul Hussain Nazir, Md. Rafiqul Islam, Md. Rezaul Karim, Md. Abu Yousuf
11:14am	11:24am	Crossbred cow's metabolic profile and its relation with retention of fetal membrane MS Arif, MMU Bhuiyan, J Bhattacharjee

Poster Presentation

Molecular detection of Pasteurella multocida Type B causing haemorrhagic septicemia in cattle and buffaloes of Bangladesh
MS Ara, MT Rahman, M Akhtar, M Rahman, KHMNH Nazir, S Ahmed, ML Hossen, MFR Khan and MB Rahman

Environmental contamination of lead in dairy feed and products in Narayangonj district of Bangladesh
Sharmin Akter, Yusuf Ali Sarker, Jannatul Ferdous, Sabbya Sachi, Zakaria al Noman, Mahmudul Hasan Sikder

In-Vitro Efficacy of Verenda (Ricinus communis) Leaves extract against ticks in cattle
Shariful Islam, Yusuf Ali Sarker, Md. Mehedi Hasan, Sudipta Talukder, Jannatul Ferdous, Mahmudul Hasan Sikder

Retrospective study of surgical cases of ruminants reported at Veterinary Teaching Hospital, Bangladesh Agricultural University
Zakaria Al Noman, Jannatul Ferdous, Sabbya Sachi, Mahmudul Hasan Sikder

Prevalence of infectious and non-infectious diseases in cattle population in Moulvibazar district of Bangladesh
Q M Monzur Kader Chowdhury, Sawrab Roy, Shahrul Alam, Juned Ahmed

Socioeconomic status of Buffalo farming communities of Jaintapur Upazilla of Sylhet, Bangladesh
Md. Farhan Rafiq, Mahfuz Rahman Adnan, Md. Abdul Baset, Khalid Hussain,. Sharmin Mostary

Value Chain Analysis and Comparative Advantage of Milk Production in Selected Areas of Bangladesh
S Islam, TN Nahar, J Begum, M Khatun and MI Hossain

Passive prevalence of bovine viral diarrhoea virus by detection of antibody in bulk tank milk samples
Pritilata Das and Sukumar Saha

Technical Session - IV : Disease

Chairperson : **Dr Mohammad Rafiqul Islam,** Professor, Dept of Pathology, BAU

Co-Chairperson : **Prof Dr Bibek Chandra Sutradhar,** Dept of Medicine & Surgery, CVASU, Chittagong

Rapporteurs : **Dr Rupaly Akhter,** Asst Prof & Chairman, Dept of Pharmacology & Toxicology, SAU, Dhaka

: **Mr Ashikur Rahman,** Assistant Professor & Chairman, Dept of Dairy Science, SAU, Dhaka

: **Dr Md Shahidul Islam,** Asst Professor, BSMRAU, Gazipur

Venue : Media Bazar, BICC, 9th March 2018, Time : 11:40am-12:23am

From	To	Title of Presentation
11:40am	11:50am	Pro-inflammatory cytokines as indicators of health status around calving in dairy cows Nusrat Jahan, Andrea Minut, Giuseppe Bertoni, Annarita Ferrari, Sadek Ahmed, Erminio Trevisi
11:51am	12:01pm	Pathological investigation of trachea and lungs of buffaloes in Gazipur district M.G. Haider and M.J. Alam
12:02pm	12:12pm	Investigations on Retained Placenta in Dairy Cows and observation of its prevention with common drugs Md. Hemayatul Islam, Md. Jalal Uddin Sarder, Md. Reazul Islam and Md Shofiqul Islam
12:13pm	12:23pm	Prevalence and risk factors of brucellosis in cattle M. A. S. Sarker, M. M. Begum, M. S. Rahman, M. T. Islam, M. F. Rahman and Kamruzzaman

Technical Session - V : Genetics

Chairperson : **Professor Dr A K Fazlul Haque Bhuiyan,** Dept of Animal Breeding and Genetics, BAU, Mymensingh

Co-Chairperson : **Professor Dr Md Azharul Hoque,** Dept of Animal Breeding and Genetics BAU, Mymensingh

Rapporteurs : **Dr Lam Yea Asad,** Associate Professor, Dept of Animal Nutrition Genetics & Breeding, Sher-E-Bangla Agriculture University, Dhaka

: **Mr Mohabbat Ali,** Assistant Professor, Dept of Animal Nutrition Genetics & Breeding, Sher-E-Bangla Agriculture University, Dhaka

: **Dr Md Tusar Chowdhury,** Managing Partner, ZAS Animal Health

Venue : Media Bazar, BICC, 9th March 2018, Time : 02:45pm-04:15pm

From	To	Title of Presentation
02:45pm	02:57pm	Candidate genes for milk production traits of Red Chittagong Cattle Md. Yousuf Ali Khan and Mst. Parvin Mostari
02:58pm	03:10pm	Genotyping of cattle based on lactoprotein genes: Kappacasein and Alpha-lactalbumin Sumaiya Rahman, Md. Imam Hossain, MM Kamal Hossain, Md. Abdul Alim, Nusrat Jahan, Md. Ariful Islam, Md. Salimullah, Jahangir Alam
03:11pm	03:23pm	Differentiation of meat origin by polymerase chain reaction-restriction fragment length polymorphism using 12S rRNA gene Md. Ariful Islam, ATM Mijanur Rahman, Salahuddin, Kamal Hossain, Nusrat Jahan, Md. Abdul Alim, Md. Salimullah, Jahangir Alam
03:24pm	03:36pm	Intrafollicular transfer of abattoir-derived immature or in vitro-matured oocytes results in blastocysts' gene expression profiles comparable to fully in vivo-produced blastocysts Mohammad Bozlur Rahman,Md Aminul Islam, Mohammed Saeed-Zidane, Franca Rings, Dessie Salilew-Wondim, Dawit Tesfaye, Michael Hölker, Karl Schellander
03:37pm	03:49pm	Characterization of sperm attributes separated by modified swim up method using hTF in crossbred cattle (Brahman X) and indigenous ram in Bangladesh. Mohammad Mofizul Islam, Mohammad Aminur Rahman, Mohammad Anwar Hossain, Farida Yeasmin Bari and Nasrin Sultana Juyena
03:50pm	04:02pm	Improvement of Fertility of Cows Using Prostaglandin F2α Analogue Md. Jalal Uddin Sarder, Md. Hemayatul Islam, Md. Reazul Islam, Soniya Akter Nishi, Mst. Ishrat Zerin Moni and Most. Rokeya Sultana
04:03pm	04:15pm	Existence of A1 and A2 allele of Beta-casein gene in cattle in Bangladesh Jahangir Alam, Sayeeda Monira Rahman, Nusrat Jahan, Mridha Md. Kamal Hossain, Shohel Mahmud1, Md Ariful Islam, Md. Abdul Alim, Md. Humayun Kabir, Salma Akter, Md. Salimullah

Poster Presentation

High yielding beef production in Bangladesh: Comparative growth performance of F1 progeny of different crossbred beef cattle
Mst. Parvin Mostari, Md. Yousuf Ali Khan, Biplob Kumer Roy, S. M. Jahangir Hossain and Khan Shahidul Huque

Determination of genetic variations among BLRI Cattle Breed-1 using microsatellite markers
Mst. Parvin Mostari and Md. Yousuf Ali Khan

Timed artificial insemination (TAI) in Bangladeshi buffaloes during the off breeding season
Mohammad Harun-Or- Rashid, Md. Nazrul Islam, Mir Md. Iqbal Hasan,Md. Mofizul Islam, Mohammad Mahbubul Hoque and Nasrin Sultana Juyena

Technical Session - VI : Feed & Nutrition

Chairperson : **Dr. Nathu Ram Sarker,** DG BLRI, Savar

Co-Chairperson : **Dr Mohammad Shahidul Islam PhD,** ULO, Osmaninagar, Sylhet

Rapporteurs : **Dr Md Muklesur Rahman,** Veterinary Surgeon, District Veterinary Hospital, Gazipur

: **Dr Ayesha Siddiqua,** Scientific Officer, DLS, Khamarbari, Farmgate, Dhaka

: **Mr Md Mahbubur Rahman,** Proprietor, Cordial International

Venue : Media Bazar, BICC, 09th March 2018, Time : 04:30pm-06:00pm

From	To	Title of Presentation
04:30pm	04:42pm	Economics and stakeholders participation in fodder production and marketing system under different climatic zones of Bangladesh Rahman S.M.R, N.R. Sarker, M. R. Amin, M. Kamruzzaman and M. R. Haque
04:43pm	04:55pm	Development of New Napier Cultivars through Gamma-ray Irradiation Alam MK, NR Sarker, KM Nasiruddin and AM Shohael
04:56pm	05:08pm	Buffaloes for dairying in South Asia: potential, challenges and way forward Md. Nure Alam Siddiky
05:09pm	05:21pm	Feeds and Fodder Production in Bangladesh: Present trend, availability,future requirements and challenges for dairying in 2030 N.R. Sarker, S.M.R. Rahman and M.Y.A. Khan
05:22pm	05:34pm	Commercialization of fodder production among smallholder dairy producers in the saline prone coastal areas of southwest Bangladesh: A Research-NGO linkage M.J. Hossain and N.R. Sarker
05:35pm	05:47pm	Feeding effect of straw based Total Mixed Ration (TMR) on milk yield, milk composition and rumen parameters in Red Chittagong cows N.R. Sarker, D. Yeasmin , F. Tabassum and M.A. Habib
05:48pm	06:00pm	Study of system modeling of Moringa (Moringa oleifera) fodder agronomy and its nutritional response to ruminants MK Bashar, KS Huque, NR Sarker, N Sultana and HPS Makkar

Poster Presentation

Dairy buffalo production system under semi-intensive condition at the Coastal area of Bangladesh
S M Rajiur Rahman, Md. Nurul Islam, Md Harun-ur-Rashid, Md. Sohel Rana Siddiki, Mohammad Ashiqul Islam

Technical Session - VII : Zoonosis

Chairperson : **Dr Bidhan Chandra Das,** Ex DD , DLS

Co-Chairperson : **Dr. K. B. M. Saiful Islam,** Chairman & Associate Professor, Dept. of Medicine & Public Health & Center-in-Charge, Zoonotic Diseases Research & Information Center, Sher-e-Bangla Agricultural University,Dhaka

Rapporteurs : **Dr Md Saiful Islam,** Chair & Asst Professor, Dept of Animal Production & Mgt Sher-E-Bang Agriculture University, Dhaka

: **Dr. Uday Kumar Mohanata,** Associate Professor & Chairman, Dept of Microbiology & Parasitology, Sher-E-Bangla Agri Culture University, Dhaka

: **Dr AHM Shahadat Hossain,** ULO, DLS, Dhaka

Venue : Media Bazar, BICC, 10th March 2018, Time : 09:15am-10:58am

From	To	Title of Presentation
09:15am	09:27am	Molecular characterization of *Bacillus anthracis* from soils in selected districts of Bangladesh Maidul Islam, Md. Shahjahan Ali Sarker, Md. ShahabUddin, Jafrul Hasan Ripon, Shurya Khanam, Taslima Akter Shapna, Md. Ariful Islam, Md. Muket Mahmud and KHM Nazmul Hussain Nazir
09:28am	09:40am	Campylobacter species from Bangladesh Agricultural University dairy farm: isolation, molecular identification and antimicrobial susceptibility profiles Most. Mostary Lubna, Md. Mehedul Islam, Shinji Yamasaki and S. M. Lutful Kabir
09:41am	09:53am	Bacteriological and Molecular detection of Staphylococcus spp. and antibiotic resistant mecA and blaZ genes in milk from selective dairy farms in Mymensingh Asmaul Husna, Md. Muket Mahmud, Md Asief Hossain Zihadi, Md. Ali Akbar and Marzia Rahman
09:54am	10:06am	Q Fever in cattle with reproductive disorders : Prevalence and molecular diagnosis A. Chakrabartty, M. S. Rahman, P.K. Bhattacharjee, M. A.S Sarker, A. K. M. A. Rahman, K. Henning, H. Neubauer
10:07am	10:19am	Microbiological quality of raw and pasteurized milk obtained from local market Salma Akter, AKM Mostafa Anower, MM Kamal Hossain, Md. Abdul Alim, Md. Salimullah, Jahangir Alam
10:20am	10:32am	Detection and quantification of Antibiotic residues in Milk Collected From Non-Commercial and Commercial Dairy Farms in Mymensingh District of Bangladesh Sabbasachi Jannatul Ferdous, Yusuf Ali Sarker, Zakaria Al Noman, Mahmudul Hasan Sikder
10:33am	10:45am	Antimicrobial resistance in food producing animal leads to human health hazard Mohammad Rafiqul Islam, Md. Kamruzzaman Khokon, Md. Jasim Uddin, Md. Muklesur Rahman
10:46am	10:58am	characterization of staphylococcus aureus from milk and dairy products sold in some local markets of mymensingh district of bangladesh Zobayda Farzana and Sukumar Saha

Technical Session - VIII : Industrial

Chairperson : **Dr. M Ali Imam,** President & CEO, Jims Tech International

Co-Chairperson : **Dr.Md.Abu Sufian,** PhD, Assistant Director (Animal health & Administration) Department of Livestock Services, Khamarbari, Dhaka

Rapporteurs : **Dr Md Rafiul Karim,** Managing Director, Doctor's Agro-Vet Ltd

: **Dr Mohammad Shakif-Ul-Azam,** ULO, DLS, Dhaka

: **Dr Rashedul Jakir,** CEO, RR Agro Traders

Venue : Media Bazar, BICC, 10th March 2018, Time : 11:15am-12:06pm

From	To	Title of Presentation
11:15am	11:27am	Use of phytobiotics to improve health in tilapia Cristina García-Diez
10:28am	11:40am	Incidence of common diseases and disorders of cattle at Kotalipara Upazila, Gopalgonj, Bangladesh. Animesh Biswas and Md. Selim Ahmed
11:41am	11:53am	Present status and problems of fish seed marketing in Sylhet District, Bangladesh Shahrear Hemal, Md. Shahab Uddin, Md. Saif Uddin, Bhaskar Chandra Majumdar, Md. Golam Rasul and Md. Tariqul Alam
11:54am	12:06pm	Biological efficacy of the mineral combination, Extramin™ on enhancing the growth by the regulation of molting and controlling disorders arising due to mineral deficiency in Pacific white leg shrimp, Litopenaeus vannamei *(Boone, 1931)* Godfred Ponraj Jeyaraja, Mohammed Tarique Sarkerb, Edward Gnana Jothi Georgea, and Veera Ravi Arumughamc

Pet

A Serological Survey of Newcastle Disease Virus in Pigeons at Chittagong Metropolitan Area, Bangladesh

Elina K.C.[1], Abdul Ahad[2], Nayem Akhter Palash[1], A.K.M. Mostafa Anower[1*]

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Abstract

Newcastle Disease (ND) is a viral disease in birds caused by Newcastle Disease Virus (NDV) which belongs to the family Paramyxoviridae. ND is one of the major killer diseases in chicken and pigeon. Pigeon is considered as a fancy bird by the rearer because of its beauty. The squab meat is very nutritious for debilitative human being as it is easily digestible. The objective of this study is to evaluate the clinical signs of Newcastle Disease in infected pigeon lofts at Chittagong Metropolitan Area and to determine serological status against NDV of these lofts. From 10 pigeon lofts, 100 blood samples were collected and examined with Haemagglutination Inhibition (HI) test. The samples were divided into ten different groups by sample collected from pigeon lofts. Descriptive statistical analysis was performed based on age, breed, housing and their association with system affected by chi-square test through STATA statistical package. Geometric mean antibody titer (GMT) for NDV in serum of pigeon was found to be $\log 2^{3.09}$ although the pigeons were not vaccinated against NDV. Some of the pigeons exhibited nervous and diarrheic symptoms. From the investigation, we can conclude that most pigeons have antibody against NDV in the bottom line of protection. This antibody level is not sufficient to give protection in the birds infected with a velogenic strain of the virus. Therefore, administration of NDV vaccine routinely and monitoring of antibody at regular intervals may be advisable to the farmers.

[**Keywords**: Pigeons, Newcastle disease, HI, Chittagong Metropolitan area.]

Introduction

Newcastle Disease (ND) ranked among the most infectious diseases of great importance by WHO for Animal Health (Linde et al., 2010). It has been identified that it can infect 200 species of birds (Wambura, 2010). Among the bird species, pigeons are fancy petbirds which are valuable to the owners of Bangladesh. Pegions are susceptible to the NDV. The pathogenicity ranges from subclinical infections to highly virulent depending on the susceptibility of the host and the pathotype of the virus (Jindal et al., 2009). In 1984, the neurotropic ND occurring in pigeons was first recorded in Europe continent, and infection has been reported being transmitted from one type of bird to another without prior adaptation (Roy et al., 2000). The avian paramyxovirus-1 (APMV-1) or NDV which is the most pathogenic serotype (Caupa and Alexander, 2009), belongs to the order Mononegavirales. The genus is Avulavirus of paramyxoviridae family and subfamily paramyxovirinae (Tan et al., 2010). The variant of APMV-1, Pigeon Paramyxovirus serotype-1 (PPMV-1), causes ND in pigeons and is characterized by the unique monoclonal antibody. NDV genome is negative sense, single-stranded RNA molecule containing 15,186 nucleotides (Munir et al., 2012). The pigeon variety of NDV is an enigma, as the virus can produce high mortality in pigeons, yet it can react as a lentogenic virus in susceptible chickens.

The only velogenic strain causes disease in pigeons (Saif, 2003) and produces greenish feces, dull and inappetence, misshapen eggs with decline in production (Numan et al., 2005) and some egg shell defects (Feberwee and Landman, 2010). PPMV-1 circulates in domesticated and wild pigeons, but strains of APMV-1 often cause little or no disease in pigeons, although there have been reports of neurological signs. Estimates of virulence for PPMV-1 from pigeons may not be accurate when assessed with routine pathotyping. Serological assays may be useful in some circumstances. Routinely HI test is carried out to monitor and evaluating of antibody response for NDV (Alexander, 2008). However, diagnosis is based on vaccination history, types of vaccine and existing disease condition (OIE, 2014). High antibody titer against NDV indicates the birds have been exposed to the virus, but it will not give any clue about circulating strain in that

area. Evaluation of exact distribution of ND is difficult due to lack of proper reporting, but the vNDV strains are prevalent in Asia, Africa along with North and South America to some extent (Miller, 2014). Contacts between feral and racing pigeons in environments favored the spread of pigeon-strain vNDV through newly introduced ones with subclinical infections or in carrier state (Tu et al., 1998).

Vaccination should be employed in conjunction with good management practice, biosecurity and good hygiene (Alexander, 2001) to control ND in pegions. However, due to lack of a specific vaccine for ND in pigeons in Bangladesh, high mortality in pigeons by ND is overviewed. Considering the potential risk of contamination of poultry species by pigeons carrying NDV, it is important to study the pathogenesis of the disease in pigeons. The survey was conceded with the goal of antibody level determination against NDV at different pigeon lofts via area based study to formulate suitable vaccination calendar in pigeons along with coordinated control strategy.

Materials and methods

A preset questionnaire was designed to collect information such as feeding, management, vaccination and signs/symptoms from sampled pigeon. The data thus recorded were stored in Excel sheet. Samples were collected from 10 pigeon lofts suspicious to infect with ND, throughout the Chittagong Metropolitan Area, Chittagong, Bangladesh. Totally 100 sera samples were collected from 10 understudy lofts: Loft I (15 samples), Loft II (5 samples), Loft III (10 samples), Loft IV (10 samples), Loft V (10 samples), Loft VI (25 samples), Loft VII (5 samples), Loft VIII (10 samples), Loft IX (5 samples) and Loft X (5 samples). The informed consent was obtained from pigeon owners. All the pigeons were between the ages of 8 to 24 months. The serum samples were tested using standard HI method for antibodies detection against NDV in the laboratory of Microbiology and Veterinary Public Health Department of the Chittagong Veterinary and Animal Sciences University, Chittagong. The antigen, Avinew® vaccine (Advance Animal Science Co. Ltd. Dhaka, Bangladesh) containing VG/GA strain of ND was used. 5 ml of chicken blood was collected with a disposable syringe containing 1 ml of 4% sodium citrate as an anticoagulant. Blood was centrifuged at the speed of 1500 rpm for 15 minutes. Buffy coat along with plasma was decanted keeping only RBC. 1% of chicken RBC suspension in Normal Saline (NS) was prepared after washing three times with NS. HI test was performed according to the method mentioned in OIE manual (2002).

In brief, two-fold serial dilution of test serum up to 10th well was made then 4 HA unit of NDV was added upto 11th well and kept at room temperature for 30 minutes. In each well, 1% chicken RBCs suspension was added. By this way, 12^{th} well was kept for RBC control and 11^{th} well was kept for virus control containing 4 HA unit. The samples showing buttoning of RBCs at the center were considered positive. The end point is a maximum dilution of each sample causing Haemagglutination inhibition. The reciprocal of the serum dilution was used to determine HI titer of each sample.

Statistical analysis

The geometric mean titer was estimated, and results were analyzed statistically by applying ANOVA and t-test for the calculation of means/ statistical significance between housing system with system affected in NDV infection in pigeons.

Results

From 100 serum samples, 34 were positive in HI test for antibodies against NDV; this gives a seroprevalence of 34%. The antibody titer levels at 4 and above were considered positive (Rahman et al., 2017). Here, HI titer level ranges from 2^2-2^5. The geometric mean titer was found to be 3.09. The average antibody titer in 10 pigeon lofts (I-X) were 3.3, 2.8, 3.1, 2.9, 3.2, 2.8, 2.4, 3.8, 3.6 and 3.0 respectively (**Figure 1**).

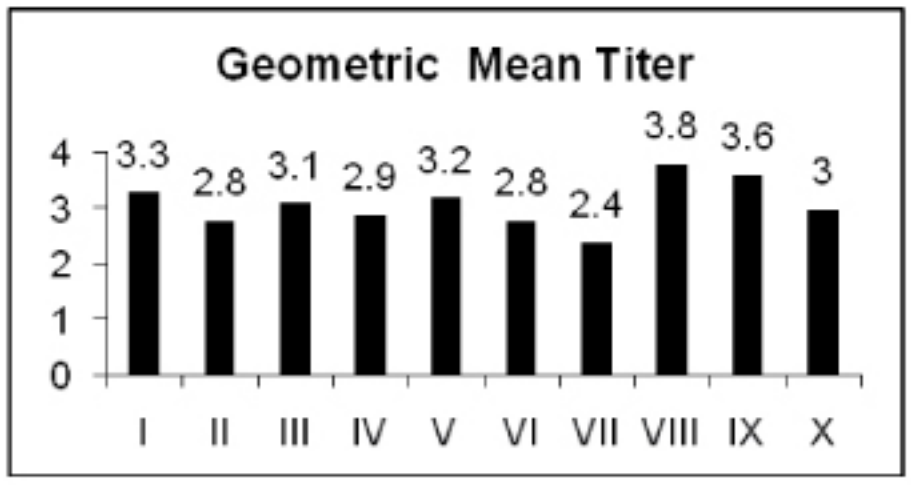

Figure 1: Geometric mean titer of different loft in Pigeon

From farm statistics, we observed that ND infections in breeds of pigeons such as Jalali (26%), Giribaz (29%), Siraji (11%), Fantail (12%) were found higher compared to other (18%). It was found that age-group of 12-24 months were mainly affected and showed the signs of ND. We found that caged pigeons were more susceptible to infections than free ranging and coop housed pigeons (**Figure 2, 3, 4**). Insignificant relation ($p>0.05$) was found between the system affected by ND and housing system of pigeons (**Table 2).** In each farm proportion of pigeons having antibody titer against NDV greater than 8 HI titers were calculated. It was observed that in farm number I, VIII and X, 80% of the pigeons have more than 8 HI titer. In farm number 2 and 7, the least 40% bird has antibody titer more than HI titer (**Table 1**).

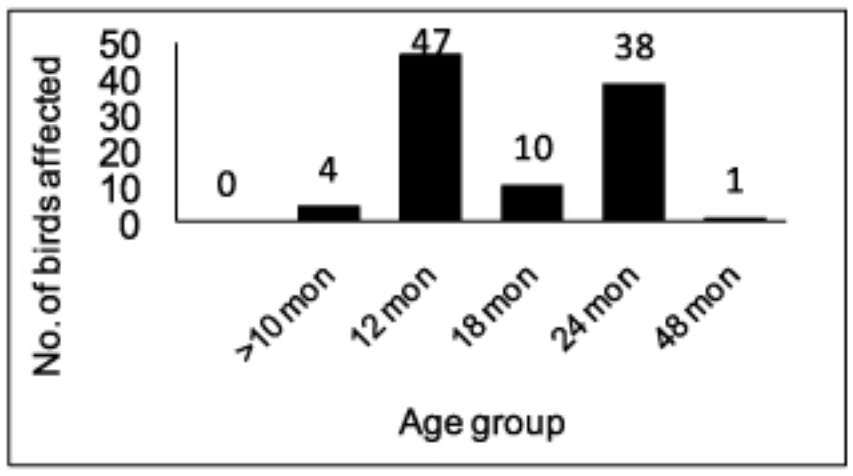

Figure 2: Occurrence of ND in relation to age

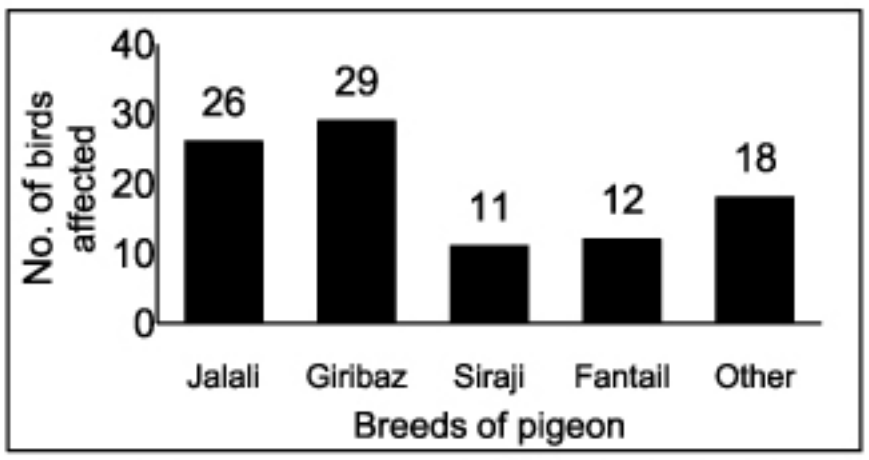

Figure 3: Occurrence of ND in relation to breed

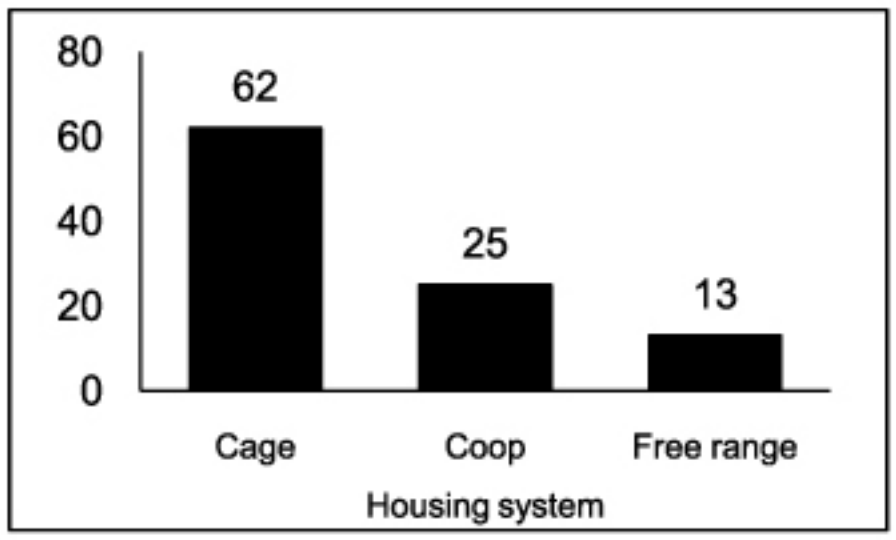

Figure 4: Occurrence of ND in relation to housing system

Table 1: Proportion of farm pigeons having antibody titer greater than 8 HI titer

Loft #	Cut off (No)	Cut off (yes)	Total
I	2(13.33%)	13(86.67%)	15
II	3 (60%)	2 (40%)	5
III	3 (30%)	7(70%)	10
IV	5 (50%)	5 (50%)	10
V	3(30%)	7(70%)	10
VI	11(44%)	14(56%)	25
VII	3 (60%)	2(40%)	5
VIII	2 (20%)	8(80%)	10
IX	7 (40%)	3(60%)	10
X	1 (20%)	4(80%)	5

Discussion

In the present study, antibodies levels in serum of ND affected pigeons were analyzed to evaluate the serological status in pigeons against ND. Of the total serum samples (n=100) collected from 10 pigeon lofts, only 34% were seropositive for Newcastle virus using HI test. 66% of birds were negative and had HI titer 2-3. 34% of birds had 2-4≤HI titer≤2-5. Most of the owners did not vaccinate their pigeons against ND though they were aware of the disease. However, many said they had faced ND as the major threat. History of a vaccination program is important in the interpretation of results. Similarly, PPMV-1 was isolated from 67.8% of tested pigeons (Mansour et al., 2017). Management practices may be responsible for the difference in titer.

From statistical analysis, it was found that age-group of 12-24 months pigeons are more susceptible. Abdu 2007 estimated that morbidity is approximately 100% in adult pigeons, with minimal mortality in the absence of co-infections. Association of housing system with system affected calculated by chi-square test showed that there was an insignificant relationship ($p > 0.05$) in antibody prevalence between groups and within groups.

Clinical signs observed were greenish white diarrhea, torticollis, incoordination and death. Similar signs were reported (Beard and Hanson, 1984). The dead pigeons brought to the hospital were subjected to postmortem examination. Primary lesions observed on necropsy were hemorrhage on proventriculus and button ulcers in the intestine. It is reported that ND as one of the major cause of impaired production (Ahlers, 2009) and increased mortality (Mavale, 2001). It was reported to cause 70% mortality in experimentally infected pigeons. However, transmission of vNDV strains from poultry to pigeons (Kapczynski and King, 2005) and vice-versa may occur.

Several serological and molecular tools are applied to diagnose the infection. HI is commonly used in diagnostic laboratories to determine antibody titers against NDV. Some level of cross-reactivity may be pragmatic among the assorted avian paramyxovirus serotypes in HI test. In this study, we observed antibody titer level ranging from 2-5 although pigeons were not vaccinated. This antibody may be cross reacting antibody with another paramyxovirus which was carried by a pigeon. Thus, most pigeons have antibody against NDV in the bottom line of protection, but it is not sufficient to give protection when there is an outbreak of NDV especially in the case of very virulent NDV.

Table 2: Association between rearing system and clinical signs

Housing system	System affected						Total	χ^2	df	p
	A	B	C	D	E	F				
1	25 (3.4%)	17 (13.6)	13 (11.8)	1 (1.24)	3 (1.86)	3 (3.10)	62			
2	7 (6.37%)	3 (2.86)	2 (2.47)	0 (0.260)	0 (0.390)	0 (0.650)	13	11.8	10	0.295
3	17 (12.2%)	2 (5.50)	4 (4.75)	0 (0.500)	0 (0.750)	2 (1.25)	25			
Total	49	22	19	2	3	5	**100**			

Note:1= Cage, 2= Coop, 3=Free range, A= No signs, B= Digestive, C=Nervous +Digestive, D=Oral, E= only Nervous, F= Respiratory; df=Degree of freedom; p=Probability

Many owners believed that ND vaccine of chicken is not safe to use in pigeons. Some research trials of ND vaccines of chicken in pigeons proved safe to use and twice vaccination at 4 weeks interval could produce protection against ND. Some field data suggested that only birds with HI titer above 16 after twice vaccination will survive a vNDV challenge (Idi et al., 1999). Therefore, it is vital to monitor the antibody titer before and after vaccination. The owners are to be advised to administered NDV vaccine routinely and monitor antibody at a regular interval.

The above findings showed that there is dire need to carry out regular vaccination in pigeons against ND. The same recommendation has also been put forward by several others (Saidu et al., 2004). Lately, the oral vaccine like oiled rice coated with I-2 ND strain in chickens (Wambura et al., 2007) has been developed. A similar practice in pigeons and other semi-domesticated birds is required. This was a small area-based study that covered only five different areas in Chittagong district, but a bigger study covering more areas throughout the country and a more thorough questionnaire about management would be necessary to get a more exact picture of the prevalence and other possible problems.

Conclusion

Newcastle disease is prevailing among pigeons in Bangladesh. Signs and symptoms of the disease were greenish or white diarrhea, respiratory distress, and torticollis. Sudden deaths, with few or no preceding clinical signs,were also seen frequently. Prevalence of low level of HI titers in the pigeons may be due to the early phase of infection whereas a high prevalence due to post-outbreak. A large percentage of antibodies against avian PMV-1 and their relatively high titers revealed the presence of virus in the pigeon population. If PPMV-1 strains circulate in chickens, it could evolve hazardous condition in the poultry industry. It needs to provide regular vaccination in pigeons against ND.

Acknowledgements

We would like to pay special thanks and appreciation to the faculty of Veterinary Medicine, Chittagong Veterinary and Animal Sciences University whose services turned our research success.

Conflict of Interest

No conflict of interests regarding the publication of this paper.

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Epidemiological investigation of clinical diseases and conditions of pet animals at Chittagong city area, Chittagong, Bangladesh.

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ABSTRACT

This study was conducted to estimate the proportional prevalence of clinical diseases and conditions in pet animals (dog, cat, and rabbit) attended at S.A. Quaderi Teaching Veterinary Hospital, Chittagong Veterinary and Animal Sciences University (CVASU), Chittagong, Bangladesh. A total of 388 cases of different clinical diseases and conditions were recorded during the study period from October 2016 to January 2017. Among them the overall proportional prevalence of clinical diseases and conditions in dog, cat and rabbit were 44.32%, 34.28% and 21.39%, respectively. The medicinal case comprises the highest percentage of diseases as 79.07% and 66.92% in dog and cat, respectively. Among the medicinal cases parasitic diseases were most frequently encountered both in dog (24.42%) and cat (23.31%). Whereas, prevalence of surgical cases were 16.28% in dog and 31.58% in cat and gynecological cases were 4.65% in dog and 1.50% in cat. In rabbit, prevalence of traumatic injuries and fractures were 16.87% followed by mange 12.06%. The parasitic cases were highest in all of the clinical diseases and conditions. So, proper prevention and therapeutic management should be taken to reduce the prevalence of parasitic diseases of pet animals. Immunoprophylaxis or vaccination and hygienic management should be performed to curb the infectious diseases. Further extensive epidemiologic, laboratory or molecular studies are also needed for the appropriate prevention and control measures of diseases and conditions of pet animals in study area.

Key Words: Dog, Cat, Rabbit, prevalence, clinical diseases, Chittagong.

INTRODUCTION

Modern society is becoming more urbanized now a days. Pets have become an integral part of the family and often considered to be extended family. The pet animals are kept by a significant percentage of people all over the world irrespective of their social status including Bangladesh. In many households, pet animals contribute to physical, social and mental well-beings of children and their owners (Dohoo *et al*., 1998; Robertson *et al*., 2000). Dog and cat have significant benefits to our society like companionship, play with children, guard the house, and alerts the owner from any adverse condition, gift to special ones and economic purposes (Parvez *et al*., 2014). Dogs are not only serving as a companion but also act as workers (Singh *et al.,* 2014). They have proven to be invaluable member of family with different roles including guide dogs for blind person, provide assistance to the disable, sniffers dogs used by police and customs and farm dogs used as shepherd. The rabbit makes mutual relationship with their owners by better understandings. A lot of diseases and clinical conditions are frequently observed in pet dog. Among the viral diseases rabies, infectious canine hepatitis, canine distemper, canine parvo viral infections are very common in Indian sub-continent like Bangladesh (Biswas *et al*., 1996). Rabies is the most important zoonotic disease worldwide including Bangladesh (Biswas *et al*., 1996) and approximately 90% of rabies in human results from dog bite. Almost all human deaths caused by rabies originated from Asia and Africa and there are an estimated 55,000 human deaths annually from rabies, with about 31000 in Asia and 24,000 in Africa (Samad, 2011). On the other hand, most common bacterial diseases include leptospirosis, brucellosis, kennel cough, clostridial infection etc. Rearing pet animals are usually connected with certain responsibilities like housing, control of diseases and responsibility of pet ownership with public health importance. Pet animals are the important reservoir of zoonotic diseases as they share the same environment with humans. Household pets also have been found to play a direct role in transmitting zoonosis (Damborg *et al*., 2016). However, a diverse range of infections, include parasitic, bacterial, fungal and viral diseases are transmitted to humans from domestic pets (Plant *et al.,* 1996 and Geffray, 1999).

The prevalence of infectious diseases and conditions of domestic animals and birds has already been performed in various regions of Bangladesh (Tarafder and Samad, 2010; Mahmud *et al*., 2014). But very few studies have been reported on the prevalence of clinical diseases and conditions in pet animals (Parvez *et al*., 2014). Hossain and Kayesh (2014) reported the overall prevalence of clinical diseases at Dhaka city area in dog, cat and rabbit as 2.39%, 3.72% and 6.66%, respectively. It has been reported that ectoparasitic infestation (tick infestation 11.88%, flea infestation 9.84% and mange 3.76%) cases were highest among the all reported clinical diseases in pet dog (Tarafder and Samad, 2010). Mahmud *et al*. (2014) reported overall prevalence of protozoan diseases was 22.42% at Sirajganj, Bangladesh. So, this study was undertaken with the following objectives:

I. To determine the overall prevalence of clinical diseases and conditions of pet animals at SAQVH, CVASU, Chittagong, Bangladesh.
II. To know the prevalence of diseases and conditions in relation to age, sex, breed, affected systems of the body and etiology of pet animal's diseases (dog, cat and rabbit).

MATERIALS AND METHODS

The study was conducted at S.A Quaderi Teaching Veterinary Hospital during the period of October 2016 to January 2017 at Chittagong Veterinary and Animal Science University (CVASU), Chittagong, Bangladesh. A total of 388 sick pet animals (dog, cat and rabbit) were admitted in hospital. The clinical examination of diseased animal was performed on basis of owner's complaints, anamnesis and clinical examinations of patients.

Owner's complaints: Complaints of owner of patients were considered during animal examination.

Anamnesis: The history of clinical diseases and patients were collected from owners to identify the predisposing factors of diseases.

Physical examination

Wounds were identified by inspection and further examined for more precise diagnosis to categorize the nature of the wound whether it might be septic, lacerated, incised, punctured, perforating, abrasions, avulsion or hematoma. Needle puncture was also performed if required. Temperature, pulse, respiratory rates were taken through clinical method. Mouth gag and general anesthesia were also used for restraining of the pet animals. In case of fracture, extension and flexion method were performed for pet animals.

Laboratory diagnosis

Fecal samples and skin scrapings were examined with compound microscope. Blood and urine samples were collected for routine and specific diagnosis. X-Ray or imaging was performed to diagnose the musculo-skeletal and chest diseases. Dead animals were subjected to necropsy examination and gross lesions were recorded. The samples were collected to diagnose the etiology of diseases.

Statistical analysis

The data collected from each patient were entered into MS excel (Microsoft office excel-2007, USA). Data management and descriptive analysis was done in Excel. Results were reported as proportional prevalence percentage and calculated by using formula.

RESULTS AND DISCUSSSION

Proportional prevalence of clinical diseases and conditions of pet animals in relation to affected system and etiology of diseases

A total of 388 cases of different clinical diseases and conditions were recorded during the study from October 2016 to January 2017. Among them the overall proportional prevalence of clinical diseases in dogs, cats and rabbits were 44.32%, 34.28 % and 21.39% (Table 1). About 79% and 67% of the total cases were medicinal in dogs and cats, respectively (Table 2 and 4). Among medical cases, the most frequently encountered disease was parasitic diseases both in dogs (16.28%) and cats (31.58%). Parvez *et al*. (2014) reported more parasitic

cases in dogs (51.54%) and cats (54%). Sarker *et al.*,(2015) reported that overall proportional prevalence of clinical diseases in dog and cat as 75% and 25%, respectively and prevalence of parasitic diseases were 14.77% in dogs and 13.33% in cats. Sarker *et al.*, (2015) observed very low prevalence of parasitic disease, because pet's owners were more concerned with deworming of pet dogs in studied area. The occurrence of clinical diseases of digestive system was 17.44% in dogs and 12.78% in cats. We observed relatively higher prevalence of digestive disorder in dogs (17.44%) which was in contrast with the results of Chaudhari and Atsanda, (2002) who reported only 6.73%. Parvez *et al.*, (2014) recorded 12.64% digestive disorder in dogs. Prevalence of loss appetite 5.81% in dogs was in contrast with the result recorded by Chaudhari and Atsanda, (2002) where it was only 2.69%. However, Sarker *et al.,* (2015) reported similar level of loss of appetite (5.11%) in dogs. The prevalence of skin diseases were 7.6% in dog and 0.8% in cats. Similar finding was also reported by Tarafder and Samad, (2010) but other authors reported lower prevalence of skin disease (Freeman et al., 2006; Chaudhari and Atsanda 2002) in dogs. The prevalence of disease in eyes and ear in dog and cat were 4.65%, 3.01%, respectively and the result were agreed with Freeman *et al.*, (2006) and Tarafder and Samad (2010). The prevalence of bacterial diseases was 5.8% in dogs and 3.0% in cats. The prevalence of viral diseases was 4.7% in dogs and 6.8% in cats. Our results were not in agreement with the result of Tarafder and Samad, (2010) who reported lower prevalence (0.08% in dog and 1.96% in cat). This variation of the result might be due to different geographical locations and periods of study. The prevalence of metabolic diseases of this study were 1.16 % and 1.50% in dog and cat, respectively which disagreed with the results of Tarafder and Samad, (2010) who reported higher prevalence of metabolic and nutritional diseases (3.13%) in pet dog. This variation might be due to small size of the sample studied. The prevalence of diseases of the respiratory system was 4.65% in dog and 4.51% in cat. Out results were in contrast with the findings of other authors who observed relatively higher prevalence in both dogs and cats (Tarafder and Samad, 2010; Chaudhari and Atsanda, 2002; Parvez *et al.,* 2014). Respiratory tract infections can be caused by viruses, bacteria and less often by fungi and sometimes from faulty medication. Prevalence of upper respiratory tract infections in dog and cat was 0.58% and 0.75%, respectively. The reported prevalence of fungal and protozoal diseases in dog was less than the result of Tarafder and Samad, (2010) who enlisted protozoal diseases as 2.02% and fungal diseases as 3.30%. In rabbits, the prevalence of traumatic injuries and fracture was the highest as 16.87% followed by mange 12.06%.

Sex wise prevalence of clinical diseases and conditions in pet animals

The proportional prevalence of clinical diseases and conditions in dog and cat in relation with their sex showed that male pets were most frequently admitted at SAQTVH. About 59 % of the clinical cases were recorded for male dog (Table 2). In case of cats, 58.65% of the clinical cases were recorded in male cat (Table 4). Similarly, 77.71% clinical cases were recorded in male rabbits (Table 5). Other authors also reported similar findings to our results (Sarker *et al.,* 2015; Parvez *et al.*, 2014). Usually the pet owner had the tendency to rear the male pet animals rather than female one to avoid the nuisance of reproductive behavior and difficulties arises from female dog, cat and rabbit.

Age wise prevalence of clinical diseases and conditions in pet animals

The proportional prevalence of clinical conditions was higher in adult (40.12%) than young dog (31.98%). The age-wise distribution of the prevalence of clinical diseases and conditions in dog, cat and rabbit are shown in the Table 2, 4 and 5. In cat, the higher prevalence of clinical cases was recorded in young (51.13%) than adult (39.09%). The higher prevalence of clinical cases in rabbit was recorded in young (49.40%) than adult (30.12%). However in dogs, Tarafder and Samad, (2010) reported higher prevalence clinical diseases in aged dogs (48.12%) as compared with young (17.55%) and adult (34.33%).

Breed wise prevalence of clinical and diseases and conditions in pet animals

Prevalence of clinical disease and conditions in dog and cat in relation to their breed revealed that the highest clinical diseases and conditions were found in the local dog (39.53%) and in the local cat (79.70%). In Persian cat, prevalence of clinical diseases and conditions were 16.54 % and in Bengal 3.76%. Among the exotic breed of dog, the prevalence of clinical diseases and conditions were highest in in German shepherd (18.02%) and lowest in Samoyed (3.49%) as shown in Tables 3 and 4. Tarafder and Samad, (2010) also reported similar finding as we observed.

Table.1. Overall prevalence of clinical diseases and conditions of pet animals

Pet animals	Clinical diseases and conditions	Prevalence (%)
Dog	172	44.32
Cat	133	34.28
Rabbit	83	21.39
Total cases	**388**	**100**

Table 2. Sex and age-wise distribution of clinical diseases and conditions of dog attended at SA Quadery Teaching Veterinary hospital during October 2016 to January 2017.

Clinical Diseases and Conditions	Overall Prevalence (%)	Sex		Age		
		Male	Female	<6months	>7 to 36 months	>36 months
1. Loss of appetite	10(5.81)	6(3.49)	4(2.33)	5(2.91)	4(2.33)	1(0.58)
2. Diarrhea	4(2.33)	2(1.16)	2(1.16)	2(1.16)	2(1.16)	0(0.00)
3. Gastritis	8(4.65)	5(2.91)	3(1.74)	2(1.16)	4(2.33)	2(1.16)
4. Dental Disorder	2(1.16)	1(0.58)	1(0.58)	0(0.00)	1(0.58)	1(0.58)
5. Others digestive problems	6(3.49)	4(2.33)	2(1.16)	4(2.33)	1(0.58)	1(0.58)
I. Digestive Disorders	**30(17.44)**	**18(10.47)**	**12(6.97)**	**13(7.56)**	**12(6.98)**	**5(2.91)**
1. Pneumonia	2(2.33)	1(0.58)	1(0.58)	2(1.16)	0(0.00)	0(0.00)
2. Bonchitis	1(0.58)	0(0.00)	1(0.58)	0(0.00)	1(0.58)	0(0.00)
3. kennel cough	5(2.91)	3(1.74)	2(1.16)	3(1.74)	0(0.00)	2(1.16)
II. Respiratory disorder	**8(4.65)**	**4(2.33)**	**4(2.33)**	**5(2.91)**	**1(0.58)**	**2(1.16)**
III. Urinary disorders	**1(0.58)**	**1(0.58)**	**0(0.00)**	**0(0.00)**	**1(0.58)**	**0(0.00)**
IV. Lamness	**2(1.16)**	**1(0.58)**	**1(0.58)**	**1(0.58)**	**1(0.58)**	**0(0.00)**
V. Metabolic disorders	**2(1.16)**	**2(1.16)**	**0(0.00)**	**0(0.00)**	**1(0.58)**	**1(0.58)**
1. Dermatitis	10(5.81)	6(3.49)	4(2.33)	2(1.16)	4(2.33)	4(2.33)
2. Alopecia	3(1.74)	2(1.16)	1(0.58)	0(0.00)	2(1.16)	1(0.58)
VI. Skin disorders	**13(7.56)**	**8(4.65)**	**5(2.91)**	**2(1.16)**	**6(3.49)**	**5(2.91)**
1. Conjunctivitis	4(2.33)	2(1.16)	2((1.16)	1(0.58)	2(1.16)	1(0.58)
2. Cataract	2(1.16)	2(1.16)	0(0.00)	0(0.00)	0(0.00)	2(1.16)
3. Otitis externa	2(1.16)	1(1.16)	1(1.16)	0(0.00)	2(1.16)	0(0.00)
VII.Eyes and Ears disorders	**8(4.65)**	**5(2.91)**	**3(1.74)**	**1(0.58)**	**4(2.33)**	**3(1.74)**
VIII.Nervous system	**5(2.91)**	**3(1.74)**	**2(1.16)**	**1(0.58)**	**3(1.74)**	**1(0.58)**
IX. Others	**5(2.91)**	**3(1.74)**	**2(1.16)**	**2(1.16)**	**0(0.00)**	**3(1.74)**
1. Canine distemper	3(1.74)	2(1.16)	0(0.00)	0(0.00)	2(1.16)	1(0.58)
2. Canine parvovirus	4(2.33)	2(1.16)	2(1.16)	4(2.33)	0(0.00)	0(0.00)
3. Pox/Herpes virus	1(0.58)	1(0.58)	1(0.58)	0(0.00)	1(0.58)	0(0.00)
X. Viral diseases	**8(4.65)**	**5(2.91)**	**3(1.74)**	**4(2.33)**	**3(1.74)**	**1(0.58)**
XI. Bacterial diseases	**8(4.65)**	**5(2.91)**	**3(1.74)**	**4(2.33)**	**3(1.74)**	**1(0.58)**
1. Ancylostomiasis	3(1.74)	2(1.16)	1(0.58)	2(1.16)	1(0.58)	0(0.00)

2. Unknown internal worms	7(4.07)	4(2.33)	3(1.74)	3(1.74)	2(1.16)	2(1.16)
3. Maggot infestation	11(11.05)	6(3.49)	5(2.91)	3(1.74)	5(2.91)	3(1.74)
4. Tick infestation	10(5.81)	5(2.91)	5(2.91)	3(1.74)	5(2.91)	2(1.16)
5. Flea infestation	6(3.49)	4(2.33)	2(2.33)	0(0.00)	4(2.33)	2(1.16)
6. Lice infestation	3(1.74)	2(1.16)	1(0.58)	0(0.00)	0(0.00)	3(1.74)
7. Mange	2(1.16)	1(0.58)	1(058)	2(1.16)	0(0.00)	0(0.00)
XII. Parasitic infestation	**42(24.42)**	**24(13.95)**	**18(10.47)**	**13(7.56)**	**17(9.88)**	**12(6.98)**
XIII. Protozal diseases	**1(0.58)**	**1(0.58)**	**0(0.00)**	**0(0.00)**	**1(0.58)**	**0(0.00)**
XIV. Fungal diseases	**3(1.74)**	**2(1.16)**	**1(0.58)**	**0(0.00)**	**2(1.16)**	**1(0.58)**
Total Medicinal cases	**136(79.07)**	**82(47.67)**	**54(31.40)**	**46(26.74)**	**55(31.98)**	**35(20.35)**
1. Endometritis & pyomerta	3(1.74)	0 (0.00)	3 (1.74)	0(0.00)	2(1.16)	1(0.58)
2. Paraphymosis	2(1.16)	2(1.16)	0(0.00)	0(0.00)	1(0.58)	1(0.58)
3. Pregnancy detection	3(1.74)	0(0.00)	3(1.74)	0(0.00)	2(1.16)	1(0.58)
Total Gynecological cases	**8(4.65)**	**2(1.16)**	**6(3.49)**	**0(0.00)**	**5(2.91)**	**3(1.74)**
1. Orthopedic surgery	10(5.81)	6(3.48)	4(2.33)	5(2.91)	2(1.16)	3(1.74)
2. Accidental wound /dog bite	12(6.98)	7(4.07)	5(2.91)	4(2.33)	4(2.33)	4(2.33)
3. Abscess	1(0.58)	1(0.58)	0(0.00)	0(0.00)	1(0.58)	0(0.00)
4. Spaying	3(1.74)	2(1.16)	1(0.58)	0(0.00)	1(0.58)	2(1.16)
5. Castration	2(1.16)	1(0.58)	1(0.58)	0(0.00)	1(0.58)	1(0.58)
Total Surgical cases	**28(16.28)**	**17(9.88)**	**11(6.40)**	**9(5.23)**	**9(5.23)**	**10(5.81)**
Total cases	**172(100)**	**101(58.72)**	**71(41.28)**	**55 (31.98)**	**69(40.12)**	**48(27.91)**

Table 3: Breed-wise distribution of clinical diseases and conditions of dog attended at SA Quadery Teaching Veterinary hospital during October 2016 to January 2017

Clinical Diseases and Conditions	Local Breed	German Shepherd	Labrador	Spitz	Puddle	Samoyed	Cross Breed
1. Loss of appetite	3(1.74)	5(2.91)	1(0.58)	0(0.00)	0(0.00)	1(0.58)	0(0
2. Diarrhea	2(1.16)	0(0.00)	0(0.00)	1(0.58)	0(0.00)	0(0.00)	1(0.58)
3. Gastritis	2(1.16)	2(1.16)	0(0.00)	1(0.58)	1(0.58)	0(0.00)	2(1.16)
4. Dental Disorder	1(0.58)	0(0.00)	1(0.58)	0(0.00)	0(0.00)	0(0.00)	0(0.00)
5. Others digestive problems	3(1.74)	0(0.00)	0(0.00)	2(1.16)	0(0.00)	0(0.00)	1(0.58)
I. Digestive Disorders	**11(6.40)**	**7(4.07)**	**2(1.16)**	**4(2.23)**	**1(0.58)**	**1(0.58)**	**4(2.23)**
1. Pneumonia	2(1.16)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)
2. Bonchitis	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(0.58)

3. Kennel cough	3(1.74)	0(0.00)	0(0.00)	0(0.00)	1(0.58)	0(0.00)	1(0.58)
II .Respiratory disorder	**5(2.91)**	**0(0.00)**	**0(0.00)**	**0(0.00)**	**1(0.58)**	**0(0.00)**	**2(1.16)**
III . Urinary disorders	**0(0.00)**	**0(0.00)**	**1(0.58)**	**0(0.00)**	**0(0.00)**	**0(0.00)**	**0(0.00)**
IV . Lameness	**1(0.58)**	**0(0.00)**	**0(0.00)**	**0(0.00)**	**0(0.00)**	**0(0.00)**	**1(0.58)**
V . Metabolic disorders	**0(0.00)**	**1(0.58)**	**0(0.00)**	**0(0.00)**	**0(0.00)**	**0(0.00)**	**1(0.58)**
1. Dermatits	4(2.33)	4(2.33)	0(0.00)	1(0.58)	0(0.00)	0(0.00)	1(0.58)
2. Aopecia	2(1.16)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(0.58)
VI . Skin disorders	**6(3.49)**	**4(2.33)**	**0(0.00)**	**1(0.58)**	**0(0.00)**	**0(0.00)**	**2(1.16)**
1. Conjunctivites	1(0.58)	0(0.00)	0(0.00)	0(0.00)	2(1.16)	0(0.00)	1(0.58)
2. Cataract	0(0.00)	0(0.00)	0(0.00)	2(1.16)	0(0.00)	0(0.00)	0(0.00)
3. Otitis	0(0.00)	1(0.58)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(0.58)
VII . Eyes and Ears disorders	**1(0.58)**	**1(0.58)**	**0(0.00)**	**2(1.16)**	**2(1.16)**	**0(0.00)**	**2(1.16)**
VIII . Nervous system	**5(2.91)**	**0(0.00)**	**0(0.00)**	**0(0.00)**	**0(0.00)**	**0(0.00)**	**0(0.00)**
IX . Others	**0(0.00)**	**0(0.00)**	**0(0.00)**	**0(0.00)**	**4(2.33)**	**0(0.00)**	**1(0.58)**
1. Canine Distemper	2(1.16)	0(0.00)	1(0.58)	0(0.00)	0(0.00)	0(0.00)	0(0.00)
2. Canine parvovirus	2(1.16)	0(0.00)	2(1.16)	0(0.00)	0(0.00)	0(0.00)	0(0.00)
3. Pox/Herpes virus	0(0.00)	0(0.00)	0(0.00)	1(0.58)	0(0.00)	0(0.00)	0(0.00)
X. Viral diseases	**2(1.16)**	**0(0.00)**	**3(1.74)**	**1(0.58)**	**0(0.00)**	**2(1.16)**	**0(0.00)**
XI. Bacterial diseases	**3(1.74)**	**0(0.00)**	**0(0.00)**	**0(0.00)**	**3(1.74)**	**0(0.00)**	**2(1.16)**
1. Ancyclostomiasis	1(0.58)	1(0.58)	0(0.00)	1(0.58)	0(0.00)	0(0.00)	0(0.00)
2. Unknown internal worms	5(2.91)	0(0.00)	2(1.16)	0(0.00)	0(0.00)	0(0.00)	0(0.00)
3. Maggot infestation	5(2.91)	3(1.74)	1(0.58)	1(0.58)	0(0.00)	1(0.58)	0(0.00)
4. Tick infestation	5(2.91)	2(1.16)	1(0.58)	1(0.58)	0(0.00)	1(0.58)	0(0.00)
5. Flea infestation	4(2.33)	2(1.16)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)
6. Lice infestation	1(0.58)	0(0.00)	0(0.00)	2(1.16)	0(0.00)	0(0.00)	0(0.00)
7. Mange	2(1.16)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)
XII. Parasitic infestation	**23(13.37)**	**8(6.98)**	**4(2.33)**	**5(2.91)**	**0(0.00)**	**2(1.16)**	**0(0.00)**
XIII. Protozal diseases	**1(0.58)**	**0(0.00)**	**0(0.00)**	**0(0.00)**	**0(0.00)**	**0(0.00)**	**0(0.00)**
XIV. Fungal diseases	**1(0.58)**	**1(0.58)**	**0(0.00)**	**1(0.58)**	**0(0.00)**	**0(0.00)**	**0(0.00)**
Total Medicinal cases	**59(34.30)**	**22(12.79)**	**10(5.81)**	**14(8.14)**	**11(6.40)**	**5(2.91)**	**15(8.72)**
1. Endometritis & pyomerta	0(0.00)	0(0.00)	1(0.58)	1(0.58)	0(0. 00)	1(0.00)	0(0.00)
2. Paraphymosis	1(0.58)	1(0.58)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)
3. Pregnancy detection	1(0.58)	0(0.00)	0(0.00)	2(1.16)	0(0.00)	0(0.00)	0(0.00)
Total Gynecological cases	**2(1.16)**	**1(0.58)**	**1(0.58)**	**3(1.74)**	**0(0.00)**	**1(0.58)**	**0(0.00)**
1. Orthopedic surgery	4(2.33)	4(2.33)	1(0.58)	1(0.58)	0(0.00)	0(0.00)	0(0.00)

2. Accidental wound /dog bite	2(1.16)	4(2.33)	2(1.16)	2(1.16)	0(0.00)	0(0.00)	2(1.16)
3. Abscess	0(0.00)	0(0.00)	1(0.58)	0(0.00)	0(0.00)	0(0.00)	0(0.00)
4. Spaying	1(0.58)	0(0.00)	2(1.16)	0(0.00)	0(0.00)	0(0.00)	0(0.00)
5. Castration	0(0.00)	0(0.00)	2(1.16)	0(0.00)	0(0.00)	0(0.00)	0(0.00)
Total Surgical cases	**7(4.07)**	**8(4.65)**	**8(4.65)**	**3(1.74)**	**0(0.00)**	**0(0.00)**	**2(1.16)**
Total cases	**68 (39.53)**	**31 (18.02)**	**19 (11.05)**	**20 (11.63)**	**11 (6.40)**	**6 (3.49)**	**17 (9.88)**

Table 4: Demographic distribution of clinical diseases and conditions of cat attended at SA Quadery Quadery Teaching Veterinary hospital during October 2016 to January 2017.

Clinical Diseases and conditions	Overall Prevalence	Sex		Breed			Age		
		Male	Female	Local breed	Persian	Bengal	<6 months	>7 to 24 months	>24 months
1. Diarrhoea	2(1.50)	0(0.00)	2(1.50)	2(1.50)	0(0.00)	0(0.00)	2(1.50)	0(0.00)	0(0.00)
2. Gastritis	3(2.26)	2(1.50)	1(0.75)	3(2.26)	0(0.00)	0(0.00)	3(2.26)	0(0.00)	0(0.00)
3. Mega colon	2(1.50)	0(0.00)	2(1.50)	0(0.00)	2(1.50)	0(0.00)	2(1.50)	0(0.00)	0(0.00)
4. Others digestive problems	10(7.52)	4(3.01)	6(4.51)	6(4.51)	3(2.26)	1(0.75)	7(5.26)	3(2.26)	0(0.00)
I.Digestive Disorders	**17(12.78)**	**6(4.51)**	**11(8.27)**	**11(8.27)**	**5(3.76)**	**1(0.75)**	**14(10.53)**	**3(2.26)**	**0(0.00)**
1. Pneumonia	5()3.76	1(0.75)	4(3.01)	5(3.76)	0(0.00)	0(0.00)	3(2.26)	0(0.00)	2(1.50)
2. Bonchitis	1(0.75)	0(0.00)	1(0.75)	0(0.00)	0(0.00)	1(0.75)	0(0.00)	1(0.75)	0(0.00)
II. Respiratory disorder	**6(4.51)**	**1(0.75)**	**5(3.76)**	**5(3.76)**	**0(0.00)**	**1(0.75)**	**3(2.26)**	**1(0.75)**	**2(1.50)**
III. Urinary disorders	**1(0.75)**	**1(0.75)**	**0(0.00)**	**1(0.75)**	**0(0.00)**	**0(0.00)**	**0(0.00)**	**0(0.00)**	**1(0.75)**
IV. Lamness	**3(2.26)**	**3(2.26)**	**(0.00)**	**2(1.50)**	**1(0.75)**	**0(0.00)**	**3(2.26)**	**0(0.00)**	**0(0.00)**
V. Metabolic disorders	**2(1.50)**	**2(1.50)**	**0(0.00)**	**1(0.75)**	**1(0.75)**	**0(0.00)**	**1(0.75)**	**1(0.75)**	**0(0.00)**
VI. Posioning	**4(3.01)**	**3(2.26)**	**1(0.75)**	**4(3.01)**	**0(0.00)**	**0(0.00)**	**3(2.26)**	**1(0.75)**	**0(0.00)**
1. Dermatits	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)
2. Alopecia	1(0.75)	0(0.00)	1(0.75)	1(0.75)	0(0.00)	0(0.00)	0(0.00)	1(0.75)	0(0.00)
VII. Skin disorders	**1(0.75)**	**0(0.00)**	**1(0.75)**	**1(0.75)**	**0(0.00)**	**0(0.00)**	**0(0.00)**	**1(0.75)**	**0(0.00)**
1. Conjunctivites	1(0.75	0(0.00)	1(0.75)	1(0.75)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(0.75)
2. Cataract	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)
3. Otitis	1(0.75	1(0.75)	0(0.00)	0(0.00)	1((0.75)	0(0.00)	0(0.00)	1(0.75)	0(0.00)
4. Protrution of eye ball	2(1.50)	2(1.50)	0(0.00)	2(1.50)	0(0.00)	0(0.00)	1(0.75)	1(0.75)	0(0.00)
VIII. Eyes and Ears disorders	**4(3.01)**	**3(2.26)**	**1(0.75)**	**3(2.26)**	**1(0.75)**	**0(0.00)**	**1(0.75)**	**2(1.50)**	**1(0.75)**
IX. Others	**4(3.01)**	**1(0.75)**	**3(2.26)**	**3(2.26)**	**1(0.75)**	**0(0.00)**	**3(2.26)**	**0(0.00)**	**1(0.75)**
X. Bacterial diseases	**4(3.01)**	**1(0.75)**	**3(2.26)**	**3(2.26)**	**1(0.75)**	**0(0.00)**	**3(2.26)**	**0(0.00)**	**1(0.75)**

1. Rabies	1(0.75)	1(0.75)	0(0.00)	1(0.75)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(0.75)
2. Feline calci virus	2(1.50)	2(1.50)	0(0.00)	2(1.50)	0(0.00)	0(0.00)	2(1.50)	0(0.00)	0(0.00)
3. Feline panleukemia virus	4(3.01)	2(1.50)	2(1.50)	4(3.01)	0(0.00)	0(0.00)	4(3.01)	0(0.00)	0(0.00)
4. Pox/Herpes virus	2(1.50)	1(0.75)	1(0.75)	2(1.50)	0(0.00)	0(0.00)	2(1.50)	0(0.00)	0(0.00)
XI. Viral diseases	**9(6.77)**	**6(4.51)**	**3(2.26)**	**9(3.01)**	**0(0.00)**	**0(0.00)**	**8(6.02)**	**0(0.00)**	**1(0.75)**
1. Toxocariasis	4(3.01)	2(1.50)	2(1.50)	4(1.50)	0(0.00)	0(0.00)	2(1.50)	2(1.50)	0(0.00)
2. Unknown Internal worms	12(9.02)	8(6.02)	4(3.01)	8(6.02)	3(2.26)	1(0.75)	10(7.52)	1(0.75)	1(0.75)
3. Myiasis	3(2.26)	2(1.50)	1(0.75)	3(2.26)	0(0.00)	0(0.00)	1(0.75)	1(0.75)	1(0.75)
4. Tick infestation	2(1.50)	2(1.50)	0(0.00)	2(1.50)	0(0.00)	0(0.00)	1(0.75)	1(0.75)	0(0.00)
5. Flea infestation	4(3.01)	1(0.75)	3(2.26)	3(2.26)	1(0.75)	0(0.00)	2(1.50)	2(1.50)	0(0.00)
6. Lice infestation	2(1.50)	1(0.75)	1(0.75)	1(2.26)	1(0.75)	0(0.00)	0(0.00)	2(1.50)	2(1.50)
7. Mange	4(3.01)	3(2.26)	1(0.75)	2(1.50)	1(0.75)	1(0.75)	0(0.00)	2(1.50)	0(0.00)
XII. Parasitic diseases	**31(23.31)**	**19(14.29)**	**12(9.02)**	**23(17.29)**	**6(4.51)**	**2(1.50)**	**16(12.03)**	**11(8.23)**	**4(3.01)**
XIII. Protozal diseases	**2 (1.50)**	**1(0.75)**	**1(0.75)**	**2(1.50)**	**0(0.00)**	**0(0.00)**	**0(0.00)**	**2(1.50)**	**0(0.00)**
XIV. Fungal diseases	**2(1.50)**	**1(0.75)**	**1(0.75)**	**1(0.75)**	**1(0.75)**	**0(0.00)**	**1(0.75)**	**0(0.00)**	**1(0.75)**
XV. Nail trimming	**1(0.75)**	**1(0.75)**	**0(0.00)**	**1(0.75)**	**0(0.00)**	**0(0.00)**	**0(0.00)**	**1(0.75)**	**0(0.00)**
XVI. Nervous system	**2(1.50)**	**1(0.75)**	**1(0.75)**	**2(1.50)**	**0(0.00)**	**0(0.00)**	**1(0.75)**	**0(0.00)**	**1(0.75)**
Total Medicinal cases	**89(66.92)**	**49(36.84)**	**40(30.08)**	**69(51.88)**	**16(12.03)**	**4(3.01)**	**54(40.60)**	**23(17.29)**	**12(9.02)**
Pregnancy detection	2(1.50)	0(0.00)	2(1.50)	2(1.50)	0(0.00)	0(0.00)	0(0.00)	2(1.50)	0(0.00)
Total Gynecological cases	**2(1.50)**	**0(0.00)**	**2(1.50)**	**2(1.50)**	**0(0.00)**	**0(0.00)**	**0(0.00)**	**2(1.50)**	**0(0.00)**
1. Orthopedic Surgery	9(6.77)	6(4.51)	3(2.26)	6(4.51)	2(1.50)	1(0.75)	8(6.02)	1(0.75)	0(0.00)
2. Accidental wound /dog bite	24(18.05)	18(13.53)	6(4.51)	20(15.04)	4(3.01)	0(0.00)	6(4.51)	14(10.53)	4(3.01)
3. Spaying	4(3.01)	0(0.00)	4(3.01)	4(3.01)	0(0.00)	0(0.00)	0(0.00)	4(3.01)	0(0.00)
4. Castration	5(3.76)	5(3.76)	0(0.00)	5(3.76)	0(0.00)	0(0.00)	0(0.00)	4(3.01)	1(0.75)
Total Surgical Cases	**42(31.58)**	**29(21.80)**	**13(9.77)**	**35(26.32)**	**6(4.51)**	**1(0.75)**	**14(10.53)**	**23(17.29)**	**5(3.76)**
Total Cases	**133(100)**	**78(58.65)**	**55(41.35)**	**106(79.70)**	**22(16.54)**	**5(3.76)**	**68(51.13)**	**48(39.09)**	**17(12.78)**

Table 5: Demographic distribution of clinical diseases and conditions of pet rabbit attended at SAQTVH during October 2016 to January 2017

Clinical Diseases and conditions	Overall prevalence	Sex		Age		
		Male rabbit	Female rabbit	Below 6 month	6 month to 1 year	1 year and above
Mange	10(12.05)	25(30.12)	5(6.02)	15(18.07)	7(8.43)	8(9.64)
Traumatic injuries and fracture	14(16.87)	10(12.05)	4(4.82)	6(7.23)	6(7.23)	2((2.41)
Respiratory tract infection	3(3.61)	3(3.61)	0(0.00)	3(3.61)	0(0.00)	0(0.00)
Conjunctivitis	4(4.82)	2(2.41)	2(2.41)	2(2.41)	1(1.20)	1(1.20)
Nutritional deficiency	8(9.64)	6(7.23)	2(2.41)	4(4.82)	3(3.61)	1(1.20)
Coccidiosis	5(6.02)	4(4.82)	1(1.20)	2(2.41)	1(1.20)	2(2.41)
Alopecia	3(3.61)	2(2.41)	1(1.20)	1(2.00)	2(2.41)	0(0.00)
Parasitic infestation	6(7.23)	4(4.82)	2(2.41)	3(3.61)	2(2.41)	1(1.20)
Abscess	1(1.20)	1(1.20)	0(0.00)	0(0.00)	1(1.20)	0(0.00)
Others	9(10.84)	7(8.43)	2(2.41)	5(6.02)	2(2.41)	2(2.41)
Total	**83(100)**	**64(77.11)**	**19(22.88)**	**41(49.40)**	**25(30.12)**	**17(20.48)**

CONCLUSIONS

Prevalence of clinical diseases and disorders of pet animals were investigated at the S.A. Quaderi Teaching Veterinary Hospital, Chittagong Veterinary and Animal Sciences University (CVASU), Chittagong, Bangladesh. The study was performed to assess the present scenario of clinical diseases and disorders of pet animals at study area. The parasitic diseases were found in highest percentage among the all clinical diseases and conditions. Proper prevention and therapeutic management should be performed to reduce the burden of parasitic diseases in pet animals. Vaccination and hygienic management also should be taken to reduce the load of infectious diseases. An extensive epidemiology, laboratory and molecular studies are also needed for the confirmation of etiology of diseases and the appropriate strategies of prevention and control measures of diseases and conditions of pet animals in mentioned study area.

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Present Status of Commercial Pigeon Farming in Selected Areas of Bangladesh

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Abstract

Objectives of the study were to investigate the status, problems and prospects of pigeon farming in Bangladesh. The study was conducted in 10 Districts throughout the country from May 2016 to April 2017. Data were collected through interviewing the pigeon farmers with a structured questionnaire. Focus group discussions were also carried out. Out of 100 farms studied, 35.0% were owned by students, 22.0% by service holder and 19.0% by businessman. Most of the farmers (59.0%) were young and aged between 20-29 years. Farmers with bachelors degree was highest (45.0%), followed by HSC (33.0%) and <SSC (10.0%). Mixed farm accounted for 70.0%, followed by fancy (23.0%) and squab production (7.0%). Highest number of farmers reared pigeons in cage system (75.0%) than floor plus wooden box (23.0%). Most of the farmers (68.0%) had <50 pairs of pigeons, followed by 50-100 (25.0%) pairs. Although 97.0% farmers experienced disease-related problems, however, these were less frequent (88.0%) in most farms, but frequent in 9.0% farms. Occurrence of diseases was more in winter (49.0%) than rainy season (29.0%) or summer (21.0%). Disease prevalence was significantly higher (<0.001) in squabs (75.6%) than adults (59.0%). However, only 26.0% farmer used drugs/additives in the feed and water regularly. Among the different diseases of pigeon, New Castle Disease (18.0%) was most endemic followed by salmonellosis (14.0%), pigeon pox (8.0%), mycoplasmosis (5.0%), crop infections (4.0%), coccidiosis (3.0%), diarrhea (2.0%), colibacillosis (2.0%), conjunctivitis (2.0%), IBD (1.0%) and bacillary diarrhea (1.0%). The overall income from pigeon farming greatly correlated with farm population size and highest profit (BDT 60,000.0) was found only in farms having more than 1,000 birds (2.0%). However, the lowest income was BDT 5,000 and most of the pigeon farmers (69.0%) had overall annual profit between BDT 5,000-10,000. On the other hand, the major constraints of pigeon farming were market instability, non-professionalism, lack of proper knowledge & training, outbreak of endemic diseases, lack of vaccines and inadequate bio-security practices. Therefore, proper training, bio-security & health management, market stability etc could make pigeon farming sustainable in Bangladesh. In conclusions, commercial pigeon farming has enormous potentiality and could be a good venture for income generation and employment opportunity. As a valuable source of meat and fancies, pigeon farming should be encouraged and promoted in Bangladesh.

Keywords: Pigeons, Status, Problems, Prospects, Bangladesh

Introduction

Worldwide pigeon are considered as the symbol of peace. Pigeon keeping is the art. They are related to human since ancient time (BC 3,000-5,000) and people have practiced pigeon keeping in almost every part of the world (Levi, 1977). Probably pigeon is the first bird species to have been reared by humans (Johnston and Janiga, 1995). In that time, mankind has substantially altered the morphology and the behavior of the domesticated descendants of the wild rock pigeon (*Columba livia*) to suit its need for food, aesthetic satisfaction and entertainment. Almost all types of people who have facilities, love to raise some pigeon in their home. They live side by side with human as a source of food, hobby and experimental purposes (Sari *et al*., 2008). People who breed pigeons are commonly referred to as pigeon fanciers (Levi, 1977).

Pigeon farming is very interesting and profitable worldwide. It requires less labor and low investment. Even one can raise and take care of the pigeon during his leisure time. The meat of squab (baby pigeon) is tender, tasty, nutritious and restorative. Squabs also have huge demand and price in the market. Squabs have never flown and have never usually eaten anything except 'pigeon milk', which is formed in the crops of both parents

during the incubation period. Squabs are ready to fly and leave their nest at about 26-30 days of age. At this age they weigh about 500 g and are ready to market for the table (Bolla, 2007). Therefore, pigeon farming became an easy source of income generation and entertainment. Raising pigeons using modern methods is very profitable than traditional way.

Keeping pigeon is almost common in the villages of Bangladesh. In this country people usually keep different local breeds for hobby and meat purpose. But now a day demand for different types of fancy breeds increased not only in Dhaka or Chittagong, but also other parts of the country. Because pigeons are now raised for racing and show purposes in addition to meat and hobby, therefore, to fulfill this demand pigeon fanciers and businessmen started raising different types of fancy breeds, varieties, and even species of pigeon. These birds are expensive to highly expensive which are sold between 10,000 (ten thousand) taka and 1,000,000 (ten lac) taka a pair. Recently, a number of commercial pigeon farms (small to big) have been established all over the country. Currently, there are more than 2,000 pigeon breeders in Dhaka city with more than 20,000 scattered across the country, especially in Chittagong, Rangpur, Dinajpur and Barisal.

Although the number of pigeon breeders (fanciers) and farms are increased, however, there is a lack of knowledge and skills on husbandry, breeding and disease management of these highly expensive birds. As raising expensive fancy breeds are not as easy as local breeds, therefore, pigeon fanciers are currently facing various problems including health problems. Squabs are mostly affected with different types of bacterial, viral and fungal infections as well as parasitic infestations which causes retarded growth, low egg production and susceptibility to other infections. This ultimately results in financial loss in this new enterprise.

Although, commercial pigeon rearing has immense potentialities as a new dimension in poultry farming, however, till now the distribution, status, disease management, problems and prospects of commercial pigeon farming in Bangladesh has not explored and evaluated. Keeping this in mind the present piece of work has been undertaken to obtain thorough and detailed information on the present status of different breeds of pigeon and their disease management in some selected areas of Bangladesh.

Reports on different aspects of pigeons (Abulude *et al.,* 2006; Tume, 2007; Pomianowski *et al.,* 2009; Ibrahim and Sani, 2010) including diseases of pigeon (Wehr and Hwang, 1964; Dranzoa, 1999; Marques *et al*., 2007; Danapat *et al.*, 2012) in different countries have been published. A review of available literatures revealed only one report on pigeon farming and several others on pigeon diseases in Bangladesh (Begum and Shaikh, 1987; Nasrin, 2004; Begum *et al.,* 2008; Islam *et al.*, 2009; Asaduzzaman *et al.,* 2009; Musa *et al.*, 2011). Using a pre-tested interview schedule Asaduzzaman *et al.* (2009) conducted a survey on status, problems and prospects of pigeon farming in rural Bangladesh. Their results revealed housing cost, height of house, duration of house, quantity of feed supplied to each pigeon/day, male female ratio, marketing age, weight, price of squab, number of squab/pair of pigeon, production of squab/year/pair of pigeon, price of each pigeon and mortality of pigeon were Tk. 113.33, 3.50 m, 5 years, 34.50 g, 1.07, 26.66 days, 258 g, Tk. 51, 1.67, 19.53, Tk. 113.33 and 6.67%, respectively. Most of the pigeon farmers raised pigeon in small scale scavenging system with supplementary feeding. They had no idea on breeds and varieties and prevention of diseases. Begum and Shaikh (1987) and Nasrin (2004) recorded parasites of pigeons in Bangladesh. Islam *et al.* (2009) found that 28.60% and 85.70% pigeons, respectively, were affected with bacterial infections and parasitic infestations in Gaibandha district of Bangladesh. Their results indicated that pigeon raised in scavenging or free-range system were more susceptible to helminth parasites. Musa *et al.* (2011) found one species of nematode and five species of ectoparasites in pigeons randomly purchased from the local market. All these reports were on small scale free range or home raised pigeons. Review of available literature revealed no reports on distribution, status, breeding, disease management, problems and prospects of different pigeon breeds and commercial pigeon farming in Bangladesh which is very much important in respect to present situation of commercial pigeon farming in Bangladesh.

The present investigation is designed with the following specific objectives:

- To investigate the status, problems including disease-related problems and prospects of commercial pigeon farming in some selected areas of Bangladesh

Materials & Methods

Study Area & Period

This study was conducted during the period from May 2016 to April 2017. Data were collected from 100 commercial pigeon farms distributed in major regions of Bangladesh including Barishal, Chittagong, Comilla, Dhaka, Khulna, Moulovibazar, Natore, Pabna, Rajshahi and Sylhet according to the farm list collected from pigeon breeders. Number of farm(s) in each district where study was carried out is depicted in the Figure 1. Bangladesh is vulnerable due to its position in the globe (Southeast Asian country). The climate is tropical; mild winter (October to March); hot, humid summer (March to June); humid, warm rainy monsoon (June to October). The country's total land area is about 147,570 km^2 and geographic position is Latitude: 20°45' to 26°40'N, Longitude: 88°05' to 92°40'E. The average annual rainfall varies from a maximum of 5,690 mm in the northeast of the country to minimum of 1,110 mm in the west. The day temperature ranges from 7 to 12 degree centigrade in the cool months and in the other months it varies between 23 to 30°C.

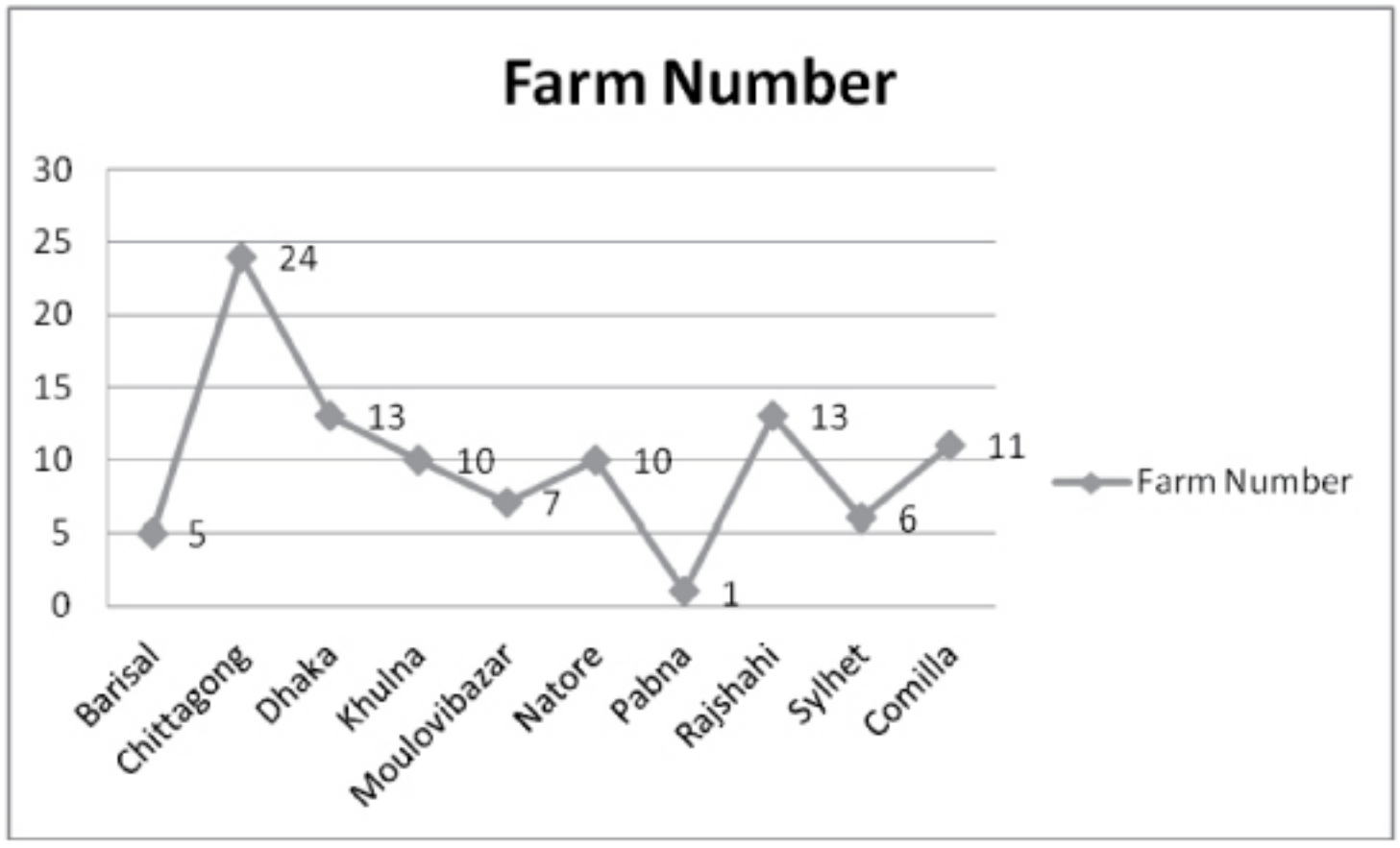

Figure 1: Number of commercial pigeon farms in selected districts of Bangladesh

Data Collection

The samples of this study were commercial pigeon farmers (small, medium and large) including, successful farmers, unsuccessful farmers those have already closed their farms, new farmers and interested peoples who want to start commercial pigeon farming. A structured questionnaire schedule was prepared for interviewing the available commercial pigeon farmers in the selected areas of Bangladesh. This questionnaire was first piloted on a very small group of farmers and then the questions had been restructured in accordance with their responses. Different pigeon farms was visited and information on the number of breeds, breeding methods, frequency of egg laying, mortality and diseases of both squabs and adult pigeons was screened and recorded. Focus group discussions were also is carried out with both successful and unsuccessful farmers whenever necessary. High resolution digital camera (Panasonic DMC FZ50, Cannon SX60HS or Cannon EOS 7D) was used to take photographs and video clips of different breeds, eggs, squabs, disease symptoms, external condition of diseased and infected pigeons, feeds etc.

Statistical Analysis:

The data generated from this experiment were entered in Microsoft Excel (2007) worksheet, organized and processed for further analysis. Analysis was performed with the help of Statistical Packages for Social Sciences, version 20.0 for windows (SPSS, Inc., Chicago, IL, USA). Descriptive statistics were used to describe the basic features of the data in the present study.

Results and Discussions

To the best of our knowledge, there are no studies available on the present scenario, prospects and major constraints of commercial pigeon farming in Bangladesh and this one might be first ever study in this regard. The study was carried out in 100 commercial pigeon farms and among these, 98 farms (98%) were operated by male and only 2 (2%) were operated by female. Pigeon farming has not been well established and industrialized like other poultry farming. Although, pigeon farming in Bangladesh was started in 1996, it remained static for more than a decade (1996-2008) since its inception, thereafter, gradually increased till 2011 (highest; 27.0% in 2011) and gradually declined thereafter (Figure 2). The reasons might be outbreak of epidemics, faulty management systems, higher price of feeds, the higher incidence of different infectious diseases, and lack of veterinary care.

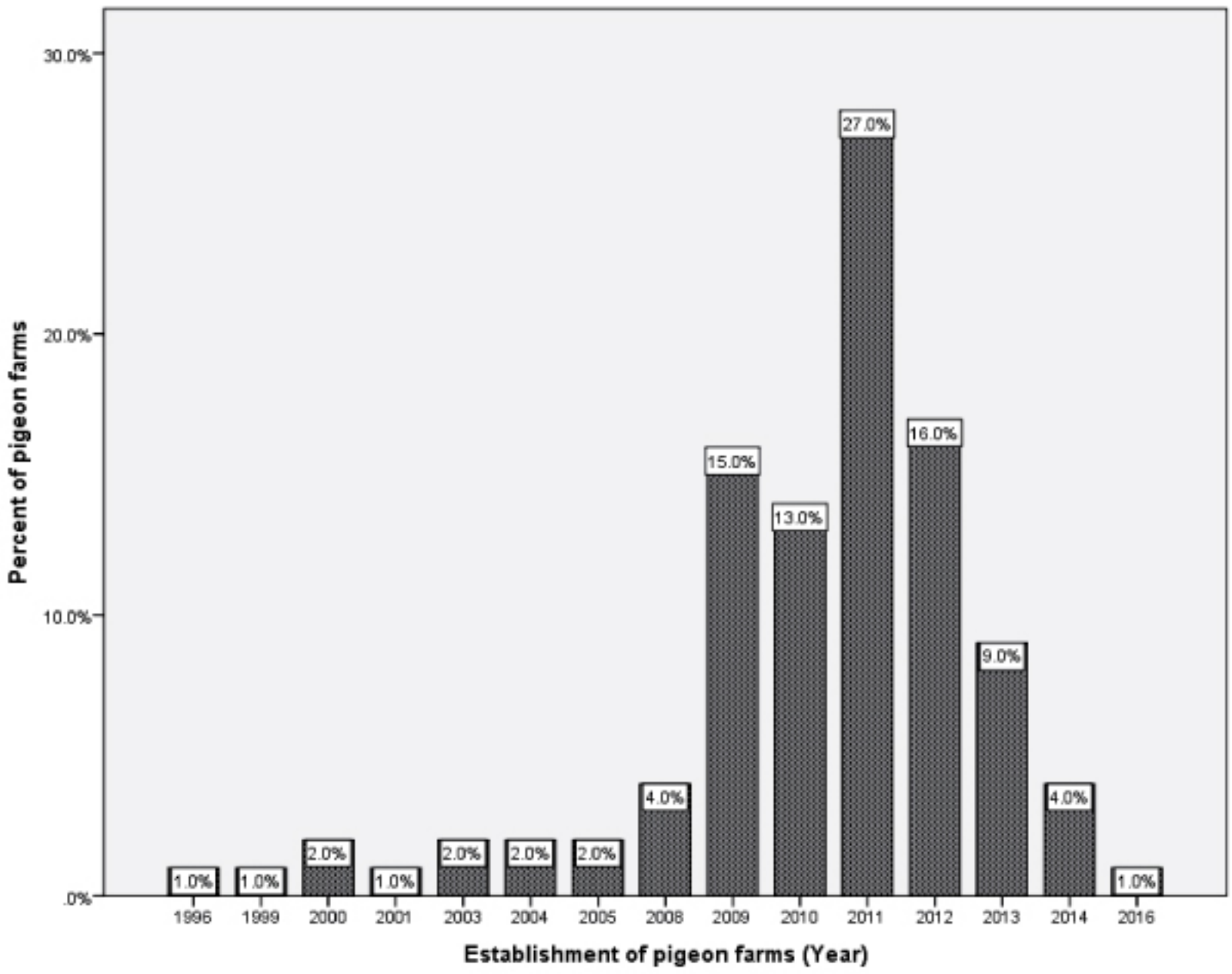

Figure 2: Establishment of pigeon farms in Bangladesh during 1996 to 2016 (n=100)

Detailed scenario of pigeon farming in Bangladesh has been depicted in table 1. In spite of having tremendous economic potential pigeon farming in Bangladesh is not yet well organized and commercialized like other poultry, and thus, there is lack of research information in particular to their present scenario, prospects and major constraints of pigeon farming in Bangladesh. The majority of the farms (59.0%) in this study were operated by the young people (20-29 years old). The pigeon farmers of this study had mixed occupation like business (19.0%), service (22.0%), students (35.0%) and others (24.0%), and of them, 33.0% and 45.0% had HSC and bachelor degrees, respectively. The farmers had potential role in spreading pigeon farming especially to motivate (62.0%) others to establishing new pigeon farms. Most of the farmers (70.0%; 70/100) rear pigeon as mixed farming (squab and fancy pigeon production) and interested in pigeon farming as their special intend to doing so; hobby (84.0%). In our current study, most of the pigeon farmers had less than 50 pigeons in their farms, and they had different housing systems (farm house; 26.0%, own flat; 9.0%, verandah; 25.0% and others; 40.0%). In this study, a good number of the farmers (62.0%; 62/100) had significant role in establishing new pigeon farms as like as other poultry industry by motivating the jobless youth and distressed women, providing training and technical support. The study also revealed that 75.0% farm holders rear pigeon in cage system, and they fed their birds with both crumble (42.0%) and grain (58.0%) feeds.

Table 1: Status of commercial pigeon faming in Bangladesh (n=100)

SL. No.	Criteria	Groups	No (%)
1.	Age of the farmer	<20	9 (9.0)
		20-29	59 (59.0)
		30-39	25 (25.0)
		40-49	4 (4.0)
		≥50	3 (3.0)
2.	Occupation of the farmer	Business	19 (19.0)
		Service holders	22 (22.0)
		Student	35 (35.0)
		Others	24 (24.0)
3.	Educational background	Bellow SSC	9 (9.0)
		SSC	10 (10.0)
		HSC	33.0 (33.0)
		Bachelor	45 (45.0)
		Masters	3 (3.0)
4.	Role in spreading pigeon farming	Provide training	0 (0.0)
		Motivate to establish farms	62 (62.0)
		Technical support	6 (6.0)
		Financial support	4 (4.0)
		Consultancy	5 (5.0)
		None	14 (14.0)
		Others	9 (9.0)
5.	Purpose of farming	Squab production	7 (7.0)
		Fancy pigeon production	23 (23.0)
		Racing pigeon production	0 (0.0)
		Mixed	70 (70.0)
6.	Why interested in pigeon farming	Business	4 (4.0)
		Inspired by neighbors	0 (0.0)
		Inspired by friends	12 (12.0)
		Hobby	84 (84.0)
7.	Farm size	<50	68 (68.0)
		50-100	25 (25.0)
		100-200	0 (0.0)
		200-500	3 (3.0)
		500-1000	2 (2.0)
		>1000	2 (2.0)
8.	Type of housing	Farm house	26 (26.0)
		Inside own flat	9 (9.0)
		Verandah	25 (25.0)
		Others	40 (40.0)
9.	Role in pigeon farming	Provide training	4 (4.0)
		Motivate to establish new farms	62 (62.0)
		Provide technical support	6 (6.0)
		Provide financial support	4 (4.0)
		Others	24 (24.0)
10.	Rearing system	Cage	75 (75.0)
		Floor + wooden box	23 (23.0)
		others	2 (2.0)
11.	Feed types	Crumble	42 (42.0)
		Grain	58 (58.0)

Several farm and management-level factors described in the data analysis section were considered in the univariable logistic regression analysis for the presence of different infectious diseases in the studied pigeon farms. Among those factors, farm type, rearing system, age of birds, use of feed additives/drugs, veterinary consultation, vaccination and bio-security were found to be significantly ($p < 0.05$) associated with the occurrence different infectious diseases in pigeon. On the other hand, size of farm, type of house and feeds did not have significant effect ($p > 0.05$) on the occurrence of pigeon diseases (Table 2).

Table 2: Univariable logistic regression analysis of the association of infectious diseases of pigeon with different risk factors

Variable	No.	No. Positive	Prevalence (%)	*p*
Type of farm				
Squab farm	7	6	86.0[a]	<0.001
Fancy pigeon farm	23	15	65.2[b]	
Mixed farm	70	56	80.0[a]	
Size of farm				
<50	68	44	65.0[a]	<0.258
50-100	25	16	64.0[a]	
200-500	3	2	67.0[a]	
500-1000	2	1	50.0[b]	
>1000	2	1	50.0[b]	
Type of house				
Farm house	26	18	69.2[a]	<0.583
Inside own flat	9	4	44.4[b]	
Verandah	25	13	52.0[ab]	
Others	40	24	60.0[a]	
Rearing system				
Case	75	51	68.3[a]	<0.028
Floor + wooden box	23	17	74.0[a]	
Others	2	1	50.0[b]	
Type of feeds				
Crumble	42	33	78.6[a]	<0.358
Grain	58	41	70.7[a]	
Age of infection				
Squab	78	59	75.6[a]	<0.001
Adult	22	13	59.0[b]	
Use of drugs or feed additives				
Yes	26	18	69.2[a]	<0.071
No	74	63	85.1[b]	
Veterinary consultation				
Regularly	7	3	43.0[a]	<0.003
At interval	10	6	60.0[b]	
When needed	50	50	100.0[c]	
Never	33	27	82.0[d]	
Vaccination				
Yes	70	24	34.2[a]	<0.035
No	30	19	63.3[b]	
Biosecurity				
Strictly followed	3	0	0.0[a]	<0.024
Normally followed	31	17	54.8[b]	
Never followed	66	48	72.7[c]	

Unlike other poultry, pigeon is more resistant to different infectious disease. However, the findings of the current study revealed that a good number of pigeon farms have had experience of different infectious disease prevalence. Data on disease management practices in different pigeon farms in selected areas of Bangladesh is depicted in Table 3. Therefore, pigeon diseases became one of the major constraints for profitable commercial pigeon farming in Bangladesh along with poor disease management practices, lack of bio-security, lack of veterinary service and environmental factors. Very narrow space, lack of flying, poor ventilation, lack of cleanliness is the main source of several diseases (Echols, 2010). Although most of the farmers (97%) experienced diseases in their farms at some time, but diseases were less frequent in most of the farms (88%). Disease occurrence was higher in winter (49%) than other seasons and squabs (76%) were mostly affected than the adult pigeons (24%).

Table 3: Disease management practices in selected commercial pigeon farms (n=100)

Criteria	Groups	No (%)
Experience diseases in the farm	Yes	97 (97%)
	No	3 (3%)
Occurrence of diseases	Summer	21 (21%)
	Rainy season	29 (29%)
	Winter	49 (49%)
	Spring	1 (1%)
Frequency of diseases	Frequent	9 (9%)
	Less frequent	88 (88%)
	Rare	3 (3%)
Drugs/additives used in feed and water	Yes	26 (26%)
	No	74 (74%)

Among the infectious diseases, viral diseases were more prevalent than bacterial and parasitic infections (Figure 3), and most of them occurred as mixed infections. Within the mixed infections, New Castle Disease (NCD) plus pigeon pox were the most prevalent disease episodes occurring in 30.0% of the studied farms, followed by NCD plus salmonellosis (10.0%). However in this work, NCD was found as the most prevalent infectious disease affecting 18.0% of the pigeon farms alone, and other infectious diseases were salmonellosis (14.0%), Pox (8.0%), mycoplasmosis (5.0%), crop infections (4.0%), coccidiosis (3.0%), diarrhea (2.0%), colibacillosis (2.0%), conjunctivitis (2.0%), IBD (1.0%) and bacillary diarrhea (1.0%). In their study, Begum and Shaikh (1987) and Nasrin (2004) recorded parasites of pigeons in Bangladesh. Islam *et al.* (2009) found that 28.60% and 85.70% pigeons, respectively, were affected with bacterial infections and parasitic infestations in Gaibandha district of Bangladesh. Their results indicated that pigeon raised in scavenging or free-range system were more susceptible to helminth parasites. Musa *et al.* (2011) found one species of nematode and five species of Ectoparasitic in pigeons randomly purchased from the local market. All these reports were on small scale free range or home raised pigeons, but not in any commercial pigeon farming system.

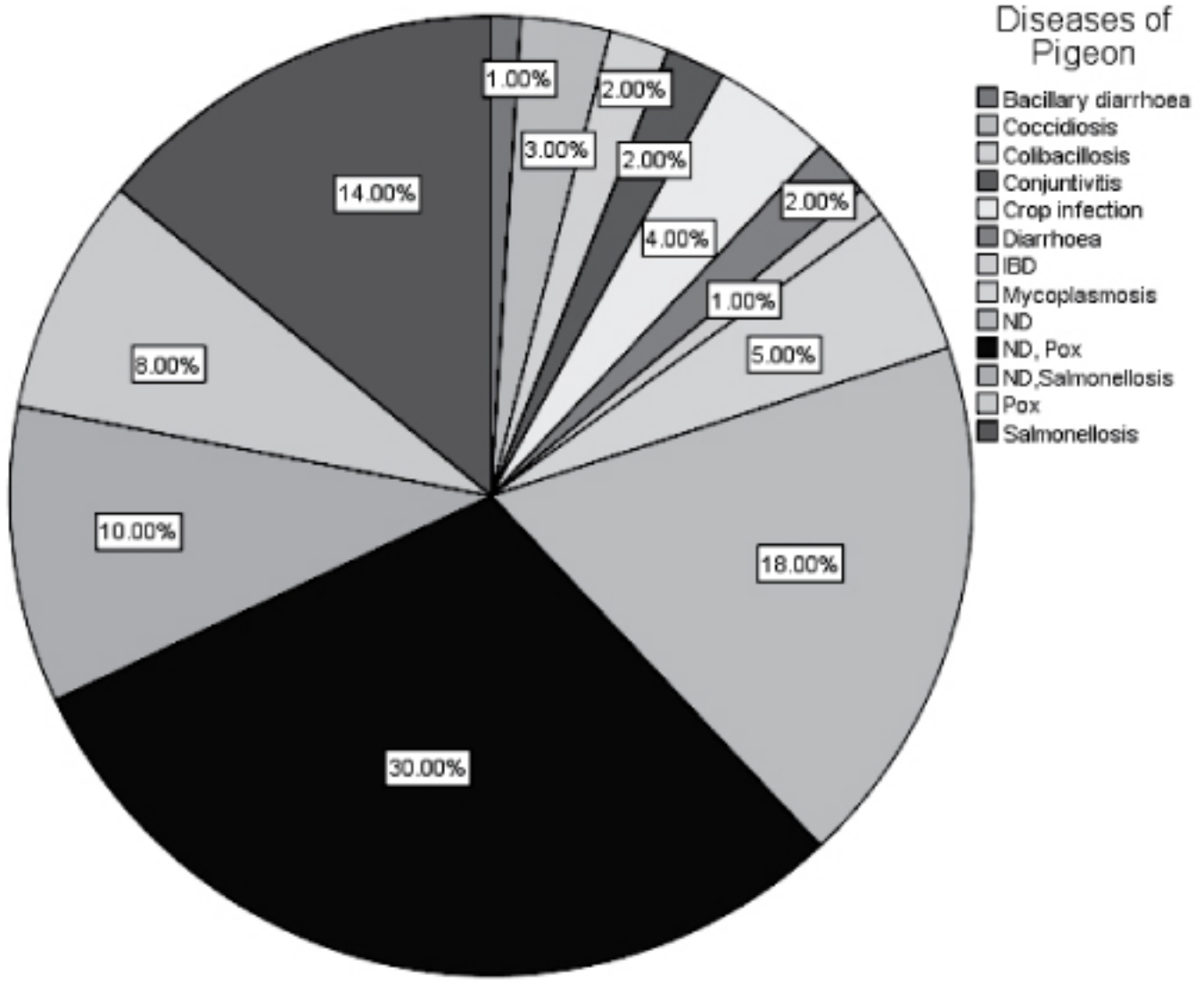

Figure 3: Occurrence of different infectious diseases and disease conditions in pigeons of Bangladesh

Economics of commercial pigeon farming in selected areas of Bangladesh is depicted in Table 4. Economic consideration is a big issue for pigeon farming in Bangladesh. Most of the farmers (88%) had an initial capital between 7,000 taka and 50,000 taka. Although farmers had to spent a huge amount of money to operate pigeon farms like permanent costs (housing costs) and running costs (feeds, medicines, labors etc), however, most of the farmers (68%) earned a net profit of 5,000-10,000 taka per month and other (32%) got net profit of 10,001-60,000 taka per month. In his study Kabir (2013) found a profit of taka 2,000 from each pair of pigeon in commercial pigeon farm. Although not in commercial enterprise, Asaduzzaman *et al.* (2009) conducted a survey on status, problems and prospects of pigeon farming using a pre-tested interview schedule in rural small scale free range or home raised pigeons. Their results revealed housing cost, height of house, duration of house, quantity of feed supplied to each pigeon/day, male female ratio, marketing age, weight, price of squab, number of squab/pair of pigeon, production of squab/year/pair of pigeon, price of each pigeon and mortality of pigeon were Tk. 113.33, 3.50 m, 5 years, 34.50 g, 1.07, 26.66 days, 258 g, Tk. 51, 1.67, 19.53, Tk. 113.33 and 6.67%, respectively.

Table 4: Economics of commercial pigeon farming in selected farms in Bangladesh (n=100)

Criteria	**Groups (taka)**	**No (%)**
Initial capital	7,000-50,000	88%
	50,001-1,000,000	12%
Current capital	5,000-50,000	71%
	50,001-7,000,000	29%
Permanent building cost	5,000-40,000	76%
	40,001-2,000,000	24%
Feed cost/month	1,500-8,000	67%
	8,001-75,000	33%
Worker cost/month	No cost	78%
	2,000-40,000	22%
Medicine cost/month	150-500	79%

Criteria	Groups (taka)	No (%)
	501-15,000	21%
Selling of squab/month	5,000-10,000	52%
	10,001-40,000	48%
Selling of adult/month	2,000-10,000	64%
	10,001-170,000	36%
Net profit/month	5,000-10,000	68%
	10,001-60,000	32%

To show the correlation of overall income from pigeon farming with farm population size Box and Whiskers plots analysis was done. The Box plot revealed that most of the pigeon farmers (93.0%) had overall profit less than BDT 20000. The findings of the present study suggests that overall income from pigeon farming greatly correlated with farm population size and highest profit (BDT 60,000) was found only in farms having more than 1000 birds (2.0%). However, the lowest income was BDT 5000 and most of the pigeon farmers (69.0%) had overall annual profit between BDT 5000-10000. The mean annual income from the pigeon farms was BDT 11600.

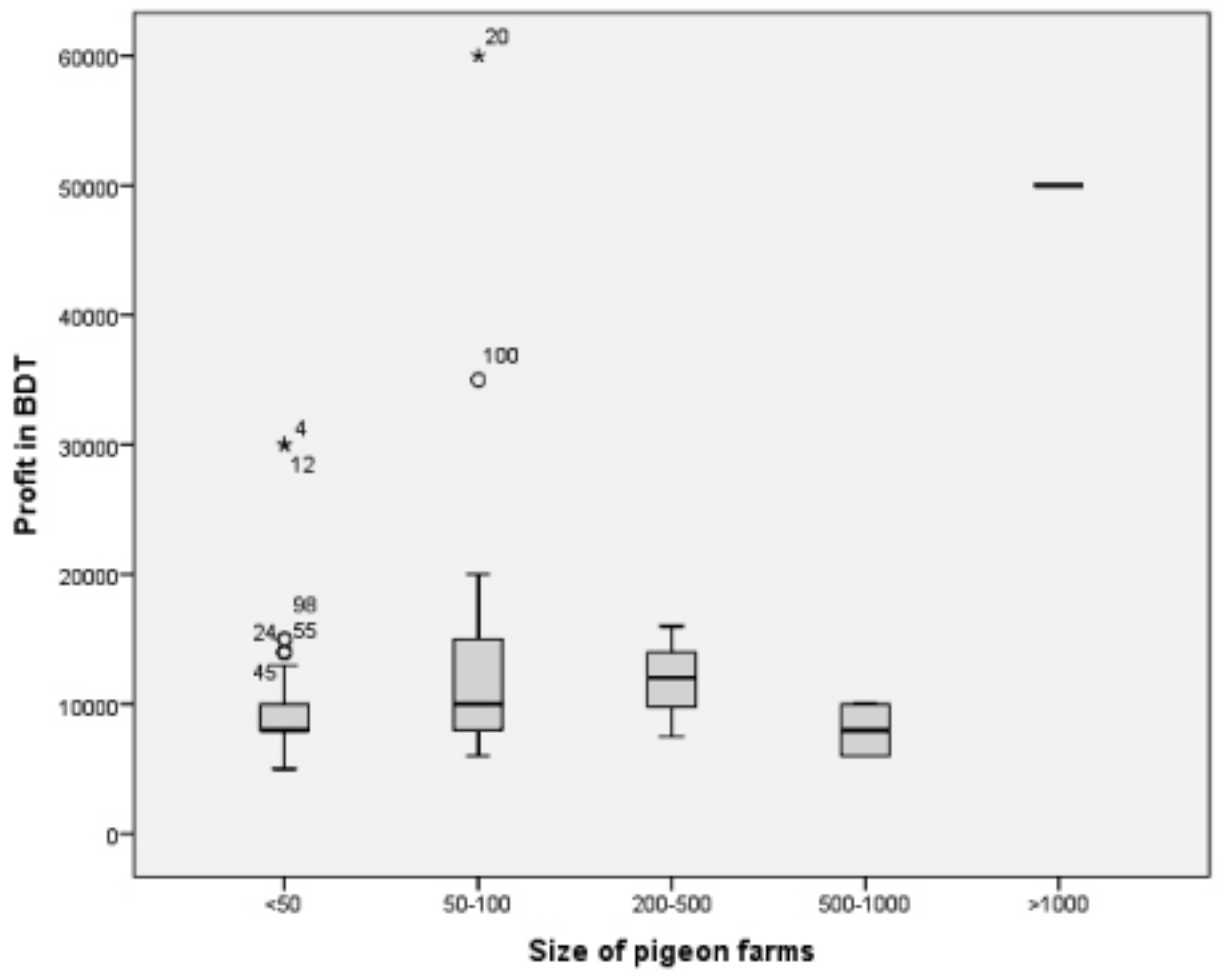

Figure 3: Box and Whiskers plots showing overall profit from pigeon farms (n=100) according to population size. The vertical axis showing profit (range; BDT 0 to 60000) per farm per year and horizontal axis representing to pigeon population per farm (range; <50 to > 1000 birds/farm). Whiskers indicate the minimum and maximum values, the upper and lower side of the box represent 75th and 25th percentile. Median values are given in the boxes.

Conclusion

Recognizing the enormous potentiality of commercial pigeon farming, both fancy pigeon as well as squab production to supplement chickens in providing gainful employment, supplementary income and as a valuable source of meat, pigeon farming should be encouraged and promoted in Bangladesh. However, the present study has explored the real status and major constraints of commercial pigeon farming in selected areas of the country which brought about many challenges to the researchers, academicians and practicing veterinarians to adopt all the strategies to make pigeon farming economically and commercially viable in near future in Bangladesh.

Acknowledgements

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Fluid Therapy in Small Animal Practice

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Abstract

Fluid therapy is an essential clinical approach in pet animal medicine. Pet animal patients are given fluids for many reasons, and the number of available fluids is growing day by day. Fluids are administered for different clinical conditions. The objective and limitations of fluid therapy, and how fluids are chosen is a key competency for small animal veterinary practice. This secondary data based review paper reviews some of the reasons fluid therapy may be prescribed for a patient, how to administer and monitor fluid therapy, and the fluid types available in general. Body water which is up to 60% of body weight is generally distributed into compartments. According to the concentration of ions and molecules in the lost fluids, dehydration is classified into hypertone, hypotone and isotone. Fluid selection is dictated by the patient's needs, including volume, rate, fluid composition required, and location the fluid is needed as for example interstitial versus intravascular. Therapy must be individualized, tailored to each patient, and constantly re-evaluated and reformulated according to changes in status. The quantity of fluids necessary for rehydration of the patient is calculated on the basis of clinical findings. Over hydration risk has to be considered more seriously in cats than dogs. Intravenous, subcutaneous, oral and intraperitoneal infusions are feasible.

Key words: Fluid therapy, small animal

Introduction

The Fluid therapy application whether it as a sole therapy or more commonly in combination with other therapies forms a vital component of veterinary medicine being used managed fluid therapy plan is increasingly being recognized as a major contributing factor to the successful outcome in the treatment of many critical veterinary conditions.

These guidelines will provide practical recommendations for fluid choice, rate, and route of administration. They are organized by general considerations, followed by specific guidelines for perianesthetic fluid therapy and for treatment of patients with alterations in body fluid volume, changes in body fluid content, and abnormal distribution of fluid within the body.

Dehydration and hypovolaemia are the general factors when assessing fluid therapy patients and it is worth clarifying what they actually mean. During dehydration sick animal has fluid depletion across multiple compartments although the predominant compartment affected in terms of actual volume lost is the interstitial compartment with variable effects on the intravascular and intracellular compartments. Hypovolaemia refers exclusively to a reduced blood volume and can result from either a direct loss from the vascular compartment such as haemorrhage or as part of a more generalized dehydration process. Whichever the cause of hypovolaemia, primary or secondary, the consequences to the animal are very similar namely reduced delivery of oxygen to the body cells (Wolfsheimer, 1989).

Therapy must be individualized and tailored to each patient and constantly re-evaluated and reformulated according to changes in status. Fluid selection is dictated by the patient's needs, including volume, rate, and fluid composition required, as well as location the fluid is needed (interstitial versus intravascular). Factors to consider include the following: a. Acute versus chronic conditions b. Patient pathology (e.g., acid-base balance, oncotic pressure, electrolyte abnormalities) c. Comorbid conditions.

A variety of conditions can be effectively managed using three types of fluids: a balanced isotonic electrolyte (e.g., a crystalloid such as lactated Ringer's solution [LRS]); a hypotonic solution (e.g., a crystalloid such as 5% dextrose in water [D5W]); and a synthetic colloid (e.g., a hydroxyethyl starch such as hetastarch or tetrastarch).

Normal body fluid distribution

An adult animal's body weight is composed of about 60% water, which is distributed throughout the intracellular and extracellular compartments. Accordingly Total Body Water (TBW) is calculated as approximately 60% of the body weight in kilograms (where 1 L H20 weighs 1.0 kg). Approximately two thirds of TBW is intracellular fluid (ICF) and one third is extracellular fluid (ECF). Three quarters of the ECF is interstitial fluid, and the remaining one quarter is intravascular fluid. Fluid administered intravenously is distributed between the intravascular and extravascular spaces in fractions determined by the compartments' protein and sodium contents.

The intracellular compartment consists of the largest volume of fluid, about two-thirds of total body water (approximately 40% of body weight). The extracellular space, which constitutes about one-third of the total body water, contains the fluid that is not in the cells. It is divided into three sub-compartments—interstitial, intravascular, and transcellular.

The interstitial compartment contains three-quarters of all the fluid in the extracellular space. The intravascular compartment contains the fluid, mostly plasma, that is within the blood vessels. The fluid in the transcellular compartment is produced by specialized cells responsible for cerebrospinal fluid, gastrointestinal fluid, bile, glandular secretions, respiratory sections, and synovial fluids.

The intracellular and extracellular compartments are separated by specialized membranes that are semipermeable to allow water to equilibrate across the membrane according to the osmotic-pressure gradient. Dehydrated patients have a water loss in the extravascular space, and when fluids are intravenously administered, they are redistributed into the other compartments until all the solutes are in equilibrium again, thus correcting the water loss in the extravascular space.

Objectives of Fluid Therapy

Fluid therapy in clinical medicine is used to fulfill the following objectives: (1) to replace dehydration deficits, (2) to maintain normal hydration, (3) to replace essential electrolytes and nutrients, and (4) to serve as a vehicle for the infusions of certain intravenous medications. Except for the urgency of treatment, the same objectives apply in the critically ill animal. The methods for providing fluids often influence the eventual outcome of the case. The clinician and staff, therefore, should familiarize themselves with the pathophysiology of the diseases they are treating and how these conditions relate to the various types of fluids that are available for general use.

General Principles and Patient Assessment

The assessment of patient history, chief complaint, and physical exam findings will determine the need for additional testing and fluid therapy. Assess for the following three types of fluid disturbances: 1. Changes in volume (e.g., dehydration, blood loss) 2. Changes in content (e.g., hyperkalemia) 3. Changes in distribution (e.g., pleural effusion),the initial assessment includes evaluation of hydration, tissue perfusion, and fluid volume/loss. Items of particular importance in evaluating the need for fluids are:pulse rate and quality, packed cell volume, capillary refill time, total protein, mucous membrane color, serum lactate, respiratory rate and effort, urine specific gravity, lung sounds, blood urea nitrogen, skin turgor, creatinine, body weight, electrolytes, urine output, BP, Mental status, arterial and venous blood gas, extremity temperature andO_2 saturation.

Guidelines for route of administration are shown in Table 1. Consider the temperature of the fluids. Body temperature (warmed) fluids are useful for large volume resuscitation but provide limited usefulness at low IV infusion rates. It is not possible to provide sufficient heat via IV fluids at limited infusion rates to either meet or exceed heat losses elsewhere.

Routes of Administration

In general, fluids can be given by the following routes: (1) oral, (2) subcutaneous, (3) intraperitoneal, (4) intravenous, and (5) intraosseous. The two latter routes are preferred for the critically ill patient because they give direct access to the intravascular space.Intravenous infusion is the preferred means of delivering fluids to severely dehydrated animals and medium to large dogs. It allows for a controlled delivery rate to meet the patient's changing needs.Intraosseous fluid therapy is a preferred route for animals weighing less than 5 kg when the intravenous approach is impossible. A comparison of the various routes of fluid administration is provided in Table 1.

Table 1. Routes of Fluid Administration for the Dogs and Cats

Route	Indications and Advantage	Technique	Complications and Contraindications
Oral	1. For anorectic patients with short-term illness 2. More conducive for small animals < 20 kg 3. Very conducive for neonates	1. Can use a stomach tube, pharyngostomy tube, small dosing syringe, or a small baby bottle and nipple, depending on the animal's size and underlying illness 2. Warm fluids to body temperature	1. Aspiration pneumonia 2. Not useful for hypovolemic shock 3. Should not be used in vomiting animals 4. Avoid air administration
Subcutaneous	1. For correction of mild to moderate dehydration 2. For maintenance in not too severely ill patients 3. Not conducive for animals weighing > 10 kg	1. Use isotonic fluids 2. Best to administer by gravity flow through an 18- to 20-gauge needle (for an adult-sized cat; use smaller needle for pediatric patients) 3. Do not deposit more than 10-12 ml/kg per injection site 4. Fluid should be deposited dorsally along the area bordered by the scapulae anteriorly and the iliac crests posteriorly 5. The average 5- to 6-kg cat can receive 150-200 cc once or twice daily	1. Avoid using hypertonic and hypotonic fluids 2. Do not deposit fluids under infected or devitalized skin 3. Not useful for hypovolemic shock 4. Do not use irritating solutions
Intraperitoneal	1. When intravenous access is unavailable 2. Provides a vehicle for delivering ample volumes of fluid over a short time period 3. Relatively rapid absorption	1. Use isotonic fluids 2. Use needle gauges 16-20, depending on the patient's size 3. Prepare a sterile injection site just lateral to the midline and midway between the umbilicus and the pelvic brim	1. Hypertonic fluids will worsen the dehydration 2. Do not use if patient has abdominal sepsis, ascites, or peritonitis 3. Do not use with pending abdominal surgery
Intravenous	1. Preferred route for severely dehydrated and hypovolemic patients 2. Best route for correcting hypotension 3. Provides for rapid delivery at the most precise dosage 4. Most effective for medium and large dogs	1. Prepare a sterile site for needle or cannula intravenous insertion 2. Amount and rate of fluid delivery depends on patient's status 3. Use isotonic fluids for volume repletion 4. Maintain completely sterile IV cannula and infusion system	1. Avoid intravenous overload caused by excess fluid delivery 2. Avoid catheter sepsis and phlebitis 3. Avoid catheter displacement and the inadvertent extravascular placement of the fluid infusion
Intraosseous	1. When intravenous access is unavailable 2. Particularly useful in small animals 3. Provides direct access to the vascular space	1. Prepare a sterile site 1 cm distal to tibial tuberosity, proximal media tibia, or trochanteric fossa of femur 2. Make a small nick skin incision 3. Insert either an 18-20 gauge hypodermic needle, a spinal needle, or a small bone marrow needle 4. Secure with tape and bandage	1. Avoid growth plates 2. Use needle proportional to bone size to avoid trauma 3. Bone infection is rare

Modified from Schaer M: General principles of fluid therapy. Vet Clin North Am 19:203, 1989.

Fluid Selection—Crystalloid

Parenteral fluid packages provide a list of the solute content and osmolality. This information is important when deciding which fluid to use in each particular clinical situation. Fluids can be conveniently classified on the basis of tonicity and fluid deficit (Table 2).

Comparison of commonly used parenteral pluids in the Dog and Cat can be analysed on the basis of osmolality (Table 3). Isotonic fluids contain the same osmolality as the extracellular fluids (approximately 290 to 310 mOsm/L). Commonly used isotonic solutions include lactated Ringer's, .9 percent (normal or physiologic) saline; Ringer's, acetated Ringer's, and 2.5% dextrose in 0.45% saline.

Lactated Ringer's solution (LRS) is a polyionic, isotonic (273 mOsm/L) solution. It is more physiologic than isotonic saline because its electrolyte concentration is similar to that of plasma. The 28 mEq of lactate anions can help reverse metabolic acidosis because of their potential for conversion to bicarbonate ions by the patient's liver (in the absence of shock). Although each liter contains 4 mEq of potassium, supplementation with this cation is usually required for the patient's maintenance needs

Table 2. A Guide to determining approximate fluid deficits in dehydration

Percentage Dehydration	Clinical Sign
0-5%	No specific clinical signs, animal may be depressed and thirsty, urine concentrated
5-7%	Sunken eyes, dry mucous membranes, some tenting of the skin
7-10%	Prolonged skin tenting, hypovolaemia symptoms (cold extremities, weak rapid pulse etc.)
10-12%	Collapse and progressive shock
12-15%	Severe shock, death imminent

An acetated Ringer's solution is also available. Its uses and restrictions are similar to those for LRS. Theoretically, sodium acetate is preferable to sodium lactate because a significant part of the latter anion is converted into liver glycogen, whereas acetate ions are not metabolized by the liver but by muscles and other peripheral tissues.

Ringer's solution is an isotonic saline solution (309 mOsm/L), with potassium and calcium ion concentrations approximately equal to those normally found in blood and extracellular water. The chloride concentration, however; is supraphysiologic (155 mEq/L). Potassium and calcium ions should be added when patients are depleted of these cations.

"Physiologic," 0.9% saline (NS) is also isotonic (310 mOsm/L) and is commonly used for rehydration. However, because of its supraphysiologic levels of sodium and chloride ions (154 mEq/L), it is not recommended for maintenance. This solution is mainly used for plasma volume expansion, for the correction of hyponatremia, and, along with potassium chloride supplementation for the treatment of metabolic alkalosis. Once plasma volume deficits are restored to normal, NS should not be used in animals with congestive heart failure or other conditions in which sodium restrictions are imposed.

Dextrose 2.5% in 0.45% saline is nearly isotonic (280 mOsm/L). Once rehydration has been accomplished and normal electrolyte balance has been restored, it is a useful maintenance solution when supplemented with potassium chloride. It is also the fluid of choice for patients whose sodium intake is restricted and those that tend to develop hypernatremia with NS (as seen in some azotemic cats with urethral obstruction).

Colloids

Whole blood, plasma, and colloidal plasma expanders are valuable for increasing the circulating blood volume when shock is present. Most of the solution is retained within the vascular system, where it increases the osmotic pressure of the blood above that of the extravascular fluid spaces. Consequently, water passes from the interstitial fluid space into the blood, increasing the circulating blood volume.

Plasma is the most commonly used colloid solution in veterinary medicine. Its main advantage stems from the colloid osmotic pressure provided by plasma proteins. It is useful for treating hypoproteinemic conditions such as chronic liver disease, protein-losing enteropathy, and glomerulopathy. The main disadvantages of plasma are that its availability is limited, its effects are temporary, and it is expensive.

Dextrans are synthetic colloids derived from sugar beets. Dextran 70 and 40 are available in 5% dextrose or saline solutions. Dextran 40 has the advantage of retarding formation of rouleaux and sludging of red blood cells, thus improving microcirculation above and beyond simple volume expansion.6 Disadvantages include coagulopathies as a result of decreased platelet function and altered fibrin clot formation. Other problems include renal failure, anaphylaxis, and depressed immune function.

Hydroxyethyl starch (Hetastarch) is a synthetic polymer derived from a waxy starch composed mostly of amylopectin. Like albumin, it expands the circulating plasma volume. Its osmolality is approximately 310 mOsm/L.

Table 3. Comparison of Commonly Used Parenteral Fluids in the Dog and Cat

Solution	Electrolyte Content (mEq/L)					Lactate	Acetate	Glucose (g/L)	Calories Kcal/L	Tonicity	Osmolality (mOsm/L)
	Na+	K+	Ca++	Cl-	Mg++						
Lactated Ringer's	130	4	3	109		28				Isotonic	273
Acetated Ringer's	131	4	3	98	3		28			Isotonic	295
Ringer's	147	4	4	155						Isotonic	310
Sodium chloride 0.45%	77			77						Hypotonic	155
Sodium chloride 0.9%	154			154						Isotonic	310
Dextrose 2.5%								25	85	Hypotonic	126
Dextrose 5%								50	170	Hypotonic	253
Dextrose 10%								100	340	Hypertonic	505
Dextrose 50%								500	1700	Hypertonic	2525
Dextrose 2.5% in half-strength lactated Ringer's	65	2	1	55		14		25	85	Isotonic	265
Dextrose 5% in lactated Ringer's	130	4	3	111		28		50	170	Hypertonic	525
Dextrose 2.5% with 0.45% sodium chloride	77			77				25	85	Isotonic	280
Dextrose 5% with 0.45% sodium chloride	77			77				50	170	Hypertonic	405
Dextrose 5% with 0.9% sodium chloride	154			154				50	170	Hypertonic	560

From Covington TR, Dipalma JR, Hussar DA, et al (eds): Drug Facts and Comparisons, 1985 edition. St. Louis, JB Lippincott, 1984

Fluid Volume Replacement

The animal requires fluids for (1) rehydration, (2) maintenance, (3) replacement of insensible loss volumes, and (4) replacement of ongoing loss volumes. Clinically, the amount of fluid needed to correct dehydration deficits can be assessed from the degree of skin turgor, capillary refill time, and pulse rate and quality. The degree of dehydration ranges from 5% to 12% (Table 2). Skin turgor assessment can sometimes be misleading in the obese animal, because adipose tissue replaces subcutaneous interstitial water and maintains elasticity despite negative water balance. Also, old, cachectic patients that have lost skin resiliency may give a false impression of marked dehydration. To clarify the diagnosis in such questionable situations, clinicians can check for the elevated packed red cell volume and plasma total solids that accompany the hemoconcentration caused by volume depletion. Discretion should be used when interpreting these clinicopathologic parameters, because lab values in an anemic animal with concomitant hypoproteinemia may seem normal even if the animal is dehydrated(Webb, 1982)

The volume of fluid needed to correct dehydration is calculated from either of the following formulas:

1. Volume (ml) of fluid needed = % dehydration x body weight (lb) x 500
2. Volume (ml) of fluid needed = % dehydration x body weight (kg) x 1000

The maintenance volume is that amount normally required in a 24-hour period by a well hydrated patient. Taking insensible fluid loss into consideration, the 24-hour maintenance volume for a dog or cat whose urine output is normal is approximately 50 to 60 ml/kg (25 to 30 ml/lb) per day. The total 24-hour fluid requirement for the dehydrated animal is the sum of maintenance volume and volume required to correct dehydration.

The initial rate and route of fluid delivery depend on the patient's status. Mildly to moderately dehydrated small dogs and cats that require short-term fluid treatment can be adequately managed with subcutaneous fluids (Table 1).

Any severely (>10%) dehydrated patient should initially receive fluids intravenously. Because of the accompanying vasocollapse, medications injected subcutaneously may not be adequately absorbed into the systemic circulation. For the mildly to moderately hypovolemic patient, it is recommended that one fourth to one half of the estimated dehydration deficit be replaced over the first two to four hours with the remaining dehydration deficit and maintenance isotonic volumes administered over the subsequent 20 to 22-hour period. The amount of subsequent fluid infusion will depend on the patient's response to treatment (Twedt et. al., 1982).

Exceptions to the recommended maintenance doses of fluids occur under the following circumstances:

1. Oliguria and anuria. After dehydration deficits are replaced, the patient's maintenance needs depend on urinary output, which should be estimated or quantitated. Providing full normal maintenance fluid volumes to oliguric and anuric patients can lead to fatal pulmonary edema or pleural effusion because of iatrogenic intravascular fluid overload. Specific treatment for low output renal failure is provided in the next section.

2. Polyuria. Polyuric animals require fluid volumes in excess of normal maintenance needs. Failure to provide these volumes can result in a sustained negative water balance if the patient is unable to drink. The maintenance needs for polyuria consist of exact urinary losses plus insensible and ongoing losses. Assurance of adequate treatment is made by weighing the patient each day as well as by assessing the physical and laboratory parameters for hydration. An acute loss of 1 kg of body weight suggests a 1 L fluid deficit (Davis, 2013).

3. Rapid internal shifts of fluid, which can occur in pancreatitis, extensive burns, enteritis, and gastrointestinal obstructions. In these conditions, the fluid needs of the patient will exceed the usual maintenance volumes by as much as three times.

The assessment of the animal's fluid requirements must always be made within the pathophysiologic context.

Conditions RequiringSpecial Fluid Therapy Consideration (See Table 4)

Anemia

Intravenous fluids are sometimes used excessively in the anemic patient when the decrease in red blood cell mass is misinterpreted as total blood volume depletion, when in fact the plasma volume might even be expanded. To compensate for decreased tissue oxygen delivery, the heart rate increases, and if these patients are subjected to large fluid volumes over a short period of time, pulmonary edema can occur.

Extracellular Fluid Volume Excess

This condition is associated with an increase in total body salt and water and occurs in a variety of clinical settings including congestive heart failure, glomerulopathies, liver fibrosis, and protein-losing enteropathy. These conditions are associated with a decrease in "effective arterial volume," which stimulates the renin-angiotensin-aldosterone cycle and the release of antidiuretic hormones to promote renal salt and water retention, respectively.

Hypovolemic Shock

Isotonic crystalloid solutions (NS, acetated Ringer's or LRS) are the most commonly used replacement fluids because they are usually effective, readily available, easily administered, and relatively inexpensive.Severely hypotensive patients might require at least one whole blood volume of replacement fluids during the first hour of treatment (Goodwin and Schaer, 1989).

Vomiting

Vomiting is the principle sign of gastric disease, but it can also accompany disorders of the small or large bowel, liver, and pancreas, as well as disorders occurring outside of the digestive system. NS supplemented with potassium chloride (3 to 10 mEq/kgBW every 24-hours) is the fluid of choice.

Gastric Dilatation--Volvulus (GDV)

The GDV complex causes hypovolemic shock as well as gastric sequestration of fluids and electrolytes. Although the hypovolemia can cause tissue hypoxia and eventually metabolic acidosis, there are several instances in which the gastric hydrogen and chloride ion sequestration can offset the acidosis.

Oliguric and Anuric Renal Failure

The urine output of all critically ill patients should be monitored, especially during periods of intensive fluid therapy. If urine production is inadequate, the following protocol is recommended:

1. Insert an indwelling urethral catheter and empty the urinary bladder of any residual urine.
2. Administer the calculated dehydration deficit fluid volume over the first two to four hours of treatment.
3. Once rehydration has occurred, administer furosemide (4 mg/kg IV push) and/or mannitol (0.5 gm/kg IV) over a 10-minute period.
4. If no urine flow occurs, readminister furosemide (8 mg/kg IV push) or administer dopamine (1 to 2 µg/kg/min IV).
5. If oliguria or anuria persists, the amount of fluids infused per day will consist of the sum of the measured urine output, the insensible water loss (5 ml/kg/day), and the extra losses caused by vomiting or diarrhea. Peritoneal dialysis will be required to rid the body of uremic toxins.

Diarrhea

The fluid deficit from massive diarrhea can be efficiently corrected with LRS or acetated Ringer's because it resembles the type of fluid lost, is readily available, and provides uniformly good results. In markedly hypotensive patients, the intravenous fluids should be given as described previously (see Hypovolemic Shock).

Hyperosmolar Conditions

The common causes of extreme hyperosmolality in the dog and cat include hyperosmolar nonketotic diabetes mellitus, hypernatremia associated with water deprivation in diabetes insipidus patients.

Hypotonic Disorders

A hypotonic disorder is one in which the serum osmolality and sodium levels are reduced in parallel. The goal of treatment in hyponatremia is to correct body water osmolality and restore cell volume by raising the sodium-to-water ratio of extracellular fluid. Acute hyponatremia occurs when the decline in serum sodium exceeds 0.5 mEq/L/hr.

Hypercalcemia

Many patients with hypercalcemia are volume depleted. Initially, NS should be infused to normalize intravascular volume. Because the renal excretions of sodium and calcium are linked, a forced saline diuresis using furosemide and isotonic saline will accelerate calciuresis. Close monitoring of serum electrolyte levels, especially potassium, is essential to detect and correct possible hypokalemia. All patients receiving rapid saline diuresis should be monitored for signs of intravascular fluid overload(Covington et al., 1984).

Fluids and Anesthesia

One of the most common uses of fluid therapy is for patient support during the perianesthetic period. Decisions regarding whether to provide fluids during anesthesia and the type and volume used depend on many factors, including the patient's signalment, physical condition, and the length and type of the procedure.

Pre-anesthetic Fluids and Preparing the Sick Patient

Correct fluid and electrolyte abnormalities in the sick patient as much as possible before anesthesia by balancing the need for preanesthetic fluid correction with the condition requiring surgery.

Fluid deficit calculation

Body weight (kg) X % dehydration = volume (L) to correct
General principles for fluid therapy to correct dehydration include the following:

- Add the deficit and ongoing losses to maintenance volumes.
- Frequency of monitoring will depend on the rate at which fluid resuscitation is being administered (usually q 15–60 min). Assess for euhydration.
- Maintenance solutions low in Na should not be used to replace extracellular deficits (to correct dehydration) because that may lead to hyponatremia and hyperkalemia when those solutions are administered in large volumes

Treating hypovolemia due to blood loss

The decision of when to use blood products instead of balanced electrolyte solutions is based on the severity of estimated blood loss. Following 15 mL/kg of hemorrhage, even 75 mL/kg of crystalloid will not return blood volume to prehemorrhage levels because crystalloids are highly redistributed (Michael, 2005).

Hypervolemia

Hypervolemia can be due to heart failure, renal failure, and/or iatrogenic fluid overload. Hypertension is not an indicator of hypervolemia.

Hyperthermia

Increased body temperature can rapidly lead to dehydration. Treatment includes administering IV replacement fluids while monitoring for overhydration.

Hyperkalemia

Suspect hyperkalemia in cases of obvious urinary obstruction, uroabdomen, acute kidney injury, diabetic ketoacidosis (DKA), or changes on an electrocardiogram.

Hypokalemia

Charts are available in many texts to aid in K supplementation of fluids and determination of administration rate.37 It is essential to mix added KCl thoroughly in the IV bag as inadvertent K overdoses can occur and are often fatal.

Hyponatremia

Hyponatremia is most commonly seen in DKA and with water intoxication. Changes in serum Na levels must occur slowly, as with hypernatremia. Monitor electrolyte levels frequently, and use a fluid with Na content similar to the measured plasma Na to keep the rate of change at an appropriate level.

Table 4. Fluid Therapy for Special Circumstances

Condition	Pathophysiologic Setting	Fluid Therapy Recommendations
Heart failure or myocardial dysfunction	- Increased RAA mechanisms	- Monitoring essential
	- Increased ADH release	- CVP measurements recommended
	- Prone to fluid overload	- Avoid excess fluid administration when in actual CHF
		- PWP is optional but difficult to do
		- Maintain on low-sodium containing solutions
Anemia	- Increased cardiac workload	- Avoid rapid volume loading with crystalloid
	- Increased plasma volume	- Infuse at maintenance rate if possible
	- Rapid heart rates	- Avoid excess amounts of crystalloid
	- Prone to fluid overload	
Hypoproteinemia	- Decreased plasma oncotic pressure	- Avoid excess crystalloid
	- Starling's forces favor fluid escaping into interstitial and 3rd spaces	- Provide colloids - Plasma preferred - Hetastarch beneficial when necessary
Pulmonary contusion and ARDS	- The viable lung is predisposed to fluid accumulation	- Try to avoid any significant systemic hypotension
	- Right to left shunting predisposes to "wet" lungs	- Avoid overhydration
	- Leaky capillary-alveolar junctions	- Crystalloid vs. colloid still debated
		- Hypertonic saline can be used for resuscitating patient with pulmonary contusion
Head trauma	- Brain fluid auto regulation disrupted	- Correct hypovolemia with crystalloid boluses, but avoid full volume loading. Do not exceed 40-60 ml/kg to correct hypovolemia
	- Brain swelling tendency	
		- Maintenance volumes should maintain normal blood pressure at the low range of normal
Oliguric renal failure	- Impaired renal perfusion	- First replace dehydration deficits
	- Inadequate renal filtration	- If still oliguric, can administer:
	- Excess maintenance fluid volumes will cause fluid overload	(a) Furosemide 4-8 mg/kg (b) Mannitol 0.5-1.0 gm/kg in the absence of co-existing hyperosmolality (c) Dopamine 1-2 µgm/kg/minute
Renal failure with congestive heart failure	- Tendency for fluid overload due to RAA and ADH mechanisms	- Under ideal circumstances monitor with CVP and urine output measuring
	- The needs of one condition complicates the other	- A Swan-Ganz pulmonary artery catheter would be ideal
		- Prognosis fair to dismal

Hypernatremia

Hypernatremia may be common, yet mild and clinically silent. Causes of hypernatremia include loss of free water. The amount of free water (in the form of D5W) to infuse over the calculated timeframe (to decrease the Na concentration by the above guidelines) can be calculated as follows:

Volume (L) of free water (D5W) needed =
([current Na concentration/normal Na concentration] – 1) X (0.6 X body weight [kg])

Hypoproteinemia/hypoalbuminemia

Colloid osmotic pressure is related to plasma albumin and protein levels and governs whether fluid remains in the vascular space. Fluid loss into the pulmonary, pleural, abdominal, intestinal, or interstitial spaces is uncommon until serum albumin is < 15 g/L or total protein is < 35 g/L.

Hyperglycemia

Hypoglycemia Initial therapy for hypoglycemia is based on severity of clinical signs more than on laboratory findings. Treatment options include oral glucose solutions, IV dextrose-containing fluids, or food (if not contraindicated).

Anemia and Polycythemia

Blood products may be needed to treat anemia. Treatment of symptomatic polycythemia involves reducing the number of red blood cells through phlebotomy and replacing the volume removed with balanced electrolyte solutions to reduce viscosity and improve blood flow and O_2 delivery.

Conclusion

Fluid therapy is important for many medical conditions in veterinary patients. It is dictated by many factors and is highly patient variable. Fluid selection for a given patient may change during therapy, depending on patient needs. The goal of these guidelines is to assist the clinician in prioritizing goals, selecting appropriate fluids and rates of administration, and assessing patient response to therapy.The reader must recognize the highly individual patient variables and dynamic nature of fluid therapy. Because fluid therapy can be highly individualized in complex cases, having a relationship with a referral facility for consultation can be helpful.

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Incidence of pigeon diseases in northern Bangladesh

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Abstract
The aim of the study was to evaluate the incidence of pigeon diseases in northern part of Bangladesh. 7489 pigeons from 45 pigeon farms (15 farms from each district) were evaluated during from April 2015 to March 2017. Selected pigeons were classified into different groups on the basis of breed, stains, age of pigeon, hosing, rearing system feeding, breeding, biosecurity, health care, farm type, location of farm, season, occupation and experience of pigeon farmers etc. The information was collected from the pigeon farmers using the structured questionnaire and specimens were collected from the diseased pigeon to identify the respective causal agents. The incidence of pigeon diseases was confirmed by the retrospective and cross sectional study. Collected data were analyzed by SPSS programme. Out of 7489 pigeon, 1592 were sick and 1176 dead within one year. The overall morbidity and mortality rate were recorded 21.25% and 15.70%, respectively. Significantly ($P<0.05$) highest mortality rate was observed in local breed (23.35%) than in exotic breed (11.80%). The strain and age of pigeon, effect of hosing, type of pigeon rearing, effect of cleaning of farm, sizes of farm, location of farms and season had significant effect ($P<0.05$) on mortality rate in pigeon. Although, the case fatality rate of pigeon was recorded higher in young (79.29%) than adult (66.96%). The local breed, gola pigeon, young age, modern rearing system, Bumboo+wood made housing system higher chance to pigeon mortality rate on the other hands good quality feed, regular vaccination and regular deworming caused less chance to diseases. Finally, it would be concluded that the mortality rate of pigeons was recorded 15.70% in northern Bangladesh which is alarming to sustainable pigeon farming in Bangladesh.

Key words: Pigeon, incidence of diseases, mortality rate and northern Bangladesh.

Introduction
Pigeon farming is very interesting, profitable and pigeons are very popular domestic bird. Worldwide, pigeon are considered as the symbol of peace. Almost all types of people who have facilities, love to raise some pigeon in their home. Pigeon farming require less labor and low investment. The meat of baby pigeon (squab) is very tasty, lean, easily digestible, and richer in protein, mineral and vitamin. It is also used as tasty, delicate and fancy meat (Richard, 2006). Squabs also have huge demand and price in the market. On the other hand pigeon farming can be a great source of some extra income and entertainment. Raising pigeons using modern methods is very profitable than traditional way. Mankind has practiced with pigeon keeping about 10,000 years in almost every part of the world (Levi, 1977). Probably pigeon is the first bird species to have been reared by humans (Johnston and Janiga, 1995). Pigeons reared in Bangladesh are predominantly belongs to Gola (native) breeds for squab production (Rahman, 1999). Pigeons are mainly reared in houses and their management, feeding and breeding is quite economical. Pigeons mainly feed on fruit seeds, plant shoots, fruits and grains. Pigeons feed their young ones by producing a thick, nutrient rich secretion produced from their crop. Nevertheless, about twenty varieties of domestic pigeon are Bangladesh e.g. Gola pigeon, While king, Tumbler, Rolling pigeon, Fantail, Lahores. Jacobin, Kokha, Pouter, etc. (Rahman, 1999). A lot of professional pigeon farms have been established in different areas of Bangladesh viz. Dhaka, Chittagong, Rajshahi, Natore, Pabna, Dinajpur, Khulna, Rangpur, Kustia, Norshindi, Jenaidah, Jessore, Naogaon, Gazipur, Potuakhali, Barguna, Mymensingh, Bandarban, Cox-bazar etc (Sarder et.al., 2016 & 2017). They kept the ornamental, high quality and fancy pigeon and their market prizes are few thousands per pair. Diseases in pigeon are comparatively lower than other poultry birds. Sarder *et al.* (2014) reported that the

Newcastle, Pigeon pox, Mycoplasmosis, Cholera, Influenza, Parasitic diseases etc were observed at northern part of Bangladesh. The mortality rate of squab was found 10-15% and adult pigeon 2-4% who they were used regular vaccination and preventive measures. Otherwise, some farm, the mortality was found more 80% due to Newcastle disease. The mortality rate was shown high in winter season. During last few years several emerging diseases like Salmonellosis, Collibacillosis, ND, Pigeon Pox, Canker, Coccidiosis, Pigeon malaria and Respiratory infections- Mycoplasmosis and some unknown cause threat the pigeon industry and causes huge damage to the farmers. There are few literatures available on pigeon diseases in our country. Till now, there is a lack of expert on pigeon disease management and control. Sometimes field veterinarians are confused to diagnose the fatal diseases and cannot suggest the farmers to take necessary measures. So, the present study has been designed to known the incidence of pigeon diseases in Northern Bangladesh.

Materials and Methods

Selection of pigeon: A total of 7489 pigeons were selected from 45 pigeon farms at three districts (Pabna n= 1702, Natore n=3940 and Rajshahi n=1847) for the study. The selected pigeons were classified according to their genetic makeup as local and exotic. The pigeons were also grouped on the basis of rearing purpose (Meat or fancy) and age (young and adult).

Data collection procedure: The data was collected directly from the pigeon owners using questionnaires. Interview schedule was carefully designed towards the farm owner and research assistant keeping the objectives in view. The schedule was containing both open and closed form of questions. The important information was collected by using questionnaire. The questionnaire included following information viz. pigeon farmers name and full address, farm's name or ID, occupation, farm size, education, experience, purposes, total number, farm type, management of housing, feeding and breeding etc as was as specific information on individual pigeon viz- breed, varieties, age etc.

Grouping of pigeons: The housing was classified according to their housing pattern, housing system, housing condition, housing environment etc. The feeds and feeding system of pigeon was recorded on the basis of quality and quantity. The vaccination programme and deworming programme was recorded under various classes viz. regular, irregular and none. The health management of the pigeons was recorded and supervised by veterinarian, quack doctor or none.

Breed: The pigeon breed broadly divided into two groups one Local & Exotic. The breed of pigeon further classified according to genetic composition such as: King, Bokora, Nun, Pouter, Short face, Art Engle, Cormona, Lakka, Homer, Helmet, Owl, Strasser, Frillback, Magpie, Jacobin, Mookee, Maltese, Oriental field, Rain, Shallow, Giribag, Gola and Gobindo.

Age of pigeon: Age was young (before sexual maturity and adults (After sexual maturity).

Farming system: Traditional and modern; **Rearing system**: Traditional and modern; **Feed quality**: Poor, medium and good; **Deworming:** Regular, irregular and none; **Biosecurity:** Strong, medium and none and **Health care:** Traditional and modern; **Sizes of farm:** Small, Medium and Large; **Study area:** It was divided into three regions such as Rajshahi, Natore and Pabna; **Occupation:** Occupation was divided into two group's viz. Group I: Main purpose and Group-II: Side business of pigeon farmers; **Training of farmers:** No training and Short & medium training

Analysis: The raw data sorted and computed according to the experiment. Collected data was analyzed by the use of some statistical computer programme such as SPSS. Simple tabular techniques were used to explain the data. Minimum, maximum, mean, standard deviation and percentage was use mainly to illustrate the results.

Results and Discussion

The incidence of pigeon diseases observed under various factors like breed, strains, age, rearing system, farming system, feed quality, treatment pattern, deworming and biosecurity of pigeon, size of farm, location,

occupation & training of farmers, of the farms are summarised in the Tables 1-11 and Figures 1-8. Effects breed on incidence of pigeon morbidity and mortality in a year at northern Bangladesh are shown Table 1. The highest morbidity and mortality was (32.14% & 23.35%) in local and lowest (15.71% & 11.80%) in exotic pigeon breed. Out of 7489 pigeon, 1592 were sick and 1176 dead within one year. The overall morbidity and mortality rate were recorded 21.25% and 15.70%, respectively. Significantly ($P<0.05$) highest mortality rate was observed in local breed (23.35%) than in exotic breed (11.80%). The strain and age of pigeon, effect of hosing, type of pigeon rearing, effect of cleaning of farm, sizes of farm, location of farms and season had significant effect ($P<0.05$) on mortality rate of pigeon. The overall morbidity and mortality rate of pigeon diseases were recorded 21.25% and 15.70%. Assaduzzaman *et al.* (2009) reported that mortality of pigeon ranged from 5 to 15%.

Table-1. Effects breed on incidence of pigeon morbidity and mortality in a year at northern Bangladesh.

Breed	No. of observation	No. of affected young	No. of dead young	No. of affected adult	No. of dead adult	Total no. affected pigeon	Total no. of dead pigeon and %	% of mortality within population
Local	2526	500	380	250	160	812 (32.14%)[a]	590 (23.35%)[a]	7.87%
Exotic	4963	413	344	429	292	780 (15.71%)[b]	586 (11.80%)[a]	7.82%
Total	7489	913	724	679	452	1592 (21.25%)	1176 (15.70%)	**15.70%**

Values are percentage (%), values [ab] with superscript level in column differ significantly ($P<0.05$) with each other.

The mortality rate of pigeon at different strains is summarized Table 2. The highest morbidity & mortality rate were in Gola (54.16±2.75 & 38.87±4.64) and lowest in Mookee (13.78±1.75 & 9.10±1.29).

Table-2. Influence of strains of pigeon on incidence of morbidity and mortality in a year at northern Bangladesh.

Varieties of pigeon	Morbidity (%)	Mortality (%)
King (n=9)	20.29±2.88	15.33±2.03
Bokora (n=14)	18.88±2.29	13.14±2.10
Nun (n=9)	15.09±4.31	10.51±3.64
Pouter (n=14)	19.59±3.01	14.74±3.10
Short face (n=14)	30.05±4.70	21.03±4.29
Art Engle (n=11)	20.49±4.77	16.00±4.82
Cormona (n=8)	23.52±6.52	11.83±3.62
Lakka (n=19)	15.87±1.91	10.68±1.55
Homer (n=14)	24.32±4.09	17.21±3.94
Helmet (n=12)	30.16±3.13	19.48±1.97
Owl (n=16)	20.30±3.28	12.08±1.63
Strasser (n=10)	20.69±4.69	16.98±4.30
Frillback (n=12)	27.94±3.75	21.84±3.76
Magpie (n=7)	40.36±4.55	22.11±0.75
Jacobin (n=14)	18.12±2.82	13.58±2.01
Mookee (n=15)	13.78±1.75	9.10±1.29
Maltese (n=12)	25.56±4.68	13.41±2.24
Rain (n=12)	23.07±3.88	17.45±3.50

Oriental field (n=6)	29.85±6.47	23.43±7.47
Shallow (n=11)	18.62±1.43	11.95±1.14
Giribag (n=10)	43.56±1.87	34.44±3.48
Gola (n=9)	54.16±2.75	38.87±4.64
Gobindo (n=10)	51.67±4.00	31.30±2.91
Total (n=268)	25.04±0.95	17.25±.76

The rates of affected dead and case fatality of pigeon in relation with age are shown in Table 4. According to age, 1592 pigeons were classified into two categories Young (squab including 1-7 weeks) and adult (>90days). The highest 913 pigeon was affected, 724 dead and 79.29% case fatality observed in young pigeon than adult, similar trend was observed by Paul *et al*., 2015.

Table-3. Effect of age on Pigeon morbidity and mortality rate among the three districts in a year.

Age of pigeon		No. of affected	No. of dead	Case fatality %
Yong	Commercial	809	644	79.60
	Traditional	104	80	76.92
	Total	**913**	**724**	**79.29[a]**
Adult	Commercial	546	356	65.20%
	Traditional	133	96	72.18
	Total	**679**	**452**	**66.96[b]**
Grand total		**1592 (21.25%)**	**1176(15.70%)**	**73.86%**

Values are percentage (%), values [ab] with superscript level in column differ significantly (P<0.05) with each other.

The rates of affected and dead pigeon effect with rearing system are shown in Table 4. The rate of affected and dead pigeon was highest (85.11%) & 85.03%) in modern rearing system but lowest in traditional rearing system (14.88% & 14.96%). The rates of affected and dead pigeon effect with housing pattern are shown in Table 5. The percentage of dead pigeon was highest 34.01% in Bamboo + wood and lowest in 13.06% in Iron cage.

Table-4. Effects of modern and traditional farming system in pigeon on morbidity and mortality rate in a year.

Rearing system of pigeon	**No. of observation**	**No. of affected young**	**No. of dead young**	**No. of affected adult**	**No. of dead adult**	**Total no. affected pigeon**	**Total no. of dead pigeon**
Modern n=30	6985	809	644	546	356	1355 85.11%	1000 (85.03%)[a]
Traditional n=15	504	104	80	133	96	237 14.88%	176 (14.96%)[b]
Total	7489	913	724	679	452	1592(21 .25%)	1176 (15.70%)

Values are percentage (%), values [ab] with superscript level in column differ significantly (P<0.05) with each other.

Table-5. Effect of housing pattern of pigeon farm on morbidity and mortality rate in a year.

Made of houses	No. of observation	Total no. affected pigeon	% of affected pigeon	Total no. of dead pigeon	% of dead pigeon
Iron cage n=23	6940	1067	17.96	776	13.06[c]
Iron + Bamboo n=13	1255	400	31.87	300	23.90[b]
Bamboo + wood n=9	294	125	42.51	100	34.01[a]
Total	7489	1592	21.25	1176	15.70

Values are percentage (%), values [abc] with superscript level in column differ significantly (P<0.05) with each other.

The rates of affected and dead pigeon effect with places of housing are shown in Table 6. The mortality rate was highest 19.29% in others than primacies which was 11.29%.

Table-6. Places of housing of pigeon farm on morbidity and mortality rate in a year.

Places of housing	No. of observation	Total no. affected pigeon	% of affected pigeon	Total no. of dead pigeon	% of dead pigeon
Premises n=23	3364	540	16.05	380	11.29[b]
Others	4125	1052	25.50	796	19.29[a]
Total	7489	1592	21.25	1176	15.70

Values are percentage (%), values [ab] with superscript level in column differ significantly (P<0.05) with each other.

The morbidity & mortality of pigeon on the basis of rearing system of farmers are shown in Figure 1. The highest morbidity and mortality was highest (44.64% & 44.64%) scavenging rearing system than give supplement feed (44.05% & 27.05%) for pigeon, the researcher Paul *et al*., 2015 was observed similar pattern and Kabir (2013) also observed Semi intensive rearing is more effective for its exercise and alternative food of corn and wheat in summer and winter season was remarkable.

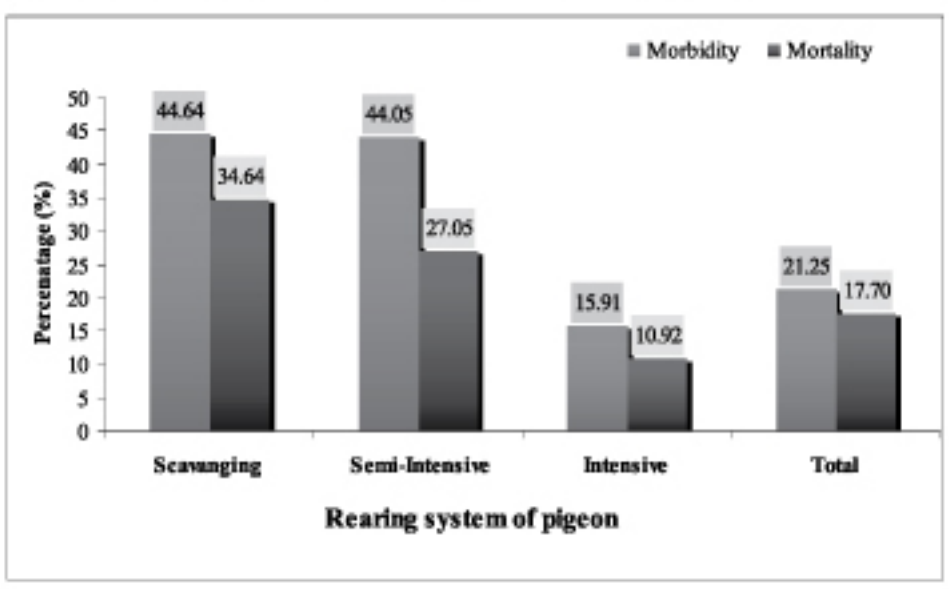

Figure 1. Effect of rearing system of pigeon farmers on pigeon morbidity and mortality rate

The rates of affected and dead pigeon effect with type of pigeon are shown in Table 7. The rate of affected and dead pigeon was highest (41.17% & 27.44%) in meat type and lowest in 17.85% & 13.70% in fancy type pigeon.

Table-7. Effect of Fancy and meat type pigeon on morbidity and mortality rate in a year.

Purposes of pigeon rearing	No. of observation	Total no. affected pigeon	Total no. of dead pigeon and %
Fancy type n=27	6396	1142(17.85%)	876(13.70%)[b]
Meat type n=18	1093	450 (41.17%)	300(27.44%)[a]
Total	7489	1592(21.25%)	1176 (15.70%)

Values are percentage (%), values [ab] with superscript level in column differ significantly (P<0.05) with each other.

The effect of feed quality with morbidity & mortality of pigeon are shown in Figure 2. The highest to lowest morbidity and mortality rate of poor, medium and good quality feed supplied were 38.34% & 29.76%, 31.90% & 26.53%, and 21.46% & 14.00%, respectively.

Figure 2. Effect of feed quality of pigeon farmers on pigeon morbidity and mortality rate

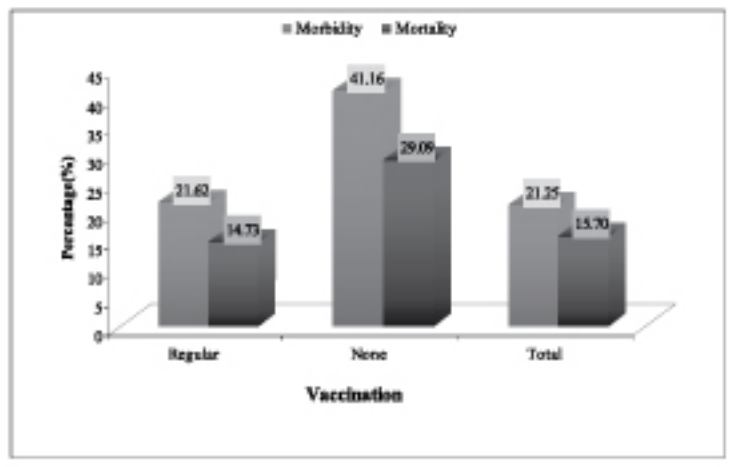

Figure 3. Effect of vaccination of pigeon farmers on pigeon morbidity and mortality rate

The morbidity & mortality of pigeon in relation with vaccination status of pigeon are shown in Figure 3. The highest morbidity and mortality was highest (41.16 % & 29.09 %) no vaccine with regular used (21.26 % & 26.87 %) for pigeon farm. The morbidity & mortality of pigeon in relation with vaccination status of pigeon are shown in figure 3. The figure 4 shown the highest morbidity and mortality was highest (50.00 % & 31.05 %) in regular, medium in irregular (50.00 % & 31.05 %) and lowest in no used (40.16 % & 18.70%) anthelmentics for deworming (21.31 % & 14.42 %) in the study pigeon farm, but Paul *et al.* (2015) sated that As causes of parasitic infestation found within those farm and pigeon loft, the owner does not use regular doses of anthelmintic and irregular cleaning of pigeon nest. The highest morbidity and mortality was highest (44.57% & 26.51%) in none bio security measure taken than moderate in medium bio security (20.75 % & 15.99 %) and highest in strong bio security measured (19.54 % & 13.59%) in the study pigeon farm (Figure 5).

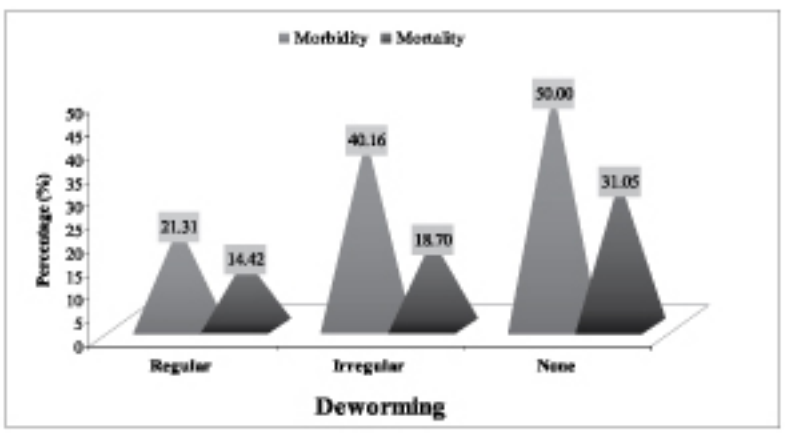

Figure 4. Effect of deworming of pigeon farmers on pigeon morbidity and mortality rate

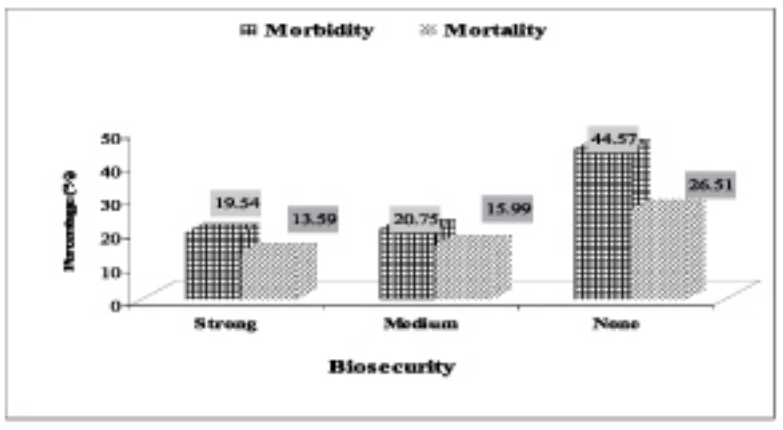

Figure 5. Effect of Biosecurity of pigeon farmers on pigeon morbidity and mortality rate

The morbidity & mortality of pigeon in relation with treatment pattern of pigeon are shown in Figure 6. The highest morbidity and mortality was highest (45.38 % & 26.87 %) traditional rearing system with modern rearing (20.94% & 15.30%) for pigeon farm.

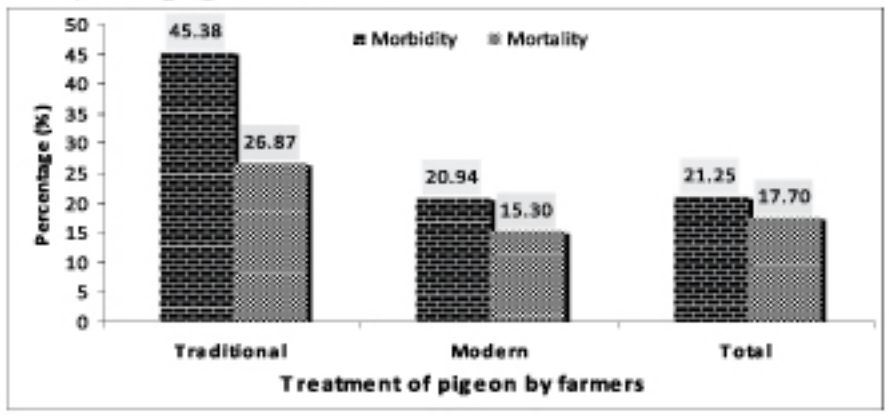

Figure 6. Effect of treatment pattern of pigeon farmers on pigeon morbidity and mortality rate

The mortality of pigeon in relation with cleaning of pigeon farm are shown in Table 8. The highest mortality was 34.00% in none group and lowest in 9.92 % in regular cleaning group. The rates of affected and dead pigeon effect with size of farms are shown in Table 9. The rate of affected and dead pigeon was highest (44.11% & 36.76%) in small and lowest in 17.90% & 13.08% in large size pigeon (Table 10).

T**able-8.** Effect of cleaning of pigeon farm on morbidity and mortality rate in a year.

Cleaning system	No. of observation	Total no. affected pigeon	% of affected pigeon	Total no. of dead pigeon	% of dead pigeon
Regularly n=25	5100	792	15.52	506	9.92^{c}
Irregularly n=13	1889	600	31.76	500	26.46^{ab}
None n=10	500	200	40.00	170	34.00^{a}
Total	7489	1592	21.25	1176	15.70

Values are percentage (%), values ^{abc} with superscript level in column differ significantly (P<0.05) with each other.

T**able-9.** Effect of sizes of farm on morbidity and mortality rate in a year.

Farm size	No. of observation	Total no. affected pigeon	Total no. of dead pigeon and %
Small n=25	544	240(44.11%)a	$200(36.76\%)^{a}$
Medium n=10	1625	400(24.60%)b	$280\ (17.23\%)^{b}$
Large n=10	5320	952(17.90%)bc	$696(13.08\%)^{bc}$
Total	7489	1592(21.25%)	1176 (15.70%)

Values are percentage (%), values ^{ab} with superscript level in column differ significantly (P<0.05) with each other.

The rates of affected and dead pigeon effect with location (districts) are shown in table 10. The rate of affected and dead pigeon was highest (53.95% & 17.00%) in Natore than moderate in Rajshahi (22.67% &

15.10%) and lowest in Pabna (21.10% & 13.33%).The rates of affected and dead pigeon effect with season are shown in Table 11. The rate of affected and dead pigeon was highest (27.04% & 21.80%) in winter season and lowest in 18.50% & 12.80% in spring season. The morbidity & mortality of pigeon relation with occupation of pigeon farmers are shown in Figure 7. The highest morbidity and mortality was highest (27.43% & 19.75%) main occupation in pigeon farming than side business (19.52% & 11.45%) for their pigeon rarer, similar fashion was observed by Paul *et al*., 2015.

Table-10. Pigeon morbidity and mortality rate among the three districts in a year.

Name of districts		No. of observation	No. of affected young	No. of dead young	No. of affected adult	No. of dead adult	Total no. affected pigeon	Total no. of dead pigeon
Rajshahi	Commercial	1675	205	168	62	31	267	199 (11.88%) b
	Traditional	172	39	33	55	47	94	80 (46.51%) [a]
	Total	**1847**	**244**	**201**	**117**	**78**	**361** (22.67%)	**279 (15.10%)[B]**
Natore	Commercial	3780	415	330	400	271	815	601(15.89%) [b]
	Traditional	160	27	27	53	42	80	69(43.12%)[a]
	Total	**3940**	**442**	**357**	**453**	**313**	**895** (53.95%)	**670 (17.00%)[A]**
Pabna	Commercial	1530	189	146	84	54	273	200(13.07%)
	Traditional	172	38	20	25	7	63	27(15.69%)
	Total	**1702**	**227**	**166**	**109**	**61**	**336** (21.10%)	**227 (13.33%)[C]**
Grand total		**7489**	**913**	**724**	**679**	**452**	**1592 (21.25%)**	**1176 (15.70%)**

Values are percentage (%), values [abc] with superscript level in column differ significantly (P<0.05) with each other.

Table-11. Effects of seasons on morbidity and mortality rate in a year.

Season	No. of observation	Total no. affected pigeon	Total no. of dead pigeon and %	% of mortality within population
Spring	1900	360(18.94%)	233(12.26%)[c]	3.11%
Summer	2000	370 (18.50%)	256(12.80%)[c]	3.41%
Rainy	1814	382(21.05%)	300 (16.53%)[b]	4.00%
Winter	1775	480 (27.04)	387 (21.80%)[a]	5.16%
Total	7489	1592(21.25%)	1176 (15.70%)	**15.70%**

Values are percentage (%), values [abc] with superscript level in column differ significantly (P<0.05) with each other.

The morbidity & mortality of pigeon at training of pigeon farmers are shown in Figure 8. The highest morbidity and mortality was highest (27.75% & 19.18%) short and medium training taken of pigeon farmer than side business (12.63% & 8.37%) for their pigeon rarer.

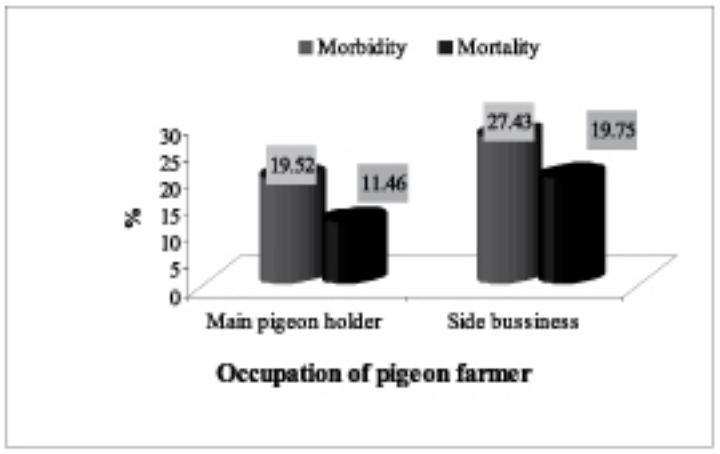

Figure 7. Effect of occupation of pigeon farmers on pigeon morbidity and mortality rate

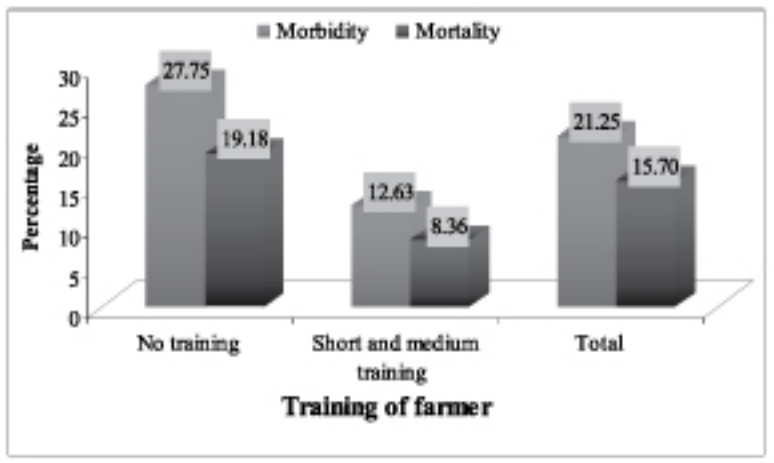

Figure 8. Effect of training of farmers on pigeon morbidity and mortality rate

Conclusion

Pigeon diseases are detrimental to development the commercial and backyard pigeon farm in Bangladesh. The morbidity and mortality rate of pigeon were compatible high in Northern Bangladesh which is alarming to sustainable pigeon farming.

Recommendations:

Farmers should aware about the pigeon diseases and it diagnosis & control.
Local Livestock department would take necessary action to develop sustainable pigeon farming.

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A Comparative Study on Canine Parvovirus Infection of Dogs in Bangladesh and India

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ABSTRACT

Canine parvovirus (CPV) infection is an infectious and contagious viral disease found in canine especially dogs. A comparative study on cases of CPV infections among the hospitalized dogs at Central Veterinary Hospital (CVH) in Bangladesh and Veterinary College and Research Institute-Madras Veterinary College (VCRI-MVC) in India was conducted throughout January to July 2015. A total of 270 hospitalized dogs (80 at CVH and 190 at VCRI-MVC) of different breeds were clinically examined and included in this study. Characteristic clinical signs were observed among the CPV infected dogs that include bloody diarrhoea (90.4%), vomition (94.5%) and dehydration (severe 85.7%, moderate 10.0% and mild 7.6%). The prevalence of CPV infection was higher in VCRI-MVC (42.7%) than CVH (31.2%). Prevalence of CPV infection varied significantly ($P<0.05$) among different age groups and between vaccinated and non-vaccinated dogs. Highest prevalence was found among younger (1-3 months of age 48.7%) dogs compared to older dogs (4-6 months 17.2% and over 6 months 8.3%). Highest prevalence was recorded in non -vaccinated dogs at CVH, Bangladesh. In VCRI-MVC, India rate of infection also varied significantly ($P<0.05$) in different age groups (57.4%, 28.9%, 10.0% among 1-3 months, 4-6 months and >6 months respectively) and 13.2% in vaccinated and 64.4% in non-vaccinated groups. Significant ($P<0.05$) variation in prevalence of CPV infection also observed in different breeds- indigenous (50.0%), Spitz (28.2%), Lhasa (18.1%), Doberman (40.0%) and German Shepherd (46.6%).

Key words: CPV, CVH, VCRI-MVC, Prevalence.

INTRODUCTION

Canine parvovirus (CPV) infection is an infectious and contagious viral disease of canine especially dogs. Parvovirus is a non-enveloped virus with a single stranded DNA genome belonging to the family Parvoviridae (Aappel et al., 1978). CPV is genetically and antigenically unrelated to Canine Minute Virus (CnMV), formerly known as canine parvovirus type 1 (CPV-1), which is responsible for neonatal death in dogs (Tattersall et al., 2005). Dogs of all age groups can be infected by CPV while puppies of 3 month of age are highly susceptible compared to adults (Behera et al., 2015). CPV infection may lead to high morbidity (as high as 100%) with frequent mortality of up to 10% in adult dogs and 91% in pups (Aappel et al., 1978). CPV infection is characterized by vomition, diarrhea and dehydration, brownish or bloody foul smelling diarrhoea and in severe cases fever (Pollock and Coyne, 1993). The clinical manifestations of CPV infection depends on the age and immune status of the dogs, virulence of the virus, dose of the virus and pre-existing or concurrent parasitic, bacterial or virus infections (McAdaragh et al., 1982). Factors that predispose CPV infection in puppies are lack of protective immunity, intestinal parasites, overcrowded, unsanitary, and stressful environmental conditions (Hoskins, 1997). Earlier reports indicated that Doberman, Rottweiler and German shepherd (GS) dogs are more susceptible to Parvovirus infection than other breed (Ling et al., 2012) Unvaccinated puppies aged between six weeks and six months are at maximum risk of developing CPV related disease (Godsall et al., 2010).The prevalence of CPV infection was reported as 77-80.4% in Thailand, 82.9% in Korea and 6% in Lithuania (Grigonis et al., 2002). The outbreak of CPV disease in dogs was also reported in Belgium and France (1977), Thailand, USA (1978), Portugal, Pakistan, Italy, Spain, Germany

(Lamm and Rezabek, 2008). In India 1st outbreak of CPV infection was reported in Madras (1981) and in Bombay (1985) (Haque and Arfa, 2012). Though diarrhea is one of common clinical features faced by the pet practitioners, there were limited reports of CPV infection in Bangladeshi dog population (Islam et al., 2014).

MATERIALS AND METHODS

Location, duration of study and study population

The study was conducted at Central veterinary hospital (CVH), Bangladesh and the clinics of Veterinary College and Research Institute (VCRI) and clinics of Madras Veterinary College (MVC), India; during the period of January to till March 2015. Only hospitalized dogs were considered as study population. A total of 270 (80 at CVH and 190 at VCRI-MVC) dogs of different breeds (Indigenous and Exotic breed i.e. Spitz, Lhasa, Doberman, and GS) were clinically examined and included in the study. The data related to age, sex, breed, vaccination, clinical history etc were retrieved throughinterviewing the owner using a standard case report sheet. Data concerning rectal temperature, heart rate and respiration rate was recorded for future use. Skin fold test was performed to estimate the degree of dehydration. Diagnosis was mainly based on characteristics clinical signs. For example, in enteritis forms, signs appear within 5 to 7 days after exposure including depression, loss of appetite, high fever (above 104˚F), vomition, bloody diarrhea and in myocarditis form which is usually found in younger puppies (less than 8 week of age). Dyspnea, Crying and retching finally death occur within 24 hours.

Statistical Analysis

All the data were Uploaded into MS Office Excel-2010. Then the data was cleaned, coded and finally analyzed using statistical software STATA version-13/C. To measure the association between categorical variables with the outcomes, chi-square test was performed with 95% level of confidence and 5% level of significance.

RESULTS AND DISCUSSION

Careful data analyses and characteristic clinical sings were assessed for positive case selection. In CVH among the 80 clinically sick dogs, 25 were found positive for CPV infection. Prevalence of different risk factors (age, sex, breed, vaccination status, dehydration and diarrhea) associated with CPV disease are summarized in Table 1.

Table 1. Prevalence of CPV Infection according to different risk factors at CVH

Variables	Category level	No of observation (N=80)	Positive Case	Prevalence (%)	P value
Age	1-3 month	39	20	48.7	0.004
	4-6 Month	29	5	17.2	
	>6 month	12	1	8.3	
Sex	Male	44	16	36.3	0.275
	Female	36	9	25.0	
Breed	Indigenous	10	3	30.0	0.079
	Spitz	34	14	41.1	
	Lhasa	14	2	22.2	
	Doberman	14	3	21.4	
	GS	8	3	37.5	
Vaccination	Yes	45	4	8.8	0.001
	No	35	21	60.0	

The study revealed that, the overall prevalence of CPV infection during the study period at CVH in Bangladesh was 31.2%.The prevalence of CPV infection in different age group differed significantly ($P<0.05$) and these were 48.7% for 1-3months, 17.2% for 4-6 months and 8.3% for above 6 months of ages. Between two different sexes the prevalence was insignificantly ($P>0.05$) higher in male (36.3%) than female (25.0%).

Table 2. Prevalence of CPV infection Cases according to different risk factors in VCRI-MVC

Variables	Category level	Prevalence (%)	Prevalence (%)	P value
Age	1-3 month	48.7	57.4	0.288
	4-6 Month	17.2	28.9	0.188
	>6 month	8.3	10	0.756
Sex	Male	36.3	33.7	0.149
	Female	25.0	49.4	0.756
Breed	Indigenous	30.0	30.0	0.685
	Spitz	41.1	41.1	0.820
	Lhasa	22.2	22.2	0.897
	Doberman	21.4	21.4	0.999
	GS	37.5	37.5	0.472
Vaccination	Yes	8.8	13.2	0.464
	No	60.0	64.4	0.633

Among the breeds a variable rate of infections were encountered as 30.0%in indigenous, 41.1%in Spitz, 22.2% in Lhasa, 21.4 % in Doberman, 37.5% in German Shephard. However a substantial difference (P<0.01) of occurrence of CPV infection which were recorded from in vaccinated dogs (8.33%) and in non-vaccinated dogs (60.0%) (Table 1).

Table 2 represents at date of VCRI-MVC, India, 190 where dogs were studied, of which 80 were found positive. The risk factors (Age, Sex, Breed and Vaccination Status) which influence the prevalence of CPV infection are summarized in Table 2. The estimated prevalence of CPV disease at VCRI-MVC in India was 42.1%.In compared with age wise distribution, prevalence was significant (P<0.05), it was 57.4% for 1-3 months, 28.9% for 4-6 months, and 10.0% for above 6 months. Sex specific prevalence was statistically significant (P<0.05) in where male 33.7%, and female 49.4%. Among the breeds of dog, prevalence were significant (P<0.05) in where 50.0% for indigenous, 30.7%for Spitz, 28.2% for Lhasa, 40.0% for Doberman and 46.6% for GS. Moreover, vaccinated 13.2%and non-vaccinated 64.4% were significantly (P<0.05) affected with CPV infection.

Table 3. Comparison on prevalence of CPV Infection between CVH, Bangladesh and VCRI-MVC, India

Variables	Category level	No of observation (N=190)	Positive Case	Prevalence (%)	P value
Age	1-3 month	101	58	57.4	0.001
	4-6 Month	69	20	28.9	
	>6 month	20	2	10.0	
Sex	Male	95	33	33.7	0.040
	Female	95	47	49.4	
Breed	Indigenous	84	42	50.0	0.001
	Spitz	39	12	30.7	
	Lhasa	22	5	22.7	
	Doberman	30	12	40.0	
	GS	15	3	46.6	
Vaccination	Yes	83	11	13.2	0.001
	No	107	69	64.4	

Table 3 represents the data of comparative study of CPV associated risk factors. Notable that the prevalence of different risk factors associated with CPV infections was insignificant (P> 0.05) between CVH and VCRI-MVC.

Table 4. Different clinical signs observed among the CPV Infection in dogs

Variables	Category	No of observation (N=190)	Positive Case	(%)
Bloody Diarrhoea	Yes	105	100	90.4
	No	165	5	3
Vomition	Yes	110	104	94.5
	No	160	1	.6
Dehydration	Severe	105	90	85.7
	Moderate	100	10	10
	Mild	65	5	7.6

We also found that clinical symptoms varied in different cases where bloody diarrhea (90.4%), vomition (94.5%) and Severe (85.71%), moderatedehydration (10.0%), mild dehydration (7.6%)Were present in positive cases.

The study revealed an overall prevalence of CPV infection in suspected dogs in CVH, Bangladesh was 31.2%.

This was consistent with the single previous reportfrom Bangladesh that reported prevalence of canine parvovirus infection in street Dogs, at Mymensingh City, in Bangladesh was 30.0% (Islam et al., 2014).Overall prevalence of CPV infection in suspected dogs in VCRI-MVC, India was 42.1%. A Similar observation were reported previously where prevalence was 40.8% in India (Behera et al., 2015). . One would consider that prevalence of CPV infection is higher in India than Bangladesh .This might be attributed to the presence of endemic infection in the population under study at VCRI-MVC in India prevalence of CPV infection was higher among 1-3 month of age group is consistent with the findings by Vivek (2011). Again younger puppies (≤ 3 months) is mostly affected which might be due to the affinity of the virus being multiplying rapidly at intestinal crypt cells at the weaning age along with higher mitotic index(Aappel et al., 1978). Prevalence of CPV infection below 3 month of age puppies was insignificantly higher in VCRI, MVC than CVH. Once again in CVH, prevalence of CPV infection was slightly higher in male (36.3%) compared with female (25.0%). The study was in agreed with Islam et al. (2014). On the contrary female dogs were more susceptible (49.4%) than the male (33.7%) in VCRI-MVC and earlier reported by Umar et al. (2015). When specific breeds of dogs were considered, the prevalence of CPV disease was higher in exotic breed than local indigenous in CVH. Among the exotic breeds Spitz, GS, Doberman were more susceptible. Among the breeds in VCRI-MVC, The occurrence of CPV infection is significantly higher in Local indigenous than the exotic breeds which is supported by research findings of Shukla et al. (2009).

This study also exposed that among the exotic breeds GS, Doberman were higher in susceptibility than the others breed due to inherited immunodeficiency. It was supported by Singh et al. (2013), where CPV infection was highest in German shepherd (70%), followed by Doberman (55%). In CVH, Spitz was more susceptible due to its small size & most preferable breed in Bangladeshi people. In MVC-VCRI Local indigenous dogs were higher susceptible due to higher population density of this breed, poor vaccination and lack of awareness. Among exotic breed GS, Doberman were highly susceptible. In non-vaccinated dogs the prevalence was higher in compared with vaccinated one. The finding was consistent with finding of Godsall et al. (2010) where unvaccinated puppies aged between six weeks and six months are at greatest risk of developing CPV infection. The higher prevalence of CPV infection in Non-vaccinated dogs could be due to lack of protective immunity. In vaccinated dogs there was also presence of CPV infection might be due to, incomplete or ineffective primary vaccination course, or failure of vaccination at the some point.

The study was performed on the basis of tentative diagnosis by observing clinical signs and symptoms. The characteristics clinical signs of CPV infections were bloody diarrhea, vomition and dehydration. These findings were similar that reported by earlier investigators (Prittie 2004, Thomson & Gagnon 1987).

CONCLUSION

Canine parvovirus is an infectious and highly contagious viral disease of dogs in Bangladesh and India. Dogs of all age groups are infected but puppies age less than 3 month are highly susceptible than adults. Both male and female can be infected with CPV. Both indigenous and exotic breed are susceptible to CPV infection. The rate of infection is high in non-vaccinated than vaccinated dogs. Further molecular study from larger areas and samples can highlight the comprehensive information regarding CPV infection in this two neighboring countries.

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Surgical management of femoral fracture in cats by retrograde intramedullary pinning

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Abstract

Long bones such as femur and humerous fractures are common in all feline and domestic animals following substantial trauma. The present study describes the outcome of the femur fracture management in three cases of cats. Case 1- an eight months old intact male indigenous cat weighing 2.8 kg, limping in right hind limb, case 2- one and half years old intact male indigenous cat weighing 3 kgs, limping left hind limband case-3, a 5months old indigenous intact female cat weighing 1.8kg, limping left hind limb were brought to Teaching Veterinary Hospital, Chittagong Veterinary and Animal Sciences University (CVASU). Clinical examination revealed all three cases were active and alert but mild weight bearing limping, pain and crepitation also noticed in affected limb. Radiographic examination confirmed the case 1-right distal diaphyseal transverse femur fracture, case 2- left proximal multiple diaphyseal femur fracture and case 3-complete distal left femur fracture. On the basis of fracture patient assessment score (FPAS), all the 3 cases were decided for internal fixation by intramedullary pinning with or without wiring. Case-1 and case -3 retrograde intramedullary pinning, case 2-similar technique with 4 cerclage wires were performed with standard surgical approach and xylazine and ketamine anaesthesia. Postoperatively cats were managed by systemic antibiotic and painkiller with protective bandage. Mild weight bearing was observed in three cases on the 7th postoperative (PO) day and sutures were removed on same day without any wound complications. Postoperative at day 18, improved weight was observed clinically and secondary bone healing was noticed on radiographic examination. Pin migration and mild seroma was noticed on PO 3rd weeks in case no. 1 and 2 and mild pin migration also noticed in case no.3 after two week of surgery. Four months after PO bone remodeling was noticed and three patients maintained a comfortable life and owners were also very happy. The present case study suggests that intramedullary pinning with cerclage wires is an easy, economic and field based effective method for internal femur fracture fixation in cat.

Key Words: Long bone, femur, fracture, Intramedullary pin, Cats

Introduction

Long bone fractures are common and account for about 50% of all feline fractures and femur and humerus fracture is one of the most common of all long bone fracture in animal. The femur is the most affected bone (50.84%) then other bone in cat. (Cardoso, 2016) The maximum incidence of fracture in femur prone due to exposure to hind quarters of the major force of impact. Fractures in cats somewhat analogous to fractures in dogs, but anatomical differences need to keep in mind such as humerus and femur are straighter, supracondylar foramen in the humerus in which brachial vessels and median nerve pass (Harasen,2004).The primary aim of fracture treatment is to achieve the fastest possible healing and enable the patient to function normally by allowing early walking (Aron, 1998; Shahar, 2000). There are several techniques applied for fracture correction. Fixation methods include external coaptation, Intramedullary Pinning (IMP) (single pin, stacked pins), cerclage wire, external skeletal fixation with IMP, bone plates, lag screw, plate rod and interlocking nails (Scott, 2005, Scott and Mclaughlin 2007). Intramedullary Pinning is a popular method for long bone fracture repair in cats, often used to stabilize fracture of the humerus, femur and tibia (Boone *at al*., 1986). Complications associated with the use of pins are secondary to instability or inappropriate placement. Damage of soft tissues can occur if pins protrude excessively from the bone or migrate over time. Temporary or permanent damage to the sciatic nerve can occur from incorrect placement or migration of femoral IM pin. Delayed healing or nonunion can occur from lack of rigid fixation (Stiffer, 2004). The present study describes the outcome of intramedullary pinning technique in three cases of cats.

Case history and observation

Three indigenous cats of 8 months, 1.5 years, and 5months old weighing 2.8kg, 3.0kg and 1.8 kg, respectively were admitted to Teaching Veterinary Hospital, CVASU with the history of fall down and got pain on limb. Limping in right hind limb in case-1 and left hind limb in case-2 and case-3 for2 days. Clinical examination revealed that cat-1 was active and alert but mild weight bearing in right hind limb, cat-2 had non weight bearing in left hind limb and case 3 had mild weight bearing on left hind limb (Fig. 1, 2, 3). Pain, mild swelling and crepitation also noticed on palpation in affected limb. Blood parameters revealed normal values. Radiographic examination of case 1 confirmed the right distal transverse femoral fracture (Fig.4), case 2 left proximal multiple femoral fracture (Fig.5) and case 3 transverse one third distal diaphyseal fracture of left femur (Fig.6). On the basis of fracture patient assessment score (FPAS) and fracture management protocol, all three cases were decided for internal fracture management by intramedullary pinning with or without wiring.

Fig.1:Case-1: mild weight

Fig.2: Case-2: Non

Fig.3: Case-3: mild weight bearing

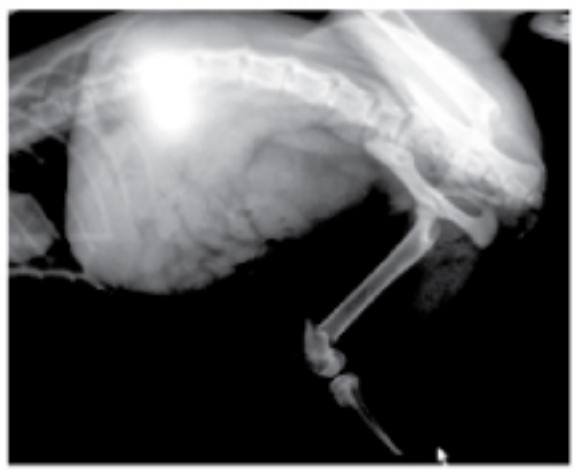

Fig.4: Case-1: Distal transverse fracture

Fig.5: Case-2: Proximal multiple fracture

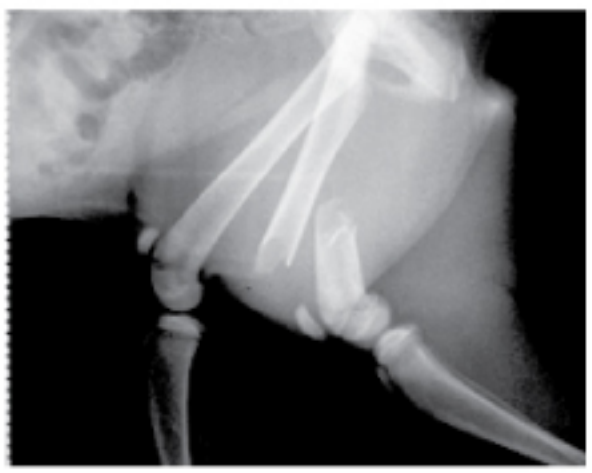

Fig.6: Case-3: Distal one third transverse fracture

Anaesthesia , surgical technique and postoperative care

Anesthesia was done by xylazine (1.0mg/kg body weight) and ketamine (10.0 mg/kg body weight) mixture by intramuscular route. In all cases retrograde IMP was performed by standard cranio-lateral approach (Fig.7) by using Steinmann pin and stainless wire. In addition 4 cerclage wires (22gauze) were used in cat-2 (Fig.11) and postoperatively, cats were managed by systemic antibiotic, antihistaminics and pain killer with protective bandage and restricted movement for one week. Postoperatively all cases were analyzed for weight bearing condition clinically and radiographic analyzed for fracture alignment, implant position and healing condition at different interval.

Fig.7: Surgical approach and draping the patient

Fig.8: Case-1: Distal diapyseal fracture

Fig.9: Case-2: Proximal multiple fracture

Fig.10: Case-3: One third distal tranverse fracture

Fig.11: Wiring the fractured fragments

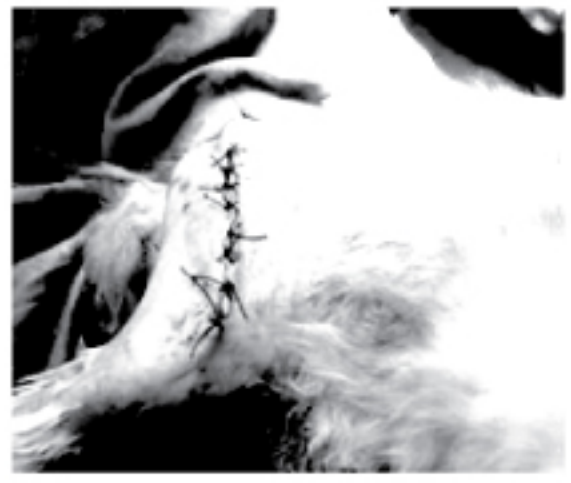

Fig.12: Closer the surgical wound

Results

Gradually improved weight bearing was observed on PO day 7, day 18 and day 30 (Fig.14). Surgical wound was also healed on PO day 7 and stitches were removed on the same day with no wound complication. Fracture alignment, reduction and implant position were normal post operatively (Fig.13). Secondary bone healing was noticed on PO day 18 (Fig.15) and 30 and finally at PO day 60. Seroma (Fig.16) and pin migration were noticed in all three cases on the proximal area of femur at postoperative day 18 and pin was removed in only case no. 3.

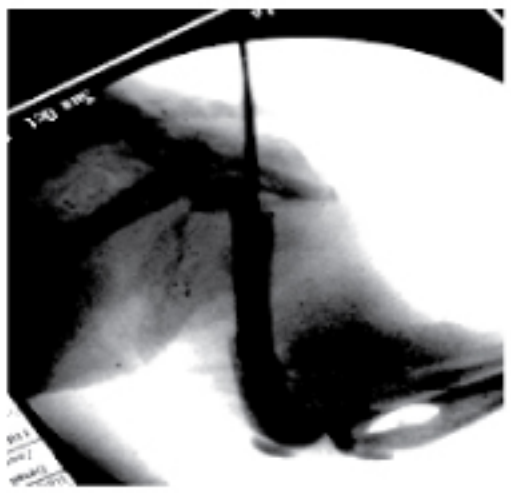

Fig. 13: Immediate post operative checkup case 2and 3

Fig.14: Case-1: PO 18 days- Weight bearing the effected

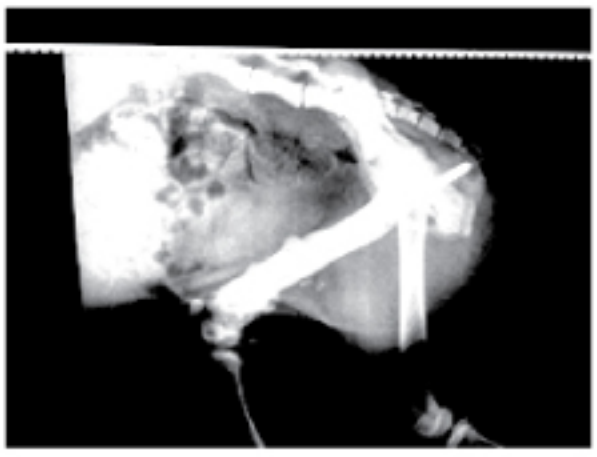

Fig.15. Secondary bone healing in case-3 (PO-18days) and case-2 (60days)

Fig.16: PO mild seroma formation 18days

Discussion

Post operative (PO) management is very important to prevent PO complications. Pin loosening, pin migration and seroma formation are very common complicationsof IMP technique (Denny and Butterworth, 2007; Arun *et al*, 2010). However, infection and non-union may alsooccur in open fracture. Appropriate pin selection is very important to prevent pin loosening and pin migration. Selection of appropriate pin depends on the medullary cavity of the bone to be repaired, the fracture configuration and application of ancillary methods of fixation. Pin diameters of 1.6 mm to 4.8mm are suitable to use in most cats and it should be big enough to fill at least 60 to 70% of the medullary cavity at its narrowest point. The present case study revealed that in all cases was well maintained and healed having appropriate gait and posture and no anatomical deformity. Finally the cats were jumping and playing normally.

Conclusion

The present case study suggests that IMP could be an easy, effective and economic method for the management of long bone fracture in cat.

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Aqua

Isolation, molecular identification and antibiotic susceptibility profile of *Aeromonas hydrophila* from diseased stinging catfish shing (*Heteropneustes fossilis*)

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Abstract

Fish play a crucial role in the Bangladeshi diet, providing more than 60% of animal source food, representing a crucial source of micro-nutrients, and possessing an extremely strong cultural attachment. In this study isolation and identification of *Aeromonas hydrophila* was done by studying cultural properties, gram staining and biochemical properties of isolates of Shing fish (*Heteropneustes fossilis*) of different upazillas of Mymensingh district. Antibiogram profile of the isolated bacteria was studied by using ten commercially available antibiotics. Quantitative study of bacteria isolated from diseased Shing fish showed variation of number in different organ. Total bacterial load was found to be 1.90×10^5, 1.19×10^5, 3.21×10^5, 2.18×10^6 and 3.14×10^5cfu/g in lesions; 2.52×10^7, 2.34×10^8, 5.41×10^8, 2.54×10^9 and 5.21×10^9cfu/g in liver; 2.54×10^8, 2.41×10^8, 1.90×10^7, 3.65×10^7 and 3.45×10^8cfu/g in spleen; 3.51×10^7, 5.28×10^7, 3.14×10^6, 1.85×10^7 and 4.52×10^7cfu/g in kidney in diseased Shing of Mymensingh sadar, Muktagacha, Tarakanda, Gouripur and Fulpurupazillas, respectively under Mymensingh districts. *Aeromonas hydrophila* was initially identified by their specific morphological, physiological and biochemical characteristics. Then molecular identification of *A. hydrophila* was done by PCR. PCR products of desired 760 bp were obtained for *A. hydrophila*. The results of the antibiotic sensitivity test is exhibited that most of the bacterial samples were sensitive against ciprofloxacin (92%) and levofloxacin (84%), intermediate resistant against gentamicin (40%) and resistant against novobiocin (84%), ampicillin (100%) and penicillin (92%). In conclusion, it is recommended that the farmers should be trained about basic fish health management techniques and there should be some support service for farmers about easy and proper diagnosis and treatment of common diseases of Shing fish.

Key Words: catfish; bacterial infection; bacterial load; *Aeromonas hydrophila*; PCR

Introduction

Aquaculture production in Bangladesh has got a considerable momentum in terms of production, nutritional supplement and livelihood improvement of a wide range of people. It has been progressively increased and its contribution to total fish production has gone up from 38.2% in 1998-99 to 52.9% in 2011-2012 (Hussain et al., 2014). Currently, Bangladesh ranked 4th position in leading aquaculture producing countries in the world just after China, India, Vietnam and Indonesia (FAO, 2013). Sing (*Heteropneustes fossilis*) is considered as one of the high value species and can benefit fish farmers because of their high productivity and profitability. This fish is locally known as Shingi or shing. It is considered to be highly nourishing, palatable and tasty and well preferred because of its less spine, less fat and high digestibility in many parts of Indian subcontinent (Khan et al., 2003). The species is very high content of iron (226 mg 100 g^{-1}) and fairly high content of calcium compared to many other freshwater fishes. Due to high nutritive value the fish is recommended in the diet of sick and convalescents. Being a lean fish it is very suitable for people for whom animal fats are undesirable (Rahman et al., 1982). But now a day's indiscriminate destructive practices have caused havoc to aquatic biodiversity in Bangladesh (Hussain and Mazid, 2001). Presently, *H. fossilis* is one of the threatened fish in Bangladesh (IUCN Bangladesh, 2000). Prevalence of disease is considered as one of the major reasons for poor fish production of the country. It is a great threat to achieve optimum production and become a

limiting factor to economic success of aquaculture in Bangladesh. A total lack of mucus, edema, enlarged abdomen, growth of nodules on the body surface, presence of yellow, white or black spots on the skin, prolapsed anus and exophthalmia are all clinical signs of fish diseases (Plumb, 1994).

Only few studies have been conducted on the health and diseases of *H. fossilis* Bangladesh. Hasan (2007) reported that an established shing fish farm was found tobe heavily affected by ulcer type disease in Mymensingh area and by investigation, *Aeromonas hydrophila* were isolated from the lesion. *H. fossilis* has been reported to be affected bysome bacterial diseases (Sahoo and Mukherjee, 1997). Sahoo et al. (1998) found anoutbreak of ulcer diseases in an organized farm having 10% of *H. fossilis* populationwith round ulcers on body surface. During the outbreaks of EUS in late 80s, *H. fossilis* was found to be one of the most severely affected species along with other air-breathing fishes (Barua et al., 1994; Roberts et al., 1994). It is still very vulnerable to EUS and other diseases. If this situation continues, these endemic fishes will be endangered in near future. There is also a sever lack of information regarding disease outbreak of *H. fossilis* under culture condition. Investigation on the diseases in *H. fossilis* is thus essential for developing its sustainable culture technology. With the countrywide rapid expansion of farming of *H. fossilis*, there has been reports of serious disease outbreaks and mortality of this fish under culture condition (Dehadrai et al., 1998). As a result farmers are suffering from huge financial loss every year. They are getting frustrated and loosing in culturing in this fish. Farmers in Mymensingh area are in need for proper health management suggestion and appropriate treatment. Control of aquatic disease is complex and relies heavily on a combination of pathogen detection, disease diagnosis, treatment, prevention and general health management. Sustainable aquaculture production can only occur when fish are healthy and free from disease. Therefore, there is an urgent need to give immediate attention to investigate diseases of *H. fossilis* and develop appropriate health management strategies. Therefore, this study was designed with a view to isolate and identify *Aeromonas hydrophila* from diseased stinging catfish Shing *(H. fossilis)* and to determine the antimicrobial resistance patterns of the isolated bacteria

Materials and Methods

Selection of fish farms and study area

Different Shing (*H. fossilis*) farms of Muktagacha, Tarakanda, Gouripur, Fulpur, Sadar upazillas under Mymensingh district located at 24°38′3″N 90°16′4″E of Bangladesh were selected to collect infected Shing fish samples for isolation and identification of *Aeromonas hydrophila* to evaluate their antimicrobial resistance patterns.The study was conducted one year starting from July, 2013 to June, 2014.

Fish sample collection

Samples were collected from a total of 20 afflicted Shing farms depending on availability of diseased fish from the study area in which Shing (*H. fossilis*) were suffering from either suspected EUS lesion or suspected MAS disease symptoms exhibited by reddish hemorrhagic external lesions and were having mortality. Moribund fishes were collected in clean sterile boxes containing ice packs and then transported to Fish Disease and Health Management Laboratory of Bangladesh Fisheries Research Institute, Mymensingh. The clinical signs and postmortem alterations were recorded according to Rashid (2008) and Ahmed et al., 2009.

Bacteriological examination

Specimens from diseased Shing's skin, liver, spleen and kidney were inoculated on trypticase soya agar (TSA) plates and then incubated at 30°C for 24 hrs. The isolated bacteria were identified according to their biochemical characteristics (Sabur et al.,2006; Narejo et al., 2005).

Clinical observation

Collected fish were examined to observe their external lesion, injury or any other abnormalities and were recorded properly.

Total viable count of bacteria

Two fish from each district were subjected to bacteriological investigation as follows. At first each fish was examined for its clinical sign of disease and disorders. A drop of blood was dissolved on TSA plate for colony counting. The fish was dissected immediately after clinical examination. The portions of kidney, liver and intestine were removed, weighed on an electric balance and kept in a sterilized pastel mortar for crushing. Each organ was crushed with physiological saline in the ratio of 0.1 g of organ: 0.9 ml of PS to make stock solution. Eight decimal dilutions were prepared by transferring 0.1 ml from the earlier test tube to the next. These eight tubes were designated as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5},10^{-6}, 10^{-7} and 10^{-8}. Two samples of 0.1 ml from 10^{-2} and 10^{-3} in case of liver and kidney; 10^{-3} and 10^{-4} in case of intestine were transferred to AIM medium to get only the colonies of *Aeromonas.* Another two samples of same dilution were transferred to TSA for total bacterial colony counting.

Calculation of total bacterial load

Total bacterial load of each organ was calculated using the following formula used by Rashid et al. (2008).

$$\text{Total bacterial load} = \frac{\text{Avarage number of colonies on plates}}{\text{Dilution factor} \times \text{Volume plated}}$$

Identification of *Aeromonas* spp.

Identification of *Aeromonas* spp. was done based on detailed morphological, physiological and biochemical characterization of the isolates. At first, the bacteria were sub-cultured onto TSA plates to obtain fresh 24 hours culture. They were then streaked onto the selective *Aeromonas* isolation medium for preliminary identification of the genus *Aeromonas* and discarding the others. Colonies grown on the selective medium were sub-cultured again onto TSA plates and subjected to biochemical tests using commercially available media after autoclaving at 121°C for 15 minutes.

Motility test

For motility test young and actively growing culture of the bacteria were collected from 24 h culture at 25°C. A single colony was mixed with 3 ml of PS. A drop of the suspension was taken on clean glass slide, covered with cover slip and placed under a luminous microscope. Bacterial motility was observed in a TV screan, adjusted with the microscope (OLYMPUS, Model CHS, Japan).

Physiological characterization

Physiological characters were studied by observing the growth of each isolate at temperature of 4°C, 37°C and 40°C. Growth of each isolate was observed in different concentrations of NaCl as 0%, 1%, 2%, 3%, 3.5% and 4%.

Biochemical characterization

Several biochemical tests were performed to evaluate the biochemical behavior of isolated bacteria. Biochemical tests are Oxidase, Catalase, Oxidative-fermentative test, 0129 test, Esculin hydrolysis test, Acid and gas production from sugars: Glucose,Lactose, Sucrose, Manitol; Methyl-Red (MR) test, Voges-Proskauer (VP) test, Indole and H2S production, Decarboxylase test, Citrate utilization test.

Molecular dectection of *Aeromonas hydrophila*

The genomic DNA was isolated asper the protocol described by Swaminathan et al.(2004). A single colony was inoculated in10 ml of Nutrient broth (NB) and grownat 29°C overnight. Culture wascentrifuged at 5000 rpm for 10 minutes. Four hundred microlitre of solution I (50mM Tris.HCl pH-8.0, 50mM EDTA pH-8.0, 25% sucrose, 1mg lysozyme), was added to the washed cell pellet and gently mixed and incubated at 37°C for 15 minutes. Thereafter 400ml of solution II (10mM Tris. HCl pH 8.0, 5mM EDTA pH-8.0, 1% SDS, 40µg Proteinase K) was added to the cells and incubated at 55 °C for three hours. The suspension was centrifuged at

6000 rpm for 10 minutes. The aqueous layer from the top was removed carefully to avoid any protein debris and transferred to a fresh microfuge tube. Double amount of chilled ethanol was added to aqueous phase so as to precipitate the DNA. The DNA was pellted by centrifugation at 12000 rpm for 10 minutes. The pellet, washed with 70% ethanol was dried and dissolved in 100 µl of TE buffer (pH 7.6). Primers used for the amplification of DNA are shown in Table 1. PCR was done as per the method described previously by Narjeo et al. (2005). Amplification was performed with a DNA thermal cycler (Mastercyclear, Eppendorf, Humburg, Germany) with some modifications as follows: The reaction mixture consisted of 1µl of Taq polymerase (1unit), 5 µl of 10X PCR amplification buffer (100 mM Tris-Hcl, 25 mM MgCl2, 500mM KCl, pH-8.3), 3 µl of deoxynucleoside triphosphate (100µM), 0.5 µl of each primer (100 pmoles) and double distilled water upto a final volume of 50 µl. A total of 40 PCR cycles were run under the following conditions: Initial denaturation at 94^0C for 4 minutes, denaturation at 94^0C for 1 minute, primer annealing at 65^0C for 1 minute, DNA extension at 72^0C for 1.5 minutes and final extension at 72^0C for 5 minutes.

Table 1. List of primers used for *Aeromonas hydrophila* genome detection.

Primers	Sequences (5′ – 3′)	Amplicon Size (bp)	Reference
Forward Primer	5′-AACCTGGTTCCGCTCAAGCCGTTG- 3′	760	Nerjeo et al., 2005
Reverse Primer	5′-TTGCCTCGCCTCGGCCCAGCAGCT- 3′		

Antibiogram profile of *Aeromonas hydrophila*

All bacterial isolates were tested for their sensitivity to ten commercially available antibiotics by the disc diffusion method (Bauer et al., 1966). The antibiotics, their codes and concentrations were as follows: ampicillin (10 µg), ciprofloxacin (5 µg), gentamicin (10 µg), oxytetracycline (10 µg), penicillin (10 µg), tetracycline (30 µg), levofloxacin (5 µg), azithromycin (10 µg), chlorotetracycline (25 µg), novobiocin (5 µg). Tested bacterial strains were classified into three categories: sensitive, intermediate, and resistant and depending on the diameters of inhibition zones and standards supplied by Himedia Laboratories and comparing with other related references (Table 2). All tests were carried out in Fish Diseases and Health Management Laboratory of Bangladesh Fisheries Research Institute (BFRI), Mymensingh.

Table 2. Interpretation standards for disc diffusion susceptibility testing for *Aeromonas hydrophila* (CLSI, 2012).

Sl. No.	Name of Antimicrobial agent	Disc concentration	Interpretation of results (zone in diameter in mm)		
			R	I	S
1	Ciprofloxacin	5 µg	≥ 16	12 – 15	≤ 17
2	Levofloxacin	5 µg	≥ 22	17 – 21	≤ 23
3	Gentamicin	10 µg	≥ 14	10 – 13	≤ 15
4	Azithromycin	15 µg	≥ 16	11 – 15	≤ 17
5	Tetracycline	30 µg	≥ 14	09 – 13	≤ 15
6	Oxytetracycline	10 µg	≥ 15	11 – 14	≤ 16
7	Chlorotetracycline	25 µg	≥ 16	13 – 15	≤ 17
8	Novobiocin	5 µg	≥ 17	14 – 17	≤ 18
9	Ampicillin	10 µg	≥ 22	16 – 21	≤ 23
10	Penicillin	10 µg	≥ 14	10 – 13	≤ 15

Sl = Serial, No. = Number, µg = Microgram, mm = Millimeter, S = Susceptible, I = intermediately resistant, R = Resistant, ≥ = Greater than or equal to, ≤ = Less than or equal to.

Results

Clinical and post mortem findings

The clinical examination of diseased Shing (*H. fossilis*) exhibited: loss of equilibrium, slight lesion on body, body and tail erosion, hemorrhage in base of fin and edge of head, move with whirling and heavy mortalities of fish occur shortly after the advent of lesions. Congestion and enlargement in internal organs were appeared in postmortem examination.

Bacterial load in skin lesions, liver, spleen and kidney

Bacterial load in skin lesions, liver, spleen and kidney of infected Shing (*H. fossilis*) are calculated and then observed to have a variation. Total bacterial load was found to be 1.90×10^5, 1.19×10^5, 3.21×10^5, 2.18×10^6 and 3.14×10^5 cfu/g in lesions; 2.52×10^7, 2.34×10^8, 5.41×10^8, 2.54×10^9 and 5.21×10^9 cfu/g in liver; 2.54×10^8, 2.41×10^8, 1.90×10^7, 3.65×10^7 and 3.45×10^8 cfu/g in spleen; 3.51×10^7, 5.28×10^7, 3.14×10^6, 1.85×10^7 and 4.52×10^7 cfu/g in kidney in diseased Shing of Mymensingh sadar, Muktagacha, Tarakanda, Gouripur and Fulpur upazillas, respectively under Mymensingh districts.

Morphological, physiological and biochemical test results

The isolated *Aeromonas hydrophila* fromdiseased Shingwas finally identified by their specific morphological, physiological and biochemical characteristics. They were Gram negative, rod shaped, motile bacteria, positive for oxidase and catalase test. They fermented glucose and were resistant to vibriostatic agent 0129 test. The results of morphological, physiological and biochemical tests are presented in Table 3.

Table 3. Results of biochemical characteristic of isolated bacteria.

Characters	Characterization by Mostafa et al. (2008)	Characterization by Sabur (2006)	Present result
Gram stain	-	-	-
Shape	Rod	Rod	Rod
Motility	+	+	+
0129	ND	ND	-
Oxidase	+	+	+
Catalase	+	+	
OF test	F	F	F
Glucose	+	+	+
Lactose	+	+	+
Sucrose	+	+	+
Maltose	+	+	+
Manitol	+	+	-
Inositol	-	-	-
Sorbitol	-	-	-
Rhamnose	-	-	-
Esculin hydrolysis	+	+	+
Methyl-red test	-	-	-
Voges-Proskaur	+	+	+
Indole	+	+	+
H2S production	+	+	-
Arginine decomposition	+	+	+
Lysine decarboxilation	-	-	-
Ornithine decarboxilation	-	-	-
Citrate utilization	+	+	+
TSI	ND	ND	'K' in slants but 'A' in butt
Growth at: 4°C	-	-	-

5°C	+	+	+
37°C	+	+	+
40°C	-	-	-

+: Negative; -: Positive; F: Fermentative; K: Alkaline; A: Acid, ND: Not done

Molecular detection of *Aeromonas hydrophila*byPCR

PCR products of desired size 760 bp were obtained in reaction mixture containing genomic DNA of the targeted organisms, *A. hydrophila* (Figure 1). In this study, all isolates gave specific amplification. No product was detected in negative control (Figure 1).

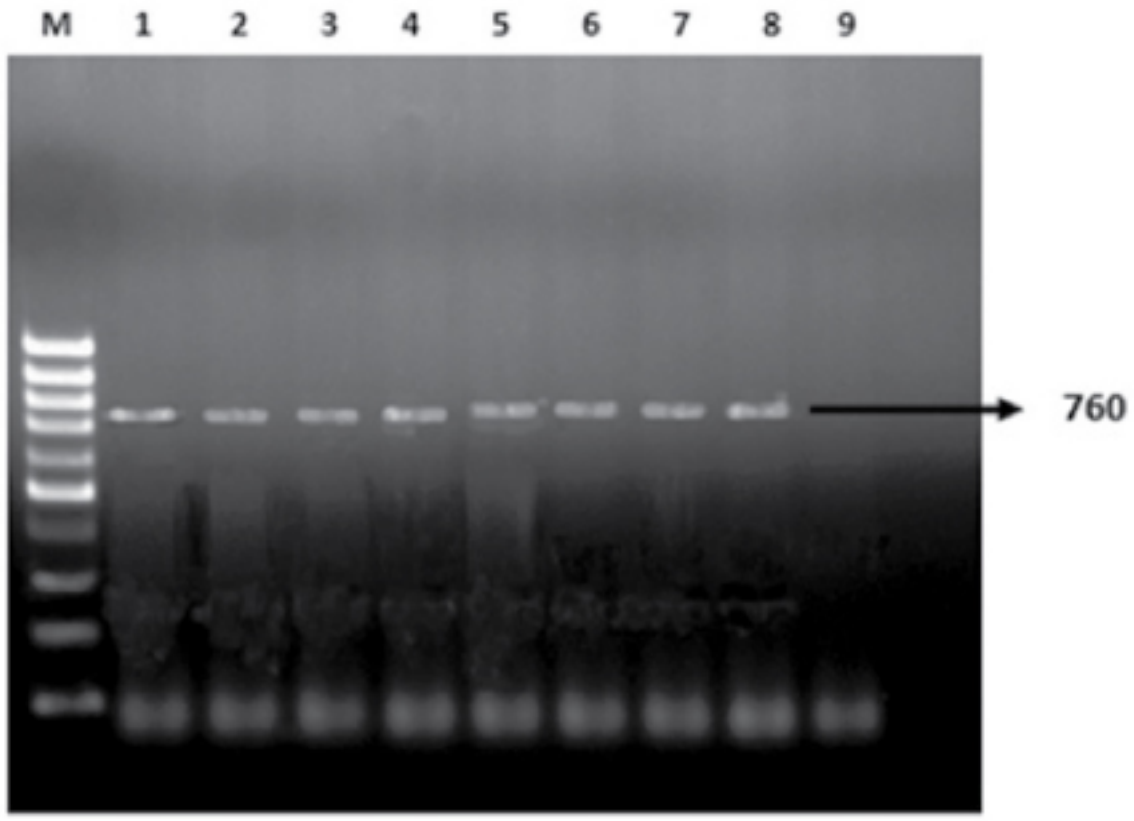

Figure 1. Agarose gel electrophoresis of PCR amplification generated by *Aeromonas hydrophila*. Lanes: (M) 100 bp DNA marker; (1-8) positive samples from field; (9) negative control.

Antibiotic sensitivity test

The isolated *Aeromonas hydrophila* were tested against ten commercially available antibiotics and the results of their sensitivity are presented in Table 4. Most of the bacterial samples were sensitive against ciprofloxacin (92%) and levofloxacin (84%), intermediate against gentamicin (40%) and resistant against novobiocin (84%), ampicillin (100%) and penicillin (92%).

Table 4. Antibiogram profile of isolated *Aeromonas hydrophila* (n=25).

Antibiotics	No (%)		
	Sensitive	Intermediate	Resistant
Ciprofloxacin (5µg)	23 (92)	2 (8)	0 (0)
Levofloxacin (5µg)	21 (84)	4 (16)	0 (0)
Gentamicin (10µg)	15 (60)	10 (40)	0 (0)
Azithromycin (15µg)	12 (48)	8 (32)	3 (12)
Tetracycline (30µg)	4 (16)	12 (48)	9 (36)
Oxytetracycline (10µg)	1 (4)	10 (40)	14 (56)
Chlortetracycline (25µg)	3 (12)	13 (52)	9 (36)
Novobiocin (5µg)	0	4 (16)	21 (84)
Ampicillin (10µg)	0	0	25 (100)
Penicillin	0	2 (8)	23 (92)

Discussion

The clinical and post mortem findings of the diseased Shing fishes in this study is quite in consonance with those that reported by Ahmed et al., 2009 and Chandra et al., 1994. The *Aeromonas hydrophila* bacteria was isolated from diseased fish from different locations such as Mymensingh sadar, Muktagacha, Tarakanda, Gouripur and Fulpur upazillas. Total bacterial load was found to be 1.90×10^5, 1.19×10^5, 3.21×10^5, 2.18×10^6 and 3.14×10^5 cfu/g in lesions; 2.52×10^7, 2.34×10^8, 5.41×10^8, 2.54×10^9 and 5.21×10^9 cfu/g in liver; 2.54×10^8, 2.41×10^8, 1.90×10^7, 3.65×10^7 and 3.45×10^8 cfu/g in spleen; 3.51×10^7, 5.28×10^7, 3.14×10^6, 1.85×10^7 and 4.52×10^7 cfu/g in kidney of diseased shing fish of different upazillas of Mymensingh district consecutively. Rashid et al. 2008 and Hasan et al., 2007 found 1.67×10^4 to 6.4×10^8 CFU/g, 1.71×10^3 to 1.18×10^9 CFU/g and 1.47×10^4 to 3.70×10^8 CFU/g of bacteria in liver, kidney and intestine of naturally infected Thai pangas respectively, those findings is partially similar with our study. Allison (2007) isolated *A. hydrophila* from Thai pangus, the bacterial load was found 2.6×10^6 to 3.6×10^7 CFU/g in liver, 4.8×10^6 to 7.2×10^7 CFU/g in intestine and 2.4×10^3 to 3.70×10^6 CFU/g in kidney, this is also in consonance with our study. Rahman and Chowdhury (1996) isolated *A. hydrophila* from kidney of carp fishes, total load of bacteria varied in the kidney of different sampled fishes were 2.6×10^5 to 1.7×10^6 CFU/g. Here the variations might be caused by different factors like temperature, p^H, chemical and gaseous composition etc. that influences the disease incidence. Ahmed (2009) was found total bacterial load to be 2.45×10^3 (koi) in blood and 8.70×10^6 (koi) CFU/g in intestine these findings are almost similar to our study. The morphological and physiological characteristics of *A. hydrophilla* observed in this study was partially in consonance with those that found by Mostafa et al., 2008 and Islam et al., 2008. Hussain et al. 2014 also found focal necrosis haemorrrhages in the liver tissue, atrophy of the renal tubule in kidney and villi missing in intestine from the naturally infected shing fish by *Aeromonas hydrophila* in a mixed infection with the *Aphanomyces invadans* elicited EUS disease.

The biochemical characteristics of the isolated *A. hydrophilla* in this study are quite in consonance with those that reported by Mostafa et al., 2008 and Saburet al., 2006. For the molecular detection of the bacterial causative agent of Shing fish diseases PCR was done by using gene specific primer according to Hasan et al., 2007. The antibiogram profile of different antibiotics against *Aeromonas hydrophila* was found similar to those that previously reported by Hussain et al., 2014, Sobur et al., 2006 and Mostafa et al., 2008. The result of this study will be beneficial for the fish farmers who are regularly culturing catfish for diagnosing and controlling diseases by the administration of specific antibiotics. Future research scopes are the pathogencity test of the bacteria for homologous susceptible fishes, identification of pathogenicity island in chromosome, production of antibiotics against *Aeromonas hydrophila*, serotyping of all *A. hydrophila*isolates.

Conclusions

The present study was conducted to identify the *Aeromonas hydrophila* from of catfish (*H. fossilis*). In addition, clinical and bacteriological studies were carried out to examine disease status of cultured Shing fish of Mymensingh district. According to the farmer's opinion, the disease occurring seasons were early and late winter and frequency of disease occurrence was 1 to 2 times in a year. Shing was found to have high mortality rate. About 80-90% mortality occurs due to diseases. Massive pathological changes were found in different organs of diseased sample. Application of lime and salt in pond were the most common treatment followed by the use of antibiotics, potassium permanganate and copper sulphate. This study also identified fish health management problems which included poor understanding on fish disease and health management, lack of suitable therapeutics and their appropriate uses and lack of assistance regarding disease treatment. Therefore, more precautionary measures need to be taken at the onset of winter season to prevent diseases. So, attention should be drawn to maintain appropriate ambient for rearing Shing fish to avoid common diseases.

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Genetic diversity and phylogenetics in *Puntias sarana* and *Barbodes gonionotus* fish populations of Bangladesh

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Abstract

Two minows, indigenous olive barb, *Puntius sarana* and exotic silver barb, *Barbodes gonionotus* are in least concern fish species, respectively. However, it is essential to identify diverse population of these fish species for selective breeding programme. Sixty olive barb fish were collected from three different natural stocks (Mymensingh, Madaripur and Sylhet) and twenty silver barb fish were collected from hatchery stock of Jessore in Bangladesh. Twenty individual fish from each population were subjected to genetic variation analysis by RAPD (Randomly Amplified Polymorphic DNA) markers. Out of forty primers tested, five decamer primers (producing best quality resolution of band pattern) were selected for the Polymerase Chain Reaction (PCR) based RAPD analysis. A total of 43 of polymorphic bands were obtained by the five primers and the highest proportion of polymorphic bands (62.79%) was found in the Madaripur populations. The gene diversity (0.2132±0.2067) and Shannon's Information index (0.3161±0.2950) within populations were highest in Jessore stock representing higher genetic diversity in silver barb stock. Among olive barb stocks, these values were higher in Sylhet stock than other two stocks. Besides, four populations segregated in two main clusters based on the Nei's genetic distance. The silver barb stock made one separate cluster while other three stocks of olive barb remained in another cluster. Though remain in the same cluster less diversity was found in populations obtained Madaripur and Mymensingh. The present study exposed a distinct pattern of genetic variation and phylogenetic relatedness that would be helpful in selecting brood fish for genetic improvement as well as in conservation of these fish species.

Key words: Genetic Diversity, Minnows, RAPD primer, Phylogenetic tree

Introduction

Olive barb, *Puntius sarana* (Hamilton, 1822) and silver barb, *Barbodes gonionotus* (Bleeker, 1850) are two important minows which are tropical and small freshwater fish belonging to the family Cyprinidae. The olive barb is a widely distributed cyprinid in the inland waters of South-East Asia (Jena *et al*., 2007) and is used both as food fish and ornamental fish. This species is omnivorous and feeds on aquatic weeds, algae, protozoan, mud and sand (Mookerjee *et al.,* 1946). Owing to habitat fragmentation, injudicious usage of fertilizers in agricultural fields and their effluents mixing to water bodies and over exploitation of water resources have reduced natural abundance of olive barb populations to the extent of placing it under vulnerable group in India (Mijkherjee *et al*., 2002 and Chakraborty *et al*., 2003) and least concern in Bangladesh (IUCN 2017). On the other hand, silver barb (Thai sarpunti), *B. gonionotus*, a herbivorous fish (Haroon and Pittman, 1997), has become increasingly popular in Bangladesh following its introduction from Thailand, due to its rapid growth and attaining marketable size in 3-4 months (Islam *et al*., 1998) and having bright silvery appearance and good taste. It has occupied a prime position in aquaculture scenario of Bangladesh within a short time after introduction. Among all the exotic fish species it is considered to be one of the best suitable species for aquaculture due to its better palatability, high yield potential and market demand.

Genetic conservation and brood stock selection of a fish species may be helpful for good practice by breeders and hatchery owners. In these aspects, genetic methods have great potential to distinguish populations of fish species that cannot be identified by morphological and meristic characters (Cadrin *et al*., 2005). Data on genetic variation reflects the genetic condition of a species that can be used in designing proper selective breeding program for genetic improvement. DNA markers are realistic and useful tools for investigation and monitoring of genetic conditions both in natural populations and in captive stocks. PCR (Polymerase Chain Reaction) based RAPD (Randomly Amplified Polymorphic DNA) technique is very simple and quick to perform, and the most important one makes it simple from others technique that genome specific sequence of the target organism is not required to design primers and only single primer is sufficient to amplify analyzable DNA fragments. The objective of this research is to characterize different populations of *P. sarana* and one stock of *B. gonionotus* at molecular level and to indicate phylogenetic relatedness among the stocks of minnows in Bangladesh.

Materials and Methods

Collection of fish sample and isolation of genomic DNA

Indigenous olive barb fish samples were collected from three sources namely Mymensingh, Madaripurand Sylhet (Hakaluki Haor). The silver barb fish samples was collected from a hatchery stock in Jessore. All the samples were collected from July 2015 to March 2016. For individual fish, a small piece of caudal fin was cut with scissors and preserved in 95% alcohol. Twenty fish samples from each stock were used in the research. Genomic DNA was isolated according to the method described by Alam and Islam (Alam and Islam, 2004) with some modifications.

Primer selection and PCR amplification

Primarily forty decamer primers of random sequence (Bioserve Biotechnologies India Pvt Ltd. ACGI Company) were screened with one sample from each stock to test the performance of RAPD primers and resolution of bands generated. Finally five primers exhibiting highest quality banding patterns and sufficient variability were selected for analysis of all samples. DNA amplifications were performed in a final volume of 25 µl containing 12.5 µl Master mix (Taq DNA Polymerase 2X-premix, GeneON), 2µl of template, 2 µl of primer and 8.5 µl of nuclease free water. The amplification was carried out in thermal cycler (Prime G Thermal Cycler, UK) programmed for initial heat denaturation in one step of 2 minutes at 94ºC. Subsequent 45 cycles of 1 minute at 94ºC, 30 seconds at 34ºC or 42ºC (OPA12: 38ºC, OPA17: 40ºC, OPB03: 34ºC, OPB07: 36ºC, OPB20: 42ºC) and 2 minutes at 72ºC; followed by one final step of primer extension at 72ºC for 7 minutes.

Agarose gel electrophoresis of PCR products

PCR products (10 µl) was subjected to electrophoresis in 1.5% agarose gel containing ethidium bromide on 1X TBE buffer at 100 V for 1 hour. Molecular marker (1 Kb Plus) was used alongside of the samplel. Finally, the DNA bands were observed on a GelDoc system and photographs were saved.

RAPD data Analysis

The obtained bands were scored separately on the basis of their presence (1) or absence (0) for each sample and primers. For accuracy, the scoring was done by two persons independently. The total scores were then used to create a single data matrix to estimate the proportion of polymorphic loci, Nei's gene diversity (Nei, 1973), gene flow and Nei's genetic distance (Nei, 1972) and to construct an unweighted pair group method of arithmetic mean (UPGMA) dendrogram among stocks with 1,000 simulated samples using the POPGENE (Version 1.31) program (Yeh,*et al*., 1999). Band sizes of the RAPD marker were estimated by using the software DNAfrag (Version 3.03) (Nash, 1991).

Results and Discussion

A total of 43 bands were scored of which 43 were polymorphic (100% polymorphism) across all populations

(Table 1). Number of bands ranged from 7 to 10 with an average 8.6 per primer. The highest (62.79%) polymorphic loci were found in Madaripur population and the lowest (51.16) in Mymensingh populations both of which are natural populations of olive barb (Table 2). The gene diversity was highest (0.2132±0.2067, Mean± SD) in Jessore followed by Sylhet (0.2021±0.2134), Madaripur (0.1850± 0.1883) and the Mymensingh (0.1597±0.1798) populations (Table 2).The Shannon's Information index was also highest (0.3161±0.2950) in Jessore stock. In this research, the highest gene flow was found between Madaripur and Mymensingh populations and the lowest between Sylhet and Jessore populations (Table 3).

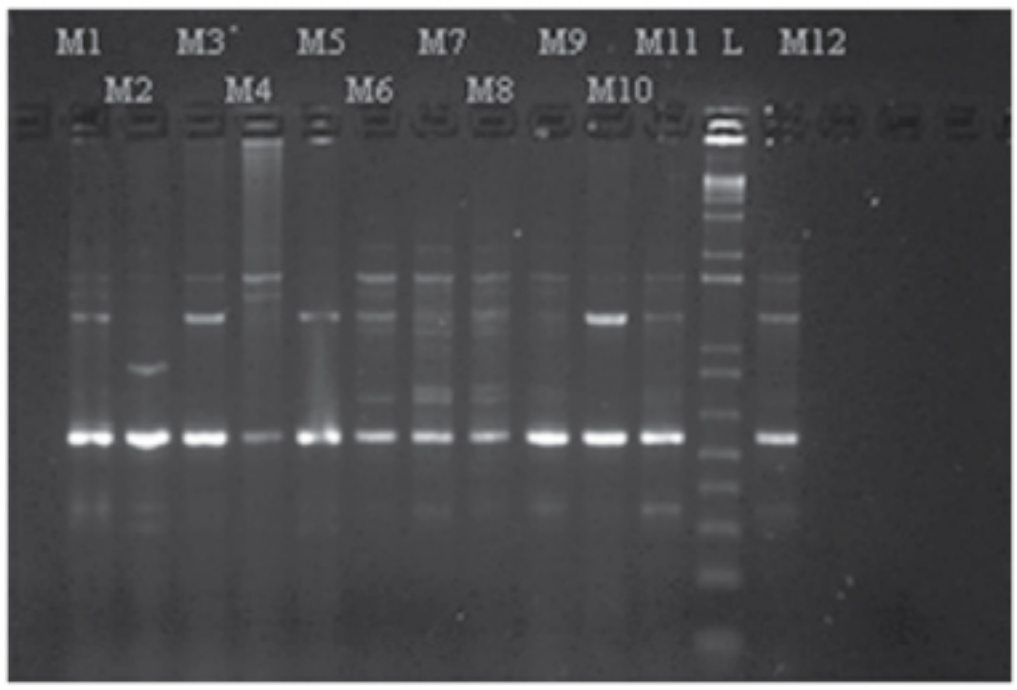

Figure 1: Band pattern of olive barb population collected from Madaripur using OPB 03 primer

Table 1: Number and percentage of polymorphic loci of each RAPD marker for four populations of *P. sarana*

Primer name	Sequence	No. of score able bands	Size range of the bands	No. of polymorphic loci	Polymorphism %
OPA012	TCGGCGATAG	8	1693-4717 bp	8	100
OPA017	GACCGCTTGT	9	1280-4956 bp	9	100
OPB03	CATCCCCCTG	10	808-3365 bp	10	100
OPB07	GGTGACGCAG	7	336-2285 bp	7	100
OPB20	GGACCCTTAC	9	195-2656 bp	9	100

Table 2: Number and percentage of polymorphic loci of each population of *P. sarana* across all RAPD markers

Population name	No. of polymorphic loci	% of polymorphic loci	Gene diversity (Mean± SD)	Shannon's Information index(I)
Sylhet	25	58.14	0.2021±0.2134	0.2994±0.2991
Madaripur	27	62.79	0.1850± 0.1883	0.2856± 0.2676
Mymensingh	22	51.16	0.1597±0.1798	0.2468±0.2649
Jessore	25	58.14	0.2132±0.2067	0.3161±0.2950

Table 3: Gene flow between populations analyzed across five RAPD primers

	Sylhet	Madaripur	Mymensingh
Madaripur	1.7514		
Mymensingh	2.3537	3.8236	
Jessore	0.8797	1.2449	0.9944

The genetic distance between Sylhet and Jessore populations was highest (0.3537) and was lowest (0.0559) between Madaripur and Mymensingh populations (Table 4). Both the values for genetic identity and gene flow between Madaripur and Mymensingh populations were highest. Based on the Nei's genetic distance, the UPGMA dendrogram indicated the segregation of all four population into two main clusters (Figure 2). The three natural and indigenous barb (Sylhet, Mymensingh and Mdaripur) population made one cluster and exotic silver barb (Jessore) remained in another cluster. The Madaripur and Mymensingh populations made one sub cluster and Sylhet population belonged to another sub cluster.

Table 4: Nei's (1972) genetic identity (above diagonal) and genetic distance (below diagonal).

	Sylhet	Madaripur	Mymensingh	Jessore
Sylhet	****	0.8630	0.9064	0.7021
Madaripur	0.1473	****	0.9456	0.8005
Mymensingh	0.0982	0.0559	****	0.7699
Jessore	0.3537	0.2226	0.2615	****

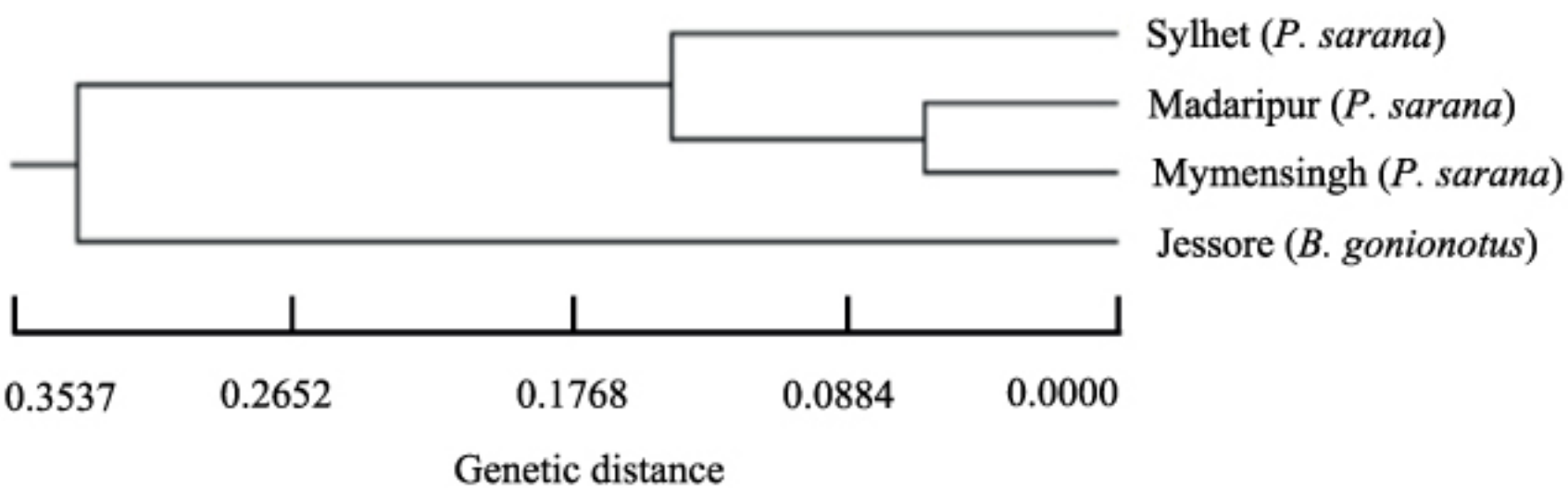

Figure 2: Phylogenetic dendrogram constructed based on Nei's (1972) genetic distance analyzed by RAPD markers

The *Puntias sarana* is a small indigenous species (SIS) in Bangladesh. Once the fish was available in natural shallow water bodies including rivers, *beels, haors* in Bangladesh but with increase of pollution, overharvesting, introduction of alien species makes it least concern. Information on the genetic structure of fish species is useful for optimizing identification of stocks, stock enhancement, breeding programs, management for sustainable yield and preservation of genetic diversity (Dinesh *et al*., 1993; Garcia and Benzie, 1995; Tassanakajon *et al*.,1997, 1998). Besides, the exotic thai silver barb became a vital candidate for aquaculture in Bangladesh due to its high and fast growth rate over the indigenous olive barb. RAPD fingerprinting offers a rapid and efficient method for generating a new series of DNA markers in fishes (Foo *et al*., 1995). DNA polymorphisms have been extensively employed as a means of assessing genetic diversity in aquatic organisms. In the present study polymorphism was 100% across all the primers. The highest proportion (62.79%) of polymorphic band was found Madaripur natural population. Akter *et al.* (2010) found

43.34% polymorphic loci in a hatchery stock of olive barb where the brood fish were collected from natural sources (a haor of Sunamgonj district) and the samples were first generations. Kabir *et al.* (2014) reported 53.84% polymorphism by RAPD marker analysis in three populations of *P. sarana* which were collected from natural sources where the primers were different from our study. The mean number of polymorphic loci was (26.6) found by Parvez *et al.* (2015) in the study of stock genetic variation of natural stock of critically endangered *P. sarana* through Allozyme electrophoresis. The higher polymorphic loci (42.31%) was repored in natural population of *Labeo kalbasu* by Mostafa *et al.* (2009) used by RAPD marker. In this study we also found the higher polymorphic loci in natural stock. The gene diversity was found to be higher (0.2132±0.2067) in the Jessore population which is the indication of higher genetic variation of this stock and it was a hatchery and silver barb stock. The higher genetic variation parameters can be used in the selective breeding programs. The higher gene diversity (0.3703±0.114, mean±SD) was found in natural population (Mohongonj haor) by Sultana *et al.* (2010) in *Heteropneustes fossilis* fish. Gopalakrishnang *et al.* (2009) found the mean gene diversity of 0.1848 from the study of genetic differentiation of Malabar carp *Labeo dussumieri* revealed by five RAPD primers. The highest genetic identity (0.9456) between Madaripur and Mymensingh populations indicates same gene shared by these two populations though they are geographically isolated. The gene flow (3.8236) was also highest between these two populations. Nei's genetic distance was highest between Sylhet and Jessore populatios. In phylogenetic dendrogram, the Jessore population made one cluster and the remaining Sylhet, Madaripur, Mymensingh populations made another cluster which clearly indicated the segregation of two different species of minows. Since Jessore population represents a captive stock of silver barb, it can be maintained in the hatchery for broodstock management. Among the olive barb populations, Sylhet population showed better genetic diversity, because this fish population belongs to the large inland water bodies, Hakaluki haor in eastern region of Bangladesh.

Conclusion and Implication

As olive barb is in least concern status, our present study recommends conserving this population to protect from extinction of the species. It can be concluded that remarkable level of genetic variable parameters like polymorphic loci, gene diversity were observed in the present study. However, these parameters can be taken in consideration for making genetically superior brood stock in selective breeding program as well as for conservation management of these species of minnow family.

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Effect of red seaweed *Hypnea musciformisas* replacement of fish meal on growth and immune response of Tilapia *(Oreochromis niloticus)*

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Abstract

Innovation and formulating quality fish feed is major challenges for aquaculture. With this view, this study was designed to find out the effect of seaweed as replacement of fish meal on growth and immune response of Tilapia. The experiment was laid out with four treatments (T_1: Control and T_2: 10%, T_3: 20% and T_4: 30% fish mealreplacement respectively) with three replications. Fish were reared in earthen pits for eight weeks from 28th April to 17th June, 2017 and fed twice a day. Sampling was done 7 days of interval to measure the growth and water quality parameter. Blood was collected at the last sampling and preserved in EDTA tube. Finally, the average weight gain in fish fed with different feed where T_1, T_2, T_3 and T_4 value was 9.95±0.05 g, 11.69±21 g, 11.72±32 g and 12.49±0.09 g, respectively, which was significantly increased ($P<0.05$) with the increased replacement rate of fish meal up to 30%. Relative percent (%) increment in weight gain of Tilapia, compare to T_1 weight gain of T_2, T_3 and T_4 was 84.26±31, 87.17±38 and 133.18±11 respectively, which was significantly different ($P<0.05$). Specific growth rate was found to be increased gradually from T_1 to and hepatosomatic index (HSI) of fish where highest in T_4 and lowest in T_1. Feed conversion ratio (FCR) of *O. niloticus*fed with T_1, T_2, T_3 and T_4 was 1.27, 1.15, 1.12 and 1.02 respectively. Regarding the immune response blood parameters such as Red Blood Cells (RBC), White Blood Cells (WBC), Hemoglobin (Hgb/ Hb), Hematocrit (HCT), Mean Corpuscle Volume (MCV), Mean Corpuscle Hemoglobin (MCH) and Mean Corpuscle Hemoglobin Concentration (MCHC) were measured and all the other parameters were showed highest result in seaweed treated feed with compare to control. From the above result, seaweed replacement feeds are showed best result compare to control feed which increased the total aquaculture production and immune response of fish.

(Key words: Seaweed, Fish Meal, Tilapia *(Oreochromisniloticus),* Growth, Immune System)

Introduction:

The most essential and major operational input in successful aquaculture is the feed and hence cheap and nutritionally balanced effective artificial feeds need to be developed. That's why we selected the seaweed as nutritional feed source. Seaweed is a colloquial term for the common name of mostly macroscopicand multicellular marine algae which do not have root systems or flowers, leaves, stems, fruits and seeds and generally grow and live attached to rock or other hard substrata below the high-water mark or remain drifted in the oceans. Those are an alternative plant feed stuffs that are increasingly being used in aqua feeds because of their nutritional quality, lower cost and availability. Seaweed includes members of green, red or brown algae families. Seaweeds, having protein, amino acids, vitamins, minerals and other trace elements, are used as different purposes such as fodder, feed, fertilizer, human food, industrial and pharmaceutical raw materials etc. Calcium and iron tend to accumulate at much higher levels in seaweed than in terrestrial plants. Seaweed promotes the production of white blood cells that are responsible for fighting off invasive bacterial and viral infections which can increase the immune system of fish. Many types of seaweed contain anti-inflammatory and anti-microbial agents.

Tilapia (*O. niloticus)*, the most important aqua cultured species has been already named as the "food fish of the 21st century" (Zhang *et al.*, 2011) and has poultry broiler like growth with a short culture period. The important characteristics of tilapia are very high tolerant to environmental condition i.e. pollution, salinity as well as diseases (Lutz *et al.*, 2010). On the other hand, many of the fish farmers are very poor

and need to cultivate fish in their ponds for their livelihood. The feed cost is one of the largest operational costs in the aquaculture industry. According to Pereira *et al.* (2012) fish feeding constitutes over 50% of operating cost in intensive aquaculture. An important approach for reducing feed costs in commercial aquaculture is to develop proper feed management, husbandry strategies and efficient broadcasting of the predetermined ration to the culture system. Proper supplementation system of commercial feed with seaweed can reduce this operational cost. Nowadays, various types of seaweeds have become the center of attention due to the possibility of these aquatic plants as an alternative source of protein for cultured fish.

For this reason, this study will be conducted to determine the effect of seaweed as a replacement of fish meal on the growth and immune system of Tilapia (*O. niloticus*).

Materials and Methods:

Experimental Site

The experiment was conducted for a period of 56 days to determine the effect of selected seaweed powder as a supplement of fish feed on growth and immune system of Tilapia *(O. niloticus)*in Pit from 28th April to 17th June, 2017. The experiment was conducted in the backyard of Faculty of Fisheries, Bangabandhu Sheikh Mujibur Rahman Agricultural University,

Design of Experiment

The experiment was conducted in a Randomized Completely Block Design (RCBD) into four different treatments with three replicationswill be done.

Table 1. Experimental design of tilapia (*Oreochromis niloticus*) fry rearing

Treatment	Replication	Pit size	No. of fish/pit	Feed type
T_1	3	75 ft^3	61	Commercial feed
T_2	3	75 ft^3	61	Supplemented feed
T_3	3	75 ft^3	61	Supplemented feed
T_4	3	75 ft^3	61	Supplemented feed

Experimental Fish

The tilapia fry was collected from a commercial hatchery at Mymensingh. The fry were transported in oxygenated bag with tranquilizer and the duration of transportation was approximately 2 hours. After transportation the fry were gently released with acclimatization into the pits. Fish were reared for seven days into pits. After this acclimatization period, then the research was started.

Selection of red seaweed (*Hypnea musciformis)*

From historical times, carrageen and alginates from seaweeds are used for medicinal purposes. Red seaweed like *H.musciformis*is selected for the experiment due to their availability and low coasting in our country. It has plenty of essential nutrients, especially trace elements and several other bioactive substances. It contains very high quality protein and has all the essential and non-essential amino acids. The lipids, which are present in very small amounts are unsaturated and thus afford the protection of fish. It has abundant vitamins, including beta-carotene, which is the precursor of vitamin A, the vitamins of the B group, including B12, vitamin C, D, E and K. In this above discussion, that's why we selected *H.musciformis*for this research.

Experimental Feed Formulation

Fresh feed ingredients were procured in dry form. Feeds were prepared with seaweed replacement at the three different concentrations of 10%, 20% and 30% respectively, where only control was prepared without seaweed. Using the ingredients such as dried fish meal, mustard oil cake, rice bran, wheat bran and seaweed, the feed was formulated following the Pearson square method. The ingredients were mixed together. The mixture was slowly mixed with hot-water (80°C) in a proportion of 50:50 (v/w) to accomplish agglutination. The dough was passed through a meat chopper (Brand-Filizola) to obtain pellets of 2 mm diameter and dried for 3 days at the room temperature and then stored at the 4°C until use.

Table 2. Percentage of feed ingredients used in Experimental diets

Ingredients	Control Feed	10% Seaweed replacement feed	20% Seaweed replacement feed	30% Seaweed replacement feed
Fish Meal	36.37	32.73	29.10	25.46
Seaweed	0.0	3.64	7.27	10.91
Rice Bran	27.78	27.78	27.78	27.78
Wheat Bran	14.15	14.15	14.15	14.15
Mustard Oil Cake	19.19	19.19	19.19	19.19
Ata (Binder)	2.5	2.5	2.5	2.5

Setting up of pit

The experiment site was first cleanup and removed the plants and weeds manually. To set up a pit, at first the soil excavated as length and wide was 5 ft and depth was 3 ft. After that polythene sheet was covered the pit and also the experiment area to hold the water and to control the runoff respectively. To control the water, outlet was setup in every pit. Water connection was done by pipe from the fisheries faculty to maintaining the water level and temperature properly.

Stocking and rearing of fish species

Tilapia (*O. niloticus*) of average 0.311±0.38g size was collected from a commercial fish hatchery at Mymensingh BSMRAU campus. Fish were reared in a 300 litre tank until experiment started. In the beginning of the experiment, fish was weighed individually, selected and distributed into 5×5×3 ft^3 pits with 61 fish per pit. Triplicate groups of fish were fed with control and seaweed treated feed. The fish was fed up twice a day up to satiation.

Fish sampling

Fish were sampled 7 days of interval. Firstly, fish were harvested by scoop net and randomly 25-30 fish were weighted by digital electric machine to know the growth. During sampling, fish were handling carefully without any harm.

Monitoring water quality

Temperatures were recorded weekly in each pit. Dissolved oxygen (DO) and pH were measured using Oxygen and pH meters (Hach Co., Loveland, Colorado).

Growth Parameter

Growth of fry in weight (g) was measured and the following formulas were used to evaluate the growth parameters:

1. Weight gain (g) = Mean final weight (W2) - Mean initial weight (W1)

2. Percent (%) weight gain = $\frac{\text{Mean final fish weight – Mean initial fish weight}}{\text{Mean initial fish weight}}$ x 100

3. Specific growth rate (SGR % per day) = $\frac{\text{LogW2 – LogW1}}{\text{Time}} \times 100$

   Where,

   W_1 = the initial live body weight (g) at time T_1 (day)
   W_2 = the final live body weight (g) at time T_2 (day)

4. Feed Conversion Ratio (FCR): $\frac{\text{Total Feed consumption}}{\text{Total body weight gain of fish}}$

5. Hepatosomatic index (HSI) = $\frac{\text{Liver weight(g)}}{\text{Body weight(g)}} \times 100$

Collection and preservation of blood

During sampling, the fish was selected randomly from every treatment to collect the blood. The fishes were collected and gently wiped with a dry cloth to remove water. Blood was collected by syringe from the caudal peduncle of fish. During blood collection, the needle was soaked in 2% heparin solution as an anticoagulant. After that the blood was transferred into the EDTA (Ethylene Diamine Tetra Acetic Acid) tube and stored at 4°Cfor determining Hematological studies.Leukocyte (WBC), erythrocyte (RBC), hematocrit (Hct), hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were measured in the FBE laboratory of the BSMRAU.

Immune Response Parameter:

Red blood cell (RBC) and white blood count (WBC)

Red blood cell (RBC) and White blood count (WBC) counts were determined using a haemocytometer with Neubauer counting chamber as described by Blaxhall, 1972.

Hemoglobin

Blood haemoglobin concentration (Hb) can be measured on the most accurate method of determining Hb is Drabkin's method (Drabkin and Austin, 1935).

Hematocrit

Blood was drawn into heparinized hematocrit pipette up to the graduation mark. The lower opening of the pipette was closed up to 2 cm depth using sealant and heating it carefully over the spirit lamp which closed the upper opening. The pipettes were centrifuged for 3 min with a speed of 3000 r/min and placed on the reading device and read-off. The hematocrit value was expressed as % blood cells in total volume of blood.

Erythrocyte indices, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated according to Stolen *et al*., 1994.

Results and Discussion:

In the present study dissolved oxygen level ranged between 7.28 mg/l and 9.80 mg/l. Artificial aeration was not provided in the pit.DoF (1996) reported that the range of suitable dissolved oxygen for fish culture would be 5-8 mg/1. From the above statement, it is clear that, the level of dissolved oxygen in the present study was within the productive range and suitable for fish culture.

The average pH level during the experimental period ranged from 7.3 to 7.5. pH level during this experimental period did not differ significantly (P> 0.05). The pH contents in the present study did not much fluctuate. Banerjee (1967) stated that the water with almost neutral reaction having pH values from 6.7 to 7.5 was the best suited for fish production.From the above statement, the content of pH in the present study was within the productive range and suitable for fish culture.

Table 3. Water Quality Parameters in pits

Parameters	T_1	T_2	T_3	T_4
Average dissolve oxygen (mg/l)	7.14 ± 0.45	7.32 ± 0.23	7.45 ± 0.12	7.63 ± 0.08
Average P^H	7.5 ± 0.41	7.5 ± 0.09	7.4 ± 0.04	7.3 ± 0.13
Average temperature (°C)	28 ± 2	27 ± 3	27 ± 1	27 ± 2

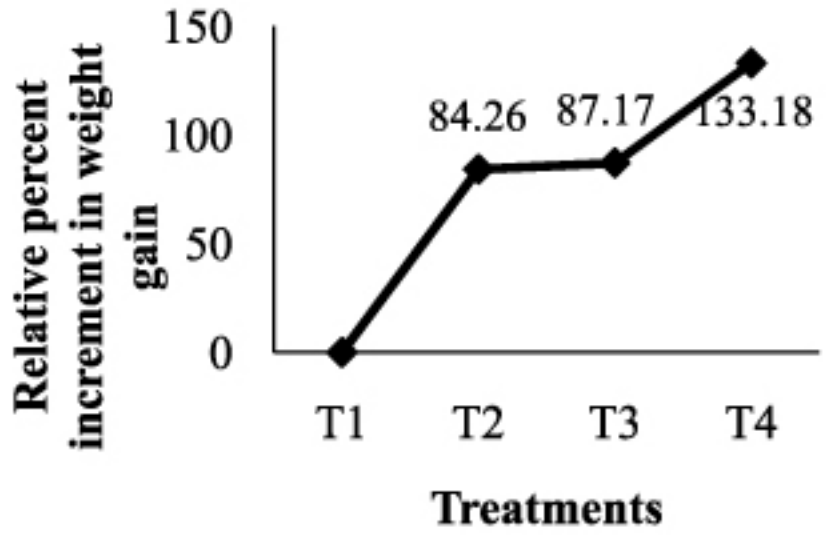
Ranges of water temperature during this experimental period were between 25°C to 32°C. At the beginning (mid- June) of the experiment the temperature was moderately high. Hossain *et al.*(2004) measured the water temperature of 12 experimental ponds for GIFT strain culture at Bangladesh Agricultural University, Mymensingh which varied between 27.4 and 33.0°C. From the above statement, it is evident that, water temperature in this study was within the suitable range.

Growth performance of *O. niloticus*

Specific growth rate (% day-1) of *O. niloticus*washigher in T_4 than control, which did not differ significantly (P>0.05).Resley*et al.* (2009) obtained average weight gain and SGR value was 8.9g to 13.5g and 4.7 (% day-1) of juvenile cobia. Rakocy*et al.* (2006) found SGR value of 4.4 (% day-1) in an intensive Nile tilapia. The present study was within the suitable range.

Table 4. Average weight gain and Specific growth rate (SGR) of different treatments

Treatment	Average weight gain	Specific growth rate (SGR)
T_1	9.95 ± 0.05	4.79 ± 0.15
T_2	11.69 ± 0.21	5.13 ± 0.08
T_3	11.72 ± 0.32	5.29 ± 0.12
T_4	12.49 ± 0.09	5.67 ± 0.06



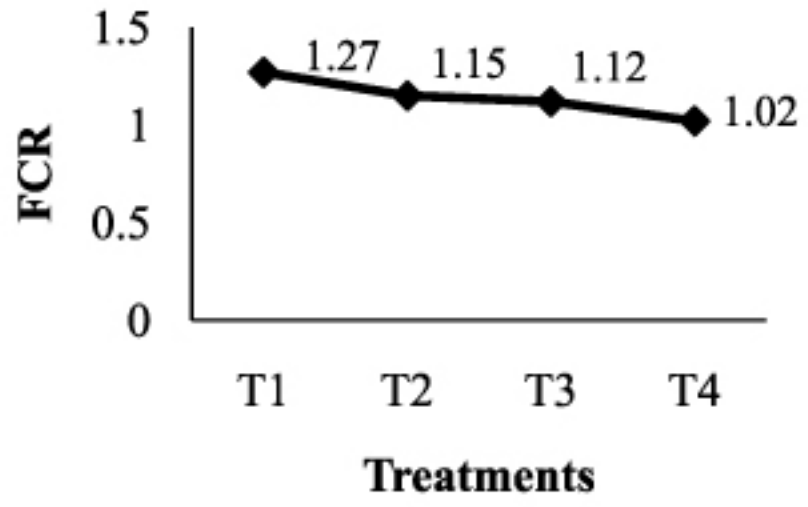


Figure 1. Relative percentincrement in weight gain **Figure 2.** Feed conversion ratio of *O. niloticus*

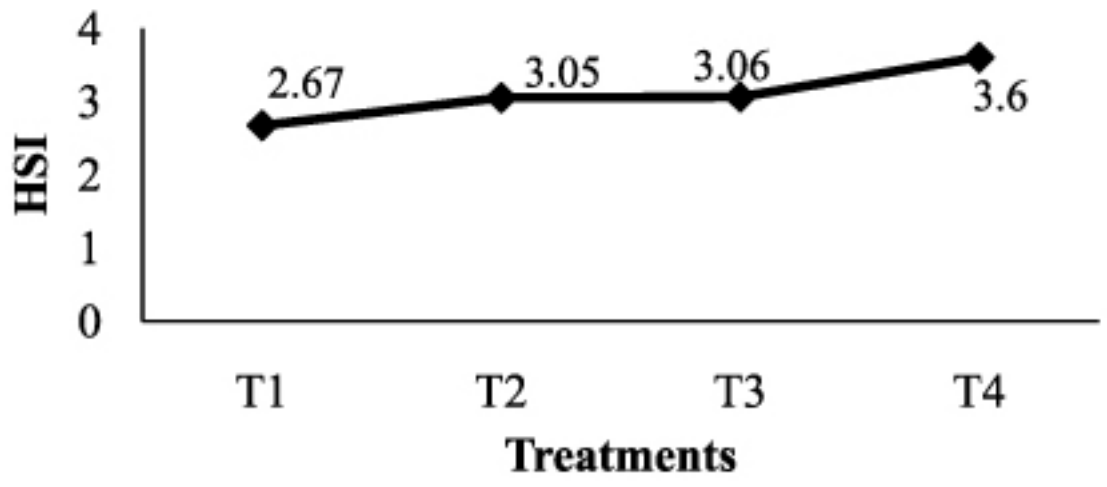


Figure 3. Hepatosomatic Index (HSI) of *O. niloticus*

Relative percent increment in weight gain of Tilapia, compare to T_1 weight gain of T_2, T_3 and T_4 was 84.26±0.31, 87.17±0.38 and 133.18±0.11 respectively, which was significantly different ($P<0.05$).

FCR value was highest in T_1(1.27) and lowest in T_4(1.02).Cruz and Ridha (2001) conducted an experiment on growth of *O. niloticus* juveniles reared in a recirculating system fed with floating and sinking pellets where the FCR value was 2.48 and 2.03 respectively. The FCR of this study was significant.

Hepatosomatic Index (HSI) is defined as the ratio of liver and body weight. The highest and lowest HIS was found in T_4 (3.6) and T_1(2.67) respectively. Ogunji*et al*. (2008) evaluate the growth performance, nutrient utilization of *O.niloticus* and found a HSI of 3.08 and 3.14 using fish meal and mageal. These previous results support the HSI value of the present study.

Immune response of *O. niloticus*

The variation degrees on the haematological parameters are an important tool for fish health diagnosis and may vary according to stressor stimulus, treatment, parasitic or infectious diseases.

Table 5. RBC counts in different treatment

Treatment	RBC (cells/mm^3)			
	1st Week	2nd Week	3rd Week	4th Week
T_1	1.00×10^6	1.21×10^6	1.42×10^6	1.64×10^6
T_2	1.15×10^6	1.47×10^6	1.95×10^6	2.32×10^6
T_3	1.31×10^6	1.68×10^6	2.13×10^6	2.98×10^6
T_4	1.54×10^6	1.85×10^6	2.57×10^6	3.64×10^6

In the present study, fish RBC count was increased in the T_2, T_3 and T_4 than Control. Highest and lowest RBC was found in T_4 (3.64×10^6cells/mm^3) and T_1 (1.00×10^6cells/mm^3) respectively(Table: 5). According to Radhika D and Mohaideen A (2016) there was a gradual increase in the RBC count from the 1st day to the 28th day the maximum being observed in the fishes fed with *G. corticata*diet on the 28th day (3.67×10^6 cells/mm3) followed by *S marginatum* (2.03×10^6 cells/mm3) and *U. lactuca*(1.50×10^6 cells/mm3). It is a good sign because increasing the RBC, it increased the Hemoglobin level and transported more oxygen in the blood and also prevent the anemia.

Table 6. WBC counts in different treatment

Treatment	WBC (cells/mm^3)			
	1st Week	2nd Week	3rd Week	4th Week
T_1	10.06×10^3	10.66×10^3	11.46×10^3	12.36×10^3
T_2	10.56×10^3	11.34×10^3	12.26×10^3	13.35×10^3
T_3	10.82×10^3	11.88×10^3	13.06×10^3	14.43×10^3
T_4	11.12×10^3	12.34×10^3	13.58×10^3	15.12×10^3

Also, WBC was increased and highest and lowest value was T4 (15.12×10^3cells/mm^3) and T_1 (10.06×10^3cells/mm^3) respectively (Table: 6).It was indicated the good sign and prevent from invasive bacteria and virus. Francesco F *et al.*(2012) were counted the WBC level that gradually increased in that study and highest and lowest value of WBC was 18.00×10^3cells/mm3 and 14.30×10^3 cells/mm3 respectively.

Table 7. Hemoglobin(Hgb/Hb) counts in different treatment

Treatment	Hemoglobin (g/dL)			
	1st Week	2nd Week	3rd Week	4th Week
T_1	5.2	6.0	6.9	7.8
T_2	6.4	7.1	8.3	9.8
T_3	7.5	8.7	9.9	11.2
T_4	8.3	9.3	10.6	12.4

In Hemoglobin (Hgb/Hb) and MCV, seaweed replacement feeds are showed best result compare to control feed which increased the total aquaculture production and immune response of fish. MCH and MCHC are not measured directly, but calculated based on the hemoglobin value (Hgb), which is the total measure of hemoglobin in the blood, and the RBC. Abnormally high or low levels of MCH and MCHC, as determined by blood testing, can be an indication of a number of problems within the body, ranging from nutrient deficiencies to chronic diseases. But this research showed a good fish health that's why the blood parameters are in good range which increased the immunity of the fish.

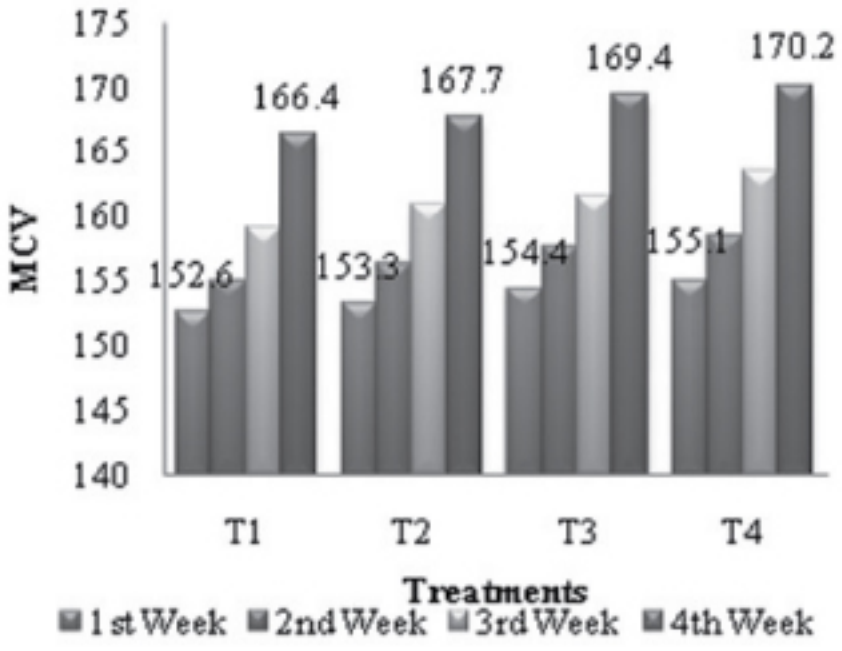


Figure 5. Mean Corpuscle Volume (MCV)

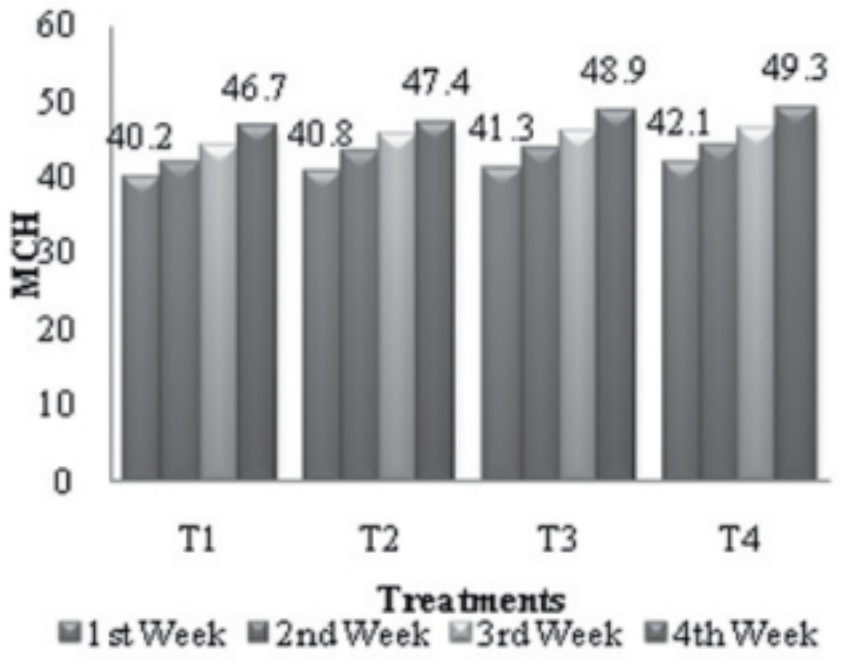


Figure 6. Mean Corpuscle Hemoglobin (MCH)

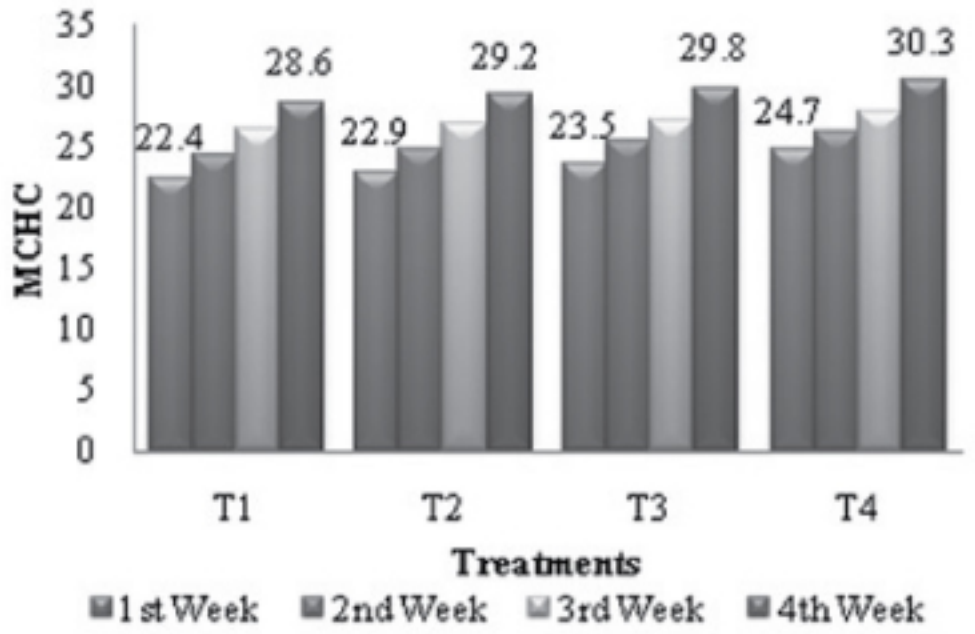


Figure 7. Mean Corpuscle Hemoglobin Concentration (MCHC)

Conclusion and Implication

Seaweed is a very important dietary component not only for human but also for fish.Through this study ithas been found that seaweed has significant effects on the different parameters of growth. The result of immunological parameters indicated that seaweed can increase the immune response of the fish. From the above results, it has been concluded that seaweed act as promising feed ingredient to increasegrowth and immune response of *O. niloticus*. This study has been provided important information to formulate new feed for the better aquaculture production.

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Occurrence of *Salmonella* and *Vibrio* species in fresh fishes collected from different markets of Mymensingh, Gazipur and Sherpur districts and their characterization

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Abstract

This study was undertaken to determine total viable count (TVC), total salmonella count (TSC) and total vibrio count (TVibC) in fresh fishes collected from markets of Mymensingh, Gazipur and Sherpur districts. Isolation and identification of *Salmonella* spp. and *Vibrio* spp. was also done from pangas (n=20), tilapia (n=20) and koi (n=20) collected from different markets of Mymensingh, Gazipur and Sherpur districts. Samples were cultured on plate count agar to determine TVC, xylose-lysine deoxycholate agar (XLD) to determine TSC and thiosulfate citrate bile salt sucrose agar (TCBS) to determine TVibC. The mean value of logarithm colony forming units (CFU±SD/g) of TVC, TSC and TVibC in pangas were log 9.09±0.616, log 5.32±0.391 & log 3.14±0.557CFU/g; log 8.46±0.441, log 5.26±0.589 & log 3.59±0.823 CFU/g and log 7.58±0.466, log 3.28±0.493 & log 2.88±0.386 CFU/g of Mymensingh, Gazipur and Sherpur, respectively. Similarly, the mean values of TVC, TSC and TVibC in tilapia were log 6.60±0.790, log 3.59±0.388 & log 3.75±0.176 CFU/g; log 6.55±0.553, log 3.26±0.502 & log 3.67±0.021 CFU/g and log 6.74±0.372, log 3.44±0.411 & log 3.05±0.609 CFU/g of Mymensingh, Gazipur and Sherpur, respectively and in koi were log 7.51±0.537, log 3.49±0.459 & log 3.35±0.390 CFU/g; log 7.66±0.752, log 3.25±0.465 & log 3.59±0.581 CFU/g and log 7.13±0.393, log 3.27±0.384 & log 3.43±0.297 CFU/g of Mymensingh, Gazipur and Sherpur respectively. The targeted *Vibrio* spp. and *Salmonella* spp. were isolated and identified from collected fishes. All the isolates of *Salmonella* were confirmed by targeting genus specific histidine transport operon gene. Antimicrobial sensitivity test was done for all the isolates of *Salmonella* and *Vibrio* species by disc diffusion method. Out of forty five isolates of *Salmonella*, seven were found multidrug resistant. The bacteria isolated from fish were of public health significance as well as responsible for spoilage of fish.

Key Words: fresh fishes; *Salmonella* species; *Vibrio* species; characterization

Introduction

Bacteria present in fish are responsible for economic loss due to mortality. Some pathogens are also known to cause zoonotic disease to the handlers and final consumers. Human infection results from contamination of hands and utensils during processing operations especially at evisceration. Fishes are also known to transmit *Salmonella* spp., *Staphylococcus* spp., *Vibrio* spp. and *Aeromonas* spp. which are the causal agent of human food-borne infection and intoxication (Gold and Salit, 1993).

Bacteria found in fish are classified into non-indigenous and indigenous bacteria. The non- indigenous bacteria include *Clostridium botulinum, Listeria monocytogenes, Staphylococcus aureus, Salmonella* spp., *Shigella* spp. and *Escherichia coli.* On the other hand, indigenous bacteria include *Vibrio* spp., *Staphylococcus aureus, Salmonella, Vibrio parahaemolyticus, Shigella, Aeromonas, Yersinia* and *Pseudomonas* (Clucas and Ward, 1996).

Pangas (*Pangasius pangasius*), Tilapia (*Oreochromis mossambicus)* and Koi (*Anabas testudineus*) are generally fresh water fishes. These fishes are found in small rivers, canal and swamp. Koi fish is the most

popular and tasty fish among all fishes of Bangladesh. Now a days commercial fish farming in pond is very popular. *Aeromonas* spp. and *Pseudomonas* spp. are deadly bacteria that live in every pond causing life threatening bacterial infection such as ulcer, fin rot and tail rot of fishes. Poor water quality cause burn off the slime coat or stress the fish making it more susceptible to bacterial infection.

Fish is involved in active and passive transfer of a wide range of bacterial infection and intoxication to humans, underscoring the need to investigate public health risk associated with fish. Prophylactic use of antibiotics and growth promoters in intensive fish feed is responsible for development of antibiotic resistance in consumers, which is an important public health issue in recent time. Understanding the main risk factors and how to reduce them is therefore essential for developing best management practice to safeguard public health. Therefore, this study was designed with a view to assess the occurrence of *Salmonella* and *Vibrio* species in fresh fishes collected from different markets of Mymensingh, Gazipur and Sherpur districts.

Materials and Methods

Collection and transportation of samples

A total of 60 samples were collected from 20 different markets of Mymensingh, Gazipur and Sherpur districts. During the collection of samples precautionary measures were maintained to avoid touch and ice box were used to maintain cool chain. The samples were then brought to the laboratory of the Department of Microbiology and Hygiene, BAU, Mymensingh.

Processing and enrichment of samples

Aseptic measures were undertaken during the sampling procedure to prevent contamination of the samples. Skin, gill, intestine of fish, mud and water samples were collected. These samples were minced and grinded together. Ten gm of samples were homogenized with 90 ml of 0.1% peptone water and 0.1 ml of homogenized sample was streaked on to selective agar media and incubated at 37ºC for 24 hours.

Determination of total viable count (TVC), total salmonella count (TSC) and total vibrio count (TVibC)

Hundred microliter (0.1 ml) of each ten fold diluted sample was transferred and spreaded to plate count agar (PCA), Xylose lysine deoxycholate agar (XLD agar) and Thiosulfate citrate bile salt sucrose agar (TCBS) using a sterile pipette and a sterile glass spreader. The incubated plates were then kept in an incubator at 37°C for 24-48 hours. The number of colonies in a particular dilution was multiplied by the dilution factor to determine TVC, TSC and TVibC, which were expressed as mean logarithm colony forming units (CFU±SD/g).

Isolation of target bacteria

Initially samples were enriched in nutrient broth at 37º C for 24 hours. The overnight cultures were streaked on XLD agar for Salmonella and TCBS agar for *Vibrio*. Inoculated plates were incubated at 37º C for 24 hours. Single well defined colony was further sub-cultured until pure culture was obtained.

Identification of target bacteria

Cultural, morphological, and biochemical characteristics were studied to identify the bacterial flora. The cultural characteristics or colonial morphology of the bacteria grown on the SS, XLD and TCBS agar were recorded. Gram's staining was performed to study the morphology and staining characteristics of the bacteria according to the method described by Cheesbrough (1985). Biochemical tests, such as sugar fermentation, motility, catalase, methyl red (MR), voges-proskauer (VP) and indole tests were performed to identify the bacteria (Cheesbrough, 1985).

Molecular identification by polymerase chain reaction (PCR)

Bacterial DNA template was prepared by using boiling method (Englen and Kelley, 2000). PCR reactions were carried out using a thermocycler (ASTEC, Fukuoka, Japan) with the following programme: initial

denaturation with 1 cycle of 5 min at 94°C, 30 cycles each consisting of denaturation with 94°C for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 45 seconds and a final extension step of 5 min at 72 °C. PCR products were separated on 2% agarose gel, stained with ethidium bromide and photographed using a gel documentation system (BioRad, USA).

Antibiotic sensitivity test

All isolates were tested for antimicrobial drug susceptibility against eight commonly used antimicrobials by disc diffusion method according to the guidelines of Clinical and Laboratory Standard Institute (CLSI), 2012. The zones of growth inhibition was compared with the zone size interpretative tables provided by Clinical and laboratory Standards Institute (CLSI, 2012). Antimicrobial testing results were recorded as susceptible, intermediate and resistant according to zone diameter interpretative standards provided by CLSI (2012).

Statistical analysis of experimental data

The data on total viable count (TVC), total vibrio count (TVibC) and total salmonella count (TSC) obtained from the bacteriological examination were analysed in completely randomised design (CRD) using SPSS Software (Version 16, 2007). The differences between means were evaluated by Duncan's Multiple Range Test. Correlation among TVC, TVibC and TSC were also evaluated.

Results and Discussion

In this study TVC of three types of fish from three districts were determined to know the bacterial load present in these samples. The mean and standard deviation of the total viable count (TVC), total salmonella count (TSC) and total vibrio count (TVbiC) in pangas, tilapia and koi of Mymensingh, Gazipur and Sherpur districts are summarized in Tables 1, 2 and 3. The result of total viable count in three districts retail markets were differed significantly ($P<0.01$). Tiamiyu et al. (2011) reported 6.09±0.65 Log CFU microbial load in skin sample of fish. Higher TVC of fish was recorded in all occasions of this present study. The permissible count of heterotrophic bacteria in the 1 cm^2 of skin ranges from 10^2 –10^7 or bacteria of log 10 CFU/cm^2 ≤ 5.70 according to International Commission on the Microbiology Specification of Foods (Zmyslowka et al., 2000). The higher TVC of fish in this study might be resulted from consumption of bacteria by the fish for long time through food and water. The survival of these bacteria is dependent on the conditions prevailing in the aquatic environment and fish are often simply their hosts (Zmyslowka et al., 2000).

Table 1. Determination of mean and standard deviation for microbiological quality of pangas at different markets of three districts.

Place of collection	TVC (Log CFU/g)	TSC (Log CFU/ g)	TVbiC (Log CFU/ g)
Mymensingh	9.09±0.616[a]	5.32±0.391[a]	3.14±0.557[b]
Gazipur	8.46±0.441[a]	5.26±0.589[a]	3.59±0.823[a]
Sherpur	7.58±0.466[b]	3.28±0.493[b]	2.88±0.386[c]
LSD	0.708	0.311	0.180
Level of sig.	**	**	**

** = Double asterisk (**) means significant at 1% level of probability
In a column figures with same letter do not differ significantly ($p>0.05$) whereas figures with dissimilar letters differ significantly (as per DMRT).
LSD= Least Significant Difference
All counts are expressed in logarithms and CFU/g.

Table 2. Determination of mean and standard deviation for microbiological quality of tilapia at different markets of three districts.

Place of collection	TVC (Log CFU/g)	TSC (Log CFU/ g)	TVbiC (Log CFU/ g)
Mymensingh	6.60±0.790[a]	3.59±0.388[a]	3.75±0.176[a]
Gazipur	6.55±0.553[a]	3.26±0.502[b]	3.67±0.021[a]
Sherpur	6.74±0.372[a]	3.44±0.411[ab]	3.05±0.609[b]
LSD	0.184	0.185	0.425
Level of sig.	NS	**	**

** = Double asterisk (**) means significant at 1% level of probability; NS = Not significant
In a column figures with same letter do not differ significantly (p>0.05) whereas figures with dissimilar letters differ significantly (as per DMRT).
LSD= Least Significant Difference
All counts are expressed in logarithms and CFU/g.

Table 3. Determination of mean and standard deviation for microbiological quality of koi at different markets of three districts.

Place of collection	TVC (Log CFU/g)	TSC (Log CFU/ g)	TVbiC (Log CFU/ g)
Mymensingh	7.74±0.610[a]	3.71±0.405[a]	3.25±.328[b]
Gazipur	7.66±0.752[a]	3.25±0.465[b]	3.59±0.581[a]
Sherpur	7.13±0.393[b]	3.27±0.384[b]	3.43±0.297[a]
LSD	0.189	0.151	0.168
Level of sig.	**	**	**

** = Double asterisk (**) means significant at 1% level of probability
In a column figures with same letter do not differ significantly (p>0.05) whereas figures with dissimilar letter differ significantly (as per DMRT).
LSD= Least Significant Difference
All counts are expressed in logarithms and CFU/g.

Two targeted bacteria *Vibrio* spp. and *Salmonella* spp. were isolated from three types of fish from three districts. The results of isolation are in agreement with the findings of Adebayo-Tayo et al. (2012).

Salmonella spp. was found in 75% of the tested samples and the highest count recorded was log 5.89 CFU/g in pangas collected from Mymensingh. Out of the 60 samples, 42 were contaminated with *Vibrio* spp. and highest count was log 4.37 CFU/g in pangas collected from Gazipur. This value is higher than the findings of Mamun (2014). This increase might be the result of exposure of fishes in unhygienic condition during different stages of cultivation and collection. It may start from hatcheries and other subsequent processes like farming area, processing station, transportation and market place. Health status of working personnels and their poor sanitation practices are also responsible. The presence of *Salmonella* and *Shigella* in the fish samples may be due to exposure to flies (Mensah et al., 2002) and prevailing poor sanitary conditions in the markets. *Salmonella* and *Shigella* are well-known important human pathogens. Their presence is unacceptable because of their attendant health risks (Mensah et al.,2002). Fishes are prone to bacterial contamination and could cause health risk to consumers (Wafaa et al., 2011). The food poisoning associated with consumption of fish and shellfish either raw or slightly cooked, contaminated with *Vibrio* spp. causes intestinal infection characterized by diarrhea, abdominal cramps, sickness, vomiting, fever and severe headache (Merwad et al., 2011, Espineira et al., 2010). The samples of fish and shellfish analyzed bacteriologically showed varying degree of *Vibrio* contamination.

Morphology of *Vibrio* spp. on TCBS agar plate recorded in this study was similar to the findings of Khan et al. (2007). In Gram's staining, bacteria exhibited curved rod shaped appearance, which was similar to the findings of Faruque et al. (2008). In this study, the colonies of *Salmonella* spp. on SS agar plate were opaque,

translucent with black centers, which were similar to the findings of Hossain (2002). In Gram's staining bacteria exhibited short rods, Gram negative, single or paired arrangement. Similar findings were also reported by Samad (2005). *Salmonella* spp. produced acid by fermenting dextrose, maltose and mannitol and did not ferment lactose and sucrose. *Vibrio* spp. produced acid by fermenting dextrose, maltose, sucrose and mannitol except lactose. *Salmonella* spp. gave positive reaction to MR and negative reaction to VP and indole production tests and *Vibrio* spp. gave positive reaction to MR and indole production tests and negative reaction to VP test that were similar to the findings described by Zaman et al. (2013).

PCR assay was used for molecular detection of *Salmonella* spp. Primers targeting histidine transport operon genes were used for specific identification of the bacteria at genus level by PCR. These findings are in agreement with the result of Nadia et al. (2011). Noah et al. (1993) also detected *Salmonella* at genus level.

Results of antimicrobial susceptibility test for *Vibrio* spp. are presented in Table 4. All *Vibrio* isolates were susceptible to azithromycin, gentamicin, norfloxacin, and tetracycline and resistant to amoxycillin. Most of the isolates susceptible to streptomycin (95.24%) and erythromycin (97.61%) and intermediate to ciprofloxacin (88.10%). Almost similar antimicrobial profiles were also reported by Hossain et al. (2012). Results of antimicrobial susceptibility test for *Salmonella* spp. are summarized in Table 5. As regards to *Salmonella* spp., all isolates were susceptible to azithromycin, ciprofloxacin, gentamicin and norfloxacin. Most of the isolates were intermediate to erythromycin (91.11%) and tetracycline (86.67%) and resistant to amoxycillin (93.33%). These findings of the present study are in agreement with the result of Frech and Schwarz (1998); and Chugh and Suheir (1983).

Table 4. Results of antimicrobial susceptibility test for *Vibrio* spp.

Name of isolates	**No. (%)**							
Vibrio spp.(n=42)	AMX	AZM	CIP	E	GEN	NOR	S	TE
Susceptible	0 (0)	42 (100)	5 (11.90)	41 (97.61)	42 (100)	42 (100)	40 (95.24)	42 (100)
Intermediate	0 (0)	0 (0)	37 (88.10)	1 (2.39)	0 (0)	0 (0)	2 (4.76)	0 (0)
Resistant	42 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
AMX= Amoxycillin, AZM= Azithromycin, CIP= Ciprofloxacin, E= Erythromycin, GEN= Gentamycin, NOR= Norfloxacin, S= Streptomycin, TE= Tetracycline								

Table 5. Results of antimicrobial susceptibility test for *Salmonella* spp.

Name of isolates	**No. (%)**							
Salmonella spp.(n=45)	AMX	AZM	CIP	E	GEN	NOR	S	TE
Susceptible	0 (0)	45 (100)	45 (100)	0 (0)	45 (100)	45 (100)	38 (84.44)	0 (0)
Intermediate	3 (6.67)	0 (0)	0 (0)	41 (91.11)	0 (0)	0 (0)	7 (15.54)	39 (86.67)
Resistant	42 (93.33)	0 (0)	0 (0)	4 (8.89)	0 (0)	0 (0)	0 (0)	6 (13.33)
AMX= Amoxycillin, AZM= Azithromycin, CIP= Ciprofloxacin, E= Erythromycin, GEN= Gentamycin, NOR= Norfloxacin, S= Streptomycin, TE= Tetracycline								

Conclusions

This study recorded the presence of multidrug resistant bacteria in fish that is alarming with regards to food safety and public health point of view.

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Growth and Production of endangered *Ompokpabda* (Hamilton) in ponds based on stocking density

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Abstract

Growth, survival and production of endangered *Ompokpabda* (Hamilton) were examined at different stocking densities. The experiment was conducted for six months in three earthen ponds from15th December to 30th June, 2017at TanoreUpazila in Rajshahi, Bangladesh. Three different stocking densities viz. 160550, 172900, 185250 piece/ha were assigned as treatment T_3, T_2 and T_1 respectively with three replication for each. The mean values of different water quality parameters such as temperature (°C), transparency (cm), Dissolved oxygen (mg/l), pH and alkalinity(mg/l) were within the suitable ranges and no significant differences were observed among the treatments. At stocking, all fingerlings were of same age group with an initial weight of 0.8±0.00g, respectively. Final weight was found to be the highest (40.01±0.56) in T_3 and the lowest (30.03±0.45) in T_1. The mean values of weight gain was highest (39.21±0.56) in T_3 and lowest (29.23±0.45) in T_1. SGR was significantly higher (2.15±0.01) in T_3 followed by in treatment $T_2$and in T_1. Highest Survival rate (%) was found in (T_3) 72.60±0.85 and treatments to treatments variation was significant. Condition Factor of three treatments were found to have range from (1.03±0.04) to (0.87±0.02). Fish production in treatment T_1, T_2, T_3 were 3249.30±50.12, 3860.60±83.42, 4358.60±62.26 respectively. The net benefit was also highest (1202300±28285.37) in T_3 followed by (796300±32099.89) in treatment $T_2$and lowest (555000±17645.45) in T_1. Despite of this, consistently higher CBR were found in treatment T_3 (1.50±0.04) than treatment T_1 (0.95±0.03) and T_2 (1.18±0.05). In the present study production of *Ompokpabda* was found to be highest in lower stocking density.

Keyword: Production, endangered, *Ompokpabda,* stocking density.

Introduction

Ompakpabda (Hamilton) popularly known as butter catfish is a freshwater species native to India, Bangladesh-Pakistan and Myanmar. It is a bony fish; belonging to the family Siluridae of the order Siluriformes is a freshwater catfish, locally known as "madhupabda" (Viswanath*et al*., 2007).It is a delicious, tasty, nutritious catfish high price because of few bones, bony structure and rich lipo-protein content (Banik*et al*., 2012). Highest retail price was recorded for *Ompokpabda* in Bangladesh and its consumption was restricted among higher class and middle class consumers only (Samad*et al*. 2010).Open beel connected with rivers are common habitat of these species (Sarker*et al*., 2005). It is also found in haors, baors, flooded water bodies, ponds, streams and river of Bangladesh. *Ompokpabda* dwells and breeds in the rivers and reservoirs (Mukherjee *et al*., 2002) but *Ompokpabda's*fry are very rare in nature because of many adverse changes in their natural breeding and growing habitats (Hossain*et al*., 2006). *Ompakpabda* has already been declared as an endangered (EN) species (IUCN, Bangladesh, 2015) criterion due to reduced abundance and restricted distribution (Lakra *etal*., 2010).Therefore, being a potential candidate species for aquaculture and its economic importance, conservation of this fish is up most important (Ayyappan*et al*. 2001).The sustainable culture operation of any fish species requires proper domestication, fry feeding and rearing and culture technique of the species concerned (Sarowar*et al*., 2010). The growth of larvae is also influenced by the quality of feed and their acceptability (Sahoo*et al*., 2010). *Ompokpabda* is a carnivorous fish; which mostly feeds on small fishes, aquatic insect, insect larvae etc. Apart from these, earthworm is also a favorite food for this catfish. As per the existing literature on food and feeding habits of *Ompokpabda*, it has been found that they are mostly piscivorous or carnivorous in nature (Booth and Alquezar, 2002; Mahmood, 2006). Improper care and lack of understanding about biotic and abiotic factors in culture system may result in mass mortality

of fish (Jhingran&Pullin, 1985). No work has yet been undertaken to evaluate the effects of stocking density on the growth and survival of *Ompokpabda* fingerlings in nursery ponds in northern region of Bangladesh. Stocking density is an important parameter in fish culture operations, since it has direct effect on the growth, survival and production (Backiel and Le Cren, 1978). The negative impact of stocking density on growth and survival has also been reported in catfishes (Sahoo*et al*., 2010; Dada *et al*., 2000). Therefore, the present experiment has been undertaken to develop a practical and economically viable methodology for grow-out production of *Ompokpabda* under controlled management system based on stocking.

Materials and Methods

Description of the experimental unit

The experiment was carried out for a period of 6 months from 15th December to 30th May, 2017 at TanoreUpazila in Rajshahi, Bangladesh. All ponds were rectangular in shape. All ponds had well organized inlet and outlet systems and also fully exposed to prevailing sunlight. Water supply was provided with the help of plastic porous pipe in each experimental pond for optimum oxygenation of water.

Design of the experiment

The experiment was designed with three treatments each contained different stocking densities such as 185250 (Treatment T_1), 172900 (Treatment T_2), 160550 (Treatment T_3) individuals per hectare and each with three replicationsfor evaluation of fingerling production of *Ompokpabda* through one stage fry rearing process. The commercial feeds namely "Bangle feed" (containing 30% protein) was supplied as supplementary feed in each treatments.

Pond preparation and stocking of fry

All the aquatic weeds were removed manually due to cheap labor cost. All ponds were treated with lime at the rate of about 1.9 kg per decimal. Then the experimental ponds were fertilized by using cowdung: 2470 kg/ha, urea: 50 kg/ha, TSP: 50 kg/ha and DAP: 40 kg/ha. Cowdung was applied to the pond in a decomposed form, TSP and DAP was wetted to previous day and spreaded over the pond and Urea was applied into the ponds after 7 days of liming. Fry of *Ompokpabda* were collected from "Sotota" hatchery, Mymensingh. Fry were transported to the experimental pond with polythene bag with proper aeration. Then the fries were acclimatized for about half an hour. Before releasing the fry to the experimental pond weight of larvae was recorded with the help of electric balance and measuring board. The initial weight of larvae was 0.8±00g .Fry were released in each experimental pond at 8.00 am.

Feed preparation and feeding

The commercial feeds were given to fry at the rate of 10% of their body weight in 20-25 days after releasing. Then the supplementary feed (Nursery floating feed) spread to the experimental pond surface at 6% in their body weight. Half of the rations were supplied after sunrise and half of the rations were supplied before sunset. The proximate composition of feed has been presented Table- 1.

Table 1: Proximate composition of feed (Company name: Bangla Feed)

Component	Protein	Lipid	Starch	Moisture	Fiber	Ash	Calcium	Phosphorus
Amount	30%	6%	28%	12%	5%	16%	2.3%	0.8%

Sampling of water quality and growth parameters

Physico-chemical parameters like water temperature (°C), transparency (cm), Dissolved oxygen (mg/1), pH, Alkalinity (mg/1) of each experimental pond (were measured at 15 days interval). Temperature was recorded by using a Celsius thermometer, transparency was recorded by secchi disc and other chemical parameters were recorded by using Hack kit box. The length and weight recorded by random sampling of 10 % *Sperataaor* fishes from each pond in every 15 days interval. To evaluate the fish growth the following

parameters were measured: Weight gain (g), length gain (cm), percent weight gain, percent length gain, SGR (%), survival rate, yield/kg/ha/3 months. Sampling was done between 10.00 am and 11.00 am.

Statistical Analysis

Collected data of water quality, growth, survival rate (%) and fish production under different treatments were analyzed statistically to show the results as mean value with standard error through analysis of variance (ANOVA). This statistical analysis was performed with the support of the computer software SPSS (Statistical package for social Sciences) program. The mean values were also compared to see the significant differences through DMRT (Duncan Multiple Range Test).

Economic analysis

Cost-benefit analysis was followed to study the economics of different treatments for the present study. Data on both fixed and variable costs were recorded to determine the total cost (BDT/ha). Total return determined from the market price of fish expressed as BDT/ha. Net benefit calculated by deducting the total return from total cost was expressed as BDT/ha. CBR was calculated as follows-

CBR = Net benefit/Total Cost

Finally the total expenditure was subtracting from the total incomes and gets the benefit.

Results and Discussion

Mean variation of physico-chemical parameters

The mean values of different hydrological parameters such as water temperature (°C), transparency (cm), dissolve oxygen (mg/l), pH and alkalinity (mg/l) with standard error (SE) under different treatments by the total of all fortnight are presented in table-2. Water parameters were within the suitable ranges of fish culture which was not significantly ($P<0.05$) different among the treatments.

Table-2: Variations in mean values of physico-chemical parameters under different treatments during the study period.

Parameters \ Treatments	T_1	T_2	T_3
Water temperature (°C)	26.00±0.35^a (20-31)	26.78±0.35^a (21-32)	27.17±0.19^a (21.5-33.5)
Transparency (cm)	22.60±0.28^a (16-35)	22.81±0.38^a (17-36)	23.08±0.21^a (18-37)
DO (mg/l)	5.20±0.8^a (3-6.1)	5.23±0.13^a (3.3-6.5)	5.24±0.14^a (3.5-7)
pH	7.00±0.05^a (6-7.2)	7.05±0.03^{ab} (6-7.2)	7.09±0.04^b (6.5-7.5)
Alkalinity	140.39±1.05^a (140-150)	142.93±1.22^b (140.2-150.5)	143.15±0.38^b (140.5-152)

Figures in a same row having same superscript have no significant different (P<0.05)

3.2 Growth parameters of fish

Growth performance (Initial weight, final weight, weight gain, specific growth rate) of fingerlings of *Ompokpabda*was significantly higher in T_3 where the stocking density of fry was low compared to those of T_2 and T_1 although same food was applied in all the treatments at an equal ratio are presented in table 3. No significant ($P<0.05$) variation was recorded in initial weight of fishes among the treatments. The lowest stocking densities provides more space ,food and less consumption ,which were reported by Narejo*et al.*, (2000).

Table 3: Variations in the mean values of fish growth performances under different treatments during the study period.

Parameters \ Treatments	T_1	T_2	T_3
Initial weight (g)	0.8±0.00[a]	0.8±0.00[b]	0.8±0.00[c]
Final weight (g)	30.03±0.45[a]	35.53±0.75[b]	40.01±0.56[c]
Total weight gain (g)	29.23±0.45[a]	34.73±0.75[b]	39.21±0.56[c]
SGR (%bwd-1)	1.99±0.01[a]	2.09±0.01[b]	2.15±0.01[c]
ADG (g)	0.16±0.01[a]	0.19±0.02[b]	0.22±0.01[c]
Condition factor	0.87±0.02[a]	0.96±0.05[b]	1.03±0.04[c]
Survival rate (%)	60.30±1.86[a]	65.33±0.95[b]	72.60±0.85[c]
Production (ha/6months)	3249.30±50.12[a]	3860.60±83.42[b]	4358.60±62.26[c]

Figures in a same row having same superscript have no significant different (P<0.05)

The weight gain of *Ompokpabda*was (39.21±0.56 g) in treatment (T_3) which is significantly (P>0.05) higher than those of the other two treatments followed by T_2 (34.73±0.75g) and T_1 (29.23±0.45g), respectively. This result was much better than the result of Malla and Banik (2015), who observed that the weight gain of*Ompokpabda* were (13.67± 1.5g) highest and lowest (8.13± 0.9g) after 28 days culture .The most probable reason of lower weight gain in their study might be high stocking density. After completion of the experiment, Sarma*et al.* (2012) recorded in their experimental pond that the highest weight gain of *Ompokpabda* fry was 0.581±0.035g respectively that was lower than the present study.

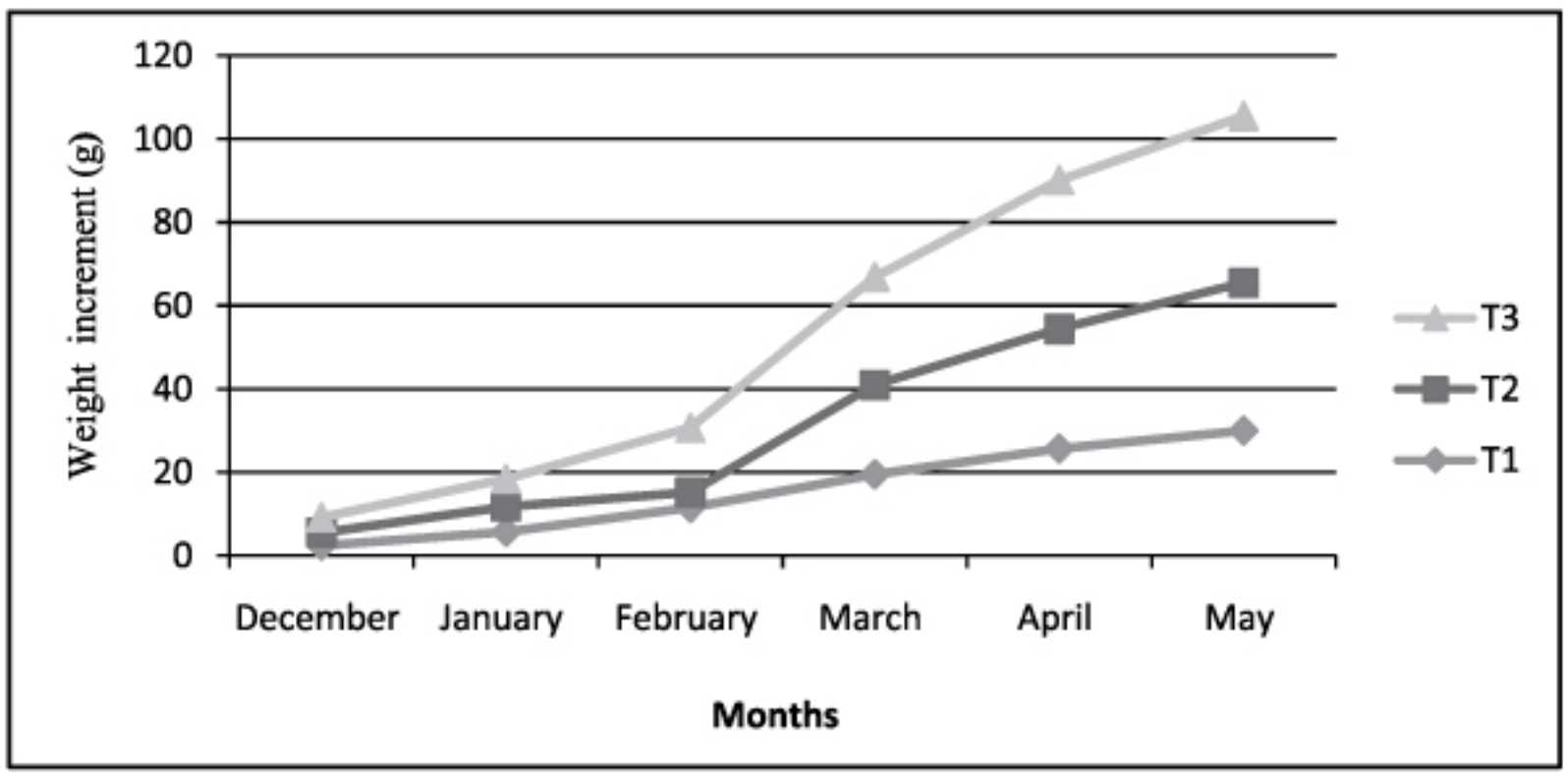

Fig. 1: Monthly weight increment *of Ompokpabda* under deferent treatment during study period.

The mean range of ADG was 0.16±0.01g (T_1), 0.19±0.02g (T_2), and 0.22±0.01 g(T_3), respectively. The mean ADG level was significantly (p<0.05) higher in T_3 and consistently lower in T_1 and T_2. Chakraborty*et al.,* (2013) pointed out that the range of ADG was 0.83±0.02 g (T_1), 0.83±0.02g (T_2) and 0.83±0.02g (T_3) which was higher than the present study. The causes might include competition for food and habitat owing to higher number of fish. The present findings was lower than the findings of Samad*et al.,*(2014) who studied that the average daily gain was highest in T_3 (0.44±0.65g) whereas, the lowest was found in T_1 (0.30±1.01 g) .

In the present study, the specific growth rates of *Ompokpabda*was ranged from 1.99±0.01 (T_1), to 2.15±0.01 (T_3). The significantly highest specific growth rate (SGR) in T_3 might be due to the fact that the fish had utilized effectively the supplied feed. Similar investigation was made by Debnath*et al.* (2016) on *Ompokpabda* larvae during culture. Malla and Banik (2015) reported that SGR of *Ompokpabda* was

4.79±0.58 to 3.46± 0.31 that was higher than the present study because of less availability of natural food and some changes in environmental parameters. According to Mondal*et al.* (2007), growth rate of fish increase with increase in the level of dietary protein till the optimum level is reached. Stocking density had previously been observed to have a direct effect on the growth of fish (Islam *et al*., 2002).In this study ,the growth and survival was might have affected to a certain extent due to carnivorous and cannibalistic mode of life of *Ompok sp.*(Rahman*et al*., 2008).

The survival rates were differed in different experimental ponds. The survival rate differed from 60.30±1.86 to 72.60±0.85.The fish had significantly higher survival in T_3 where the stocking density was lower than T_1, T_2. The reason for reduced survival rate in these treatments was due probably due to higher stocking density of fingerlings as well as competition for food and space in the experimental ponds. Similar results were obtained by Rahman*et al.* (2011) for fingerlings of various fish species. The negative influence of increased stocking densities on survival rates might be due to higher competition for food and space among the fishes. Similar kind of situations was also reported by Sahoo*et al.* (2010) in yellow catfish. Debnath*et al*., (2016) revealed that *Ompokpabda* larvae obtained survival rate 69.22±1.78 (T_1), 51.15±1.10 (T_2), 44.79±0.44 (T_3) respectively and the survival rate was lower than that of the present study.The production of *Ompokpabda*significantly ranged between T_1 (3249.30±50.12) to T_3 (4358.60±62.2). Fish production in treatment T_3 in the present study was higher than the rest of the other two treatments T_1 and T_2.The production of present study was lower than those of Rahman*et al*., (2008) who reported that production of *Ompokpoabda*were 127333 to 147670 fingerlings ha^{-1} for 8 weeks nursing.Debnath*et al*., (2016) stated that the production of *Ompokbimaculatus* were 2224.39±18.57, 1677.72±103.66and 1395.99±38.26 in treatment T_1, T_2 and T_3 respectively during 8 weeks culture that was much lower than the present study. The probable reason of lower production in their study might be due to earlier observation. Kohinoor *et al*. (2009) had experiment to culture *Mystuscavasius*with Indian Major Carps and *Ompokpabda*; and had concluded that it could be cultured effectively in low input carp polyculture management. Kohinoor *et al*. (2006) have obtained production of 1370 to 1535 kg/ha in six months of culture of *Mystuscavasius*inmonoculture management that was much lower than the present study.

Economic analysis

According to the cost-benefit analysis, all treatments in the present study were economically profitable. The cost of different input and economic return from the sale of fishes in different treatments are summarized in table 4. The cost input was lowest in T_1 treatment followed by T_2 and T_3. The net profitwas highest in $_{T3}$ treatment and lowest in T_1 treatment which was significantly different among the treatments, in the present experiment CBR of three treatments combined was 1:0.95 (T_1), 1:1.18 (T_2), 1:1.58 (T_3). Treatment T_3 is higher than those of T_1 and T_2.

Table-4: Cost and benefits from the rearing of *Ompokpabda* fry under different treatments (based on 1 ha).

Treatments / Economics	T_1	T_2	T_3
Pond preparation (BDT/ha)	15000±0.00[a]	15000±0.00[a]	15000±0.00[a]
Cost of lime (BDT/ha)	5631±0.00[a]	5631±0.00[a]	5631±0.00[a]
Cowdung	4940 ±0.00[a]	4940 ±0.00[a]	4940 ±0.00[a]
TSP	27170±0.00[a]	27170±0.00[a]	27170±0.00 [a]
Urea	55575±0.00 [a]	55575±0.00 [a]	55575±0.00 [a]
Cost of fry (BDT/ha)	160510±237.98 [a]	172880±118.98[b]	185230±97.89[c]
Feed cost (BDT/ha)	304130±134.16[a]	380230±294.08[b]	456170±173.19[c]
Harvesting cost	2100±0.00[a]	2100±0.00[a]	2100±0.00[a]
Labour cost	7200±0.00[a]	7200±0.00[a]	7200±0.00[a]
Total cost (BDT/ha/6 months)	582250±103.8[a]	670730±407.29[b]	759020±269.63[c]
Total return (BDT/ha/6months)	1137200±17542.10[a]	1467000±31701.21[b]	1961400±28015.75[c]
Benefit (BDT/ha/6months)	555000±17645.45[a]	796300±32099.89[b]	1202300±28285.37[c]
CBR	0.95±0.03[a]	1.18±0.05[b]	1.58±0.04[c]

[Lime = 190kg = 1.9kg/dec = 469.3ha; Price 12BDT/kg; Fertilizer = Cowdung 10kg/dec = 2470ha; Price 2BDT/kg; TSP = 5kg/dec = 1235ha; Price = 22BDT/kg, Urea = 15kg/dec = 3705ha; price = 15BDT/kg, Cost of feed = 10.25kg/dec = 2531.752ha; Price = 120BDT/kg; 150BDT/kg; 180BDT/kg; Cost of Fry = 1BDT/piece].

In the cost-benefit analysis, all input costs were strictly considered despite the fact that, in reality, most of the small scale fish farmers use their own resources like lands and labors etc. In this case, cash input costs would be lower and profit would be higher than the present analysis. In this study, benefit was higher in treatment T_3 than T_1, and T_2. Rahman*et al*., (2009) analyzed the cost and benefits of Reba carp (*Cirrhinusariza*) nursing and got the benefit of BDT 692777.32/ha for 8 weeks that was higher than those of present study. Samad*et al*., (2014) obtained net profit BDT 3,54,061/ha to 713542/ha for 120 days of *Clariasbatractus* that was much lower than present study. Rahman and Monir (2013) got the benefit BDT 240757 to 571112/ha for 7 weeks nursing of thai koi (*Anabas testudineus*).In this experiment, the benefit was higher than the above findings. The cost benefit Ratio (CBR) in treatment T_3 was significantly higher. The better performance of fish in treatment T_3 might be due to low stocking density. The cost benefit ratio (CBR) in treatment T_3 (1.58±0.04) in the present study was higher than T_2 (1.18±0.05) and T_1 (0.95±0.03) which is less similar to the findings of Khan *et al.* (2003). The present finding is coincide with the findings of Samad*et al*., (2016) in *Labeobata*nursery pond. The present findings is higher than the findings were obtained from Samad*et al*.,(2014) who studied that the CBR was highest in T_3 (1: 1.24) the lowest was found in T_1 (1:0.56) for *Clariasbatractus* in ponds of north western region of Bangladesh.

Conclusion

From the study, it can be concluded that the culture of *Ompokpabda* in one stage rearing with the application of appropriate density to get higher benefit in the short period of time for fingerlings production. The research has made significant contributions to the farmers have become more aware of the benefit of low stocking density. The findings of this experiment can help the farmers a lot in rearing and culture of this endangered species of *Ompokpabda* in captive condition.

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A review on commercially available methionine sources for aquaculture

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Abstract

In aquaculture feed formulations, Methionine (Met) is usually the 1st limiting essential amino acid (EAA) especially in low fish meal (FM) diets. Hence it is required to include a supplemental source of Met in order to meet the feeds' specification, targeting the animals' requirements for this particular EAA. When it comes to supplemental nutrients or additives there are three parameters that have to be seriously evaluated (i) nutritional value (biological effectiveness) of the supplemental nutrient, (ii) stability, homogeneity etc. during the feed production process and (iii) physical properties of the nutrient source during feeding practice. A recent critical review, updating the original publication entitled 'Relative bioavailability of methionine sources in fish' (Lemme, 2010), with all the latest scientific publications and industry trial data on both fish and shrimp.The current talk will provide a critical overview of the studies reporting on the nutritional value of different Met sources in aquaculture species.

Keywords: Methionine sources, supplemental Met, aquaculture nutrition, biological effectiveness

Introduction

All living organisms, including fish and crustaceans, do not require protein per se, but amino acids (AA), the building blocks of proteins. All AA structurallycontain 3 common parts: a central carbon bond to a hydrogen, a nitrogen containing amino group and a carboxylic group. Proteins, consisted of few up to thousands of AA, having numerous structural and metabolic functions. In animal production, the most direct result of AA deficiencies translates to reduced growth.Traditionally, it was only the economic incentive, which resulted in the use of supplemental AA in diet formulation. However, there has been a gradual evolution with more emphasis directed towards sustainability and total nutrient supply.

In aquaculture feed formulations,Methionine (Met) is usually the 1st limiting essential amino acid (EAA) especially in low fish meal (FM) diets. It is therefore, required to include a supplemental source ofcrystalline Metin order to meet the feeds' specification, targeting the animal's requirement for this particular EAA. While evaluating supplemental nutrients or additives for use in feed formulation, three parameters needs to be considered: (i) nutritional value (biological effectiveness)of the supplemental nutrient, (ii) stability, homogeneity etc. during the feed production process and (iii) physical properties of the nutrient source during feeding practice.

Commercially available Methionine sources

There are several commercially available Met sources in the market like DL-Met (DL-Methionine for Aquaculture), DL-Methionyl-DL-Methionine (AQUAVI® Met-Met), L-Met (L-Methionine), Methionine Hydroxy Analogue-free acid (MHA-FA or liquid MHA) and Methionine Hydroxy Analogue calcium salt (MHA-Ca). Both terrestrial as well as aquatic animals can utilize all existing Met sources however, their biological availability differ greatly. The differences in biological availability is a reflection of differences in product matrix, digestibility, transport mechanism and metabolic conversion requirements.

DL-Met, as well as the dipeptide DL-Methionyl-DL-Methionine (Met-Met),are the racemic mixture of D- and L-isomer of Methionine andis commercially available as feed additive with 99% DL-Met and 95% Met-Metpurity, respectively.As only the L-isomer can be utilized for the protein synthesis by the animals' body, D-isomer is metabolically converted to L-isomer first through oxidationto keto-Methionine by the enzyme D-

amino oxidase and then transaminated by transaminase enzyme to L-Met. The enzymes required in the conversion of D-form to L-form are not at arate-limiting factor in fish and shrimp alike poultry and swine.

On the other hand MHA-Ca and MHA-FA are also racemic mixtures of its D- and L-isomer. MHA-Ca consists of about84 % MHA monomer, 12% calcium and 4% water and MHA-FA ,65% monomer, 23 % dimers/trimers and the remaining 12% water.Cchemically both MHA-Ca and MHA-FA cannot be classified as AA. AA contains both a carboxyl (COOH) and amino group (NH2), however, in MHA, the NH2 group is replaced by a hydroxyl (OH) group and for that reason cannot be classified as an amino acid(Dibner 2003). MHA has to undergo a series of metabolic transformation in order to be utilized by the animals. Through a dehydrogenase reaction, it is first converted to a-keto analogue of methionine and then to the utilizable L-methionine via a transaminase reaction.

Finally L-Met is also commercially available as a feed additive with 99 % purity and do not require any conversion as the L form can be utilized by the body. It is however clearly stated by the National Research Council based on nutritional studies (NRC, 2011), fish and shrimp can use D-Met to replace L-Met on an equimolar basis.

Evonik Animal Nutrition published a recent critical review, updating the original publication entitled 'Relative bioavailability of methionine sources in fish' (Lemme, 2010), with all the latest scientific publications and industry trial dataon both fish and shrimp.

Several studies comparing the nutritional value of MHA products with DL-Met conducted in fish concluded that bothMHA-FA and MHA-Ca are significantly less available than DL-Met (Lemme 2010; Lemme et al. 2012; Figueiredo-Silva et al. 2014; Powell et al. 2017). By applying regression analysis and comparing the slopes for weight gain between Met sources, revealed nutritional value of MHA-Ca relative to DL-Met varied between 22% in Nile tilapia and channel catfish to 62% in red drumon weight to weight basis (wt/wt), as presented in Table 1 and Figure 1.

At this point it is worth explaining the meaning of equimolar and wt/wt basis with a simple example. According to NRC (2011) "on the basis of available experimental evidences, the committee considers it reasonable to assume that the biological efficacy of HMB (2-hydroxy-4-(methylthio)butanoic acid known as MHA) for fish is about 75 to 80% that of DL-Met on a equimolar basis. The equimolar ratio, results from in vivo scientific experiments determining the relative bioavailability of the tested nutrient sources through the analysis of key growth performance parameters of a dose response trial using regression analysis. The translation to the wt/wt basis is by multiplying the equimolar ratio of the two Met sources with the active ingredient of the product. As mentioned above DL-Met has >99% Methionine purity and MHA-Ca 84% 2-hydroxy-4-(methylthio)butanoic acidhence,with an equimolar basis of 77%,MHA-Ca has 65% biological efficacy on a wt/wt basis compared to DL-Met [77 (equimolar) x 0.84 (MHA content in the MHA-Ca product) = 65%].

Further studies comparing DL-Met and L-Met showed anon-statistically significant but slightly lower bioavailability of L-Met (82-83%) relative to DL-Met in salmonids. This requires further investigation, but agrees with earlier data obtained in salmon (Sveier et al. 2001), rainbow trout (Kim et al. 1992) and in hybrid striped bass (Keembiyehetty and Gatlin III, 1995), showing that D- and/or DL-Met are at least as effective as L-Met. A study in Indonesiashowed that *L. vannamei* fed with supplemented Met-Met has a 193% higher bioavailability in terms of biomass gain and 207% higher for FCR compared to the group fed with L-Met. Further studies in *L. vannamei* with Met-Met show a higher bioavailability ranging from 178% up to 298%compared with DL-Met establishing an average minimum of 200% bioavailability of Met-Met relative to DL-Met as well as 65% bioavailability for MHA-CA compared with DL-Met as it was validated in the Thailand trial (Table 1, Figure 2).

It is not only the nutritional value of the different Met sources but the physical characteristics of the products are equally important. Feedmillingcalls for extra attentionlooking atthe caking tendency and flowability of the different Met sources as well as the mixability and homogenous distribution of the critical and costly nutrients

in the feeds. Hence, the average size of the particlesis considered as one of the main factors for the mixing homogeneity in the feeds.

Finally, a critical parameter in aquaculture feeds is the solubility and leaching of the supplemental nutrients, like Met, especially since the feeding takes place exclusively in salt or fresh water. In vitro tests proved that the water solubility levels of each of the commercially available Met sources also vary significantly. As presented in Figure 3, the dipeptide (Met-Met)being 5 to 10 times less water soluble compared to other commercially availableMet sources. Since the nutrients' leaching from the pellets is strongly related to the feeding behavior of the targeted aquaculture species,Met-Met it is more suitable to crustaceans where the feed stays for a prolonged time in the water before it is fully consumed.

Table 1. Nutritional Value of different Met sources relatively to DL-Met (wt/wt comparison to DL-Met)

Nutritional Value of different Met sources relatively to DL-Met (wt/wt comparison to DL-Met)							
Species	**Response criteria**	**Analysis**	**MHA-Ca**	**MHA-FA**	**L-Met**	**Met-Met**	**Publication**
Fish							
Channel catfish	Weight gain	Slope-ratio	22		100		Robinson et al. 1978
Hybrid striped bass	Weight Gain	Slope-ratio		86			Li et al. 2009
Hybrid striped bass	Weight Gain	Slope-ratio*	53				*reanalysed by Lemme 2010 (Keembiyehetty and Gatlin, 1995)
Red drum	Weight Gain	Slope-ratio*	62	47			*reanalysed by Lemme 2010 (Goff and Gatlin, 2004)
Hybrid striped bass	Weight Gain	Slope-ratio*		38			*reanalysed by Lemme 2010 (Kelly et al. 2006)
Nile Tilapia	Specific Growth Rate	Slope-ratio	22				Lemme et al. 2012; Facts & Figures 1615
Common carp	Weight Gain	Slope-ratio	46				Facts & Figures 1617
Atlantic salmon	Weight Gain	Slope-ratio			83		Figueiredo-Silva et al. 2014; Facts & Figures 1620
Rainbow trout	Weight Gain	Slope-ratio	58		82		Powell et al. 2017
Species	**Response criteria**	**Analysis**	**MHA-Ca**	**MHA-FA**	**L-Met**	**Met-Met**	**Publication**
Shrimp							
L. Vannamei	Specific Growth Rate	Slope-ratio				178	Facts & Figures 1623
L. Vannamei	Specific Growth Rate	Slope-ratio				298	Facts & Figures 1624
L. Vannamei	Specific Growth Rate, FCR	Validation study	65			200	Facts & Figures 1631
L. Vannamei	Biomass Gain				193**		Under preparation (Facts & Figures -trial in Indonesia)

* *wt/wt comparison to Met-Met [Met-Met 193%]

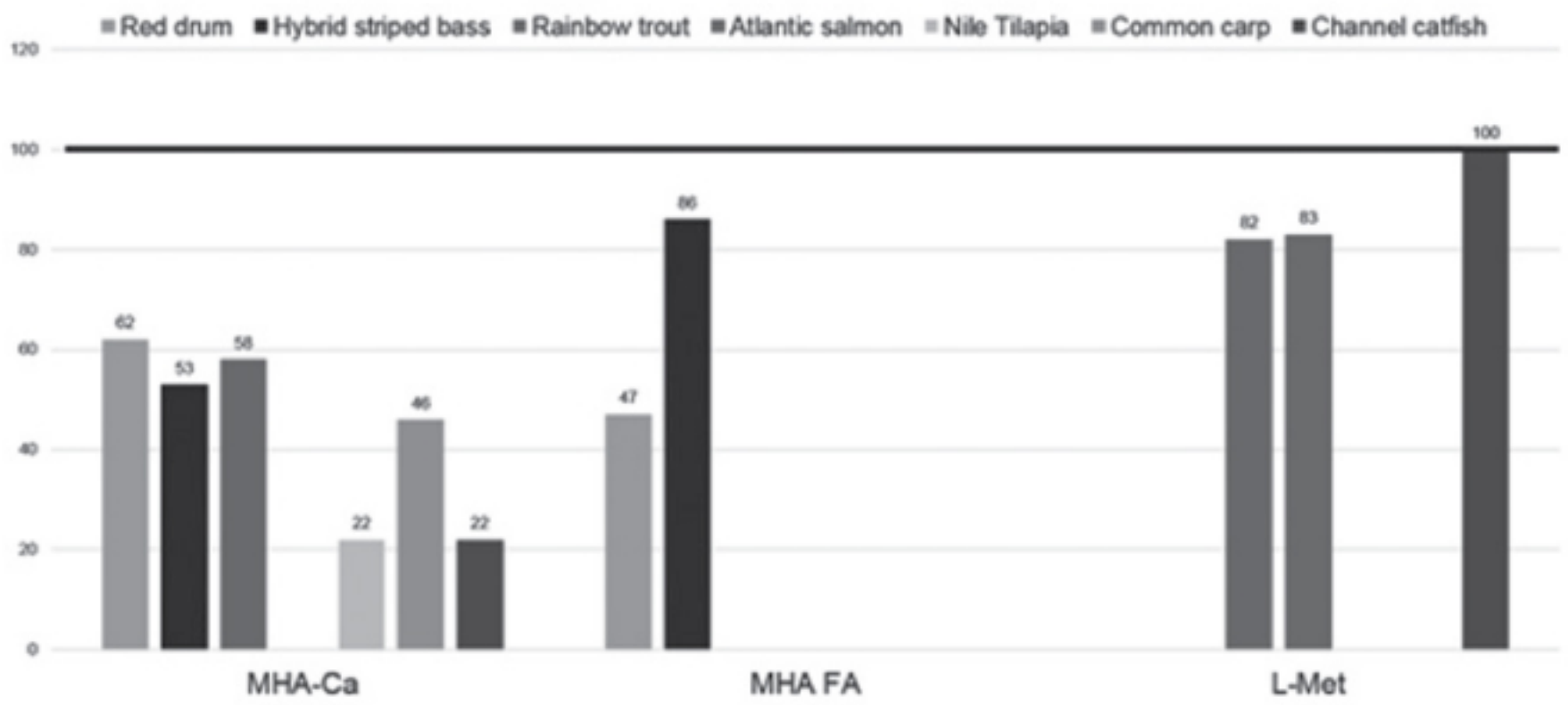

Figure 1: Nutritional Value of different Met sources (wt/wt comparison to DL-Met) on several fish species.

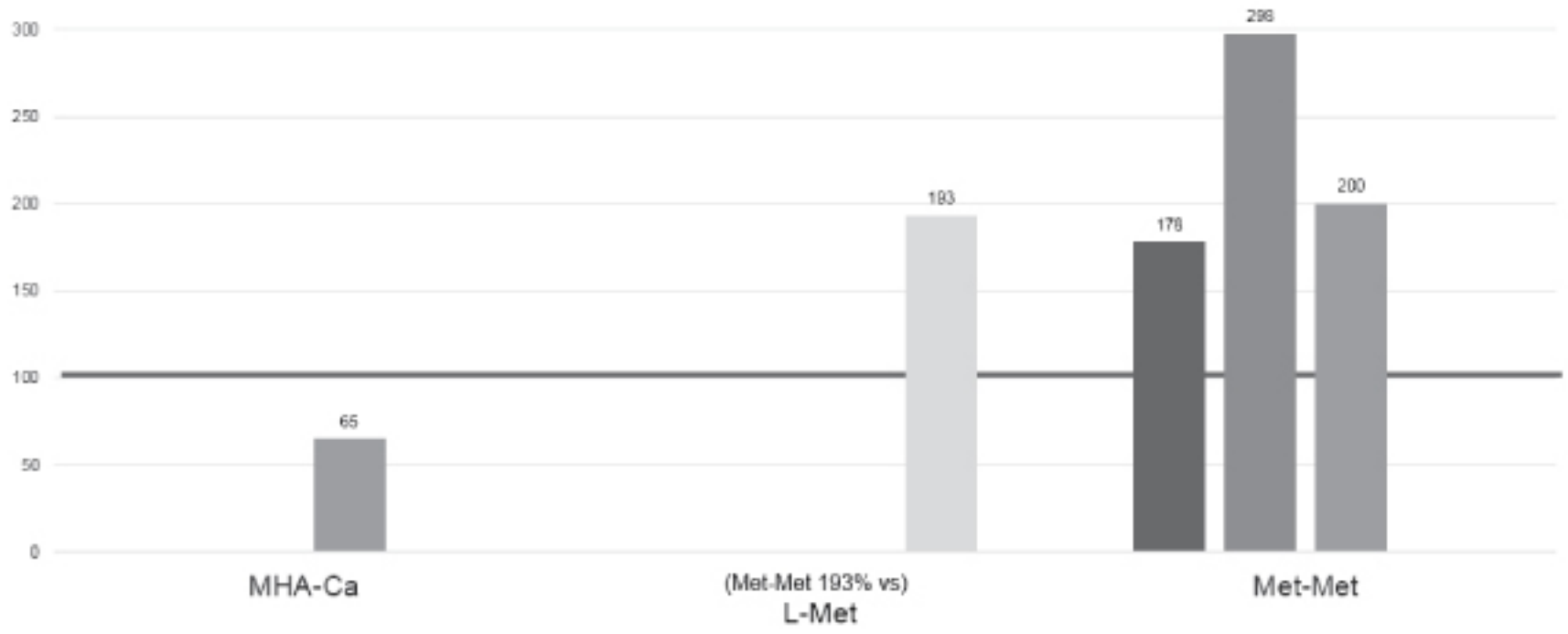

Figure 1: Nutritional Value of different Met sources (wt/wt comparison to DL-Met) on shrimp (*L. vannamei*)

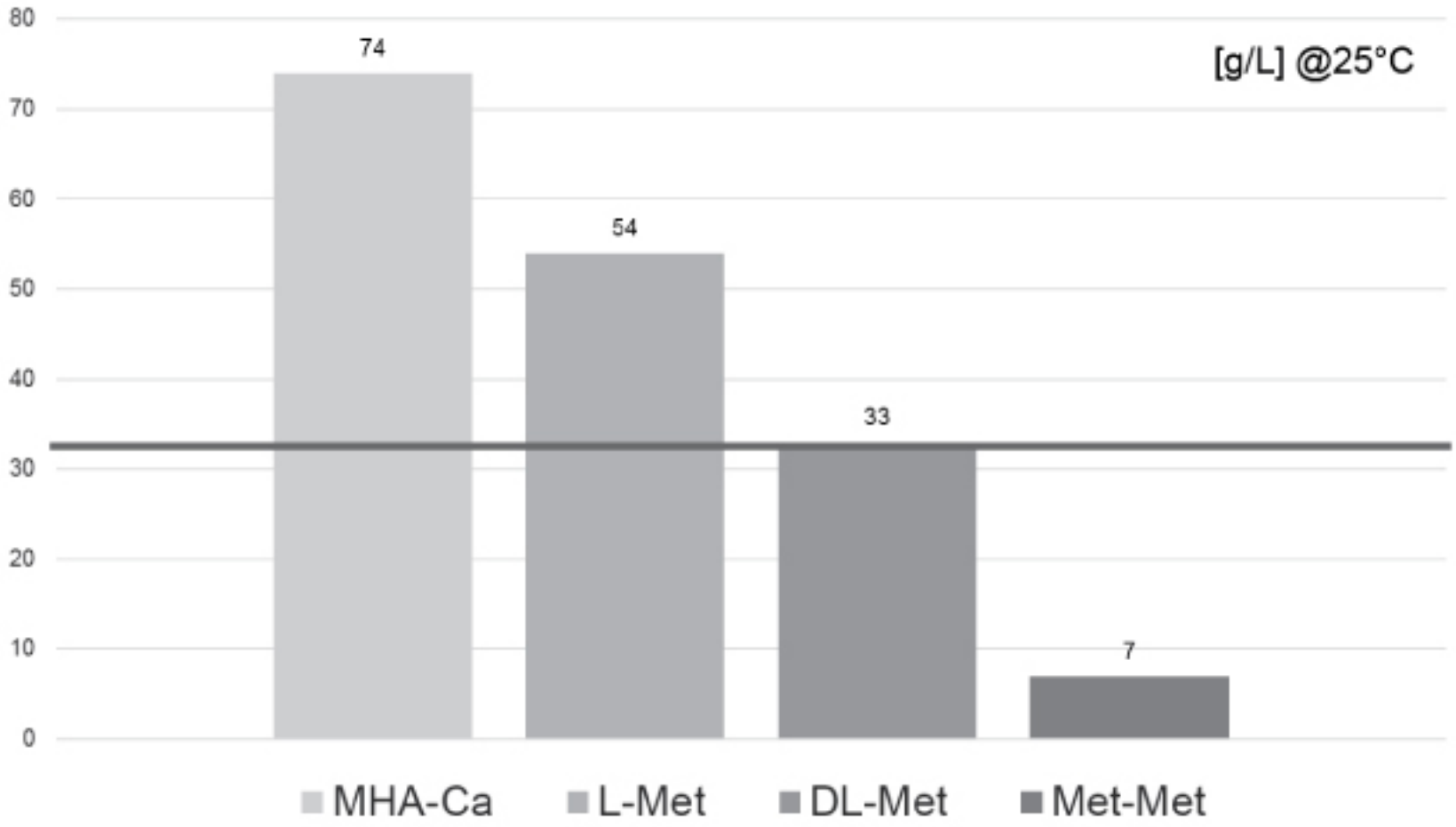

Figure 3. Water Solubility of different commercially available Methionine sources.

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Role of Micro organisms in Aquaculture

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Introduction

Aquaculture is an industrial process of raising aquatic organisms upto final commercial production within properly partitioned aquatic areas. Bycontrolling the environmental factors and positive administration the life history of the organism it has to be considered as an independent industry from the fisheries hitherto.

Faced with one of the world's greatest challenges – how to feed more than 9 billion people by 2050 in a context of climate change, economic and financial uncertainty, and growing competition for natural resources. An aquaculture is a promising source of food in the future, with incidences of high antibiotics residues and hormonal residues; the live stock protein source may not be suitable for consumption whereas the aquatic protein source is highly rich in Poly unsaturated fatty acids and protein present are highly nutricious when compared to other animal food sources.

Since 1980's fishery production was relatively static and has been responsible for the impressive growth in the supply of fish for human consumption Whereas aquaculture provided only 7 percent of fish for human consumption in 1974, this share had increased to 26 percent in 1994 and 39 percent in
2004.

Growth in the global supply of fish for human consumption has outpaced population growth in the past five decades, increasing at an average annual rate of 3.2 percent in the period 1961– 2013, double that of population growth, resulting in increasing average per capita availability. World per capita apparent fish consumption increased from an average of 9.9 kg in the 1960s to 14.4 kg in the 1990s and 19.7 kg in 2013, with preliminary estimates for 2014 and 2015 pointing towards further growth beyond 20kg.

Problems associated in Aquaculture

Deep-Green Pond Water

Water color turns to deep green because of the presence of excessive algae in water, which is the result of overabundance of nutrient like phosphorus, particularly when the water is warm with good climate. Even heavy fish load contributes to green pond water because fish waste is broken down by beneficial bacteria through a series of steps into nitrate, which is food for plants and algae. As per the World Health Organization (WHO) standard when the population of algal cells exceeds 100,000 cells/ mL, the condition is termed as Algal Blooms. Appearences of blooms are generally observed in the summer season. In general, indirect negative effects of algae are foremost in this context. Excessive algae growth can lead to a lack of dissolved oxygen in the water, particularly, drops during night (due to excessive consumption of O2 and absence of photosynthesis). In this situation, due to the heavy shortage of DO, fish start grasping on the water surface and sometimes face mass mortality. Phytoplankton blooms can also cause large diurnal fluctuations in water quality variables (e.g. very high pH and NH3 levels; due to excessive utilization of free CO2 for photosynthesis) in mid-afternoon. Such conditions are stressful to fish. Blue-green algae (Cyanobacteria) can also produce toxic substances that are responsible for lethal to some fishes. They can also produce compounds that impart a strong offflavour to fish. In addition, the dead algae accumulate at the pond bottom and create toxic gasses due to decomposition.

Muddy Pond Water

Muddy water can hinder the feeding ability of fish and even reduce their growth. Additionally, phytoplankton

growth and abundance is reduced in muddy water as it hinders sunlight penetration into the water. This may compound the problem of poor fish growth in muddy ponds by reducing the amount of food available through the entire food chain. Even fish gill can be damaged due to excess turbidity.

Oxygen Depletion In Pond and Fish Grasping For Air

Oxygen depletion is a commonly encounter problem by the fish farmers. At very early morning floating and grasping of the fishes on the water surface has been observed. It may happens due to the shortage of DO in the water. At extreme less DO with prolonged peroid, fishes start to die and dead fish could be found with wide mouths. In this context large fish may die first, but all sizes of fish are usually affected. While mollusks and crustaceans may gather at the pond edges. The causes behind low dissolved oxygen content in pond water may be:

- Dense plankton blooms or dense stands of pond weeds.
- Several days of cloudy weather that reduces plant oxygen production.
- High temperatures, which decrease the solubility of oxygen in water and increase oxygen consumption by plants and animals.
- Sudden die-off of plants or algae, especially associated with herbicide use.
- Unusual weather patterns, such as storm fronts and heavy cold rain.
- Overstocking fish, excessive fertilization, or high feeding rates.
- Decomposition of organic matter, leaves and branches of trees in water
- Excessive application of wet cow dung in pond
- Excessive turbidity in water
- High iron content in soil

Excess Ammonia

In ponds, ammonia is produced by the activities like excretion of fishes and crustaceans; microbial decomposition of organic materials; urea and ammonium fertilizers; supplementary feed etc. Unionized ammonia fraction is more toxic to fish. A concentration of 0.02-0.05 mg / L of unionized ammonia is safe for fishes. The concentration of unionized ammonia increases with increasing pH and temperature it is lethal at higher concentration to fishes. Besides pH and temperature, some other parameters are also having indirect effects on the ammonia toxicity-

- Ammonia is more toxic when dissolved oxygen concentration in fish ponds is low;
- Toxicity of ammonia decreases with low pH and increased CO_2 concentration in fish ponds;
- Toxicity of ammonia decreases slightly by high concentration of calcium.

Hydrogen Sulphide

The absence of Hydrogen sulphide (H_2S) in freshwater fish-ponds is mandatory. Any detectable concentration of H_2S in water creates stress to fish. Hydrogen sulphide is produced by chemical reduction of organic matter that accumulates and forms a thick layer of organic deposit at the bottom. Unionized hydrogen sulphide is toxic to fish.

Black Mud at Pond Bottom

Due to buildup of extra feed and organic matter at the pond bottom, soil of the bottom turns to dark and smelly. Particularly, the problem can be severe in a pond where fish culture is going on for long time. As a result, fish may face mass mortality due to the toxic gases at the bottom.

Role of Probiotics in Aquaculture

The term “probiotic” was first introduced by Parker in 1974 and the original definition was “organisms and

substances which contribute to intestinal microbial balance." The definition of probiotic was redefined as "live microorganisms which, when administrated in adequate amounts, confer a health benefit on the host" (FAO/WHO 2001). Most probiotics are supplied as live supplements in feed to benefit the host by inhibiting pathogenic microbes, improving immune response, improving survival and growth rates, enhancing digestion and increasing feed utilization, promoting antimutagenic and anticarcinogenic activity, and improving water quality in culture systems.

Mechanisms of Action

Competitive Exclusion

Probiotics can bind to colonic cell lines and to mucin, which is thought to aid colonization of the animal gut system (Schiffrin et al. 1995, Ouwehand et al. 2000, Juntunen et al. 2001). The microflora of the gastrointestinal tract (GIT) of aquatic animals can be modified by ingestion of other microorganisms. However, the dominant bacteria in fish intestine are quite different from those observed in mammals (Gatesoupe 2005). The composition of microbial communities is greatly influenced by husbandry practices and environmental conditions, e.g. culture water and abiotic or biotic factors, which stimulate the proliferation of selected bacterial species. Therefore, addition of beneficial bacteria to culture water or via feed supplementation during initial egg fertilization or pre-larval stages could have a distinct advantage through the mechanism of competitive exclusion for attachment sites on egg surfaces or in the GIT (Irianto and Austin 2002). Competitive exclusion by potential probiotic bacteria can be evaluated by in vitro antagonistic screening against multiple strains of pathogenic bacteria (Figs. 3A and 3B).

Bacterial antagonism is a common phenomenon in nature. Probiotics may prevent opportunistic pathogens from colonizing the surface of eggs or the GIT by producing antimicrobial compounds or by outcompeting them for nutrients or mucosal space (Bandyopadhyay and Mohapatra 2009). Certain pathogens produce proteolytic enzymes that can dissolve and digest the bacteria that approaches them, the most remarkable being Streptococcus sp and Bacillus pyocyaneus (Rettger 1905).

Many studies have addressed the use of probiotics in aquaculture, such as the improvement of the survival of portunid crab larvae Portunus trituberculatus through the application of probiotic Thalassobacter utilis in the rearing water (Nogami and Maeda 1992, Nogami et al. 1997). Lactic acid bacteria, notably Lactobacillus sp., Bifidobacterium sp. and Streptococcus sp. are effective against diseases caused by Vibrio sp. (Gatesoupe,1994; Olsson et al., 1998). Bacillus subtilis BT23 has inhibitory effects against vibriosis in tiger shrimp Penaeus monodon (Vaseeharan and Ramasamy 2003). Application of a Bacillus strain to the common snook Centropomus undecimalis increased larval survival, promoted better growth and decreased the number of suspected pathogenic bacteria in the fish gut (Kennedy et al., 1998). Alteromonas sp. can reduce the infection of Vibrio sp. in Pacific oyster Crassostrea gigas larviculture (Douillet and Langdon 1994). Some Pseudomonas fluorescens strains reduce the mortality of rainbow trout Oncorhynchus mykiss fingerlings infected with a pathogenic V. anguillarum (Gram et al., 1999).

Nutrient and Enzymatic Contributions to Digestion

It is well established that probiotics are beneficial to the intestinal health of host animals. Among the beneficial bacteria, Agrobacterium sp., Brevibacterium sp., Clostridium sp., Microbacterium sp., Pseudomonas sp., and Staphylococcus sp. can contribute to the host's nutrition, especially in supplying fatty acids and vitamins to host cells (Sakata,1990, Ringø et al., 1995). Some fish gut microbiota may participate directly in the digestion processes of fish. Enzyme-producing microbiota such as Bacillus and Enterobacteriacea (Acinetobacter sp., Aeromonas sp., Flavobacterium sp., Photobacterium sp., Pseudomonas sp., Vibrio sp., Microbacterium sp., Micrococcus sp., Staphylococcus sp.), and some unidentified anaerobes and yeasts are potential contributors (Ray et al., 2012).

The metabolic and physiological roles of fish gut microbiota have been the subject of several studies. These microbiota are able to stimulate gut epithelial differentiation and proliferation, gut motility, protein uptake,

nutrient metabolism, and innate immunity (Rawls et al., 2004, Rawls et al., 2006, Bates et al., 2006). However, these functional roles are mostly limited to the initial stage of fish fry. In bivalves and crustaceans, microbiota in the gut facilitate digestion by producing extracellular enzymes such as proteases and lipases, as well as providing necessary growth factors (Prieur et al., 1990, Wang et al., 2000). Despite numerous studies demonstrating that microbial activity in the digestive tract may be an important source of nutrients and enzymes to the host, it is difficult to attribute the exact contribution of gastrointestinal microbiota in exothermic animals because of the complexity and variable ecologies of the digestive tracts of different fish species (Ray et al., 2012).

Immune Response Enhancement

Numerous studies using in vitro models and rats indicate that probiotics have potential benefits to the non-specific immune system. As in many animal studies, the intimate association of probiotics with the intestinal mucosa, alongside intestinal cellular components, appears to improve immunological function in the GIT (Klaenhammer, 2007). The immune response is largely specialized to gut- associated lymphoid tissues (GALT) (Famularo et al., 1997). Certain infections are protected by serum, an effect called humoral immunity. The humoral immunity reaction is mediated by circulating antibodies known as immunoglobulins (Ig). Five immunoglobulins (IgA, IgD, IgE, IgG and IgM) play different roles in the humoral immune response system (Devereux 2002). Similar immune responses also have been observed in aquatic animals, when the disease protection mechanism is activated by both cellular and humoral immune defenses (Rengpipat et al., 2000).

The immune response of fish can be up-regulated through supplementation of probiotics, either in the form of monospecies or multispecies mixtures (Nayak, 2010). Phagocytic, lysozyme, complement, respiratory burst activity and the expression of various cytokines in fish can be stimulated by different probiotics (Nayak, 2010). Phagocytic activity of leucocytes increased after oral administration of Clostridium butyricum bacteria to rainbow trout, which subsequently enhanced the resistance of fish to vibriosis (Sakai et al., 1995). Similarly, administration of a mixture of bacterial strains (Bacillus and Vibrio sp.) positively influenced the protective effect of white shrimp against the pathogens V. harveyi and white spot syndrome virus (WSSV) (Balcázar 2006). This protection is largely attributed to the stimulation of the immune system by phagocytosis and antibacterial activity. Administration of a lactic acid bacterium Lactobacillus rhamnosus (strain ATCC 53103) stimulates respiratory burst activity in rainbow trout Oncorhynchus mykiss (Nikoskelainen et al., 2003). Some bacteriocin-like inhibitory substances (BLIS) such as antimicrobial peptides, proteins, or protein complexes excreted/synthesized by probiotics are also effective to control several fish diesease, including V. parahaemolyticus (Carraturo et al., 2006), Flavobacterium sp. (Balakrishna and Keerthi, 2012) and Aeromonas hydrophila (Selvendran and Michael, 2013).

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Dairy Health

In silico analysis of VP1 gene for prediction of peptide vaccine against three serotypes (O, A & Asia1) of Foot & Mouth Disease Virus circulating in Bangladesh

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Abstract

Foot and mouth disease (FMD) is a severe, highly contagious, and economically devastating viral disease affecting domestic cloven-hoofed animals. Etiological agent of this life threatening disease is positive sense RNA virus named Foot and Mouth Disease Virus (FMDV). This virus lacks the proofreading activity of the viral replicase. As a result, the viral genome becomes subjected highly to genetic diversity. For this reason, FMDV can escape the protective immunity induced by conventional vaccine, which also may induce allergic responses. Besides, the stability and longevity of conventional vaccines are affected due to frequent antigenic drift of the virus. Therefore, the study focused on not only in epidemiology of FMD in Bangladesh, but also on designing safer and alternative peptide vaccines. The computational analysis of the sequence data, from GenBank and study isolates, was performed to detect desirable T and B cell epitopes located in the VP1 of all serotypes circulating in the country. Among 109 clinically suspected samples, the FMD virus was detected in 78 samples, which belonged to only three serotypes O, A and Asia 1. Highest outbreak rate of FMD was found in Sirajganj during 2015-2016. From amino acid alignment of the study isolates and GenBank sequences, the motif **DLXXLA** was found conserved for all the three serotypes. The linear B-cell epitopes were detected in amino acid sequence position 39-43 for serotype O, position 49-65 for serotype A and position 1-9 for serotype Asia1 through computational analysis using IEDB and BCPREDS webserver. IEDB MHC-1 binding prediction tool has predicted T-cell epitopes interacting with different type of MHC class 1 Bola alleles. **TSFNYGAIK** (for BoLA-N:01201 allele), **ASFNFGAIK** (for BoLA-N:01301 allele) and **QMMNFDLLK** (for BoLA-N:05501 and BoLA-N:00301 allele) were predicted as T-cell epitope for serotype O, A & Asia1 respectively using IEDB resources as their percentile rank is lower than 1.00. The 3D structure of VP1 protein has showed all the predicted epitopes. However, these predicted B-cell and T-cell epitopes were expected for efficient induction of either humoral and cell mediated immunity.

Keywords: Foot and Mouth Disease Virus, Serotypes, VP1 gene, peptide vaccine.

Introduction

Foot and mouth disease (FMD) is a severe, highly contagious, and economically disturbing viral disease affecting domestic cloven-hoofed cattle, pigs, sheep, goats as well as more than 70 species within 20 families of mammals (Barnett and Cox,1999; Brown, 2003, Grubman and Baxt, 2004; Du et al., 2007). Every year this disease causes economic loss of US$60–150 million in Bangladesh (Momtaz et al., 2014). The etiological agent FMDV is small non-enveloped virus and belongs to the genus Aphtho virus of the family Picornaviridae. FMDVs are classified into 7 serological types based on antigenic properties of the capsid proteins. The serotypes are O, A, C, Asia-1, SAT-1, SAT2, SAT3 (Mittal et al., 2005). Presently, 6 serotypes of FMD virus (O, A, Asia-1, SAT-1,-2, and -3) are circulating worldwide, and serotype C has not been recorded since 1995 (Biswal et al., 2012). The genome of the FMDV is a positive sense single stranded RNA of about 8.3 kb.The genome contains a long open reading frame (ORF) that encodes four structural proteins (VP4, VP2,VP3, and VP1), and 10 non-structural proteins (L, 2A, 2B, 2C, 3A, 3B1, 3B2, 3B3, 3C, and 3D)

(Grubman and Baxt, 2004; Du et al., 2007). The structural proteins VP1, VP2, and VP3 are surface exposed, while VP4 is completely internal. Among them VP1 is the key antigen of FMDV capsid protein that contains the major B-cell epitope, which is the major immunodominant epitope prompting protective humoral immunity (Gurumurthy et al., 2002; Brown, 1995).Genetic heterogeneity within FMDV may arise as a result of normal genetic drift (Dopazo et al.,1998), high mutation rate and genetic recombination. Genetic variability of viruses also influence by molecular host factors and/or selective pressures indirectly incurred from lab methodologies (Drake and Holland, 1999; Eigen, 1971; Martinez, 1988; Carrillo et al.,1998; Martinez, 1988). As a result conventional vaccination is insufficient to prevent the disease (Kitching, 2005; Domingo et al., 1992, Domingo et al., 1990). The study of the genetic variation and distance of VP1 gene as well as antigenic variability is thus important for proper vaccine design and to prevent obstacles in control of the disease (Tayo, 2011).

Materials and Methods

Detection and Sequencing of FMDV: A total 109 clinically positive samples were collected from 8 different districts of Bangladesh. Then the samples were processed and nucleic acid (RNA) was extracted using using QIAamp® Viral RNA kit (Qiagen, Germany). Extracted RNA was first subjected to RT-PCR using universal primers 1F/1R. Serotype specific primers were used after initial confirmation. Several representative samples were sequenced using sanger sequencing method. Overlapping regions of forward and reverse sequences were determined and aligned using to obtain the entire sequence CLC MAIN WORKBENCH 7.6.2. Accession number of this study isolates are KX772232-36, KX784484-89 & KP119763.

Amino acid sequence alignment and variability index: A number of sequences of envelope proteins of FMD virus and reference sequences from NCBI database were used to predict the amino acid sequence variability envelope protein. GENEIOUS R6 and CLC MAIN WORKBENCH 7.6.2 were used to translate the nucleic acid sequences into respective amino acid sequences and verified by EBI, EMBOSS Bioinformatics Tools (Transeq). We used ClustalW to align the amino acid sequences and reference sequences and analysis was performed using SeqManII (Lasergene 8.0; DNAStar Inc., WI, USA) for discerning amino acids sequence variability (Garcia-Boronat et al., 2008). Wu–Kabat variability coefficient was used to calculate protein variability index with the help of Protein variability server (PVS). The determination of variability coefficient was performed using the following formula: variability = N*k/n, where, N is the number of sequences in the alignment, k is the number of different amino acids at a given position, and n is the times that the most common amino acid at that position is present (Kabat et al.,1977).

Modeling and validation of envelop protein 3d structure: SWISS-3D MODEL was used for generating 3D structure of envelop protein applying homology modeling Method (Alam et al., 2003; Arnold et al., 2006). The 3D structure was validated by PyMol.

Epitope Prediction: We used antibody epitope prediction server to predict continuous B-cell epitope of FMD virus envelope protein based on amino acid sequences. Chou and Fasman Beta-Turn Prediction (Chou and Fasman, 2009) , Emini Surface Accessibility Prediction (Emini et al.,1985), Karplus & Schulz Flexibility Prediction (Karplus and Schulz,1985), Kolaskar & Tongaonkar Antigenicity (Karplus and Schulz,1985), Parker Hydrophilicity Prediction (Parker et al.,1986), and Bepipred Linear Epitope Prediction (L) (Larsen et al., 2006) methods were employed for this predicttion. The secondary structure was determined using Garnier–Robson (Garnier and Robson,1989) and Chou–Fasman (Chou and Fasman, 2009) methods. The peptides with good hydrophilicity, high accessibility, flexibility and strong antigenicity were selected according to the results of these methods. Additionally, we used BCPREDS server to predict continuous, linear & flexible length B cell epitope prediction. DNAStar Protean system was used to predict continuous B cell epitopes on VP1 of FMDV, by using amino acid sequences. The secondary structure was pre-dicted using Garnier–Robson (Garnier and Robson,1989) and Chou–Fasman methods (Chou and Fasman, 2009). Surface properties of the VP1 protein, such as hydrophilicity, flexibility, accessibility, and antigenicity, were analyzed by Kyte–Doolittle (Kyte and Doolittle, 1982), Karplus–Schulz (Karplus and Schulz, 1985), Emini (Emini et al., 1985), and Jameson–Wolf (Jameson and Wolf, 1988) methods, respectively. Besides, BCPred (Bui et al.,

2005) and BepiPred (Saha and Raghava, 2004) were used to evaluate linear B cell epitopes (Haste et al., 2006).

T cell epitopes were predicted using IEDB analysis for MHC-I prediction. At present, about 60 full-length validated cattle MHC class-I cDNA sequences are available (http://www.ebi.ac.uk/ ipd/mhc/bola), and based on that data, alleles were selected for MHC-I prediction. IEDB analysis resource using Aver-age Relative Binding (ARB) was utilized to predict IC50 values for peptides binding to specific MHC molecules (Bui et al., 2005).

Results

RT-PCR Detection of FMD Virus:

A total of 109 samples from 8 different regions were collected and tested for the presence of FMDV RNA. Out of 109 samples, 78 (71.6%) samples were positive for FMD virus. Different serotypes of FMDV were detected by RT-PCR using specific primer sets. In this study three serotypes; O type, A type and Asia 1 type has been identified. Among these isolates serotype Asia 1, A and O type accounts for about 46.8% , 0.01% and 48.6%, of the total isolated samples, respectively 27 samples representing 24.8% of the total positive samples was found to be responsible for mixed infection. The co-infection were of FMDV serotype O+Asia1 and O + A . In this study 23.9% of the total samples were found to be responsible for O+Asia1 mixed infection whereas only 0.01% samples were responsible for O+Asia1 mixed infection.

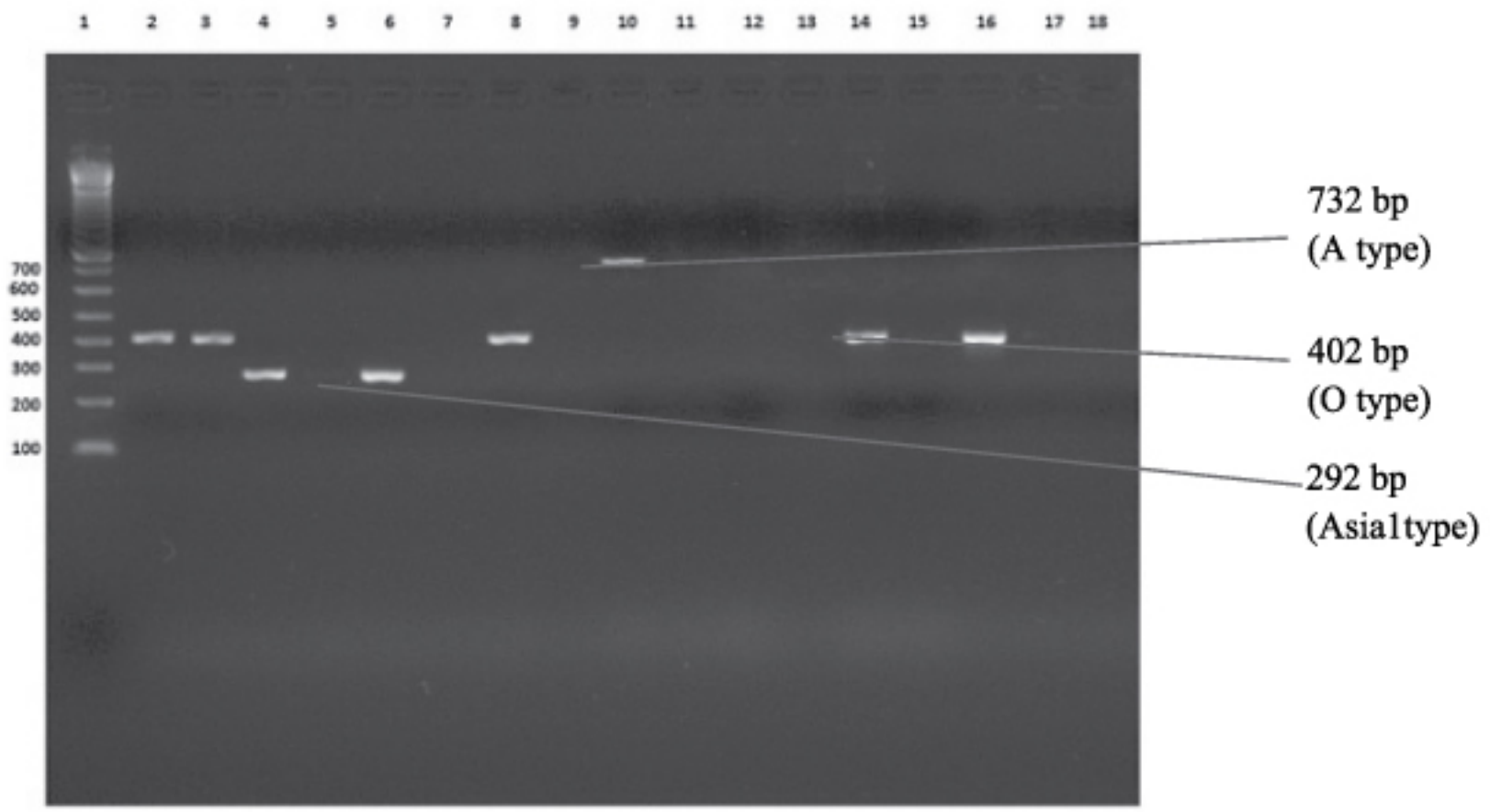


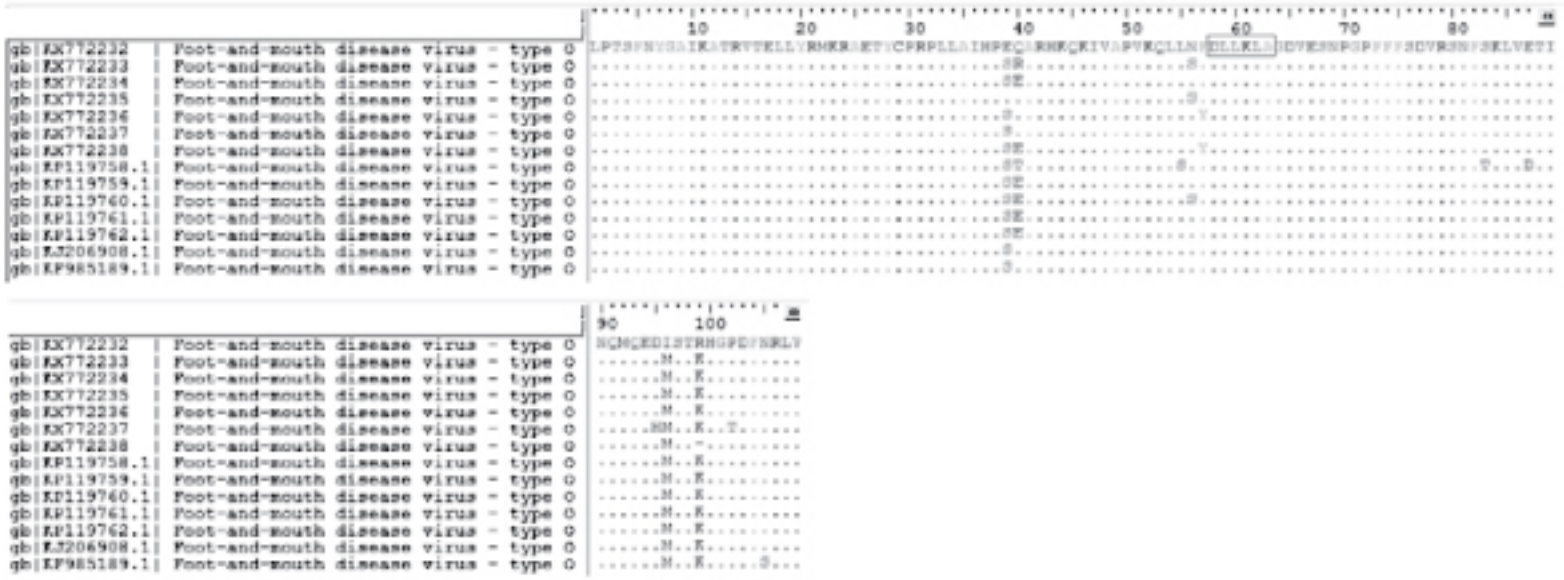
Figure 1: Electrophoresis of RT-PCR products on 2% agarose gel stained with ethidium bromide. Lane 1: 1 kb plus DNA ladder (Invitrogen, USA), lane 2,38,14,: FMDV O serotype, lane 4,6: FMDV Asia 1 serotype, lane 10: FMDV A serotype), lane 16: Positive control (Asia 1 Slhamir, Inact antigen, IAH PIRBRIGHT LABORATORY, UK), lanes 17: Negative control (RNase-free water)

Table 1. The distribution of samples and their diagnostic RT-PCR test status.

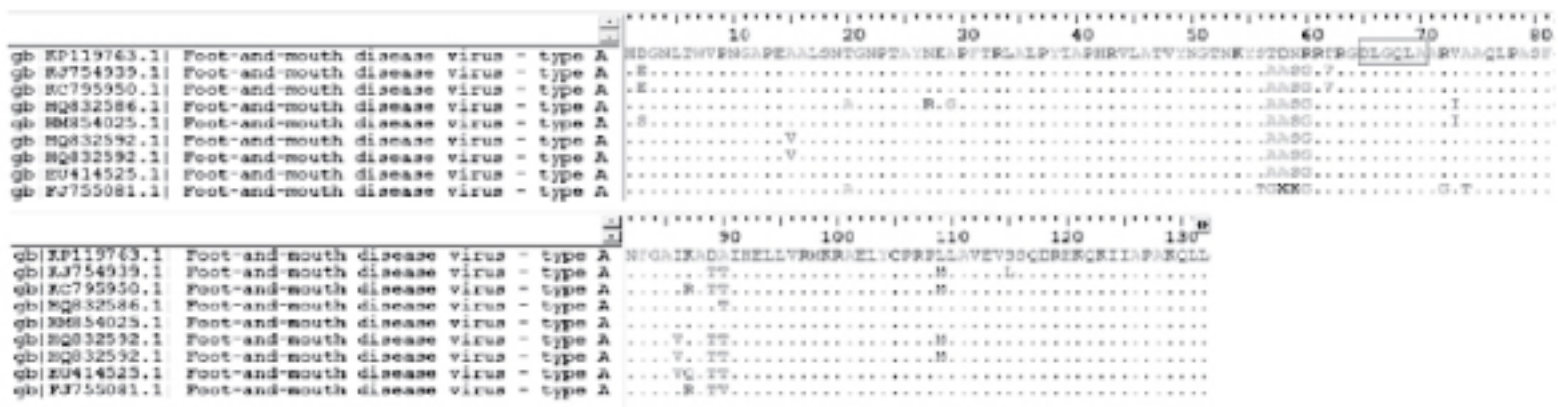
Region/ District	Year of Sample Collection	Total Samples	Positive samples	Serotype			Mixed Infections		
				O	A	Asia1	O + Asia1	O + A	A + Asia1
Lalmonirhat	2015	3	2	2	-	-	-	-	-
Kurigram	2015	9	6	6	-	-	-	-	-
Munshiganj	2015	11	11	2	-	11	2	-	-
Gazipur	2015	4	4	4	1	-	-	1	-
Dhaka	2015	9	7	5	-	4	2	-	-
	2016	4	0	-	-	-	-	-	-
Sirajganj	2015	22	22	22	-	22	22	-	-
	2016	28	21	7		14	-	-	-
Chittagong	2016	9	1	1	-	-	-	-	-
Bandorban	2016	10	4	4	-	-	-	-	-
Total		**109**	**78**	**53**	**1**	**51**	**26**	**1**	**0**

Amino Acid Alignment of VP1 Sequence of FMDV: Deduced amino acid sequences of the major VP1 protein for various FMD serotype O, A and Asia1 were aligned and compared with other field and gene bank sequences. The conserved motifs of all isolates of serotype O were position 1-38 (**LPTSFNYGAIKATRVTELLY RMKRAETYCPRPLLAIHP**), position 41-54 (**ARHKQKIVAPVKQL**) , position 58-83 (**DLLKLAGDDVESNPGPPFFFSDVRSNF**) whereas**,** the conserved regions among the isolates of serotype A were position 30-50 (**PFTRLALPYTAPHRVLATVYNG**), position 92-108 (**HELLVRMKRAELYCPRP**) , position 116-132 (**SQDRHKQKIIAPAKQLL**) and in case of Asia1 the conserved regions were (**DRRKEIIAPEKQ,NFDLLKLAGDVESNPGP** and **FFSDVRSNF**).

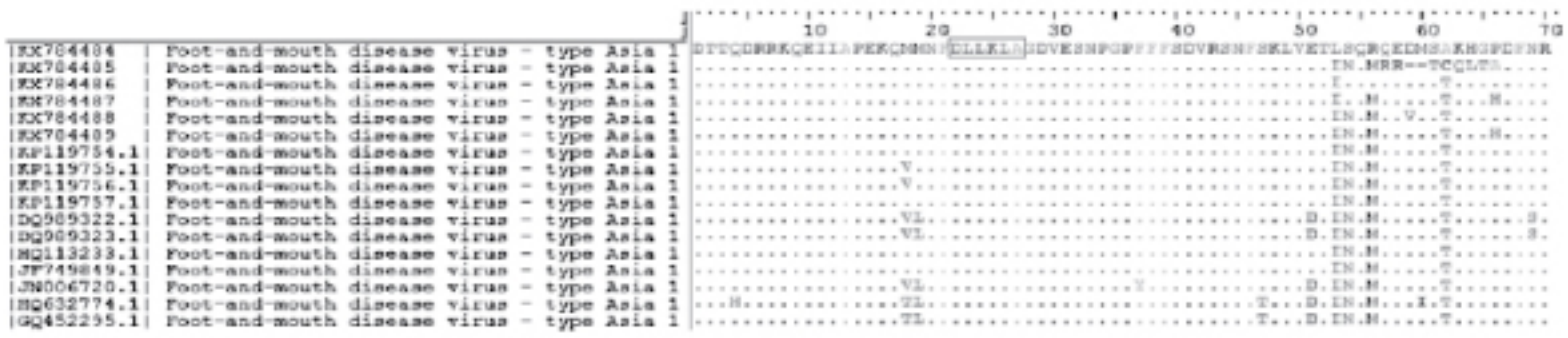
Calculation of protein variability index:
Multiple amino acid sequences of serotypes O, A & Asia1 derived from both field sample and data bank were used to calculate the variability index by using protein variability web server (PVS). Absolute sequence variability estimates 'per site' in an MSA as determined by the Shannon Entropy, the Simpson Diversity Index and the Wu-Kabat Variability Coefficient . The Wu-Kabat's coefficient, possibly the most popular sequence variability metric, was effective in resolving the highest diversity positions, but as it has been noted, underestimates the diversity in the MSA. On the other hand, Shannon and Simpson methods were statistically more sound for quantifying a system diversity. The concensus sequence of Serotype O (LPTSFNYGAIKAT RVTELLYRMKRAETYCPRPLLAIHPSQA RHKQKIVAPVKQLLKLAGDDVESNPGPPFFFSDVRSNFSKLVETINQMQEDMSTKHGPDFNRLV); serotype A (HDGNLTWVPNGAPEAALSNTGNPTYNKAPFTRLALPYTAPHRV LATVYNGTNKYSAASGRTRGDLGQLAARVAAQLPASFNFGAIKATTHELLVRMKRAELYCPRLLAV EVSSQDRHKQKIIAPAKQLL) and serotype Asia1(DTTQDRRKQEIIAPEKQM MNFDLLKLAGDVESNPGPPFFFSDVRSNFSKLVETINQMQEDMSTKHGDFNR) were determined by this webserver. In spite of having the amino acid variability, conserved motif (DLXXLA) was found in the serotypes O, A and Asia1. Both serotypes O and Asia1 contained DLLKLA motif but serotype A contained different DLGQLA motif. Protein variability found in the position 52-62 for A, 55-57 for O, 56-66 for Asia1 by all method.



A. Amino Acid Alignment of VP1 Sequence of FMDV Serotypes O



B. Amino Acid Alignment of VP1 Sequence of FMDV Serotypes A



C. Amino Acid Alignment of VP1 Sequence of FMDV Serotypes Asia1

Figure 2. Amino acid alignment of VP1 sequences of FMDV serotype O, A and Asia1.

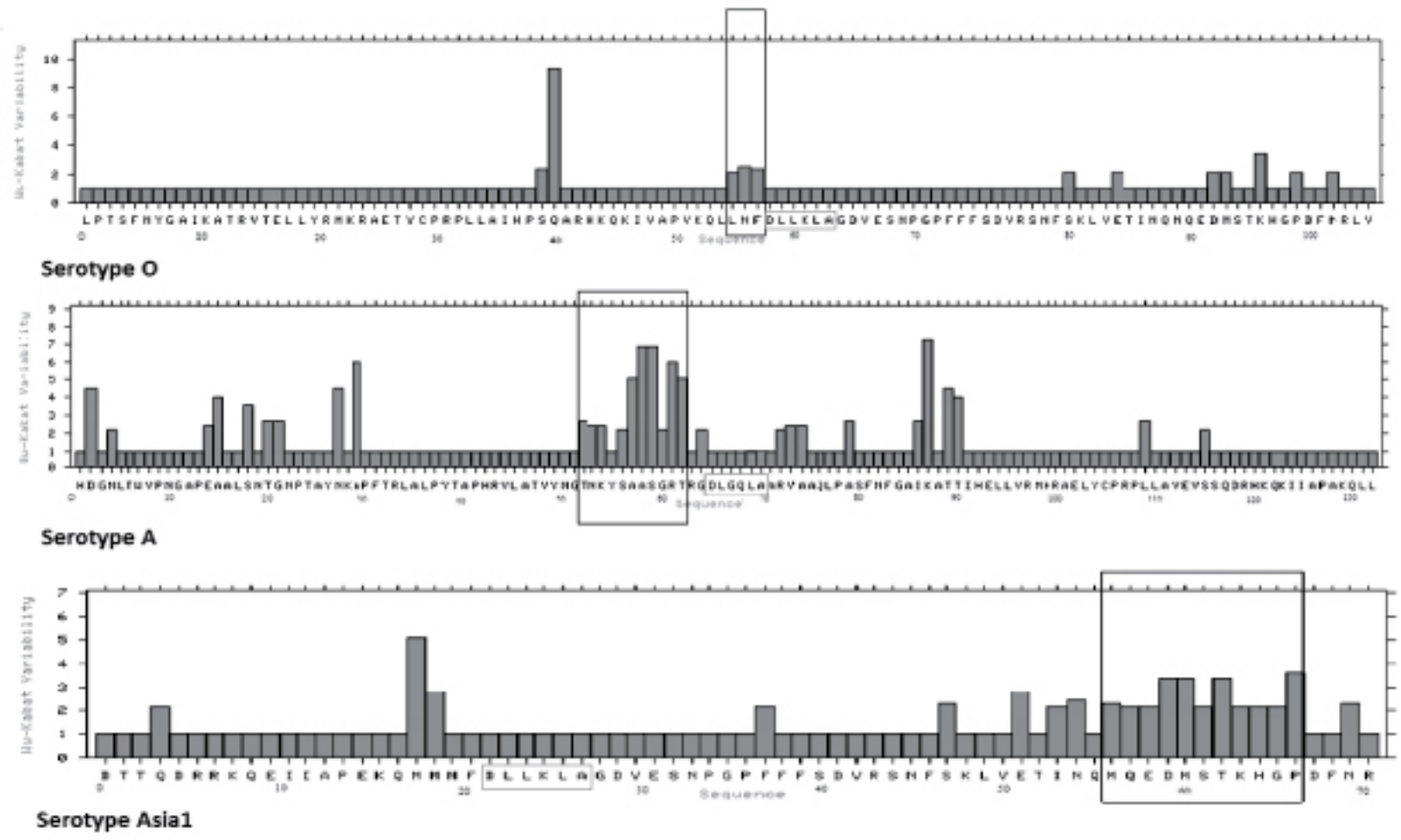


Figure 3. Wu-kubat variability index for VP1 protein of the FMDV serotype O, A and Asia1. Black rectangle shows protein variability and red rectangle shows conserve motif.

Homology modeling and validation of FMDV VP1 protein 3D structure: Construction of 3D structure of VP1 capsid protein were essential to predict plausible conformational epitopes and find distinct antigenic determining sites. The sequence alignment and template structure were used to produce a structural model of the target. The detectable levels of sequence similarity usually imply significant structural similarity. 3D structures of VP1 capsid protein for serotype O, A and Asia1 were constructed using SWISS MODEL of Expasy web server.Templates (PDB id: 1fod.1.A for type O , PDB id: 4gh4.1.A for type A and , PDB id:3k33.1.D) were taken considering better identity (94.55% – 1fod.1.A ; 92.86% – 4gh4.1.A and 24.14%-3k33.1.D . The PROSA analysis of the model showed maximum residues to have negative interaction energy, and the overall *Z*-score is −0.01 for type O; −0.26 for type A and −1.56 for type Asia1 model (Figure 5).

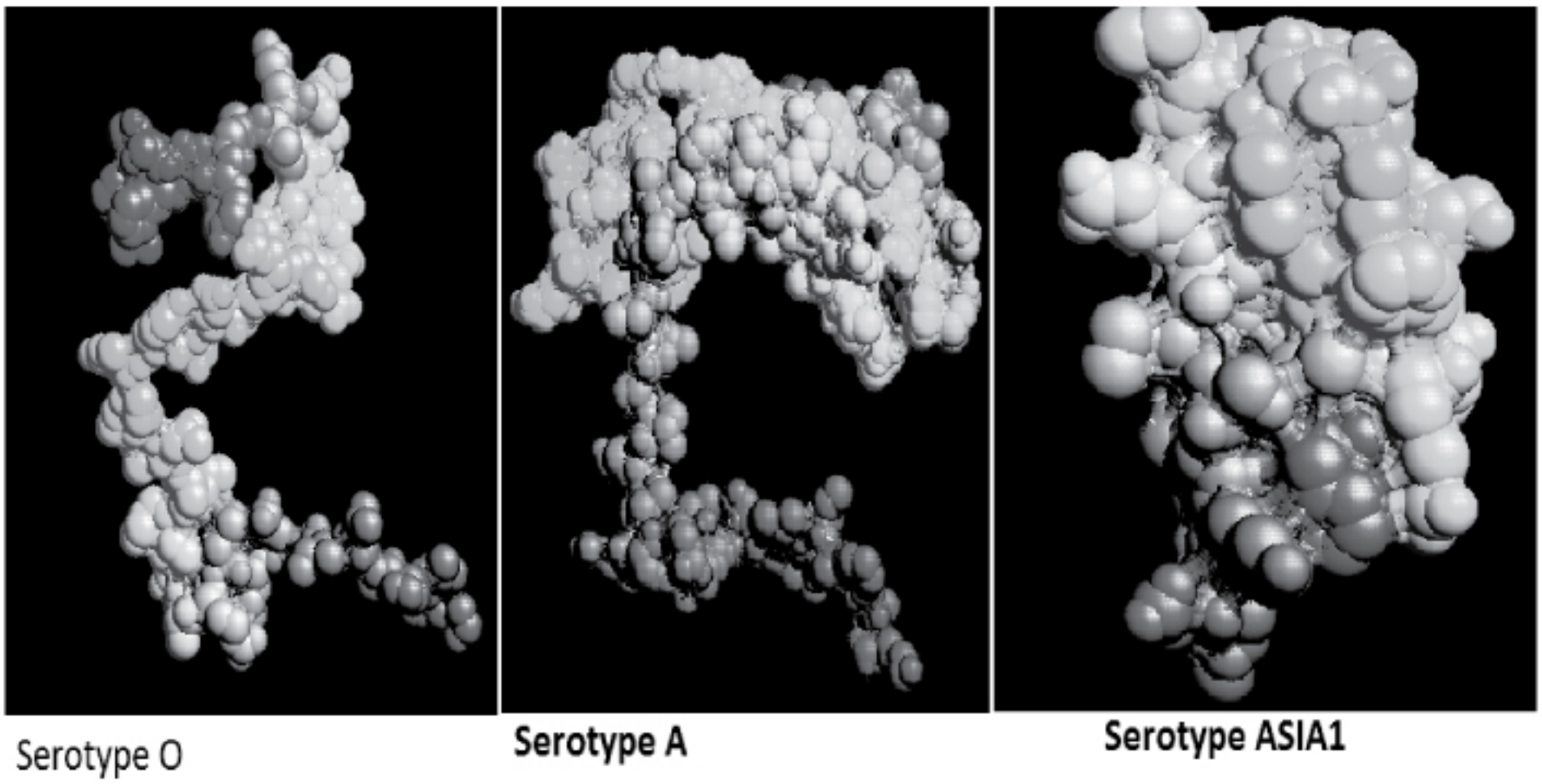


Figure 4: Protein 3D model of capsid protein VP1 of FMDV serotype O, A and Asia1.

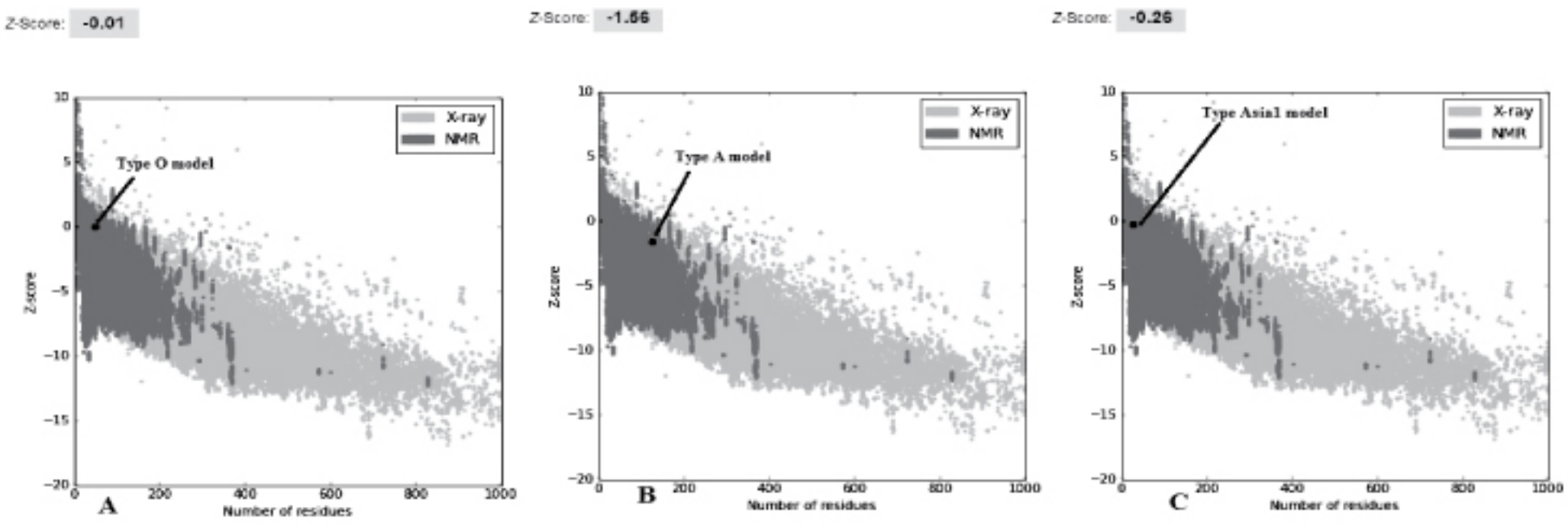


Figure 5. PROSA Z-Scores for VP1 model of type O (-0.01), A (-1.56) & Asia1 (-0.26), which residue in a zonal of experimentally derived overall Z-Score. Plot (A) shows type O-VP1 model; Plot (B) shows type A O-VP1 model and Plot (C) shows type Asia1-VP1 model.

B-cell epitope prediction by IEDB server: Capsid protein VP1 was subjected to Bepipred linear epitope prediction, Emini surface accessibility, Kolaskar and Tongaonkar antigenicity, Karplus & Schulz Flexibility , Parker hydrophilicity and Chou and Fasman beta turn prediction methods in IEDB, that predict the probability of specific regions in the protein to bind to B cell receptor, being in the surface, being immunogenic, being in

a hydrophilic region and being in a beta turn region, respectively (Figure 6). Continuous B cell epitopes were predicted by the methods of Karplus–Schulz (flexibility plot), Kyte–Doolittle (hydrophilicity plot), Emini (accessibility), and Jameson–Wolf (antigenicity) using DNAstar Protean system (Figure 7). The contiuous B-cell epitopes predicted by DNAstar Protean system was summarized in the Table:. B-cell epiopes were also predicted using different prediction methods such as, ABCpred, FBCpred, BCpred and AAP**.** FBCPred, a novel method for predicting flexible length linear B-cell epitopes using the subsequence kernel. ABCpred allowed the prediction of continuous B cell epitopes. BCPred evaluated the existing Linear B cell prediction method based on physicochemical properties**.** AAP predicted linear B-cell epitopes using amino acid pair antigenicity. Prediction results with position and score in different methods were summarized in Table 2, Table 3 & Table 4.

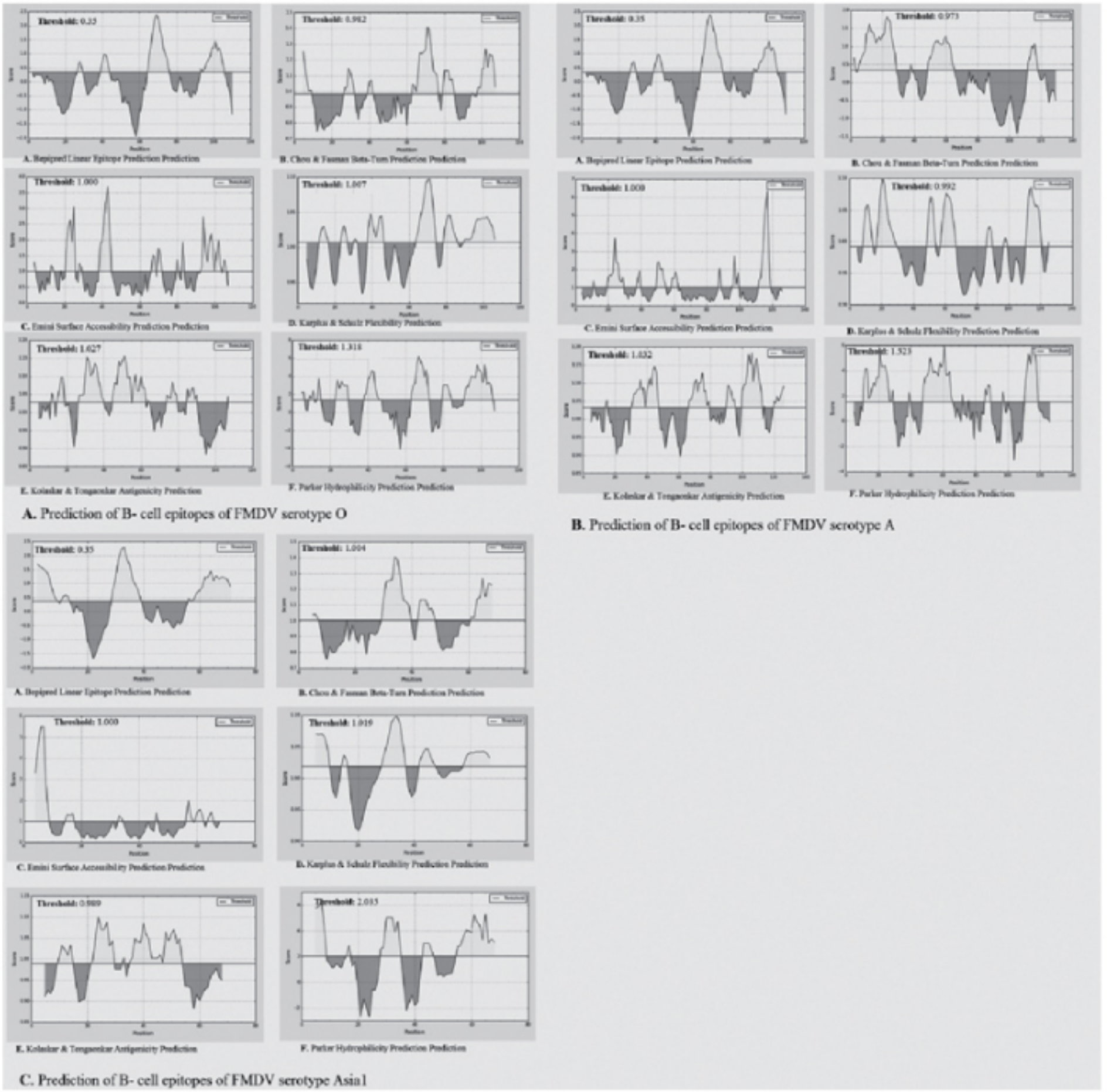


Figure 6. B-cell epitope predicted by different scales using IEDB webserver (A) serotype O, (B) serotype A & (C) serotype Asia1

Table 2. List of B-cell epitope predicted by different scales using IEDB webserver

	Bepipred Linear Epitope Prediction			surface accesability score	hydrophilicity score	antigenicity score	Beta turn score	Flexibility score
	Epitope	Position	Length					
Serotype O	TYC	27-29	3	1.01	2.99	1.05	1.064	0.99
	SQARH	39-43	5	2.64	3.81	1.02	1.0112	1.03
	DDVESNPGPPFF	65-76	12	1.23	3.64	0.98	1.2105	1.08
	QMQEDMSTKHGPD	93-105	13	1.67	4.42	0.93	1.078	1.04
Serotype A	LTWVPNGAPEAALSN TGNPTYNKA	5-28	24	1.13	3.04	0.97	1.15	1.10
	NGTNKYSAASGRTRGDL	49-65	17	1.47	4.35	0.96	1.16	1.03
	SSQDRH	12-17	6	0.471	1.92	1.05	0.89	0.98
Serotype Asia1	DTTQDRRKQ	1-9	9	4.05	6.03	0.91	1.06	1.07
	IIA	11-13	3	0.347	1.29	1.02	0.81	0.98
	DVESNPGPPFF	29-39	11	0.86	3.24	1.23	1.32	1.08

Table 3. Continuous B-cell epitopes predicted by DNAStar Protean System

FMDV Type	Prediction Items			
	Hydrophilicity	Accessibility	Flexibility	Antigenicity
O	20-29,39-46,65-72,81-83,92-106	13-14,21-25,28-29,39-44,67-72,93-106	12-16,23-27,30-32,38-46,63-74,79-106	23-27,39-45,63-73,79-87,94-108
A	1-7,10-11,17-30,38-40,49-65,98-102,112-123	20-29,38-39,50-55,59-62,112-118	9-14,18-28,49-54,58-66,87-89,111-120	1-4,11-13,16-29,50-65,95-99,111-120
Asia1	31-33,55-71	31-34,57-62,64-65,69-70	5-10,14-16,27-37,42-68	2-8,30-34,42-47,59-71

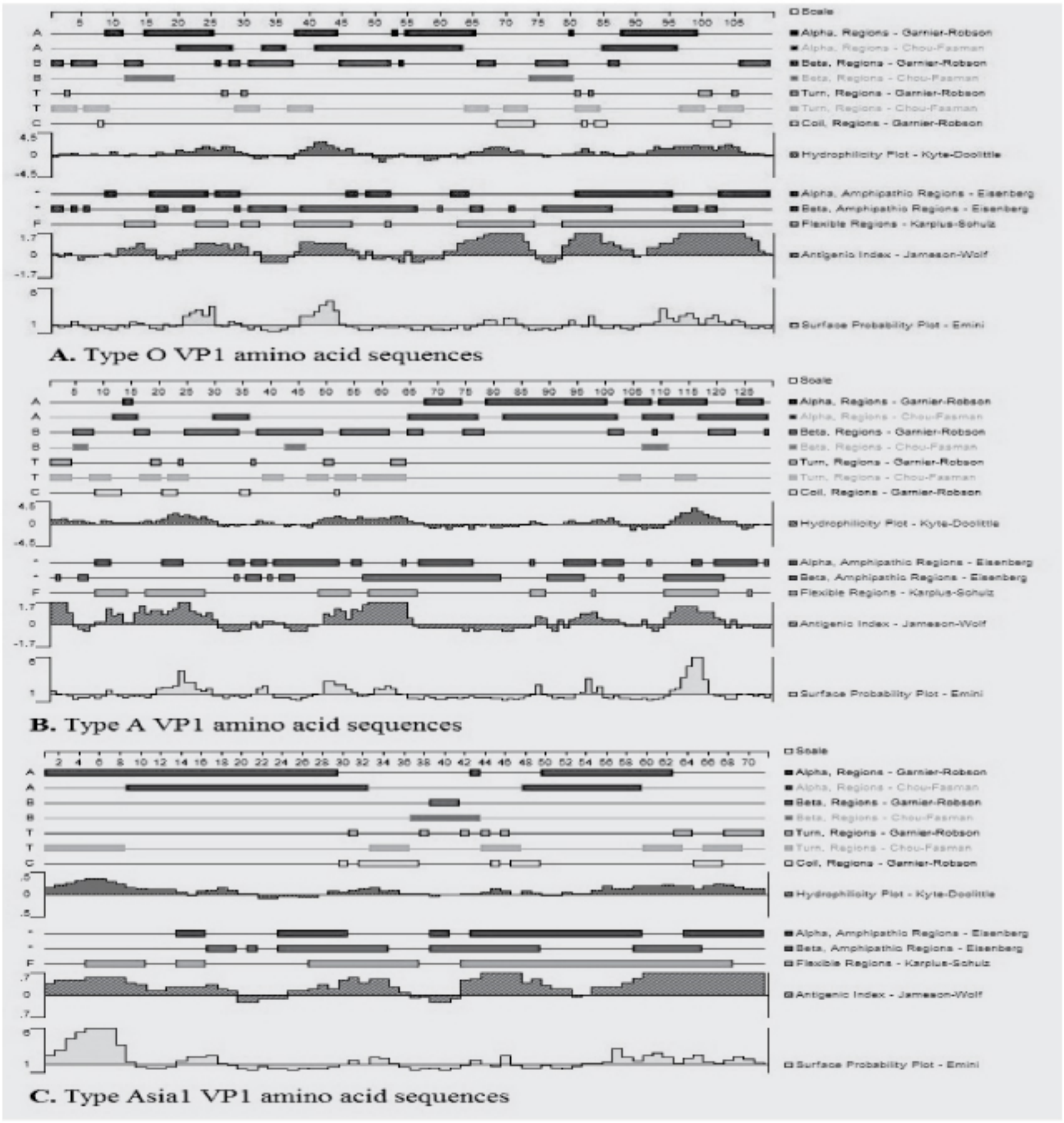


Figure 7. Secondary structures, flexibility plot, hydrophilicity plot, surface probability plot and antigenicity index for VP1 polyprotein of FMDV type O,A and Asia1.

Prediction of cytotoxic T-cell epitope and interaction with MHC class1: Capsid protein VP1 from serotype O,A &Asia1 were analyzed using IEDB MHC-1 binding prediction tool to predict T-cell epitope suggested interacting with different type of MHC class 1 Bola alleles. The MHC class 1 binding predictions were done with IEDB analysis resource (TepiTool) using consensus method which employs SMM,NN and Combinatorial library methods and NetMHCpan. Better knowledge about T-cell epitope is required to understand the protective immunity mediated by CD8+ and CD4+ T lymphocytes. A number of T-cell epitopes with their position and scores for different Bola alleles are summarized in Table. In case of FMDV serotype O peptide sequence **TSFNYGAIK** from the position 3-11 and **ETYCPRPLL** from the position 26-34 were showed percentile rank 0.2 for BoLA-N:01201 and 0.3 for BoLA-N:05301 allele respectively.**ASFNFGAIK** from the position 78-86 and **NPTYNKAPF** from the position 22-30 were predicted as T-cell epitope for FMDV serotype A and their percentile rank are 0.1 and 0.3 respectively. Peptide sequence **QMMNFDLLK** from 17-25 and **KQMMNFDLL** from 16-24 were predicted as effective T-cell epitope for serotype Asia1 for BoLA-N:05501 and BoLA-N:00301, BoLA-N:01501 respectively. The prediction result was showed in Table 5.

Table 4. List of B-cell epitope predicted by different methods using BCPREDS webserver

FMDV serotype	Method	Position	Sequence	Score
Serotype O	BCpred	15-26	VTELLYRMKRAE	0.73
		67-78	VESNPGPPFFFS	0.999
		89-100	ETINQMQEDMST	0.967
	ABCpred	8-19	GAIKATRVTELL	0.79
		13-24	TRVTELLYRMKR	0.77
		71-82	PGPPFFFSDVRS	0.76
	AAP	61-72	KLAGDDVESNPG	1.00
		93-104	QMQEDMSTKHGP	1.00
	FBCPred	42-53	RHKQKIVAPVKQ	0.72
		64-75	GDDVESNPGPPF	1.00
		88-99	VETINQMQEDMS	0.995
Serotype A	BCpred	4-15	NLTWVPNGAPEA	0.887
		18-29	SNTGNPTYNKAP	0.982
		94-105	VRMKRAELYCPR	0.628
	ABCpred	1-12	SAASGRTRGDLG	0.62
	AAP	19-30	NTGNPTYNKAPF	1.00
	FBCPred	4-15	NLTWVPNGAPEA	0.983
		18-29	SNTGNPTYNKAP	0.998
		55-66	SAASGRTRGDLG	0.963
Serotype Asia1	BCpred	2-13	TTQDRRKQEIIA	0.95
		30-41	VESNPGPPFFFS	0.999
		52-63	VESNPGPPFFFS	0.967
	FBCPred	1-12	DTTQDRRKQEII	0.64
		22-33	DLLKLAGDVESN	0.80
		32-43	SNPGPPFFFSDV	0.78
	AAP	2-13	TTQDRRKQEIIA	1.00
		25-36	KLAGDVESNPGP	1.00
		58-69	QEDMSTKHGPDF	1.00
	FBCPred	10-21	EIIAPEKQMMNF	0.886
		28-39	GDVESNPGPPFF	1.00
		51-62	VETINQMQEDMS	0.995

Table 5. List of T-cell epitope with their percentile rank that had binding affinity with MHC class1 alleles.

Serotype	Peptide start	Peptide end	Peptide	Percentile rank	Allele
Serotype O	3	11	TSFNYGAIK	0.2	BoLA-N:01201
	26	34	ETYCPRPLL	0.3	BoLA-N:05301
	52	60	KQLLNFDLL	0.5	BoLA-N:01302
	39	47	SQARHKQKI	0.5	BoLA-N:01501
	69	77	SNPGPPFFF	0.5	BoLA-N:05301
	26	34	ETYCPRPLL	0.6	BoLA-N:05201
	52	60	KQLLNFDLL	0.6	BoLA-N:01301
	12	20	ATRVTELLY	0.7	BoLA-N:00801

	39	47	SQARHKQKI	0.7	BoLA-N:01302
	12	20	ATRVTELLY	0.7	BoLA-N:05101
	26	34	ETYCPRPLL	0.8	BoLA-N:05001
	47	55	IVAPVKQLL	0.8	BoLA-N:05201
	76	84	FFSDVRSNF	0.9	BoLA-NC4:00201
	39	47	SQARHKQKI	0.9	BoLA-N:01301
Serotype A	78	86	ASFNFGAIK	0.1	BoLA-N:01201
	22	30	NPTYNKAPF	0.3	BoLA-N:00602
	22	30	NPTYNKAPF	0.4	BoLA-N:00601
	93	101	LVRMKRAEL	0.4	BoLA-N:01001
	45	53	ATVYNGTNK	0.4	BoLA-N:01201
	27	35	KAPFTRLAL	0.5	BoLA-NC2:00101
	27	35	KAPFTRLAL	0.5	BoLA-NC2:00102
	74	82	AQLPASFNF	0.6	BoLA-N:00901
	74	82	AQLPASFNF	0.6	BoLA-N:00902
	39	47	APHRVLATV	0.7	BoLA-N:01001
	37	45	YTAPHRVLA	0.7	BoLA-N:00501
	68	76	LAARVAAQL	0.8	BoLA-N:00501
	46	54	TVYNGTNKY	0.8	BoLA-N:00802
	94	102	VRMKRAELY	0.8	BoLA-N:00902
	27	35	KAPFTRLAL	0.9	BoLA-N:00601
	72	80	VAAQLPASF	0.9	BoLA-NC4:00201
	72	80	VAAQLPASF	0.9	BoLA-NC4:00201
	99	107	AELYCPRLL	1	BoLA-N:01401
	46	54	TVYNGTNKY	1	BoLA-N:00801
	60	68	RTRGDLGQL	1	BoLA-NC2:00101
	60	68	RTRGDLGQL	1	BoLA-NC2:00102
Serotype Asia1	17	25	QMMNFDLLK	0.1	BoLA-N:05501
	16	24	KQMMNFDLL	0.3	BoLA-N:00301
	16	24	KQMMNFDLL	0.3	BoLA-N:01501
	32	40	SNPGPPFFF	0.5	BoLA-N:00401
	32	40	SNPGPPFFF	0.5	BoLA-N:00402
	32	40	SNPGPPFFF	0.5	BoLA-N:05301
	16	24	KQMMNFDLL	0.7	BoLA-N:00201
	16	24	KQMMNFDLL	1	BoLA-N:00102

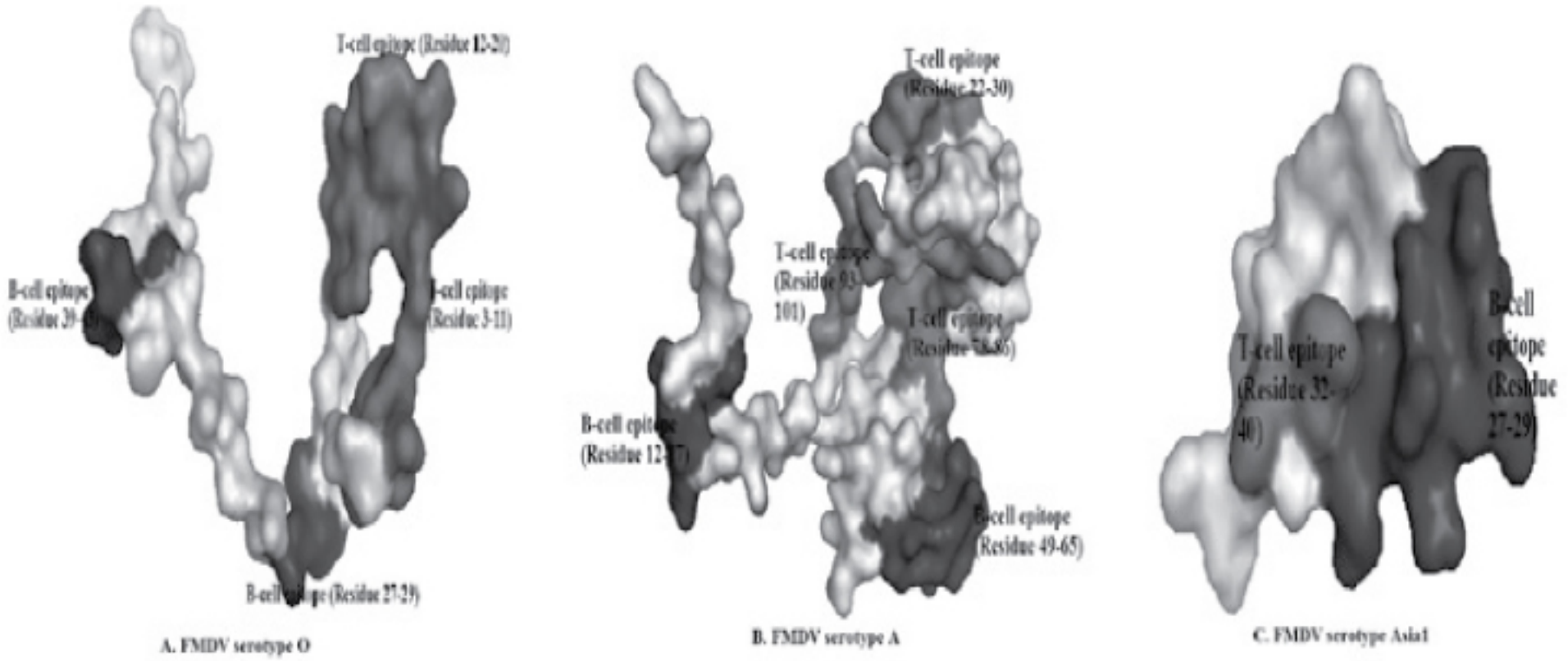


Figure 8. Predicted B and Tcell epitopes mapped onto the protein 3D structure using the PyMOL visualization tool. Gray colour indicates VP1 protein structure, whereas green shows B-cell-specific epitope and red color shows T cell (CD8+) epitope

Discussion

Economically devastating foot and mouth disease causes several economic losses throughout the world. Completely eradication FMD is not possible yet through conventional vaccine as highly variable antigenicity was found in all serotypes. Therefore, this study was conducted to detect the serotypes currently circulating in Bangladesh & their antigenic heterogeneity for B-cell and T-cell epitope prediction. In this study, a total 109 samples were collected & among them 78 samples showed positive result. Only three serotypes 0, A & Asia1 were detected in this study. The amino acid sequences derived from nucleotide sequences of the field isolates and databank was aligned using ClustalW and protein variability plot was calculated by the webserver PVS. Absolute sequence variability was estimated by the Shannon Entropy , the Simpson Diversity Index and the Wu-Kabat Variability Coefficient. Protein variability was found in the position 52-62 for A, 55-57 for O, 56-66 for Asia1 by all method. Conserved motif (**DLXXLA**) was also found in the serotypes O, A & Asia1.The protein 3D structure was estimated by SWISS model and modified using pymol. To determine a potential and effective peptide antigen for B cell, epitopes should get above threshold scores in Bepipred linear epitope prediction, Emini surface accessibility, Parker hydrophilicity, Kolaskar and Tongaonkar antigenicity and Chou and Fasman beta turn prediction methods in IEDB. The linear epitope **SQARH** (position 39-43) for serotype O, **NGTNKYSAASGRTRGDL** (position 49-65) for serotype A and **DTTQDRRKQ** (position 1-9) were found to have highest score in all scales. The B-cell epitope for these three serotypes also predicted by BCpred, ABCpred, FBCpred and APP methods.The highest score for serotype O,A &Asia1 in BCpred method were **VESNPGPPFFFS** (position 67-78), **SNTGNPTYNKAP** (position 18-29) and **VESNPGPPFFF S** (position 30-41) respectively.The best prediction result of B-cell epitope in ABCpred method for serotype O, A & Asia1 were **GAIKATRVTELL** (position 8-19), **SAASGRTRGDLG** (position 1-12) & **SNTGNPTYNKAP** (position 18-29) correspondingly. The effective B-cell epitope in FBCpred method were **GDDVESNPGPPF** (position 64-75), **SNTGNPTYNKAP** (position 18-29) and **DLLKLAGDVESN** (position 22-33) for serotype O,A and Asia1 respectively. The highest score Asia1 in APP method for serotype O,A &Asia1 were **KLAGDDVESNPG** (position 61-72), **NTGNPTYNKAPF** (position 19-30) and **TTQDRRKQEI IA** (position 2-13) correspondingly.The immune response of T cell is long lasting response comparing with B cell, where the antigen can easily escape the antibody memory response (Shawan et al., 2014) and CD8+ T and CD4+ T cell responses play a major role in antiviral immunity (Sant and McMichael., 2012), designing of vaccine against T cell epitope is much more favorable. MHC class 2 specific T-cell response may play vital role for induce protective immunity against FMD but no such prediction tools were available for Bola alleles. IEDB MHC-1 binding prediction tool has predicted T-cell epitope interacting with different type of MHC class 1 Bola alleles. In case of FMDV serotype O peptide

sequence **TSFNYGAIK** from the position 3-11 and **ETYCPRPLL** from the position 26-34 were showed percentile rank 0.2 for BoLA-N:01201 and 0.3 for BoLA-N:05301 allele respectively. **ASFNFGAIK** from the position 78-86 and **NPTYNKAPF** from the position 22-30 were predicted as T-cell epitope for FMDV serotype A and their percentile rank are 0.1 and 0.3 respectively. Peptide sequence **QMMNFDLLK** from 17-25 and **KQMMNFDLL** from 16-24 were predicted as effective T-cell epitope for serotype Asia1 for BoLA-N:05501 and BoLA-N:00301, BoLA-N:01501 respectively. The 3D structure of predicted epitopes were showed in Figure 8. However, these predicted B-cell and T-cell epitope were expected for efficient induction of either B-cell or cytotoxic T-cell response.

Conclusion and Implications

This findings of this study suggested that only three serotypes (O, A & Asia-1) of Foot and Mouth Disease virus are currently circulating in Bangladesh. Mixed infection among these serotypes were also found. Conservancy of the peptide sequences were recognized and B and T-cell vaccine epitopes were predicted and mapped in this study. These predicted epitopes could be considered as candidates for designing trivalent peptide vaccines, which could provide specific protection against FMDV serotypes O, A & Asia1 found in Bangladesh. The result of the present study hopefully will be helpful in controlling and eradicating foot and mouth disease in Bangladesh.

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Control of infectious diseases in dairy cattle through biosecurity and vaccination programmes

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Abstract

Infectious disease is one of the major health problems in dairy cattle. Infectious diseases are mainly caused by bacterial and viral pathogens. Common bacterial diseases in dairy cattle are anthrax, brucellosis, pasteurellosis, streptococcal mastitis, and tuberculosis. Infectious diseases of dairy cattle caused by viruses are Foot and Mouth disease, bovine viral diarrhoea, ephemeral fever, rabies etc. Bio-security and vaccination are the most effective methods for controlling infectious diseases of animals including dairy cattle.Bio-security is a process to prevent entry of disease causing micro-organisms into the farms. Several biosecurity measures are practiced in the farm. Purchasing disease free new animal, use of semen from disease free bull for AI, visitor control, use of disinfectant are some of the biosecurity measures in a dairy farm. All of the biosecurity measures need to be implemented in a dairy farm to achieve infectious disease control goal. Vaccination is an effective tool to control infectious diseases in dairy cattle. Vaccines confer active immunity against a specific disease. Live attenuated, inactivated and genetically engineered vaccines are being used to prevent and control of diseases in dairy cattle. Passive immunity through colostrums can be transferred to the calf following vaccination of dam. Emergency ring vaccination strategies are used to confer immunity in dairy herds during outbreaks of infectious diseases. It may be concluded that biosecurity along with vaccination programmes can help controlling outbreak of infectious diseases in dairy cattle.

Key words: Infectious disease, Dairy cattle, Biosecurity, Vaccination

Introduction

The dairy industry of Bangladesh holds great potential for sustainable development of the country.Every year, Bangladesh import 65-70 thousand metric tons powder milk, equal to 17 lakh litres of liquid milk, worth 2 to 2.5 thousand crore taka. So there is a good opportunity for the local dairy industries to contribute to this gap.

Dairy cows produce milk and meat for human consumption, which are very rich in nutrient contents. It provides cash income and creates employment opportunity for the rural people particularly for small farmers and landless labourers through sale of animals and various animal products. Cow dung is an important source of manure for crop and fuel for domestic use. The people with small means rear dairy cows as the major source of their family income and in this sense, dairy farming helps in poverty alleviation. Development of this sub-sector may be considered as an important strategy of poverty alleviation.

Infectious diseases are one of the major challenges in dairy farms. The diseases can be transmitted from infected to susceptible animal by direct contact. Indirect transmission of diseases may occur through contact with contaminated object, equipment, feed and water. Infectious agents enter into animal through oral, inhalation, skin, reproductive tract and blood vascular system. The common infectious diseases of dairy cattle with methods of transmission and signs of diseases are listed in Table 1.

Table 1. Infectious diseases of dairy cattle with mode of transmission and clinical signs

Disease	Methods of spreading	Common symptoms
Anthrax	Oral, inhalation, blood	Staggering, trembling, collapse, terminal convulsions, bloody discharge
Bovine brucellosis	Oral, reproduction	Abortions, stillbirths, weak calves
Leptospirosis	Skin, oral, inhalation, reproduction	Abortions, stillbirths, weak calves, fever, decrease in performance
Campylobacteriosis	Oral, reproduction	Abortion
Salmonellosis	Oral	Diarrhea
Bovine tuberculosis	Inhalation	Lesions in organs and body cavity, weight loss
Pasteurellosis	Oral, inhalation	Pneumonia
Bovine paratuberculosis (Johne's disease)	Oral, reproduction	Diarrhea, weight loss, bottle jaw
Streptococcal mastitis	Teat	mastitis
Bovine leukosis	Blood, reproduction	Enlarged lymph nodes
Bovine viral diarrhea (BVD)	Oral, reproduction	Severe diarrhea, fever, abortions
Foot and mouth disease	Aerosol, direct contact	Salivation,
Ephemeral fever virus	Vector borne, mosquito	Fever, lameness
Infectious bovine rhinotracheitis (IBR)	Inhalation, reproduction	Abortions, cough, eye infections, genital infections
Rabies	Skin, blood	Anorexia, apprehension, nervousness, hyperexcitability, altered temperament, death

Infectious diseases in dairy cattle cause morbidity and economic losses due to decreased milk production, abortion, infertility and stillbirth. Some of the infectious diseases (Brucellosis, Bovine tuberculosis) arezoonotic which can be transmitted to human through direct contact with infected animals and consumption of contaminated milk and its products. Therefore control of infectious diseases in dairy cattleis important. Bio-security and vaccination are important tools to control infectious diseases. The objectives of this paper are to describe bio-security and vaccination program to control infectious diseases in dairy cattle.

Biosecurity

Biosecurity refers to management practices that reduce the chances of infectious diseases will be carried onto the farm by animals or people. Biosecurity also reduces the spread of infectious disease on farms. Risk assessment of diseases is important before adopting biosecurity plan. Identification of transmission route of a disease on the farm is an important factor for selecting effective biosecurity plan. Biosecurity of a dairy farm can be broadly divided into two broad categories such as: general dairy practices and general biosecurity practices.

1. General dairy practices

1.1. Protection of calf

In order to protect calf dam should be vaccinated during dry period. The purpose of this vaccination is to confer immunity to calf against infectious diseases. Dam needs to be provided with dry and clean area for calving. Naval of calf must be dipped immediately with tincture of Iodine after birth. Calf need to be fed with colostrums at 10% of their body weight within 12 hours of birth.

1.2. Purchasing animal

To expand a dairy farm sometime purchasing of new cattle are required. Newly purchased cattle should be kept under quarantine for at least 3 weeks. They should be tested to know vaccination as well as disease status. A veterinarian can inspect the health condition of animal before purchased.

1.3. Replacement heifers

The replacement heifer becomes the genetic building block for the cow herd. The producer hopes that a replacement heifer will become a fertile cow that produces a calf, annually, for a long time. They should be reared in an isolated shed. They need to place under quarantine for 2 weeks when returning from growers.

1.4. Lactating cows

Disease susceptibility of lactating cows is increased due to calving and lactation stresses. They should be monitored daily for any signs of disease. A separate isolation area for sick cow is necessary to minimize contact with healthy lactating cows. Fresh cow need to house separately from hospital pen. Use of separate equipment for primary herd and quarantine herds need to be practiced to minimize disease transmission.

1.5. Dry cows

Dry cows are resting before calving. These animals have to be watched closely before they return to milking. Use of dry cow therapy with appropriate antibiotics is recommended to reduce the incidence of mastitis. Application of teat sealants may be required to stop frequent mastitis. Dry cow should be housed separately from sick animals. Adequate supply of a balanced ration is required to improve immune status of dry cows.

1.6. Bulls

Bulls are required in a dairy farm for natural service. They should be tested periodically for contagious and venereal diseases such as brucellosis and campylobacteriosis etc. Implementation of vaccination program for bulls is required against major venereal diseases.

1.7 Importation of semen

Most dairy farms use semen for artificial insemination (AI). Semen has to be imported or collected from a certified well known producer who have infectious disease control program. The bull health history should be known. Semen tank should be locked and only qualified people are allowed to handle semen.

2. General biosecurity practices

2.1. Visitors

A separate designated area should be kept for the visitor. They should not allow to coming into contact with animals, equipment or barns. Only limited number of people will enter the premises after writing in a log book including consultants, salesmen, deliverymen, maintenance workers and veterinarians. Feed,forageand other products should be kept away from visitors and monitor closely. Vehicle of visitor should be disinfected using baths or sprays. Disposable boots or disinfectant footbaths should be kept at the entry for visitors.

2.2. Owner practices

A clean pair of shoes should be kept to wear only around the animals on one premise. Disposable boots should available for visiting other premises. Washing of hands with soap and water is necessary before and touching any animals. All feed delivery equipment should be cleaned and disinfected. Animal feed should be tested periodically for mycotoxins and other adulterants.

2.3. Security

The main gate should be kept locked at all times. The premises and building should be marked with “No Trespassing” and “Do Not Enter” signs. A good perimeter fences are required. Feed, nutrients and hazardous

materials such as pesticides and fertilizers should be kept in a secured place. Wildlife, especially fox, street dogs and birds should not enter into the premises.

2.4. Transportation

Disinfectant such as bleach is used to kill viruses and bacteria. A mixture of 1 part bleach to 10 parts water is sufficient for killing microbes. Scraping or scrubbing of mud and manure from both the interior and exterior of the trailer, truck and equipment should be performed. Vehicles and equipment should be washed with water and detergent or disinfectant. Wheels, wheel arches, mud guards and undercarriages of vehicles and trailers need to be washed thoroughly. Disinfectants should be left on surfaces for 20 to 30 minutes to kill all pathogens.

2.5. Employees

Employees should be trained to report sick animals. Regular safety and security meetings should be conducted with everyone who lives or works on the premises. Employees should be provided with specific guidelines for sanitation procedures.

3. Vaccinations

Vaccination is an essential component of disease prevention plans. It can protect certain diseases but not 100 percent effective. Vaccination is likely to be most effective when it is planned to meet the particular needs of a farm. Setting up a strategic vaccination program means determining what diseases to vaccinate against, identifying who will most benefit from vaccination, and finding out when they will most need the protection that vaccines provide. Table 2 represents a list of vaccines commonly used in dairy farm.

Table 2. Vaccines and treatment of common diseases

Name of diseases	Causal agents	Vaccines available	Treatment option
Bovine brucellosis	*Brucella abortus*	Yes	No
Bovine tuberculosis	*Mycobacterium bovis*	No	No
Bovine paratuberculosis	*Mycobacterium paratuberculosis*	No	No
Campylobacteriosis	*Campylobacter foetus*	No	Yes
Leptospirosis	*Leptospirahardjo*	Yes	Yes
Salmonellosis	*Salmonella* spp.	Yes	Yes
Pasteurellosis	*Pasteurella multocida*	Yes	Yes
Foot and mouth disease (FMD)	Virus	Yes	No
Ephemeral fever	Virus	No	No
Rabies	Virus	Yes	No
Bovine viral diarrhoea(BVD)	Virus	Yes	Yes
Infectious bovine rhinotracheitis (IBR)	Virus	Yes	Yes

Conclusion and recommendations

Dairy farming has a potential impact on the national economy of Bangladesh. Production losses such as milk and calf in a dairy farm can be caused by infectious disease. Prevention and control of these diseases are important to make the dairy enterprise economically viable. Some of the infectious diseases are zoonotic in nature. Biosecurity and vaccination are two important strategies that are widely practice in dairy industries to control infectious diseases in dairy cattle. Two broad categories of biosecurity practices can be applied to a

dairy farm such as biosecurity in dairy practice and general biosecurity practices. The goals of the biosecurity are to prevent the entry of causal agents of disease in a dairy farm. Vaccination is an effective method for controlling infectious diseases in dairy cattle. Vaccine induces protective immunity to animal against specific diseases. Vaccines are available for most of the infectious diseases of dairy cattle. Implementations of biosecurity practice are essential for controlling infectious diseases in dairy cattle. Farm owner and employees should be trained for effective implementation of biosecurity program. Effective vaccination programs need to be adopted for dairy cattle on the basis of prevalence of diseases in a particular geographical area. Regular vaccination program should be carried out by the skilled veterinarian. The protective antibody titers of the vaccinated animals need to be monitored on regular basis by serological tests. Biosecurity and vaccination programmes should be simultaneously practiced for effective control of infectious diseases in dairy cattle.

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Advances of immunogenomics and targeted genome editing for disease resistance in livestock: application of CRISPR/Cas9 technology

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Abstract

Emerging concerns of antimicrobial drug resistance, food safety, zoonoses and animal welfare issues put the breeders, producers and veterinarians under huge pressure to explore sustainable control approaches for livestock diseases. Improvement of the host genetic resistance through genome editing is one of the most promising options. Next generation sequencing, as a cutting edge technique, enables producing high throughput transcriptomic and genomic profiles resulted from host-pathogen interactions. Immunogenomics combine the transcriptomic and genomic data linked to host resistance to disease, and predict the potential candidate gene and its genomic location. Genome editing, which involve insertion, deletion or modification of one or more genes in the DNA sequence, is advancing rapidly and may be poised to become commercial reality. The clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated protein 9 (Cas9) (CRISPR/Cas9) system is one of the latest genome editing tools, originally developed from the bacterial adaptive immune system. The CRISPR/Cas9 system uses a single-guide RNA to target the Cas9 nuclease to induce DNA double-strand break (DSB)in a predetermined site of genomic sequence. The DSB can be subsequently repaired either by nonhomologous end joining or by homology directed repair to produced desirable change. Due to its efficiency, specificity, versatility and high degree of precision, the CRISPR/Cas9 system has recently been emerged as a powerful tool for genome engineering in agricultural food production including livestock. CRISPR/Cas9 mediated insertion of *NRAMP1* gene for producing tuberculosis resistant cattle, and deletion of *CD163* gene for producing pigs resistant to porcine reproductive and respiratory syndrome are two groundbreaking applications of genome editing in livestock. In this review we highlighted the technological advances of livestock immunogenomics and the principles and application of CRISPR/Cas9 mediated genome editing in livestock breeding for disease resistance. The ethics, regulation, social acceptance and future prospects of genome editing for livestock health and production are also discussed.

Keywords: Immunogenomics, Next generation sequencing, Genome editing, CRISPR/Cas9, Disease resistance, Livestock

Introduction

Food from the animal origin is inevitable for healthy life. Efficient production of quality and quantity livestock products is one of the major issues for food safety, a long desire for disease free life. Occurrence of infectious diseases in livestock affects not only the farm production and economics, animal health and welfare; but also increasesthe risk of zoonosis. Outbreak of infectious disease in food animals is therefore a direct threat for food safety and public health. Emergence of antimicrobial drug resistance along withunavailability of effective vaccines and spontaneous genetic mutation of infectious pathogens are the major peril for breaking down the disease control strategies.Therefore, there is a growing needfor producing safe animal food through securinganimal welfare standards.Combinedefforts from farmers, breeders and veterinarian have been continued to explore effective disease control approaches for sustainable livestock production.The farm management with optimum housing, ration, biosecurity and vaccination are taken into account for maintenance of health and welfare of farm animals. In addition, improvement of host genetic resistance to disease could contribute in strengthening the profitability of food animal system, improving

animal welfare and reducing xenobiotic usage in livestock production (Davies et al., 2009).Disease resistance is the host's ability to restrict/inhibit the replication of infectious pathogens. The contribution of host genetic factors to disease outcome is one of the fundamental issues in understanding disease pathogenesis and host resistance. Immunogenomics include integrated analysis of immunologic and genomic data on host response to infectious agents and contributeto identifying potential candidate genes for disease resistance in livestock (Zhao et al., 2012). The single nucleotide polymorphisms (SNPs)within the candidate gene associated with host resistance to infection can subsequently be confirmed as DNA marker for disease resistance.

Genome editing involves insertion, deletion or modification of specific section of DNA sequence in the genome. The gene editing technology encompasses an enzyme known as nuclease for cutting the DNA sequence, in addition to a targeting mechanism that guides the enzyme to a specific location on the DNA strand (Petersen 2017).The clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated protein (Cas) (CRISPR/Cas) system is one of the latest genome editing tools, has become widely used in the laboratory in recent years (Pellagatti et al., 2015). Genome editing of livestock has been advancing rapidly as a technical field and may be poised to become commercial reality. Recent advances in genome editing with programmable nucleases have opened up new avenues for multiple applications, including animal breeding for disease resistance.In this review, we tried to discussthe technological advances inimmunogenomics research aiming to identifythe disease resistant candidate genes and the latest development of CRIPS/Cas9 system for applying genome editing technique for disease resistant livestock.The future prospects, regulations and social acceptance of genome editing technology in regard to improve the livestock health and production are also discussed.

Advances of immunogenomics

Immunogenomicsis an information science that uses tools of genomics in order to illustrate the interactions between pathogens and host immune system.Immunogenomicscombines the transcriptome, DNA variants/SNPs and quantitative trait loci (QTL) mapping data resulted from host-pathogen interaction through a series of bioinformatics, and has been considered effective genomic tool for identification of disease resistance gene (Zhao et al., 2012).Transcriptomeprofile provides anoverview of expressed genes associated with particular phenotypes to guide subsequent analyses by proteomics, metabolomics, and other methods.In another direction, transcriptome can explore the relationship between genotype and phenotypeof an organism. Therefore, transcriptomics has been considered the most informative assay to start with, for functional genomics.Real-time quantitative PCR (qRT-PCR), microarray and next-generation sequencing (NGS) are some cutting edge technologies for immunogenomics, as in many areas of the life sciences(Chris 2015).Some other advancing technologies are equally impactful; for example, flow cytometryand/or mass cytometry can now provide a better picture of the phenotypic diversity among immune cell subsets (Spitzer and Nolan, 2016). The establishment of induced pluripotent stem cell (iPSC) model from peripheral T cells has opened up another exciting avenue of clinical immunology (Nishimura et al., 2013).

NGS technologies have rapidly evolved over the past 10 years, resulting in dramatically increased amounts of sequencing data produced per instrument run at ever decreasing costs (Mardis 2017). NGS-based RNA sequencing (RNA-Seq) can qualitatively and quantitatively investigate any RNA type in the samples including messenger RNAs (mRNAs), microRNAs, small interfering RNAs, and long noncoding RNAs; and enables researcher to discover novel RNA forms and variants. NGS methodology has been extended through development of DNA sequencing, single-cell transcriptomics and in-situ RNA sequencing (Lee et al., 2014; Loweet al., 2017; Mardis, 2017).Nevertheless, direct sequencing of RNA using nanopore sequencing represents the current state-of-the-art RNA-Seq technique (Garalde et al., 2016).

Though sequencing hasbecome straight forward in NGS platform, the upstream sample preparation and the downstream data analysis are still labor-intensive.Data from both microarray and RNA-Seq experiments require rigorous bioinformatics to make meaningful interpretation. Though most of the RNA-Seqdata analysis algorithms are run from a command-line interface, either in a Unix environment or within the R/Bioconductor environment (Huber et al., 2015), aweb-based platform called Galaxy (www.usegalaxy.org) supports the analysis of NGS data. Detection of DNA variants/SNPs linked to traits including disease resistance, from

NGS reads has been an area of rapid development (Krøigard et al., 2016). Once the potential candidate gene or gene sets have become available from transcriptome analysis, data from DNA variants (SNPs) or from genome-wide association study can be integrated to explore the link between genomes and traits. In order to make bridge between genotypes and phenotypes, the Animal QTL database (QTLdb) has been designed to house all publicly available QTLs and SNP/gene association data on livestock animal species (Hu et al. 2013).To date, about 17 566 QTLs including 99,625for cattle, 25,610for pigs,7,812 for chicken, 1658for sheep and 1278 for horse have curated(https://www.animalgenome.org/cgi-bin/QTLdb/index, Release 33, Aug. 26, 2017). Bioinformatics approaches, as summarized in (Greiff et al., 2015) are important components for the analysis, annotation, and visualization of genomic data for phenotype likeimmune repertoiresincluding disease resistance. To this end, IMGT (ImMunoGeneTics)(Giudicelli et al., 1997)and InnateDB (Breuer et al., 2013) are two widely cited database for the in-silico prediction and annotation of immunogenomic data.However, standardized laboratory workflows, data formats, experimental designs and bio-statistical methods are necessary and, are progressing, and will likely place immunogenomics among the biggest of 'Big Data' enterprises in the life sciences (Holt, 2015). The advent of immunogenomics offers more robust identification of disease resistance candidate genes which are likely subjected for intervention with genome editing technique.

Genome editing

Genome editing is a type of genetic engineering in which DNA is inserted, deleted or replaced in the genome of a living organism using programmable nucleases or editors. The commonly used genome editors are zincfinger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 (reviewed by Petersen 2017). CRISPR-Cas9 has been developed from bacterial adaptive immune system that relies on using molecular 'scissors' to introduce changes into existing DNA sequence, as opposed to classical genetic modification which often involved moving genes from one species to another. Unlike ZFNs and TALENs that use proteins to recognize specific sequences in the genomic regions, CRISPR/Cas9 system uses a single-guide RNA (sgRNA) to target the Cas9 nuclease to a specific genomic sequence. The Cas9 protein binds to the sgRNA scaffold and generates a DNA double-strand break (DSB)in the presence of the sgRNA complementary sequence and the porto-spacer adjacent motif (PAM) sequence(Pellagatti et al., 2015). The DSB triggers the endogenous cellular DNA repair machinery that catalyzes non-homologous end joining or homology directed repair (Figure 1).The CRISPR/Cas9 system has elevated genome editing from being a technical challenge to a practical reality due to the simplicity of the method.

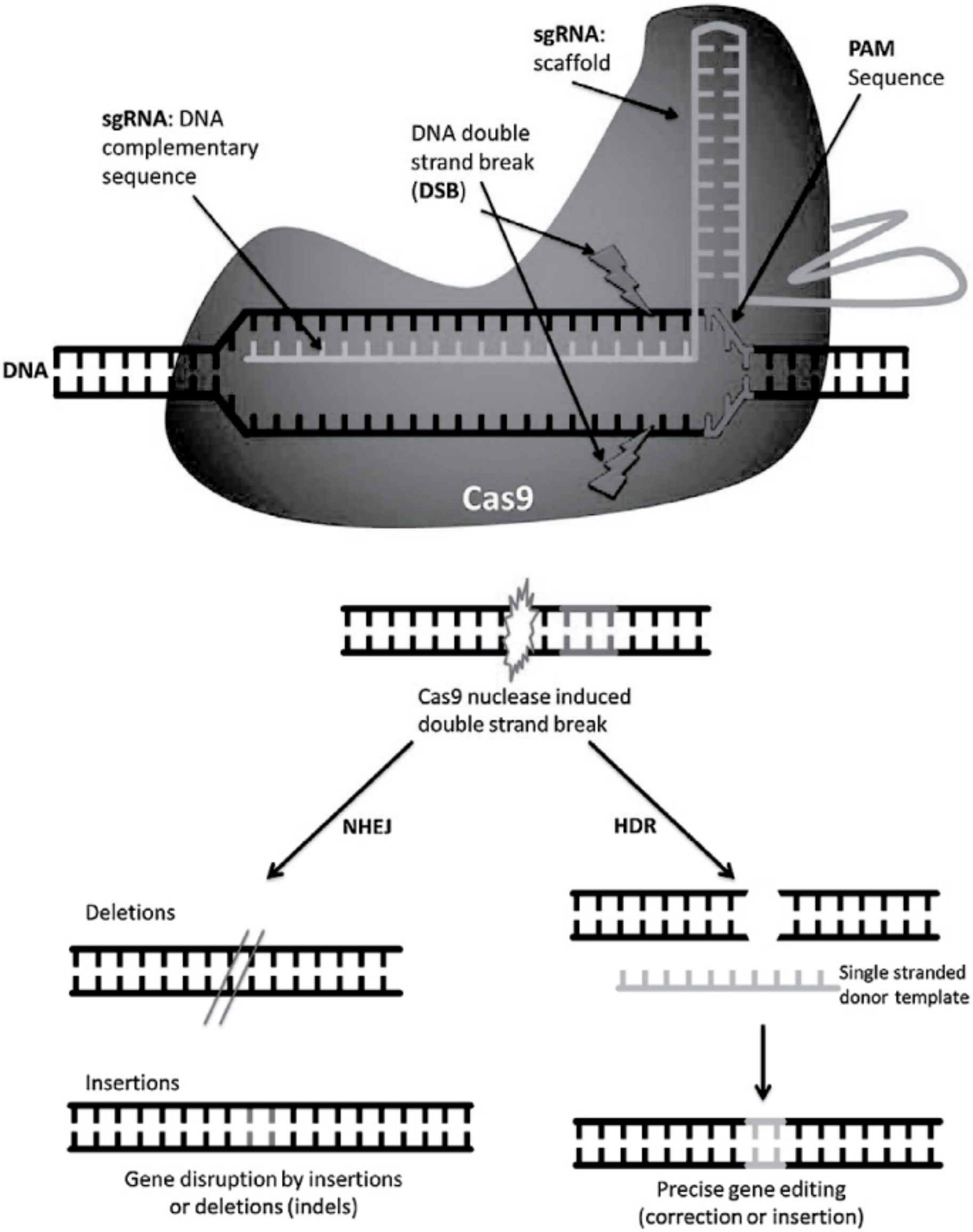

Figure 1. Schematic diagram showing genome editing using CRISPR/Cas9 system.Cas9 induces double-stranded DNA breaks which are repaired either by imperfect nonhomologous end joining (NHEJ) to generate insertions or deletions (indels) or, if a repair template is provided, by homology directed repair (HDR) (Adapted from Pellagatti et al., 2015).

Application of genome editing in livestock

Genome editing has been successfully applied in the livestock species to improve different traits like growth and production performance, animal welfare and disease resistance. The genome editing was successfully applied to knock out the myostatin(*MSTN)* gene, a negative regulator of skeletal muscle growth, from the genome of cattle, pig, sheep and goat (reviewed by Petersen 2017).The gene encoding the bovine whey protein β-lactoglobulin (BLG),which is a major milk protein and a dominant allergen,was knocked out in cattle using ZFNs (Yu et al., 2011).Another genome editing study has described the generation of pig with mutation the RELA (p65) geneusing ZFNs, which confer the tolerance of African swine fever (Carlson et al., 2012).A recent study reported the production of a hornless strain of dairy (Holstein) cattle by copying the pooled gene (P) of beef cattle (Angus) through homology directed repair path of genome editing (Carlson et al., 2016).Two prominent examples of application of CRISPR/Cas9 for genome editing to produce disease resistant livestock are porcine reproductive and respiratory syndrome (PRRS) in pig (Whitworth et al., 2016) and tuberculosis in cattle (Gao et al., 2017).

PRRS resistant pig by CRISPR/Cas9

PRRS is the most costly disease of swine industry worldwide affecting swine production, reproduction, health and welfare. Cluster of differentiation 163 (CD163)is cell surface receptor gene, a member of the scavenger receptor cysteine-rich superfamily (SRCR), consists of an intracellular domain and nine extracellular SRCR domains. CD163 facilitates the PRRS virus to enter into the pulmonary alveolar macrophage through endocytosis, where the virus replicatesand induce disease pathogenesis. The CD163 knock out pigs were completely protected against all symptoms of the PRRS infection with a single PRRSV isolate (Whitworth et al., 2016). A second study determined the SRCR5 domain from exon 7 as a critical component of infection (Burkard et al., 2017). Use of such genome-edited pigs in livestock could substantially reduce PRRS-related economic losses.

Tuberculosis resistant cattle by CRISPR/Cas9

Bovine tuberculosis is a chronic bacterial disease of cattle caused by *Mycobacteriumbovis.* Having a wide host range, *M. bovis*causes significant economic hardship for livestock farmers, with estimates of >50 million cattle infected worldwide. This disease is significant zoonosis that can transmit to human primarily via ingestion on non-pasteurized dairy products or close contact with infected cattle. The natural resistance to infection with intracellular pathogens 1 (NRAMP1) gene has reported a strong candidate gene for tuberculosis resistance. The recent report employed Cas9 nickase to introduce the *NRAMP1* (natural resistance to infection with intracellular pathogens 1) gene into the genome of bovinefoetal fibroblasts(Gao et al., 2017).These cells were then used as donor cells through somatic cell nuclear transfer, where the nucleus of a donor cell carrying the new gene is inserted into cow's ovum. Ova were nurtured in the lab up to embryos before being transferred into mother cows for a normal pregnancy cycle. The inserted *NRAMP1* was correctly expressed and provided cattle with increased resistance to infection with *M. bovis,* which is the mycobacterial pathogen that causes bovine tuberculosis (Gao et al., 2017). Wu et al. (2015) transferred the mouse *SP110* gene via TALENs in an intergenic region in a genome neighbourhood containing genes normally expressed in the macrophage and achieved resistance against *M. bovis*.

Ethics, regulations and social acceptance of genome-edited livestock products

Genome editing of livestock is poised to become commercial reality; however, questions remain as to appropriate regulation, potential impact on the industry sector and public acceptability of products. The social acceptance of genome-edited farm animal products at global level would depend on whether animal breeding by genome editing is performed after due considerations with regard to people's sense of ethics as well as animal welfare issues (Ishii, 2017). Food products derived from genome-edited livestock are expected to enter to the market immediately after confirming by the regulations of a country. However, previous controversy over genetically modified (GM) animals and cloned animals suggests that many people will be unlikely to accept the products from genome-edited animals. Psychological investigations have suggested that GM animals are viewed as less acceptable than GM plants and that people's sense of ethics has a more significant effect on the acceptance than other factors such as the perceived risks, the recognized benefits, or the trust in

regulators and researchers (Bruce, 2017). The negative attitude of people towards GMOs (GMorganisms) is, in part, based on a lack of trust in researcher and regulators (Araki andIshii 2016). Off-target mutagenesis is a major concern for the wide spread application of CRISPR/Cas9 genome editing technology, especially with regard to disease resistant animal breeding. As the history of cloned animals suggests, the investigation of off-target mutations seems vital to the use of genome editing in livestock breeding from the viewpoint of animal welfare (Ishii, 2017).Thus, the further consideration of animal welfare by reducing the risk of off-target mutations might enhance people's trust and eventually foster thesocial acceptance of products from genome-edited livestock.

Potentials and prospects of CRISPR/Cas9 technology

CRISPR-Cas9 offers the potential for substantial improvement over other gene editing technologies in ease of use, speed, efficacy, and cost. The genome-editing technique has been extensively using to illuminate gene function in disease pathogenesis and host responses. Indeed, the CRISPR/Cas9 based correction of gene mutations in mouse models of human inherited disease and in primary adult stem cells derived from patients with monogenic hereditary defects may well lay the foundations for future gene therapy approaches (Pellagatti et al., 2015). CRISPR/Cas9 knockout libraries could potentially also be used to target regions of interest in the noncoding genome, such as promoters and enhancers. Moreover, the use of CRISPR/Cas9 for genome wide studies will enable large-scale screening for disease resistance. CRISPR/Cas9 based genome editing also enables a much wider-range of changes, for example, gene knock-out, base pair substitution, targeted insertion/deletion of larger genomic regions, and modulation of gene expression (Petersen 2017). Despite the challenges, the huge advances in the field of gene therapy and genome editing by CRISPR/Cas9 achieved over the past few years pave the way for the development of exciting disease control strategies for human, plants and livestock, is hope for the production of safe animal food. Though unlike crops, where the whole population can be rapidly replaced, introducing genome editing into a livestock population is a much more complex and time consuming process. Moreover, improvement of the efficiency of CRISPR repairing process, in particular to increase the rate of gene correction and reduce undesired off-target effects and the development of more effective delivery methods will be required for its broad spectrum application.

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Prevalence and Molecular detection of the causal agents of Sub-clinical Mastitis of dairy cows in Sirajganj and Pabna Districts

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Abstract

Mastitis is a most devastating disease condition in terms of economic losses occurring throughout the world. Due to the involvement of multiple etiological agents it always remained a challenge to veterinarian all over the globe. Depending upon the climatic condition, animal species and disease management practices, etiological agents may vary place to place and case to case. Subclinical mastitis (SCM) is a serious problem in dairy industries because there are no gross changes occur in udder or glandular tissues with as a continuous source of infection to herd mates. The present research work was undertaken with the objectives to investigate the prevalence and molecular detection of the causal agents of sub-clinical mastitis (SCM) in cows at milk shed areas in Sirajganj and Pabna districts, Bangladesh. A total of 300 milk samples were randomly collected from Baghabari milk shed areas of Sirajganj and Pabna districts. The milk samples were subjected for California Mastitis Test (CMT) for identifying SCM. Total 81 positive samples were then used for the isolation and identification of associated bacteria and fungi using conventional microbiological examination and biochemical tests, followed by confirmation by polymerase chain reaction (PCR) using specific primers. Besides, universal primers were used for amplification and sequencing of PCR products where specific primers were not used. The overall prevalence of SCM was 51% (n=153/300). Based on bacteriological examination and biochemical tests, several bacteria were identified in this study; the orgnaisms included *Staphylococcus sp.* (45.68%), *Streptococcus uberis* (14.81%), *Escherichia coli* (9.88%), *Proteus sp.* (19.75%), *Salmonella sp.* (1.23%), *Acinetobacter sp.* (7.41%), and fungus (1.23%). PCR technique confirmed the bacteria as *Staphylococcus aureus* (279-bp), *Streptococcus uberis* (884-bp), *E. coli* (16SrRNA 585-bp, *stx*1 606-bp, rfbO157 497-bp) and *Salmonella sp.* (*Inv-A* gene796-bp). This study reveals that SCM in dairy cattle is persisting in Sirajganj and Pabna districts of Bangladesh. Hygienic practices should be improved, and providing technical intereventions may reduce the rate of SCM in the study areas.

Keawords: *Acinetobacter sp.*; Cow; *rfb*O157; PCR; *Proteus sp.*; *Salmonella sp.*

Introduction

Mastitis is an inflammatory condition of mammary gland characterized by physical, chemical and microbiological changes in milk (Seegers et al., 2003). However, sub-clinical mastitis (SCM) reveals no apparent change in the milk but somatic cell number is markedly increased. In dairy farms, SCM contribute up to 70%-80% of the total losses. A number of pathogens (>135) are involved for the onset of mastitis in cattle. The associated pathogens included bacteria, mycoplasma and yeast (Egwu et al., 1994). The SCM in dairy cows is crucial because it reduces milk yield (Seegers et al., 2003). The prevalence of mastitis in cattle is higher in the farms having larger herd size as compared to those in smaller herd sizes (Radostits et al., 2000). The major etiological agents of SCM included *Staphylococci*, *Streptococci* and *E. coli* (Singh and Baxi, 1982; Rahman et al., 2014). However, from clinical case of mastitis different types of bacteria like *Staphylococci, Streptococci, Corynebacterium, E. coli* and *Bacillus* sp. have been isolated and identified by Mahbub-E-Elahi et al. (1996) at Manikganj and Bangladesh Agricultural University (BAU) Dairy Farm, Mymensingh. Sub-clinical mastitis is a serious problem in dairy industries because there is no gross changes found in udder or glandular tissues and act as a continuous source of infection to herd mates. Depending upon the climatic condition, animal species and disease management practices, etiological agents may vary place to

place and case to case. Thus, the control and prevention of mastitis is a challenge and despite of the continuous efforts, causes severe economic losses to dairy industry. Early detection of mastitis with low cost and rapid screenings in field level, hygienic farm management, biosecurity and awareness building among farmers will be helpful to control the clinical and SCM of dairy cows. California Mastitis Test (CMT) is widely used for identifying SCM based on the presence of somatic cell number (SCN) in the milk. The present research work was undertaken to investigate the prevalence and molecular identification of causal agents of mastitis of cows in Sirajganj and Pabna districts in Bangladesh.

Materials and Methods

Selection of study area, duration, and study animal: A total of 60 farmers from 13 villages at Sahjadpur of Sirajgonj and Shathia of Pabna district were selected randomly during July 2016 to June 2017. A structured questionnaire was developed for screening the animals. Number of cows per farm was between 3 and 50. Only apparently healthy crossbred dairy cows were considered for this study.

Sample collection: In this study, a total of 300 milk samples were collected. Once animal was considered as one sample. Before collection of milk, the teat and tips were washed with clean water, antisepsis was done with a swab soaked with 70% alcohol and then milk samples were collected aseptically from the udder during morning. All the milk samples were collected in vials which were labeled with identification number of cow.

Physical examination of milk sample: The milk samples just after collection were observed for any abnormalities in consistency, color, and presence of any other clotted blood flakes.

Detection of SCM by CMT tester: For the detection of SCM, CMT was performed as the instructions of manufacturer (CHEIL BIO Co. Ltd.). In brief, 2 mL milk and 2 mL CMT solution were mixed together in test paddle. Rotate the paddle to mix, and changes in color and gel formation was observed within 10 to 15 Sec.

Isolation of associated bacteria and fungi: The milk samples of positive results (n=81) in CMT were transferred to Department of Microbiology and Hygiene, BAU for microbiological analyses. Before incubation, the sample was allowed in normal temperature. Then 100 µL of milk sample was inoculated into 10 mL nutrient broth. Then the milk sample containing broth was incubated at 37°C for 24 h. After incubation, one loop of incubated sample was streaked on EMB agar, Mannitol Salt Agar, Salmonella-Shigella agar, and again incubated at 37ºC for 24-48 h. The bacterial isolates were primarily identified through a series of bacteriological and biochemical examinations (Rahman et al., 2014; Chandrasekaran et al., 2014). The classification and specification of organism was based on the scheme presented in Bergey's Manual of Systemic Bacteriology (Halt et al., 1985). Fungus was isolated by spreading the sample on Potato dextrose agar, supplemented with broad spectrum antibiotic for prevening the growth of bacteria.

Oligonucleotide primers, PCR and sequencing: Polymerase Chain Reaction (PCR) was done for final confirmation of the isolated microorganisms. The oligonucleotide primers were purchased from Bioneer, Korea (**Table 1**). The oligonucleotide primers were used for the amplification of specific gene fragments for the confirmatory identification of the isolated bacteria (**Table 1**) from the SCM cases (Tanzin et al., 2016). The total volume of PCR mixture was 25 µL consisting of 12.5 µL PCR master mixture, 1 µL of each primers from 20 pmol stock and 5 µL of template DNA. The thermal profile of PCR of 35 cycles was consisted with an initial denaturation at 950C for 5 min followed by 940C for 30 Sec as denaturation, annealing at different temperature for different bacteria (**Table 1**) for 1 min, 720C for 1 min for elongation and finally 720C for 10 min as final extension using Thermal Cycler (Applied Biosystem, Germany). The PCR fragements werevisualized by UVsolo TS Imaging System (Biometra, Jena Company) after staining with ethidium bromide. The PCR fragements of *Proteus* sp., *Acinetobacter sp*. and *Strep. uberis* were sequenced (Bioneer, Korea) using universal primer set (8F 5'-AGTTGATCCTGGCTCAG-3'; 1492R 5'-ACCTTGTTACGACTT-3') for final confirmation as the specific primers were not used in these cases.

Table 1: Oligonucleotide primers used in this study

Name of organism	Name of primer	Oligonucleoide sequence (5′-3′)	Annealing temp (0C)	Expected band size (bp)	References
E. coli	EC16SrRNA F EC16SrRNA R	GACCTCGGTTTAGTTCACAGA 5'CACACGCTGACGCTGACCA	58	585	Schippa et al. (2010)
	EC *Stx*-1 F EC *Stx*-1 R	CACAATCAGGCGTCGCCAGCGCACTT GCT TGTTGCAGGGATCAGTCGTACGGGGA TGC	56	606	Heuvelink et al. (1995)
	EC *Stx*-2 F EC *Stx*-2 R	CCACATCGGTGTCTGTTATTAACCACA CC GCAGAACTGCTCTGGATGCATCTCTG GTC	56	372	Heuvelink et al. (1995)
	rfbO157F rfbO157R	AAGATTGCGCTGAAGCCTTTG CATTGGCATCGTGTGGACAG	47	497	Sanchez et al. (2010)
Salmonella spp.	Inv-A F Inv-A R	CGGTGGTTTTAAGCGTACTCT T CGAATATGCTCCACAAGGTTA	58	796	Fratamico et al. (2003)
Staph. aureus	nuc-F nuc-R	GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAACTAAAGC	55	279	Dewanand et al. (2007)
Strep. uberis	S.uber-F S.uber-R	TTGTACACACCGCCCGTCA GGTACCTTAGTTTCAGTTC	52	884	Chanter et al. (1997)
Strep. agalactiae	S.agal-F S.agal-R	AAGGAAACCTGCCATTTG TTAACCTAGTTTCTTTAAAACTAGAA	62	587	Anjali and Kashyap (2017)
Strep. dysgalactiae	S.dysg-F S.dysg-R	GAACACGTTAGG GTCGTC AGTATATCTTAACTAGAAAAACTATT G	62	403	Anjali and Kashyap (2017)

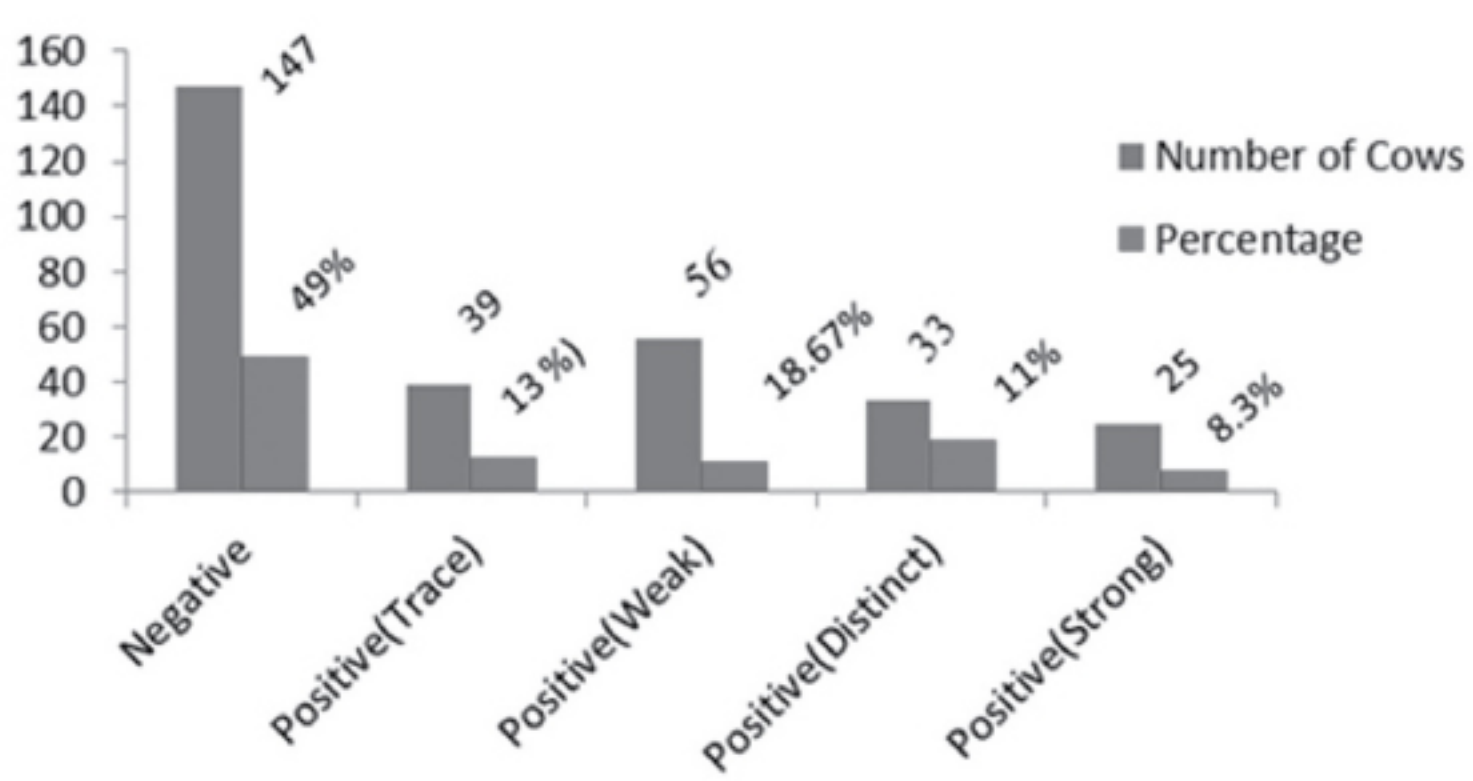

Figure 1. Prevalence of SCM in dairy cows by CMT. Except negative (49%; n=147/300) samples, 51% (n=153/300) samples were positive for SCM.

Results and Discussion

Among the 300 cows, CMT was found to be positive in 51% (n=163/300; trace - 13%, weak 11%, distinct 18.67%, strong 8.33%) samples. Bacteriological examinations and biochemical test followed by PCR confirmation revealed that several bacterial species and fungus were associated with the SCM. The number of samples positive for the SCM cases and the associated bacteria and fungus are illustrated in the **Figure 1** and **2**. Only one sample was found to be associated with fungus; however, the fungus was unidentified. The *E. coli* produced metallic sheen on EMB agar. The *Salmonella sp*. produced black colonies on SS agar. The *Staphylococcus sp*. grown on mannitol salt agar fermenting mannitol and produced characteristics yellowish colonies, and showed grapes like appearance under microscope after Gram stain. The *Acinetobacter sp*. produces blue colonies on EMB agar that reveals Gram positive bacteria arranged in pair under microscope; the bacterial genus was confirmed by sequencing. The *Proteus sp*. produces pink color colonies on SS agar, which was also confirmed by sequencing. *Strep. uberis* was confirmed by PCR (**Figure 3**) followed by sequening of amplified amplicons. However, no other *Streptococcus spp*. were detected. In this study, 51% (n=153/300) samples were confirmed as SCM. Kader et al. (2002) reported similar report who described the prevalence as 44.61% SCM in Bangladesh. On the other hand, higher prevalence (54%) of SCM was recorded in India by Singh and Baxi (1982). As comparedwith early lactation, prevalence of SCM was higher in late lactation period, as reported by Radostits et al. (2000). Prevalence of SCM was higher in high milk yielding animals as compared to low milk yielding animals (Khanal and Pandit, 2013). Previously, in Bangladesh, prevalence of SCM in dairy cows was recorded as 72.07, 66.67, 64.86 and 61.26% by CMT, SCC, WST and SFMT, respectively, as reported by Badiuzzaman et al. (2015). The highest prevalence was found for CMT (67%) and WST (62%) in the animals aging 3.5-4.5 years (Khokon et al. 2017).

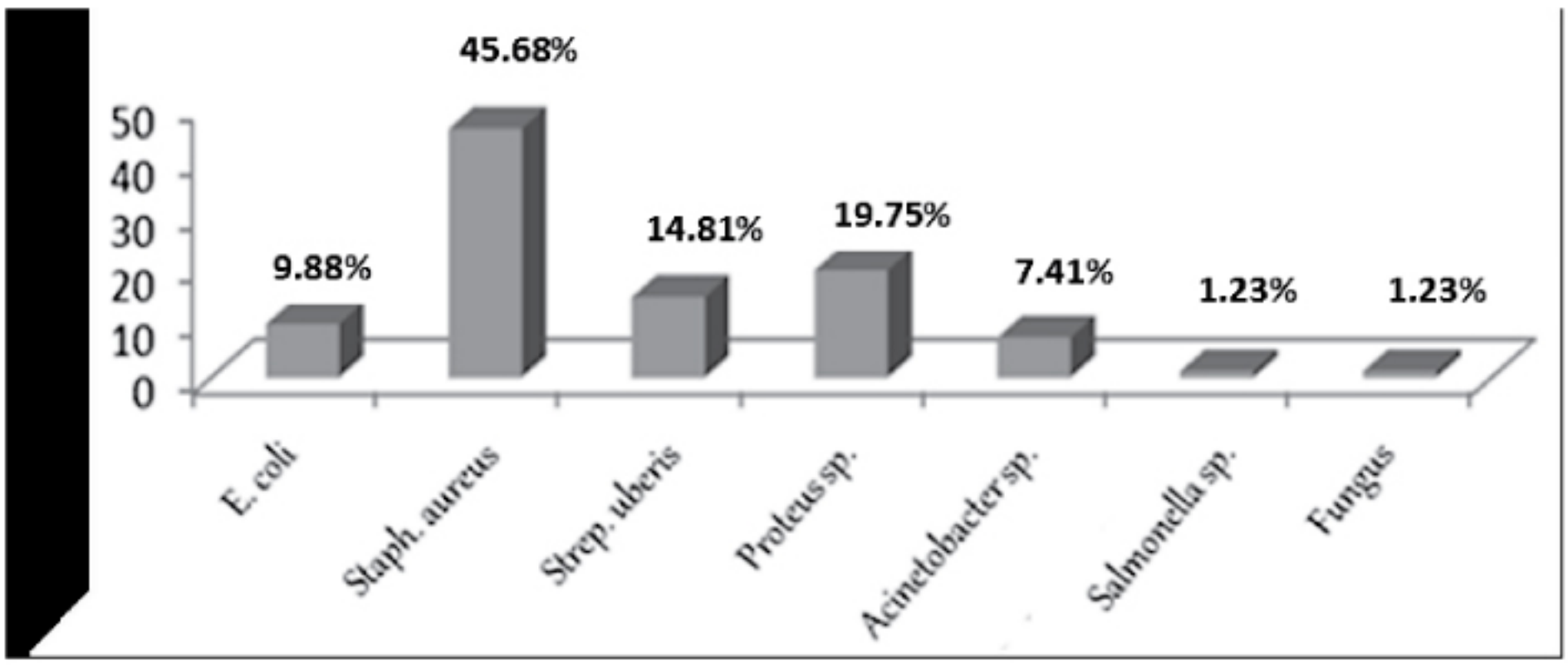

Figure 2. Percentage of different types of microorganisms prevalent in SCM. The calculation has been done based on 81 CMT positive samples.

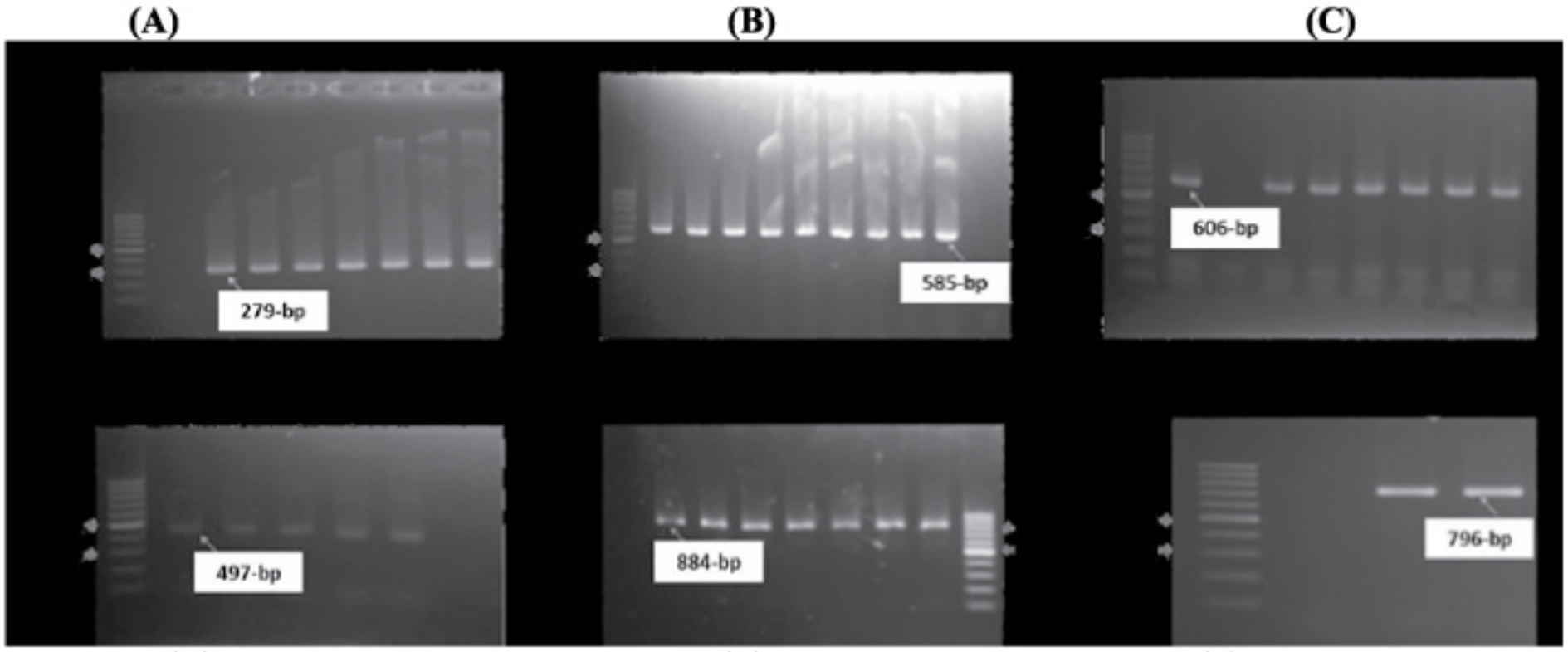

Figure 3. Molecular detection of different bacteria isolated from sub-clinical mastitis. **(A)** Amplification of *nuc* gene (279-bp) for *Staphylococcus aureus*; Lane 1: 100-bp DNA ladder; Lane 2: Negative control; Lane 3: Positive control; Lane 4-9: Test sample, **(B)** Amplification of *E. coli* 16SrRNA (585-bp) specific genomic primer; Lane 1: 100-bp DNA ladder, Lane 2-9: positive for 16SrRNA; Lane 10: Positive control; Lane 11: Negative control (*E. coli*), **(C)** Amplification of *stx*-1 (606-bp) genes; Lane 1: 100-bp DNA ladder, Lane 2-6 test samples; Lane 3: Negative control; Lane 4-9 test samples, **(D)** Amplification of *rfb*O157 (497-bp) genes; Lane 1: 100-bp DNA ladder, Lane 2: positive control; Lane 7: Negative control, **(E)** PCR picture for *Streptococcus uberis*; amplification size around 884-bp, **(F)** Amplification of *Inv-A* gene (796-bp) for *Salmonella spp*.; Lane 1: 100-bp DNA ladder; Lane 2: Negative control Lane 3: Positive control; Lane 4: Test sample.

In this study, 9.88% cases were associated with *E. coli*; whereas, Bradley et al. (2000) reported 14.5% of the *E. coli* mastitis, followed by *Staph. aureus* (11.8%) and *Streptococcus* species (7.9%) and *Strep. agalactiae* mastitis (13.2%), as reported by Al-kuzaay and Kshash (2013). This variation might be due to improved hygienic practices in this study area and awareness of the farmers. Gangwal and Kashyap (2017) showed prevalence of bacteria in mastitis as 28% *E. coli*, 24% *Staph. aureus*, 18% *Pseudomonas aeruginosa*, 10% *Klebsiella pneumoniae* and 2% *Strep. agalactiae*. Valbak (1990) and Olivares-Pérez et al. (2015) could isolate *Salmonella sp.* from milk samples taken from cows, as supporting our study. PCR was applied to detect the presence of shiga-like toxin in six *E. coli* (*stx*-1 genes) isolates among eight *E. coli* strains (**Figure 3**). No *stx*2 gene could be identified in this study. However, *stx*-1 and *stx*-2 genes were reported together by Sayed (2016). Among the *E. coli* isolated, 4 harvored *rfb*O157 genes (**Figure 3**), indicating the the *E. coli* were highly pathogenic, as reported by Caine et al. (2014). In another study, Islam et al (2014) revealed that 26.71% of milk samples were associated with *Staphylococcus sp*.; whereas, 5.48% cases were found to be positive for SCM by molecular identification. In this study, culture was done from the milk samples that demonstrated positive reaction in CMT. Mahbub-E-Elahi et al. (1996) isolated *Staphylococcus sp.*, *Streptococcus sp.*, *E. coli* and *Bacillus*, whereas Rahman et al. (1968) isolated and identified different strains of *Staphylococci* from mastitic and apparently healthy mammary glands of cows. These findings also corresponded with Shrestha and Bindari (2012) who reported highest prevalence of *Staphylococcus* followed by *E. coli*, *Streptococci* and *Corynebaceterium*. Chanda et al. (1998) reported that *Staphylococcus* was the principal organism of mastitis. *Staphylococcus* is the opportunistic bacterium which can survive the skin of the udder can infect via teat canal. In addition to *Staphylococcus*, *E. coli* was identified in this study, which is an environmental opportunistic pathogen. Similar report was also reported by Mahbub-E-Elahi et al. (1996). *Acinetobacter sp*. was isolated from mastitis in our study, as supported by Marimuthu et al. (2014). Similary, *Proteus sp*. was identified in this study, as described by Olivares-Pérez et al. (2015). Several fungal species have been isolated from milk by Pachauri et al. (2013), as we are reporting here.

Conclusion

The prevalence of subclinical mastitis indicates that it is a major threat for dairy industry. The overall prevalence of sub-clinical mastitis in the study area is 51%. The associated bacterial species with the cases were *Staphylococcus sp., Streptococcus uberis, Escherichia coli*, *Proteus sp., Salmonella sp., Acinetobacter sp*. Early detection and ensuring proper preventive measures are suggested for controlling the sub-clinical mastitis in the study area.

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Crossbred cow's metabolic profile and its relation with retention of fetal membrane

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ABSTRACT

The study was designed to determine the blood serum Calcium, Phosphorus, Magnesium, Total protein and Glucose value in different stages of crossbred cows (heifer, 1st, 2nd, 3rd trimester of pregnancy and after parturition) to find out the relation of these metabolic factors with retention of fetal membrane. One hundred and two pregnant cows and ten heifers were examined. Animals were categorized into cows with retention of fetal membrane (RFM) and cows without RFM. Blood samples were collected at different stages of pregnancy, before pregnancy (Heifer) and after parturition. Serum was analyzed by auto blood analyzer. Cows having retention of fetal membrane (RFM) had significant variation ($p<0.05$) of their calcium concentration in different period of pregnancy and after parturition. RFM affected cows' Mg level was continuously decreasing in different trimesters of pregnancy and after parturition. Interestingly, Cows in which placenta were expelled normally also showed significant difference in calcium, magnesium, total protein and glucose concentration in their different trimester of pregnancy and after parturition. Serum calcium, total protein and glucose level after parturition in cows with retention of fetal membrane was significantly lower than healthy cows. In heifers, serum calcium (12.40 mg/dl) and glucose (85.83 mg/dl) level was significantly ($p<0.05$) higher than pregnant cows. There was significant variation of calcium concentration in different trimester of pregnancy in heifers. Phosphorus (9.77 mg/dl) was also higher in heifers than pregnant cows. Protein levels also significantly ($p<0.05$) varied in different trimesters of pregnancy and were higher at 1st trimester pregnant cows. It can be concluded that serum calcium, glucose and total protein levels may have relation with retention of fetal membrane in crossbred cows. (Funded by the University Grant Commission (UGC), Agargaon,Dhaka, Bangladesh and Canada-HOPE Scholarship, CIHR, Canada).

Key Words: Placenta retention, metabolites, crossbred cows

INTRODUCTION

Milk production of dairy cows is increasing in the world steadily; however their reproductive performance has declined. Decreased reproductive performance is closely associated with the production diseases, commonly occurring in the periparturient period (Brydl et al., 2003; Ingvartsen, 2006; Thatcher et al., 2006; Brydl et al., 2008). Importantly, profitable dairying is affected by the production and reproduction performance of the animal (Norman et al., 2009). Many factors may independently or cumulatively affect the reproductive performance of dairy cattle including nutrition, herd management, accuracy of estrus detection, insemination and inseminator skill and genital diseases such as retained placenta (López-Gatius et al., 2006). Retained placenta is one of the important reproductive disorder which occur in 4-18% of calving (Han and Kim, 2005), although the percentage may vary from region to region and breed to breed. Placenta is the vital fetal organ, which connects the developing fetus to the mother's uterine wall. Placenta acts as a site of nutrient, waste and gas exchange between mother and fetus. In bovine, cotyledonary placenta usually exists until 3 to 8 hours after calving. If placenta does not expel until 12 hours of parturition then the condition is called retained placenta (Sammin et al., 2009). The etiology of retained placenta still is not completely clear. Many factors may contribute to the occurrence of retained placenta including dystocia, still birth, twining, length of gestation (Sloss and Dufty, 1980; Han and Kim, 2005), parity and nutritional problems. Oxidative stress may also contribute to retained placenta. However, still now very few data are available on host metabolic profile on retention of placenta in crossbred animals.

Retained placenta in cattle is closely associated with different other diseases including uterine infections (Larson et al., 1985; Bruun et al., 2002; Bell and Roberts, 2007), vaginal infections (Muller and Owens,

1974), lameness (Larson et al., 1985), mastitis (Larson et al., 1985) etc. All those diseases leads to reduced milk production (Bicalho et al., 2008) and increase postpartum interval (Fourihon et al., 1999) results in reducing the efficiency of first insemination after calving and results in losses in dairy industry. Ultimately in retained placenta affected cow, the overall reproductive performance is decreased (López-Gatius et al., 2006). The above-mentioned information signifies the detrimental effect of retained placenta in successful dairying. Literature also shows, the heritability of retained placenta trait is very low (0.07-0.08) (Heringstad et al., 2005; GhaviHossein-Zadeh and Ardalan, 2011). This implies that retained placenta may cause by the factors, most of them may be environmental origin or individual host specific factor(s) other than the genetics. Many studies have been performed on the incidence and treatment of retained placenta. Effect of environmental factor on the incidence of retained placenta was also examined in Holstein cows (Binabaj et al., 2014). Very few studies have been performed on the effect of host metabolic profile in retained placenta especially in crossbred cows. Therefore, it is important to know the metabolic profile of crossbred cows to determine whether it is related to retention of fetal membrane. This present study was conducted with the following objectives

- ✓ To determine the selected metabolic profile of pregnant crossbred cows
- ✓ To determine the relation of pregnant animal's metabolic profile and retention of fetal membrane (retained placenta) in crossbred cows
- ✓ To determine difference between non-retention and retention group on metabolic profile

MATERIALS AND METHODS

One hundred and two pregnant cows were selected randomly. Pregnancy was confirmed by rectal palpation prior to selection. The previous history of retained placenta and data related to management was recorded. Blood samples were taken from selected animal at different stages of pregnancy and after parturition. Samples were taken to laboratory and serum was separated and kept in 4^0C until analysis. Serum samples were analyzed by auto blood analyzer. Results for different trimester of pregnancy were recorded. Animals were categorized on the basis of placental retention. Animals with retained placenta were considered as positive case and normal placental expulsions were considered as control cases.

Brief description of data and sample collection

The present study was favorably designed for blood sample collection to determine the metabolic status of pregnant crossbred cows at different stages of pregnancy. Management and environmental factors associated with the prevalence of retained placenta in dairy cows were also recorded. Blood sample was mainly collected at first, second, trimester of pregnancy and after parturition. Blood sample taken from cows having retained placenta was considered as positive sample and from cows without retained placenta was treated as control sample. About 2 ml - 3 ml blood was collected using 10ml syringe and 18G needle. Sterilized syringe and needle was used for each sample collection. After collection of sample it was taken into sterilized 4ml NMC vacuum tube (clot activator) (Shandong Chengwu Medical Products Factory, Shandong, China). Animal identification number, owner address and date of collection were recorded on the surface of the tube. Epidemiological data were also collected based on some parameters such as breed, age, parity, body weight, feed given, date of service, BCS at AI, first, second and third trimester of pregnancy, date of parturition, any medication given during pregnancy, any complication during parturition, previous history of retention of fetal membrane. All these sample and data were collected from randomly selected farms of Potiaupazilla where CDVF is operating their activities in Chittagong. The animals were mainly reared for milk production (dairy purpose) and managed under semi conventional system. Then collected data were organized to find out the occurrence of retained placenta and sample was preserved and sent to laboratory for analysis.

Sample preservation

After collection of Blood in NMC vacuum tube (clot activator), the tubes were kept at 40C immediately. Then the samples were transported to the laboratory within 4 hours of collection. Those with retained placenta were treated according to the existing treatment regime.

Serum separation

Blood sample was kept at 4^0C for four to five hours in the laboratory to activate the clot. After complete

clotting of blood clotted portion was separated and remained at the bottom of the tube and serum on the upper part of the tube. Serum was separated manually and kept in a separate tube. Then the serum sample was centrifuged for further removal of clot.

Serum analysis to determine Calcium (Ca), Phosphorus (P), Magnesium (Mg), Glucose, Total protein
Blood serum was taken to the laboratory and analyzed by a chemical analyzer. Serum Ca, P, Mg, Total protein and glucose concentration were analyzed.

Statistical Analysis
All the data were entered in the Microsoft Excel Sheet and analyzed by GraphPad Prism and SPSS 11.5 statistical software.

RESULTS

One hundred and two pregnant crossbred cows and ten heifers were subjected to blood analysis. Calcium (Ca), Phosphorus (P), Magnesium (Mg), Total protein (TP), Glucose levels for different trimester of pregnancy, after parturition in cows and in heifers was recorded. Among 102 cows and 15 showed retention of fetal membrane. Prevalence of RFM was 14.71%. There were significant differences in serum Ca, TP and, Glucose levels between RFM cows and healthy cows.

Blood serum level of Ca, P, Mg, Total protein and Glucose in crossbred cows and in heifers regardless of occurrence of RFM:
In crossbred heifers, serum calcium (12.40 mg/dl) and glucose (85.83 mg/dl) level was significantly ($p < 0.05$) higher than pregnant cows (Table 1). However, magnesium concentrations (1.67 mg/dl) had a lower trend in heifers compared to pregnant cows. In pregnant cows, the serum calcium concentration was lower in second trimester (5.89 mg/dl) compared to its 1st and 3rd trimester of pregnancy. There was a significant variation of calcium concentration in different trimester of pregnancy (Table 1). Phosphorus (9.77) was also higher amount in heifers than pregnant cows. It was significantly different ($p < 0.05$) from first and second trimester of pregnancy. Results showed no significant ($p > 0.05$) difference of heifer's serum magnesium level with that of 2nd and 3rd trimester of pregnancy but were significantly ($p < 0.05$) lower compared to 1st trimester of pregnancy. There were significant ($p < 0.05$) differences in protein levels in different trimesters of pregnancy and was higher at 1st trimester pregnant cows. There were no ($p > 0.05$) significant differences in blood glucose levels at pregnancy but was higher in heifers compared to pregnant animals of any trimester (Table 1).

Table 1: Serum Ca, P, Mg, Total protein and Glucose level (Mean±SD) in heifers and pregnant cows in different stages of pregnancy regardless of the occurrence of RFM

Group	Calcium mg/dl (Mean±SD)	Phosphorus mg/dl (Mean±SD)	Magnesium mg/dl (Mean±SD)	Total Protein g/dl (Mean±SD)	Glucose mg/dl (Mean±SD)
Heifer	12.40±2.33 [a]	9.77±2.81 [a]	1.67±0.37 [b]	6.03±1.05 [bc]	85.83±30.72[a]
1st trimester	6.72±1.81[c]	6.73±2.82 [c]	2.81±1.17 [a]	8.04±2.68 [a]	64.14±30.97[a] [b]
2nd trimester	5.89±1.40 [d]	6.64±1.82 [c]	2.28±0.95 [b]	5.52±1.75[c]	54.88±20.55[b]
3rd trimester	7.97±2.25 [b]	7.76±2.06 [ab]	2.23±0.82 [b]	6.54±1.71 [b]	58.4±19.1[b]
Level of significance	*	*	*	*	*

P values < 0.05 were considered statistically significant. The values in the same column with different superscripts differed significantly ($p<0.05$). ** = Highly significant ($p<0.01$),* = significant ($P<0.05$), NS = Not significant.

Blood serum level of Ca, P, Mg, Total protein and Glucose in cows with RFM
Cows having retention of fetal membrane (RFM) had significant variation ($p<0.05$) of their calcium concentration in different period of pregnancy and after parturition. It showed lower serum calcium

concentration during 1st (7.14 mg/dl) and 2nd (5.87 mg/dl) trimester of pregnancy compared to 3rd trimester and after parturition (Table 2). Magnesium level was continuously decreasing in different trimesters of pregnancy and after parturition in animals suffered from RFM. This decrease was significant in 2nd trimester of pregnancy. Total protein levels showed a fluctuating concentration in different trimester of pregnancy and after parturition in the RFM affected animals. There was no significant difference in phosphorus and blood glucose levels in different period of pregnancy and after parturition in cows with RFM (Table 2).

Table 2: Serum calcium, phosphorus, magnesium, total protein and glucose level (Mean±SD) in cows with RFM

Group (RFM)	Calcium mg/dl (Mean±SD)	Phosphorus mg/dl (Mean±SD)	Magnesium mg/dl (Mean±SD)	Total Protein g/dl (Mean±SD)	Glucose mg/dl (Mean±SD)
1st trimester	7.14±1.55 [b]	8.13±4.35	3.11±1.16 [a]	8.18±3.26 [a]	67.4±35.6
2nd trimester	5.87±1.65 [c]	6.75±1.68	2.49±1.19 [b]	6.56±1.38 [b]	60.62±16.98
3rd trimester	7.65±2.28 [a]	8.20±2.26	2.24±0.97 [a]	7.06±2.05 [a]	50.94±16.47
After parturition	9.12±1.92 [a]	7.73±2.13	1.80±0.42 [a]	5.95±1.19 [a]	53.42±14.73
Level of significance	*	NS	*	*	NS

P values < 0.05 were considered statistically significant. The values in the same column with different superscripts differed significantly ($p<0.05$). ** = Highly significant ($p<0.01$),* = significant ($P<0.05$), NS = Not significant.

Serum level of Ca, P, Mg, Total protein and Glucose in cows without RFM

Cows in which placenta were expelled normally showed significant difference in calcium concentration in their 1st, 2nd, 3rd trimester of pregnancy and after parturition. Serum calcium value decreased during 2nd trimester (5.68 mg/dl) of pregnancy than 1st and 3rd, but significantly increased after parturition (11.16 mg/dl). Serum phosphorus concentration did not vary in different period of pregnancy and after parturition. Magnesium level was decreasing continuously from first trimester of pregnancy to postpartum. Total protein value was significantly different in 1st and 2nd trimester from other period of time. There was a significant increase of blood glucose level in 3rd trimester of pregnancy (Table 3).

Table 3: Serum calcium, phosphorus, magnesium, total protein and glucose level (Mean±SD) in cows without RFM

Control (without of RFM)	Calcium mg/dl (Mean±SD)	Phosphorus mg/dl (Mean±SD)	Magnesium mg/dl (Mean±SD)	Total Protein g/dl (Mean±SD)	Glucose mg/dl (Mean±SD)
1st trimester	6.78±2.10 [c]	6.11±1.27	2.91±0.96[a]	8.45±3.65[a]	56.2±32 [b]
2nd trimester	5.68±1.58 [d]	7.12±1.55	1.79±0.71 [b]	5.61±2.10[b]	54.74±10.73 [b]
3rd trimester	8.16±1.78 [b]	8.35±2.07	2.19±0.66 [b]	6.62±1.18[a]	72.66±21.17 [a]
After parturition	11.16±1.74 [a]	6.49±2.34	1.87±0.36 [b]	8.27±2.11[a]	50.2±9.7 [b]
Level of significance	**	NS	**	*	*

P values < 0.05 were considered statistically significant. The values in the same column with different superscripts differed significantly ($p<0.05$). ** = Highly significant ($p<0.01$),* = significant ($P<0.05$), NS = Not significant.

Comparative level of postpartum blood metabolites (Calcium, Phosphorus, Magnesium, Total protein and Glucose) in healthy cows and cows with RFM

Serum calcium level in cows with retention of fetal membrane was significantly lower than healthy cows (Table 4). There was no significant difference in magnesium and phosphorus level between two groups. Total protein was higher in healthy cows than cows with retention of fetal membrane. Serum glucose level was significantly higher in healthy cows (62.65±20.76) than cows with RFM (51.04±13.11) (Table 4).

Table 4: Blood metabolites (Calcium, Phosphorus, Magnesium, Total protein and Glucose) level (Mean±SD) in healthy cows and cows with RFM

Group	Calcium mg/dl (Mean±SD)	Phosphorus mg/dl (Mean±SD)	Magnesium mg/dl (Mean±SD)	Total Protein g/dl (Mean±SD)	Glucose mg/dl (Mean±SD)
Cows with RFM	9.11±1.92 ^{b}	7.72±2.13	1.79±0.43	5.95±1.18 ^{b}	51.04±13.11 ^{b}
Healthy cows	11.16±1.74 ^{a}	6.45±2.4	1.86±0.35	8.22±2.15 ^{a}	62.65±20.76 ^{a}
Level of significance	**	NS	NS	*	*

P values < 0.05 were considered statistically significant. The values in the same column with different superscripts differed significantly ($p<0.05$). ** = Highly significant ($p<0.01$),* = significant ($P<0.05$), NS = Not significant.

DISCUSSION

To the best of our knowledge, it is the first study to determine the blood serum calcium, phosphorus, magnesium, total protein and glucose levels in different stages of pregnancy (1st, 2nd, 3rd trimester) and after parturition in Frisian crossbred cows and heifers. We found significant relations of those metabolites with the occurrence of retention of fetal membrane in Frisian crossbred cows.

After examining all the animals with or without retention of placenta, we found serum calcium level was significantly higher in heifers than pregnant cows. Prakash (2013) also reported higher calcium value in jersey crossbred heifers. It might be due to higher absorption and lower utilization of calcium in heifers. In pregnant cows serum calcium level decreased during 2nd trimester then 1st trimester. However, in 3rd trimester of pregnancy calcium level was increased than the other two trimesters of pregnancy. Hussain et al. (2001) found highest calcium concentration at 8th month of pregnancy, which was 9.30 mg/dl that is similar with the present observation. Calcium concentrations at three stages of pregnancy differed significantly, which might be due to alteration of calcium demand in cows. In 2nd trimester of pregnancy, cows have high calcium demand for development of organs and ossification of fetal bones. Lactating pregnant cows have higher calcium demand than dry pregnant cows reported by Serdaru et al. (2011). This result might be due to increased absorption of calcium, decreased metabolic activity and cessation of milk production at pre-partum stage.

Cows having retention of fetal membrane (RFM) had significant variation of calcium concentration in their different periods of pregnancy and after parturition. It showed lower serum calcium concentration during 1st (7.14 mg/dl) and 2nd (5.87 mg/dl) trimester of pregnancy compared to 3rd trimester and after parturition. Sheetal et al. (2014) found lower level of calcium at last stage of pregnancy than after parturition in RFM cows which is different from the present study. The lower level of calcium in cows with RFM might be due to excessive mobilization of calcium to the fetus during the last stages of pregnancy resulting in less availability to uterine tissues (Mohanty et al., 1994).

Specifically in Cows, whose placenta expulsions were normal also showed significant differences in calcium concentration in their 1st, 2nd, 3rd trimester of pregnancy and after parturition. Those cows' serum calcium

value decreased during 2nd trimester (5.68 mg/dl) of pregnancy than 1st and 3rd, but significantly increased after parturition (11.16 mg/dl). Hussain et al. (2001) found highest calcium at 8th month of pregnancy. On the other hand, the lowest value of calcium was observed immediately after parturition, which is not in agreement with the present finding. Serum calcium level after parturition in cows with retention of fetal membrane was significantly lower than healthy cows. The level of serum calcium was found significantly lower in RFM cases when compared to control cases (sheetal et al., 2014). This is similar with the present finding. This decreased level of serum calcium might have caused atony of uterus resulting in RFM (Arthur et al., 1992). Our study indicates that calcium may have positive impact on normal expulsion of fetal membrane.

Phosphorus was also higher in heifer than pregnant cows among all the examined animals. There was significant difference between 1st and 2nd trimester of pregnancy but not with 3rd trimester of pregnancy. Prakash (2013) reported higher mean phosphorus level in oestrous heifer and Hussain et al. (2001) indicated that the highest level of phosphorus at the last stage of pregnancy which is similar with our present study. Hagawane et al. (2009) found that the serum phosphorus level in early stage of lactation (4.64 mg/dl) was significantly lower than the normal healthy control buffaloes. Moderate depression in the levels of phosphorus might be due to colostrum synthesis (Rook and Thomas, 1983) and enhanced carbohydrate metabolism. There was no significant difference in phosphorus and blood glucose levels in different period of pregnancy and after parturition in cows with RFM. This indicates that the phosphorus and glucose remain similar at pregnancy and after parturition in cows with retention of fetal membrane.

These results might be due to interrelation of phosphorus and calcium metabolism in healthy animal, as serum calcium levels raise, those of phosphorus fall. Control of phosphorus levels is in part accomplished by regulation of renal excretion. However, fairly rapid fluctuations in serum inorganic phosphate can occur because the serum inorganic phosphate concentration is influenced by carbohydrate metabolism (Wilson et al., 2015).

We also found that there was no significant difference in heifer's serum magnesium level with 2^{nd} and 3^{rd} trimester of pregnancy but were different with that of 1st trimester of pregnancy. A significant difference in the levels of calcium, phosphorus, magnesium and iron was observed in case of serum minerals in heifers (Prakash, 2013). These results might be due to different functions of magnesium. Magnesium is used as an activator for more than 300 enzymes, especially those involved in oxidative phosphorylation, glycolysis, cell replication, and protein synthesis (Metzler, 2003). The absorption of magnesium in the intestinal tract and its reabsorption in the renal tubules are directly associated with the body's need, balance and health (Swaminathan, 2003).

Magnesium level was continuously decreasing in different trimesters of pregnancy and after parturition in animals suffered from RFM. This decrease was significant in 2^{nd} trimester of pregnancy. Blood Mg concentration was lowest at second trimester of pregnancy, compared to cows before calving, after calving and 3rd trimester. Peri parturient dairy cows were found to have the lowest values for blood Mg, which were within the physiological limits (adequate supply), suggesting a somewhat higher degree of Mg utilization in the blood by the mammary gland during early lactation. The results are in disagreement with the findings reported in a number of studies (Puls, 1998; Wang and Beede, 1992; Sevinc et al., 1997; Sharma et al., 2006). This result might be due to decreased absorption of magnesium and increased utilization during 2nd trimester of pregnancy. There was no significant difference in magnesium and phosphorus level after parturition between RFM cows and healthy cows. Sheetal et al. (2014) also found similar result that the level of serum inorganic phosphorus was non-significantly lower in RFM cases when compared to control cases. In the contrary, Ray et al. (2004) reported low serum inorganic phosphorus levels in cows with RFM. Low phosphorus level during the pre-calving and post-calving periods predispose the dam to retain fetal membranes (Tillard et al., 2008). This may also be attributed to decreased contraction of phosphorus in uterine muscles.

There were significant differences in protein levels in different trimesters of pregnancy and was higher at 1st trimester pregnant cows when we considered all the animals. Higher level of total protein has also been reported by Yadav et al. (2006) in buffaloes. Total protein is needed for the optimum secretion of

gonadotropin releasing factors and number of other hormones needed in the culmination of the pregnancy. Mir et al. (2008) found that, significant increase in total plasma protein during mid and late pregnancy when compared to the values observed in early pregnancy in buffalo cow, which is not in agreement with the present study. The alteration of results might be due to species variation, changes in feed, environmental changes and other management factors. Total protein value was significantly different in 1st and 2nd trimester from 3rd trimester. Consistent with our findings, increasing pattern of serum total protein was also found in non-lactating yaks by Pouroucholtamane et al. (2005). This phenomenon could be attributed to the non-lactating state of the cows in late pregnancy.

Total protein levels were fluctuating in different trimester of pregnancy and after parturition in the RFM affected animals. This result is similar with the findings of Mir et al. (2008). The fluctuation of total protein values indicate that the demand of protein in different stages of pregnancy and after parturition vary significantly.

There were no significant differences in blood glucose levels at pregnancy but was higher in heifers compared to pregnant animals of any trimester. Singh and Singh (2006) reported that the mean plasma glucose concentration was significantly higher in oestrus heifers than that of anoestrus heifer, which is similar with the finding of our present study. The increased concentration of plasma glucose level could elevate the progesterone production directly by increasing the LH production (Kaneko, 1989). According to Arthur et al., (1982) the temporary cessation of oestrus in cows and delayed puberty in heifers occur when they were exposed to negative energy balance. Abdulkareem (2013) found that plasma glucose concentration remained steady at calving and postpartum periods. On the other hand, no significant differences in glucose concentrations during calving and postpartum periods may explain the decline in maternal circulating insulin concentrations. The insulin level in the blood of cows is low during the initiation period of lactation (Accorsi et al., 2005). Mir et al. (2008) found that, the level of plasma glucose was significantly lower during late and mid pregnancy when compared with early pregnancy in buffalo. But in present study, we found no significant difference in different stages of pregnancy. These alterations might be due to species variation, diabetes, hypoglycaemia, adrenal dysfunction and various other conditions.

Elitok et al. (2006) reported exponent decrease in serum glucose concentration as the parturition approached in dairy cattle and the significant decrease in blood glucose level during late pregnancy signifies rapid utilization of glucose towards the end of the pregnancy but we found that there was a significant increase of blood glucose level in 3rd trimester of pregnancy in cows with normal expulsion of fetal membrane. The blood concentrations of glucose decrease after calving. The decrease in blood glucose is theoretically caused by the rapid and sustained increase in glucose demand for milk production. Mechanisms that determine the circulating concentration of blood glucose at or near the time of calving are not very well understood.

Total protein and glucose levels after parturition were significantly higher in healthy cows than cows with retention of fetal membrane. Peripheral blood concentrations of glucose, total protein, total cholesterol, blood urea nitrogen, non-esterified fatty acid (NEFA) and beta-hydroxy butyric acid (BHBA) were determined by Kumari et al. (2015) in cows that expelled fetal membranes normally and in cows that developed retention of fetal membranes. They found that the cows that developed RFM had significantly lower concentrations of cholesterol, glucose and total protein which is similar with the finding of our present observation. These results might be due to decreased dietary crude protein and carbohydrate intake by cows with RFM. Higher serum total protein concentration might have been associated with infectious processes i.e. mastitis, metritis or have had improved because of dietary intake of concentrates (Hagawane et al., 2009). The blood concentrations of glucose and total protein decrease after calving. The decrease in blood glucose is theoretically caused by the rapid and sustained increase in glucose demand for colostrum and milk production.

CONCLUSIONS

The prevalence of retention of fetal membrane in Frisian crossbred cows is about 14%. Regardless of the occurrence of retention of fetal membrane, the serum levels of Ca, P, Mg, Total protein and glucose level was different in different stages of pregnancy in crossbred cows. Those metabolites were found to be related with

the occurrence of RFM. Therefore, care should be taken during pregnancy and regular monitoring for different metabolites is essential to overcome the occurrence of retention of fetal membrane.

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Molecular detection of *Pasteurella multocida* Type B causing haemorrhagic septicemia in cattle and buffaloes of Bangladesh

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Abstract

Hemorrhagic septicemia (HS) is an acute septicemic disease that primarily affects cattle and buffaloes. Thedisease is caused by *Pasteurella multocida* sero types B:2 and E:2. The objective of this study was to isolate *P.multocida* from clinical cases and to confirm its identity using polymerase chain reaction (PCR) basedapproach. Clinical samples of two suspected cases of haemorrhagic septicemia of cattle and buffalo from Mymensingh and Rajshahi districts respectively were collected. Two isolates were isolated from thesesuspected cases and primarily identified as *P. multocida* based on morphological study, staining properties, and cultural and biochemical characteristics. The isolates were confirmed initially as *P. multocida* at genus level by PCR using genus specific primers. Later, the isolates were confirmed as *P. multocida* type B, the causal agent of haemorrhagic septicemia, by PCR with primers specific for *P. multocida* type B. Theseisolated organisms can be used as vaccine candidate for the production of effective vaccine against haemorrhagic septicemia.

Key words: Haemorrhagic Septicemia, cattle, buffaloes, *Pasteurellamultocida* type B, PCR

Introduction:

Haemorrhagic septicaemia (HS) is an acute and highly fatal septicemic disease of cattle and buffaloes caused by *Pasteurella multocida*. The disease has ahigh morbidity and mortality in cattle, particularly inbuffaloes. *P. multocida* are classified into several serotypes based on their capsular and somaticantigens. According to the capsular polysaccharides *P. multocida* according are classified into fiveserotypes designated A, B, D, E and F, whileaccording to the cell wall lipopolysaccharides typingthey are classified into 16 somatic serotypes (Cartet,GR., 1955; Carter and Alwis, 1989). The general andbiochemical properties of the various strains are verysimilar, and from this point of view these organismsall belong to the single species, but differentserotypes show different pathogenicity when testedin various hosts. The etiology of HS is *P. multocida* serotype B: 2 and E: 2 (Annas*et al*., 2014; Chung *et al*., 2015; Marza et al., 2015). *P. multocida,* based on pathogenicity could be eitherthose causing a HS or that causing nonhaemorrhagic septicaemia. In addition, pasteurellosis causes great economic losses in sheep, goats, pigs,dogs, poultry, quails, ducks and sometimes in wildanimals such as tiger, lion, leopard, panther, etc (DeAlwis, 1996). In cattle, the is characterized by a rapidcourse, fever, oedematous swelling in the head throat-brisket region, swollen and haemorrhagic lymph nodes and presence of numerous subserouspetechial haemorrhages (Carter and De Alwis, 1989). *P. multocida*is a small, non-motile, Gram negative coccobacillary rod, inhabiting the nasopharynx andgastro-intestinal tract of many wild and domesticanimals and produces disease when the animals areunder stress (De Alwis, 1996). High ambienttemperature, overcrowding, inadequate ventilation,transportation and malnutrition are factors, linkedwith the onset of the disease (Stahel*et al*., 2009). Although there are reports on the occurrence of HSin Bangladesh (Hafiz, 2012; Akhtar, 2013), however, as far as we know, none of the studies have identifiedthe etiological agent of HS using molecular basedapproach. The aim of the present study was to isolateand identify accurately *P. multocida* from suspected cases of HS by molecular technique *i.e.,* PCR, so thatthe isolates could later be used for the developmentof effective vaccine to control the diseases inBangladesh.

Materials and Methods

Isolation and identification of Pasteurella

For the isolation of *P. multocida* edematous fluid was collected from the throat of buffaloes of Rajshahi (N=1) and cattle of Mymensingh (N=1) regions that showed characteristics signs of HS. The sampleswereimmediately transported to the Bacteriologylaboratory of the Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh. Isolation and identification of *P. multocida* was carried out based on morphology, staining, cultural and biochemical characteristic, as described by Cheesbrough (2006).

Polymerase Chain Reaction (PCR) for Pasteurella multocida type B

Conformation of the isolated organisms as *P. multocida* type B, the causal agent of haemorrhagic septicemia in buffaloes and cattle were done basedon PCR as described by Townsend *et al.* (1998), Panna *et al.* (2015) and Akhtar *et al.* (2016). InitiallyPCR was carried out to confirm the isolate as *Pasteurella* spp. using the primer KMT1T7 5′-ATCCGC-TAT-TTA-CCC-AGT-GG-3′ and KMT1SP65′-GCT-GTA-AAC-GAA-CTC-GCC-AC-3′. Thiswas then followed by conformation of the isolates as*P. multocida* type B using the specific primers pairsKTT72 5′-AGG-CTC-GTT-TGG-ATT-ATG-AAG-3′ and KTSP61 5′-ATC-CGC-TAA-CAC-ACTCTC-3′. Briefly, for PCR bacterial DNA was firstextracted using Wizard genomic DNA Purification Kit (Promega, USA) according to the instruction ofthe manufacturers to use as PCR template. Extractionof DNA and its quality were checked by running 5 µL suspension of the extracted DNA in a 1% (w/v) agarose gel. All the PCR was done in a final 25 µL volume containing 12.5 µL PCR mastermix (Promega, USA) , 1 µL of each primer (10 pmol), PCR grade water 8.5 µL and DNA template 2 µL. The thermal profile used for the *Pasteurella* genus specific PCR was performed as follows with slightmodification from Townsend *et al.* (1998): initialdenaturation at 94°C for 5 min, followed by 30cycles of denaturant at 94°C for 1 min, annealing at 49°C for 1 min, and elongation at 72°C for 1 min andfinally a final extension at 72°C for 9 min.

The thermal profile used for the *P. multocida* type B specific PCR was also done as per protocol describedby Townsend *et al.* (1998) with slight modification:initial denaturation at 95°C for 5 min, followed by 30cycles of denaturant at 95°C for 1 min, annealing at49°C for 1 min, and elongation at 72°C for 7 min andfinally a final extension at 72°C for 9 min. Followingthe completion of PCR, 5µL PCR products were loaded into 2% agarose gel (w/v) along with 1 µL 6Xloading dye for electrophoresis in 1X TBE buffer at100 V for 35 min. A standard 100 bp DNA ladder (Promega, USA) was also loaded in the same gel tocheck the size of the amplified PCR products. Priorto casting the gel, ethidium bromide (0.5 µg/mL) wasadded to the gel. The PCR products were visualizedunder UV light in an image documentation system (Bio Rad, USA).

Results and Discussion

Haemorrhagic septicemia caused by *P. multocida* is adevastating disease of domestic and wild animals.The disease is often associated with severe economicloss in the livestock industries. HS, together withanthrax and black quarter, are responsible for anestimated economic loss of US$148.6 million each year (Ahmed, 1996; Mondal and Yamage, 2014). Inthis study, *P. multocida* was isolated and identified from suspected cases of HS of cattle and buffalo byconventional bacteriological method. On blood agarmedia, the isolated bacteria produced small, round, whitish colonies with no hemolysis. Gram's stainingrevealed presence of Gram negative small rod shaped bacteria (Figure 1). The isolated organismsfermented dextrose, sucrose and mannitol but notmaltose and lactose. These fermented sugarsproduced acid without gas. The organisms also gavepositive indole test and negative methyl red (MR),voges-proskauer (VP) test. All these findings aresimilar to those reported by Cheesbrough (2006) asspecific for *Pasteurella*spp.

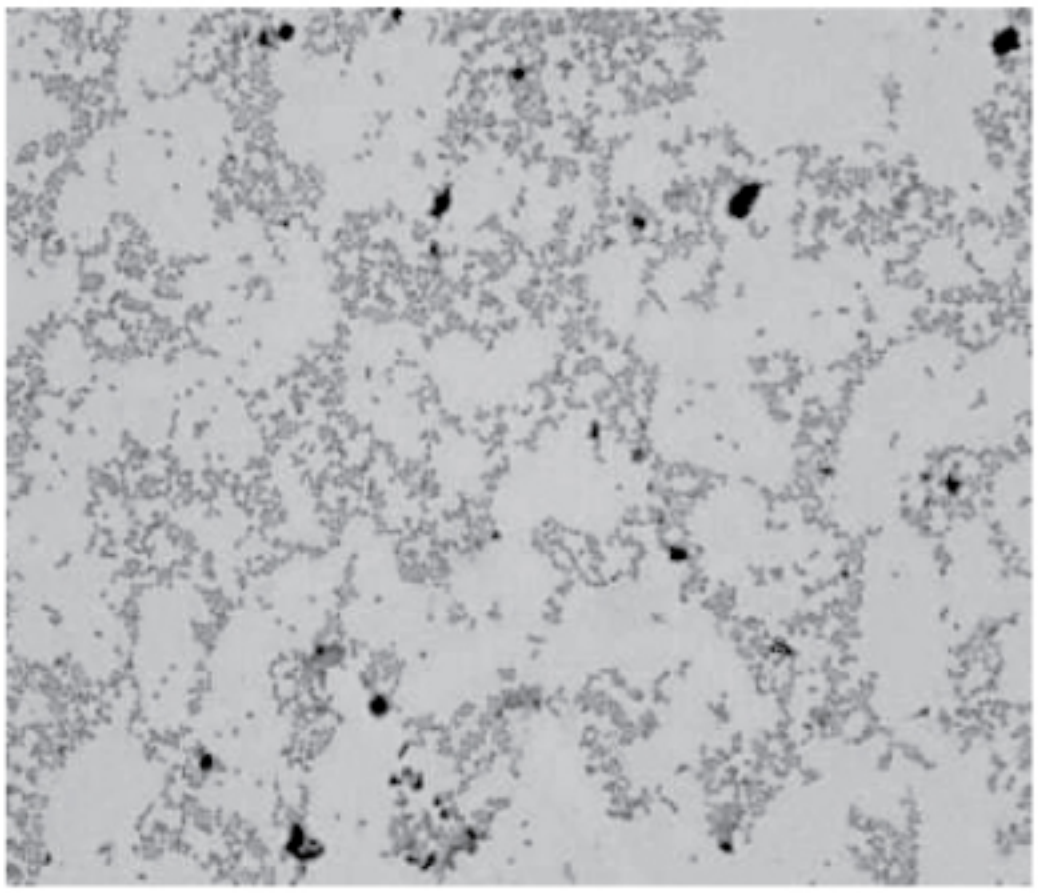

Figure 1. Gram's staining showing small Gramnegative rod bacteria (100X)

In the past, several studies have been carried out inBangladesh on the isolation and identification of *P. multocida* by conventional microbiological methods from filed cases, vaccine development and its efficacy determination (Choudhury *et al*., 1985; Hafez, 2012). However, as far as we know, none of these studies were carried out at molecular level in Bangladesh. Tothe best of our knowledge, this is probably the firststudy in Bangladesh describing the PCR baseddetection of *P. multocida* from suspected clinical cases of HS in Bangladesh.The isolated organisms were found Gram negativeand morphologically they were coccobacillary in shape. On Blood agar the isolated organismsproduced whitish, opaque, circular, translucent colonies and with no hemolysis that resembles thecharacteristics colonies of *P. multocida*, as described by Choudhury *et al*. (1985), Cheesbrough (2006) and Rahman *et al*. (2016). Biochemically the isolatedorganisms were found positive for oxidase, catalase, indole tests, negative from MR and VP test. Theorganisms were found positive for sucrose, dextrose, mannitol and negative for lactose and maltose.Results of these biochemical tests suggested that theisolated organisms could be considered as *P. multocida*. Shivachandra *et al*. (2011) also reportedsimilar biochemical characteristics for *P. multocida* type B.

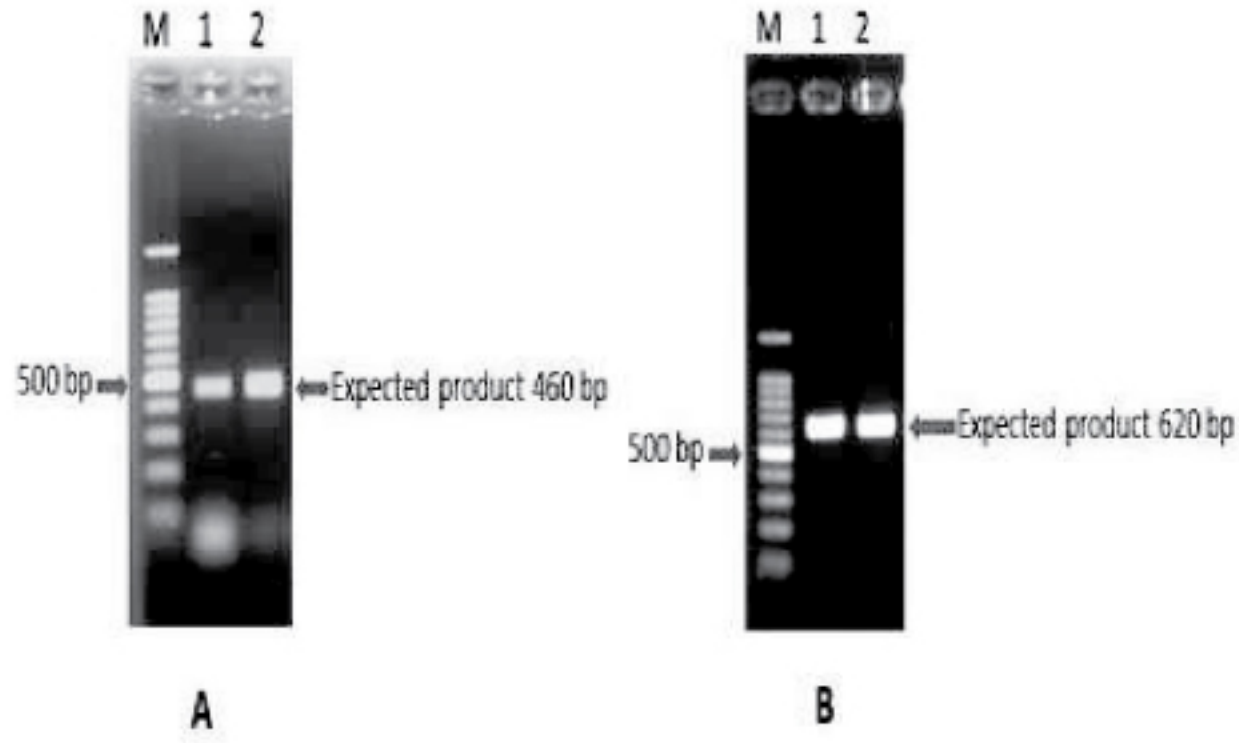

Figure 2. PCR amplification of *Pasteurella multocida*. A. Amplification using the primers KMT1SP6 and KMT1T7 specific for the genus *Pasteurella*. B. Amplification using the primersKTSP61 and KTT72specific for the *Pasteurella multocida*. Lane 1= Isolatefrom cattle, Lane 2= Isolate from buffalo, M=100 bp DNA marker

PCR is a rapid, robust and highly specific moleculartechnique for confirmatory detection of many speciesof bacteria including *P. multocida* (OIE Manual,2008).The development of molecular tools has pavedthe way for rapid and specific identification ofinfectious agents, thus PCR was used foridentification of *P. multocida.* During the presentstudy, both the buffalo and cattle isolates of *P. multocida* exhibited species specific amplification ofapproximately 460-bp size using primers KMT1SP6and KMT1T7 (Townsend *et al*., 1998). Thesefindings confirmed the results obtained by Townsend*et al*. (2000), Javia (2004) and Patel (2004), whoreported the specificity of this primer pair for all *P.multocida* isolates. Townsend *et al.* (1998) reportedthat the primer pairs gave amplification from allstrains of *P. multocida* (all serotypes and capsular serogroups), the three subspecies *i.e.*, *P. multocida* subsp. *multocida*, *P. multocida* subspp. *gallicida*, *P.multocida* subspp. *septica* and also *P. canis*biotype2. Dutta*et al*. (2001) also carried out PM-PCR usingvarious serotypes of *P. multocida*.

Presumptive identification of HS caused by type-Bspecific *P. multocida*is also possible by PCR amplification (Townsend *et al*., 1998). To date, *P. multocida* type-B-specific PCR performed by Townsend *et al*. (1998) remains 100% specific forthe detection of this organism. In the present study *P. multocida* type B cultures both from buffalo andcattle with the predominant somatic antigen beingeither type 2 or 5 are identified by the amplificationof a 620-bp fragment with the KTSP61 and KTT72primers. Results of the PCR are presented in Figure2. A standard PCR protocol which is slightlymodified from Townsend *et al.* (1998) successfullywas optimized to detect *P. multocida* type B circulating in Bangladesh.

Conclusion

P. multocida has been isolated from the field cases of haemorrhagic septicemia of cattle and buffalo inBangladesh. Besides, molecular identification of the isolated organism as *P. multocida* type B is confirmed by PCR. The isolate can be used ineffective vaccine development.

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Environmental contamination of lead in dairy feed and products in Narayangonj district of Bangladesh

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Abstract

Narayangonj district is heavily exposed to environmental pollution due to over population, rapid industrialization and urbanization during the last few decades. The present work assessed the level of environmental contamination of lead (Pb) in dairy feed and products at Narayangonj district of Bangladesh. Soil, water, forage and milk samples were collected from 5 upazilas (Narayangonj Sadar, Bondor, Rupgonj, Araihazar and Sonargoan) of Narayangonj district. The samples were digested by acid digestion procedure. Pb concentration in the samples were detected byFlame ASS and GF-AAS. The concentration of Pb varied widely in different samples. The highest concentration was detected in soil samples; 26.667, 33.333, 31.667, 38.333, 26.667 µg/kg for Narayangonj Sadar, Bondor, Rupgonj, Araihazar and Sonargoan respectively.In water samples, the concentration of Pb was 0.023, 0.058, 0.058, 0.023, 0.017 µg/L respectively. The concentration of Pb in forage samples was below detection limit (BDL), 0.017, 0.033, 0.217, BDL µg/kg respectively and in milk samples, the concentration was 0.068, 0.068, 0.067, 0.043, 0.038 µg/L respectively. Although no pathway correlations of Pb concentration could be established between different samples, concern should be made regarding environmental contamination of Pb in dairyfeed and products and measures should be taken to cease the residue in human food chain.

Keywords: Lead, Water, Soil, Forage, Milk, Feed

Introduction

Environmental pollution with heavy metals and metalloids is now being considered as a major problem in developed, developing and undeveloped countries (Islam *et al.,* 2014, 2015a). The term "heavy metals" refers to any metallic element that has a relatively high density, greater than 4 g/cm3 and is toxic or poisonous even at low concentration (Nriagu, 1989; Lenntech, 2004). Elements from natural and anthropogenic sources continuously enter the ecosystems where they pose serious threat because of their toxicity, long persistence, bioaccumulation and bio-magnification in the food chain (Alturiqi and Albedair, 2012). Elevated concentration of metals and metalloids in the environment is an ultimate result from waste disposal, smelter stacks, industrial discharge, fertilizer, tannery waste, manure, pesticides, fossil fuel, municipal waste, mining waste, ship breaking yards waste, animal waste, contaminated water and sewage sludge in the arable land (Hossain, 2010; Pandey *et al.,* 2012).

Pb is a common cause of poisoning of humans and domestic animals throughout the world. Pb and Pb-containing compounds are commonly used in Pb-acid storage batteries, sheet metal, piping, ammunition and paints. Pb exposure can occur via ingestion, inhalation and dermal contact. The most common route of exposure of the general population to Pb is the ingestion of contaminated water and food. In workplace, the most significant route of Pb exposure is inhalation (Wexler and Shayne, 1998b).

Livestock may find Pb in rubbish dumps and around farm buildings and machinery. Other sources of Pb include paint, linoleum, grease, Pb weights, Pb shot, and contaminated foliage growing near smelters or along

roadsides (Siddiqui & Rajurkar, 2008).The World Health Organization (WHO) concluded that even low levels of As, Pb, and Cd can cause diseases in humans (WHO 2001). Consumption of water contaminated with high levels of heavy metals (e.g., Hg, Pb, and Cd) can lead to cancer or damage to the central nervous system and kidney (WHO, 1996). The functionality of the thyroid and the adrenal glands is also affected by Pb. Reproductive effects, impairment of the immune system, miscarriage, male infertility and neonatal morbidity and mortality may also be seen (Wexler and Shayne, 1998b).In animals, Pb poisoning causes colic, staggering gait, rolling eyes, muscle spasms, blindness, uncoordinated attempts to climb obstacles, excessive response to external stimuli, head pressing and convulsions, abdominal pain, diarrhoea and finally death (Siddiqui & Rajurkar, 2008).

Pbfrom industrial wastages may retain in soil or leach out through the soil and may contaminate ground water along with the soil itself and finally enter the food chain and causes health hazards in human and animals. However, there are not available published reports for environmental contamination of Pb in animal feeds and products at Narayangonj district in Bangladesh. So the present study was conducted to investigate the level of Pb in soils, water, forage (environmental samples) and in milk (animal product) at Narayangonj district of Bangladesh, which is one of the largest industrialized area of Bangladesh. The objective of this study was also to explore a forward linkage how environmental Pb is leaching into human food chain through food products of animal origin such as milk.

Materials and Methods

Selection of the study area

The study was operated in order to detect the contamination of Pb in certain selected areas of Bangladesh. Five different upazilas of Narayangonj district were selected as site for collection of samples in order to estimate of the concentration of Pb in the environment and animal products. The samples were collected from industrial areas of Narayangonj Sadar, Bondor, Rupgonj, Araihazar and Sonargoan upazila of Narayangonj district. A total of 30 samples were collected from each upazila and finally, a total of 150 samples were obtained from those 5 upazilas.

Collection and Preparation of Samples

The Samples (water, soil, forage, milk) were collected from the selected Upazilas from February to March 2016 which wereexamined for Pb contamination in the Department of Pharmacology, Faculty of Veterinary Science and Department of Agricultural Chemistry, Faculty of Agriculture, Bangladesh Agricultural University.

Water Samples

A total of 25 industrial waste water samples from marshy land, lakes, and canals connected to the industrial effluents were collected in acid washed sterile screw cap vials from each selected area. The vials were filled with industrial waste water upto 100ml carefully and recap the test tube securely.0.5ml nitric acid was previously added in vials prior to fill up to 100ml. The vials were also marked by identifying marks by permanent marker pen. The samples were sent to the laboratory as soon as possible in unchanged condition.

Soil Samples

25 soil samples were collected in zip lock plastic bags (500gm) at depth of 0-15 cm from each upazila and labeled the samples location with permanent marker pen. The soil samples were air dried at room temperature, finely powdered, and sieved through a 2-mm nylon mesh to remove large debris, stones, and pebbles. Then the samples (500 g of soil) were dried at 70°C for 2 days to remove all the moisture content. The lower particle size fraction was homogenized by grinding in a mortar and pestles and stored in plastic container for chemical analyses. The dried samples were wet digested.

Forage Samples

Natural grasses (*Cylodon dactylion*), about 500gm were collected at random from the fields of the sampling sites by hand using vinyl gloves, carefully packed into zip lock bags, identified by permanent marker and the whole plant body was brought to the laboratory. The cleaning (removal of soil) of plant samples was

performed by shaking and by means of a dry pre-cleaned vinyl brush. Then the whole plant bodies were divided into different parts. The samples were washed with tap water several times and subsequently dipped in 0.01 N HCl acid for 5 min followed by thorough washing in distilled and de-ionizedwater to remove airborne pollutants. Then the samples were cut into 2-cm pieces and dried in a hot air oven at 70–80°C till the constant weight was achieved. The dried samples were ground in a stainless steel blender and then passed through a 2-mm-size sieve.

Milk Samples

Raw milk samples were collected from 25 dairy farms, situated near or around industrial area or waste disposal area. Milk samples (100 ml) were collected from cow farms during the morning milking directly into in nitric acid-washed glass vials and 0.5% of nitric acid was added to preserve its oxidation status. The vials containing the collected samples were also marked by identifying marks by permanent marker pen. The samples were brought in ice packs for further processing in the laboratory, where it was kept in -20°C till use.

Digestion of Samples

Soil Samples

One gram each of soil samples were digested (wet acid digestion) with 10 mL of di-acid mixture (concentrated nitric acid and perchloric acid in 2:1) and 2ml sulphuric acid at 120°C until a transparent solution was obtained. Digestion system 6 1007 Digester used for digestion of samples. Final volume of digested samples was 3 ml. The digested samples of soil were filtered through Whatman no. 42 filter paper (pore size 25 µm) and the filtrates were diluted to 97 mL with distilled and deionized water. Final solution was made 100 ml. Then the solution transferred in plastic container marked by identifying marks by permanent marker pen and stored in 4°C for further analysis.

Water Samples

Fifty ml of mixed effluent-contaminated water sample was digested with 10 mL of di-acid mixture (concentrated HNO_3 and $HCLO_4$ 2:1) at 120°C until the solution became transparent and volume reduced at one-third (20 ml) of total solution. Digestion system 6 1007 Digester used for digestion of samples. Digestion and filtration procedure was same as explained with soil sample. Final solution was made 50 ml. The sample we stored in 4°C for further analysis.

Forage Samples

One gram each of forage samples were digested (wet acid digestion) with 10 mL of di-acid mixture (concentrated HNO_3, and $HCIO_4$ in 2:1) at 120°C until a transparent solution was obtained. Digestion system 6 1007 Digester used for digestion of samples. Digestion and filtration procedure was same as explained with soil sample. Final solution was made 100 ml. The sample was stored in 4°C for further analysis.

Milk Samples

Ten milliliters of milk sample was digested with 10 mL of di-acid mixture (concentrated HNO_3 and $HCLO_4$ 2:1) at 120°C until the solution became transparent and volume reached at 2 ml of total solution. Digestion system 6 1007 Digester used for digestion of samples. Digestion and filtration procedure was same as explained with soil sample. Final solution was made 100 ml. The sample was stored in 4°C for further analysis.

Analysis of Heavy Metals

Samples were analyzed using a Graphite Furnace Atomic Absorption Spectrophotometer (Model: SHIMADZU, GFA-7000, Japan). Mono element hollow cathode lamp was employed for the determination of Pb. At first, the Atomic Absorption Spectrophotometer was calibrated following the manufacturer's recommendation. Magnesium nitrate and Palladium nitrate was used as matrix modifier to prevent evaporation loss of metals during analysis. Exactly 1.45 ml of filtered samples and 0.29 ml of matrix modifier was taken in a tube and run for the determination Pb in the samples and then data for each element was recorded.

Data Processing
After the analysis of Pb, the all recorded data were collected and processed using spread sheet software.

Results& Discussion

Pb in Soil Samples
Pb concentration found variable in different upazilas (Table 1). The lowest concentration was recorded in samples collected from Sadar and the highest concentration was recorded in Araihazar. The concentrations of Pb in the study areas were lower than maximum acceptable concentration of 50 µg g-1 for crop production (Kabata & Pendias 1992). In the present study, the concentrations of Pb in soil samples were less than the others data collected from the different countries; Iran, 22.2 mg/kg (Mirzaei R *et al.,* 2015) and China, 448.73 mg/kg (Yekeen TA *et al.,* 2016). This variation of result might probably be due to the variations of Pb concentrations in irrigation water and other agronomic practices of the respective area. The low concentration of Pb in the soil may be ascribed to its continuous removal by vegetables grown in the designated areas.

Pb in Water Samples
The concentration of Pb in water samples has been shown in Table 1. The lowest concentration was recorded in samples collected from Sadar and the highest concentration was recorded in Bondor and Rupgonj. Considering the reference health standard (mgL^{-1}) by WHO (2008), the permissible concentration of Pb in water samples is $0.05mgL^{-1}$. But in our present study the concentration of Pb was lower than the recommended values by WHO (2008). The study represents the lower concentration of Pb in water sample than the others data collected from the different countries; Pakistan, 16.0 µg/L (Nawab *et al*., 2016). There are many reasons to differ in Pb concentration in water samples. Season is most important factors. During dry seasons Pb present in high concentration in environment comparatively the rainy seasons. Other factors such as location of sample collection, distance of location from contaminated sources such as industrial waste, road sides' wastes, and battery factories etc. may play an important role in contamination of environment with Pb.

Pb in Forage Samples
The lowest concentration was recorded in samples collected from Sonargoan and the highest concentration was recorded in Rupgonj (Table 1). It is very difficult to discuss about Pb in forage specially *Cynodon dactylon.* Because of data are not available for discussion about *Cynodon dactylon.*But many data are available about heavy metal contamination in vegetables from industrial wastages. Data collected from different countries; Italy, 0. 294mg/kg (Beccaloni *et al.,* 2012), and Nigeria, 0.25 to 4.56mg/kg (Akan *et al.,* 2013) denotes higher concentration of Pb in different vegetables than the measured Pb concentration in *Cynodon dactylon*of our present study. Accumulation pattern of Pb in forages may be varied due to seasonal varriation, varriation in accumulation pattern, species variation, and distances of samples from contaminated area etc.

Pb in Milk Samples
The lowest concentration was recorded in samples collected from Sonargoan and the highest concentration was recorded in Narayangonj Bondor (Table 1).The concentrations of Pb in cow milk in this study were lower than the authorized limit set by FAO/WHO standards of 20 ng g−1 (Codex Alimentarius Commission 2011). In the present study, the concentrations of Pb in cow milk samples were less than the others data collected from the different countries; Pakistan, 0.014 mg/kg (Ismail *et al.,* 2015a), and Croatia, 10.8-12.2 µg/kg (Bilandzic N *et al.,* 2016). This variation of result might probably be due to different Pb concentrations in feed given to cattle, as affected by natural or industrial contamination. Although Pb plasma or tissue levels could be high and cows could show clinical signs of Pb toxicity, ingested Pb is poorly (0.0003%) transferred to milk (Antoniou *et al.,* 1989).

Table 1: Concentrations of Pb (µg/kg or µg/L) in different samples in five upazilas

Samples	Concentrations of Pb (µg/kg or µg/L) in different upazila				
	Sadar	**Bondor**	**Rupgonj**	**Arihazar**	**Sonargaon**
Soil	25.96± 3.31	35.75± 3.47	31.90± 2.02	38.22± 1.07	26.65± 2.49
Water	0.0228± 0.00376	0.059± 0.00495	0.0598± 0.01075	0.0256± 0.00622	0.0246± 0.0047
Forage	0.022± 0.123	0.0176± 0.0035	0.0346± 0.0020	0.0229± 0.0066	0.0114± 0.0087
Milk	0.0628± 0.007	0.0687± 0.0092	0.066± 0.012	0.0412± 0.008	0.0374± 0.0113

Correlation of lead concentration in soil, water, forage and milk

Pearson's correlation coefficient was calculated to establish any relationship between samples collected from Narayongong district of Bangladesh(Table 2). The hypothesis is if higher concentration of Pb is found in soil and water, it could be transferred into forage and milk. However, no relationship could be established as statistically significant. However, the highest concentration of Pb was observed in soil followed by water and forage samples. It might be due to variation in sources of sample. Though measures was taken to collect all the samples from the vicinity of industrial area, the concentration of each sample doesn't correlate.

Table 2: Correlation of lead concentration in soil, water, forage and milk

Water **r-value (V) p-value**	**Forage** **r-value (V) p-value**	**Milk** **r-value (V) p-value**
-0.08 (S/W) 0.89	-0.52366(S/F) 0.36	-0.70903(S/M) 0.17
	0.477721(W/F) 0.416	0.719044(W/M) 0.17
		0.501773(F/M) 0.38

r= correlation co-efficient, V=variables, S=soil, W=water, F=forage, M=milk

Conclusion

The results of the present study show analysis of Pb concentrations in soil, water, forage and milk samples in and around industrial areas at Narayangonj district of Bangladesh. All the samples was positive for Pb but lower than recommended tolerable levels proposed by Joint FAO/WHO Expert Committee on Food Additives and other authors.

These observations indicate the future threat of Pb accumulation in the environment, and therefore, adequate and effective measures should be taken to cease Pb residue in dairy feed and products, and to protect the ecology and environment as a whole.

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IN-VITRO EFFICACY OF VERENDA (*Ricinus communis*) LEAVES EXTRACT AGAINST TICKS IN CATTLE

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Abstract:

Tick infestation is commonly found in every commercial cattle farm and domestically reared cattle. Farm generally used acaricides to treat tick infestation; however finding new, cheap and alternative source of acaricides is a prime concern. Here, we investigated the *in-vitro* efficacy of Verenda (*Ricinus communis*) leaves extracts to treat tick infestation. We prepared aqueous, methanolic and ethanolic extract of Verenda leaves to apply on ticks. A total of 90 ticks (both hard ticks and soft ticks) was collected from cattle in local area of Mymensingh region and divided into 3 treatment groups: A (Aqueous), B (Ethanol), C (Methanol) and D (Control). All groups were sub-divided into 3 sub-groups on the basis of concentration of 1%, 2% and 3% treatment. Ticks were treated with different concentration of extract and observation of tick was performed 12, 24 and 36 hours interval. The whole experiment was repeated twice. Our data suggests 3% methanolic extract confer highest efficacy against ticks and Verenda could be used alternatively as acaricides.

Keywords: *Ricinus communis*, Acaricidal activities, Tick, Cattle

Introduction

The livestock sector of Bangladesh that contributes 15% of GDP is facing a number of obstacles including ectoparasite infestation. Among ectoparasites, Tick (*Boophilus microplus*) are most common in Bangladesh including Mymensingh. Ticks and Ticks born disease causes severe economic loss in this sector leading to retarded growth, loss of weight, decreased milk and meat production and vector to other pathogens. Chemical acaricides, vaccination, biological control by pathogens or predators, pheromone- assisted control, host resistance and botanical acaricides are the various approaches for the controlling of ticks (Benelli et al. 2016). But there is need to search for alternative control methods with the principles of sustainable agriculture as recent report says about the development of resistance against several acaricides (Singh et al. 2014a; Jyoti et al. 2015). Suitable alternative for synthetic acaricides is botanical acaricides with low toxicity and rapid biodegradation as well as prevention of development of resistance against active substances due to various mechanisms of action (Singh et al. 2015a). *Ricinus communis* is a plant that grows in tropics and warm temperature regions of the world. It possesses many medicinal activities including acaricidal effect. The acaricidal effect has also been studied in many parts of the world such as in Bangalore, California, Egypt (Adbel-Shafy and Zayed, 2002), Mexico (Muro et al., 2003), Australia (Fletcher, 1999), Namibia (Kayaa, 2000). But the acaricidal effects of Verenda plant has not yet been studied in Bangladesh. From this point of view, this research work has been designed to develop a simple protocol for in vitro screening of verenda (*Ricinus communis*) leaves having acaricidal efficacy.

Materials and Methods

Study area

The experimental study was conducted in the ADM-2 laboratory of department of pharmacology, Bangladesh Agricultural University.

Study design

The experimental study was conducted from January 2016 to June 2016 to evaluate in-vitro efficacy of

verenda (*Ricinus communis*) leaves extract against ticks.

Study plant collection and identification

Studied plants *Ricinus communis* (verenda) was collected from the field of Isa khan Hall nearest Karimbhaban, surrounding area of Bangladesh Agricultural University. Plant materials were selected on the basis of reports against various ectoparasites (Bekele et al., 2012). Five plants were identified and verified according to its physical characteristics.

Plant preparation and extraction

Plant extraction of *R. communis* leaf was conducted at the laboratory of pharmacology Department (Bangladesh Agricultural University) by the help of Soxhlet (Kumar et al., 2013) 12 cycles or till the solvent in the siphon tube of an extractor became colorless (Kumar et al., 2011). After washing the plant leaves were cut into small pieces, spread out on paper sheets, dried in shaded area at room temperature for one weeks and finely dried plant materials was crushed using a pestle and mortar to provide a greater surface area. Following this, the solvent (80 ml of ethanol, methanol or water) was added to a round bottom flask, which was attached to a Soxhlet extractor. The crushed plant materials were loaded into the thimble and placed inside the Soxhlet extractor. The condensate was dripped into the reservoir. The process runs for a total of 16 hours. Once the process had finished, extracted plant material were in the beaker.

Preparation of stock solution

Each of the samples was then categorized as 1, 2 and 3 percent concentration according to petri dish size and number of ticks. We measured 0.15gm extract for 1%, 0.3gm extract for 2% and 0.45gm extract for 3% concentration by electric balance and added with 15 ml of distilled water then mixed by the help of orbital shaker.

Study parasites collection, transportation and identification

Ticks were collected from naturally infested cattle grazed at the field of Bangladesh Agricultural University. Freshly collected parasites were transported in plastic cups covered by cotton net gauze to the Pharmacology Department laboratory BAU, Mymensingh. Species identification was done based on morphological identification keys given by (James, 2013). Collected ticks were put in vials and the vials were wrapped in cotton net gauze and transported and identify in Bangladesh Agricultural University, Parasitology laboratory. Ticks from naturally infested cattle, were identified according to (Walker et al., 2007).

In vitro acaricidal efficacy test

Three replicates for each concentration of 10 adult ticks were dipped in the respective dilutions of extracts and control solutions for 2 min of exposure. After immersion, the ticks were recovered from tube and filtered with filter paper and placed in separate Petri dishes (Zaman et al., 2012). Fifteen ml of concentration was taken for each petridish and for each concentration of methanolic, ethanolic and aqueous extract i.e. for 1,2and 3% concentration. Ten adult ticks for each petri dish was allowed to escape out. Cotton was soaked into each concentration and applied to the petri-dish to prevent desiccation. In case of positive control we used 1% Ivermectin pour on and distilled water for negative control. After incubation in an incubator at 30°C with 80% relative humidity and death of ticks were recorded after 12hr and 24hr interval. The viability of ticks was checked regularly with a blunt needle and ticks were recorded as dead if no reaction was shown. All the experiment was then repeated thrice.

The percentage mortality was calculated by the formula previously used by Krishnaveni and Venkatalakshmi (2014) as follows:

$$\text{Mortality\%} = \frac{\text{No dead ticks}}{\text{Total number of ticks}}$$

Statistical analysis

Collected raw data were stored in Microsoft Excel database system used for data management. SPSS windows version IBM 20 was used for data analysis. Results of the study were expressed as a mean of mortality percentage ± standard error (Mean ± SE). Statistical significance was determined by one way analysis of variance (ANOVA) with multiple comparison tests (Post Hoc/Turkey's test/HSD) to compare parameter within and between groups. All significant levels set at $P<0.05$.

Results and Discussion

*D*uring the present study in vitro acaricidal effect of *Ricinus communis* was studied in different preparation (aqueous ethanol and methanolic extract at various concentrations 1%, 2% and 3%) and in different time intervals. The result of different applications is described under the following Tables. Tick status regarding time elapse, solvent types and concentrations is shown in Table 1 and Table 3 and statistical analysis is shown in Table 2 and Table 4. Comparison of efficacy of different extracts is shown in Figure 1.

In Positive control group, all ticks are died within 2 hours of Ivermectin treatment whereas in Negative control group, not a single tick was died even in fourth day of application.

Table 1. Tick status regarding time elapse, solvent types and concentrations

Time elapsed (hr.)	Status of Tick for		Concentration		
			1 %	2 %	3 %
12	Aqueous	Dead Count	1[a]	5[a]	4[a]
		Percent (%)	3.3	16.7	13.3
	Ethanolic	Dead Count	15[a]	17[a]	16[a]
		Percent (%)	50	56.7	53.3
	Methanolic	Dead Count	16[a]	20[a]	20[a]
		Percent (%)	53.3	66.7	66.6
24	Aqueous	Dead Count	10[a]	17[a]	14[a]
		Percent (%)	33.3	56.7	46.7
	Ethanolic	Dead Count	18[a]	22[a]	22[a]
		Percent (%)	60	73.3	73.3
	Methanolic	Dead Count	21[a]	24[a]	27[a]
		Percent (%)	70	80	90
36	Aqueous	Dead Count	20[a]	19[a]	27
		Percent (%)	66.7	63.3	90
	Ethanolic	Dead Count	23[a]	28[a]	27[a]
		Percent (%)	76.7	93.3	90
	Methanolic	Dead Count	27[a]	28[a]	30[a]
		Percent (%)	90	93.3	100

*[a] No significant variation among each concentration group

Table 2. Chi square (Pearson Chi-Square) value among Extract concentration

Time elapsed (hr)	Concentration	Value	P value
12	1%	20.463[b]	.000
	2%	16.875[c]	.000
	3%	18.720[d]	.000
24	1%	8.690[b]	.013
	2%	4.126[c]	.127
	3%	13.650[c]	.001
36	1%	4.757[b]	.093
	2%	12.96[c]	.002
	3%	3.214[d]	.200

Table 3. Tick status and comparison among different preparation in Different intervals with Cross tabulation

Time elapsed (hr)	Status of Tick for		Concentration Aqueous	Ethanolic	Methanolic
12	1%	Dead Count	1a	15a	16a
		Percent (%)	3.3	50	53.3
	2%	Dead Count	5a	17[b]	20[b]
		Percent (%)	16.7	56.7	66.7
	3%	Dead Count	4[a]	16[b]	20[b]
		Percent (%)	13.3	53.3	66.7
24	1%	Dead Count	10[a]	18[b]	21[b]
		Percent (%)	33.3	60	70
	2%	Dead Count	17[a]	22[a]	24[a]
		Percent (%)	56.7	73.3	80
	3%	Dead Count	14[a]	22[b]	27[b]
		Percent (%)	46.7	73.3	90
36	1%	Dead Count	20[a]	23[a,b]	27[b]
		Percent (%)	66.7	76.7	90
	2%	Dead Count	19[a]	28[b]	28[b]
		Percent (%)	63.3	93.3	93.3
	3%	Dead Count	27[a]	27[a]	30[a]
		Percent (%)	90	90	100

Table 4. Chi square value among the treated Test group

Time elapsed (hr)	Test group	Value	P value
12	Aqueous	2.925[b]	0.231
	Ethanolic	0.267[c]	0.874
	Methanolic	1.512[d]	0.469
24	Aqueous	0.000[b]	1.00
	Ethanolic	0.000[b]	1.00
	Methanolic	0.000[b]	1.00
36	Aqueous	6.477[b]	0.039
	Ethanolic	4.038[c]	0.132
	Methanolic	2.964[d]	0.227

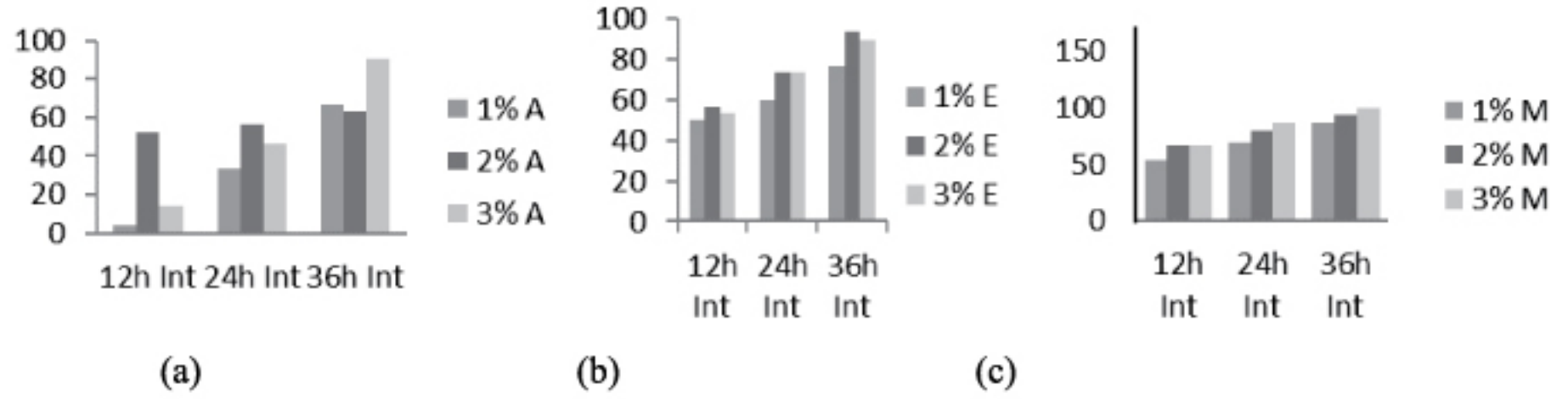

Figure 1: Acaricidal activity of *R.communis* plant extract. a) Efficacy of aqueous extract, b) Efficacy of ethanolic extract, c) Efficacy of methanolic extract. Here, x-axis indicates Percentage of death ticks using extract whereas Y-axis indicates time interval when different concentration of extract has been used.

The present study was aimed to assess the in vitro efficacy of Verenda (*Ricinus communis*) leaves extracts against ticks, Ivermectin was used as a positive control to evaluate the efficacy.

From the results it is revealed that, methanolic extract showed the highest efficacy (100% in 36 hrs. with 3%conc.) followed by the ethanolic (93.33% in 36 hrs. with 2% conc.) and aqueous (90% death in 36 hrs. with 3% conc.) extract. In case of methanolic extract, 3% concentration within 12 hours (66.7) showed a good result and in 24 hours interval with 3% concentration (90% death percentage in 24 hours) showed a better result and in 36 hours interval with 3% concentration (100%) showed the best result. methanolic extract of *R. communis* leaf had strong accaricidal activity (100%) against the tick at 0.45mg/14ml concentration. In line with this finding, Kumar et al. (2013) has reported that similar methanolic extract of the plant has potent activity on *Rh. decoloratus* even at lower concentrations. So we can say among methanolic extracts 3% extract showed perfect result but 2% methanolic extract is cost effective. Whereas Rahman (2002), found that ethanol extract showed higher efficacy against parasites. Probably, most of the active ingredients of these plants are soluble in methanol showing the higher efficacy against adult ticks than the other preparations.
Here, after methanolic extract; 3 % ethanolic extract showed best result (93.3% death in 36 hours) , within 12 hours with 2% conc. showed a good efficacy(53.3%), and in 24 hours again with 2% extract showed higher efficacy(73.3%) and in 36 hours repeatedly 2% extract showed best result again. So we can say 3% extract solution showed best result comparing 1% and 2% extract. This is similar with the ethanolic extracts of Ata (*A. squamosa*) where (Chungsamarnyart et al. 1988) reported that ethanol extract of *A. squamosa* showed high in vitro larvicidal activity (90-100%) against *B. microplus*. After ethanolic extract aqueous extract showed higher efficacy against ticks (90% death in 36 hrs with 3%conc.)

In aqueous extract within 12 hours with 2% conc. showed a very less tick mortality (16.7%) and in 24 hours with 2% extract showed higher tick mortality (56.7%). Highest tick mortality had been found in 36 hours with 3% extract (90%). So, among aqueous extracts 3% extract showed best result even, aqueous extract is cost effective than the others. This concentration of aqueous extract matched with Abdel-Shafy et al., (2002). They studied the effects of Neem seed oil against eggs, nymphs and adult stages of *H. anatolicum excavatum*. They recommended that 1.6% and 3.2% concentration might be used.

It has been reported that the high toxicity of *R. communis* extracts against ticks was due to the presence of ricin in the extracts (Tounou et al., 2011) and ricin is reported as one of the most poisonous natural compounds (El-Nikhely et al., 2007).

Same tick mortality that was obtained by positive control group (Ivermectin) might be possible with *Ricinus communis* leaves extract, although there is great variation in time between them, where only 2 hours is needed for 100% tick mortality by Ivermectin, *Ricinus communis* needs 36 hours. This is our limitation, but we are optimistic for its cost effectiveness and scarcity of drugs (acaricides), especially in rural areas where verenda (*Ricinus communis*) is available.

Conclusion

In conclusion, the acaricidal effect of Verenda (*Ricinus communis*) was studied preparing their aqueous and ethanol extract and methanolic extracts by giving in vitro trial on adult ticks. In this case only the adult parasites were used. So, further study should be conducted to determine the acaricidal effects of Verenda (*Ricinus communis*) against other parasites and also against their various developmental stages (e.g. egg, larvae or L3) with assessment of environmental fate, species toxicity and skin toxicity of verenda plants species before they can be considered as a treatment against ticks. Besides that in vivo trial should be given in field condition and the acaricidal effects of Verenda (*Ricinus communis*) should be tested.

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Retrospective study of surgical cases of ruminants reported at Veterinary Teaching Hospital, Bangladesh Agricultural University

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Abstract:

A retrospective survey was designed to determine the prevalence of surgical cases at Veterinary Teaching Hospital (VTH), Bangladesh Agricultural University from June 2014 to June 2017. 1042 surgical cases of food animal (564 for large ruminants and 493 for small ruminants), 26 for non-food animal (monogastric animal) were recorded from patient registered book and case recording card. Data were analyzed by Epi Info software and frequencies were calculated for different variables usingStatistical Package for Social Science (SPSS) software.In total, 564 (54.12%) animal reported were cattle. Hernia (16.13%) ranked top (90% umbilical & 10% lateral) followed by fracture (14.89%), abscess (14.54%), umbilical myasis (10.46%), atresia ani(5.85%) and naval ill (4.07%). About 87.41% were crossbred and 12.59% were indigenous cattle. Calf, heifer and adult cattle were 55.32%, 10.29% and 34.39%respectively whereas 43.26% were male and 56.74% were female. At surgical classification 37.56% were reported for general surgery whereas 28.71% for congenital, 19.92% for orthopedics, 11.18% for gynecological and 2.65 % for andrological problems. Sedative (Diazepam) was commonly used for pre-anesthetic medication and xylazine or ketamine was used for induction and maintenance of anesthesia. 2% lidocaine HCl was commonly used for local anesthesia. Around 5% surgical cases were returned for secondary surgery due to complication of initial surgery. In case of small ruminants, 419 (40.21%) were small ruminant the black Bengal breed showed more prevalent than others.Castration [32.94% (138)] was ranked top followed by myasis (10.55%) naval ill (10.31%), abscess (7.44%), dystocia (6.24%), for and urolithiasis (5.49%). Male (60.38%) and female (39.62%) ratio was about 2:1. About 42.50% was attended with general surgery including parasitic cause; 37.23 % with andrological; 13.12% with gynecological and 7.15% for congenital disorders. This study stresses the factors related to surgical cases management at VTH and could help to control surgical related cases in Mymensingh region of Bangladesh.

Introduction

Bangladesh 's economy is vibrant due to sustainable agricultural development and livestock is an integrated part of it (world Bank, 2016) that provides 14.21% in agricultural GDP and 3.40% in national GDP (Bangladesh economic review, 2017). Currentlylivestock population density in Bangladesh is the highest (145/sq. km^2) in compared to that of India (90/sq.Km^2) and Brazil (20/sq.Km^2) (BARC, 2015). In total, the animal population (cattle, buffalo, goat & sheep)is 54.787 million and bird (chicken & duck) is 32.64 million).It is estimated that about 20% of earning population of Bangladesh are directly and 50% indirectly engaged in livestock production (Begum et al, 2011 &karim et al, 2010).

The management practice and geo-climatic condition of Bangladesh favors a lot for occurrence of diseases of animal (Onneshan, 2014). Veterinary teaching hospital (VTH), Bangladesh Agricultural University, Mymensingh- 2202, is an ideal point of contact to study the animal diseases pattern as animal owned by people of surroundings and different complexed surgical cases from neighboringupazilla veterinary hospitals are regularly reported to the VTH every day. Therefore, case record analysis could provide a comprehensive scheme for local animal cases.

A number of retrospective study was reported in different parts of Bangladesh like Clinicalcase analysis reports from VTH , Bangladesh Agricultural University (Das and Hashim, 1996; Samad, 2001; Samad*et*

al., 2002; Ali *et al.*, 2011;Sarker *et al.*, 2013; Sarker*et al.*, 2015;MN Islam et al., 2015), Sylhet Agricultural University Veterinary Hospital,Sylhet ,(Lucky et al.,2016) Haluaghat Upazilla Veterinary Hospital,Mymensingh (Sarker*et al.*, 1999) and Dairy Cooperatives in Pabna district (Pharo,1987), Ulipur Upazilla Veterinary Hospital, Kurigram (Kabir*et al.*, 2010), ChandanaishUpazilla of Chittagong (Pallab*et al.*, 2012) , Patuakhali Science and TechnologyUniversity Veterinary Clinic (Rahman *et al.*, 2012), Hajee Mohammad Danesh Science and Technology University Veterinary Teaching Hospital (juli et al.,2015). Here we present the epidemiologicalsurvey on surgical cases reported at VTH, Bangladesh Agricultural University, Mymensingh- 2202.

The present study was undertaken to determine theprevalence of surgical cases in animal attended at VTH, Bangladesh Agricultural University and to analysis the distribution based on species,breed, age, sex, surgery classification,case type, anesthesia, prognosis study and seasonal variation from July 2014 to Jun 2017.

Materials and Methods

Collection of data

The retrospective survey was designed to determine the prevalence of surgical cases at VTH, Bangladesh Agricultural University from June 2014 to Jun 2017. Data were collected from patient register book and case recording card with authority's permission and recorded 1042 surgical cases of food animal (564 for large ruminant & 493 for small ruminant), 26 for non-food animal (monogastric animal) and 33 for bird.The data were analyzed retrospectively and interpreted to determine the occurrence of diseases; seasonal pattern and distribution of diseases

Statistical analysis

The data were manually patterned for recording errors or missing data and obvious discrepancies. The potential errors were assessed and corrected. Data with apprehensive values were excluded. The data were analyzed by Epi Info software version 7.2.1.0 (CDC, Atlanta) and frequencies were calculated for different variables and Statistical Package for Social Science (SPSS) software 20 version was used for Chi-square test to know the relation among groups in respective surgical cases. We considered three seasons according to Ali et al. (2011) as summer from March to June, rainy from July to October and winter from November to February.

Results & discussion

Breed, age, sex and distribution of surgical cases of large ruminants

The prevalence of surgical cases are shown in Table 1. In total 1042 surgical cases, among them 564 (54.12%) were cattle;16.13% (91) of cattle were reported with hernia (90% umbilical & 10% lateral) followed by fracture (14.89%), abscess (14.54%), umbilical myasis (10.46%), atresia ani(5.85%) and naval ill (4.07%). About 3%-4% cases were others injury, retention of placenta, pyometra, upper patellar fixation. Cases like dystocia, mastitis, gangrene were also reported.

Breed-wise 87.41%(493) were crossbred and 12.59%(71) were indigenous cattle. Age-wise calf [55.32%(312)], heifer [10.29%(58)]and adult cattle [34.39%(194)] were reported. Female [56.74%(320)] was predominant than male[43.26%(244)]. About37.56% of the cases was responded with general surgery whereas 28.71% with congenital, 19.92% withorthopedics, 11.18% withgynecological and 2.65 % withandrologicalproblems.

Temporal distribution of surgical cases are presented at Table 3.Temporal distribution shows that about 15%(85) surgical cases were referred from neighboring upazilla veterinary hospitals of Mymensingh region and 85% (479) admitted to VTH, Bangladesh Agricultural University. During handling of different surgery of cattle sedative (Diazepam) was commonly used for pre anesthetic medication and xylazine or

Ketamine was used for induction and maintenance of anesthesia and 2% lidocaine HCl was commonly used for local anesthesia supplied by VTH, Bangladesh Agricultural University with payment and other appliances (suture, post-operative medication, saline etc.) cost were carried out by owner form outside of VTH. Around 5% surgical cases were returned for secondary surgery due to complication of initial surgery.

Congenital defect is more prominent in crossbred cattle than other species in Bangladesh (Hasan et al.,2015) and different reports stated that umbilical hernia in crossbred male calf was more prevalent in summer (Salim et al.,2015; Rahman et al.,2001)which are echoed in our findings.In our findings the ratio between male and female is 1:1 for atresia ani (6.38%), naval ill (5.85%) and dermoid cyst (1.24%) which contrasted finding with Hossain, 2015 where they reported 5:2, probably both male and females have similar risk factors for these problems.

Reproductive tract problems (both gynecological & obstetrical) were more prominent in crossbred cattle rather than indigenous which completely merged with previous research findings (Sarder*et al.*, 2010; Talukder et al., 2005). The orthopedic surgery ratio in between male and female was 1:1 and more prevalent in summer followed by winter and rainy season which also agreed with the previous research findings (Arju et al., 2014). The relations were significant in between surgical cause and breed (χ^2 = 69.07); surgical cause and sex (χ^2=194.58) and surgical cause and age (χ^2 = 106.38)

At temporal distribution,Sarker*et al.*, (2015); Islam et al., (2015), Samad et al. (2011) reported the highestoccurrence of diseases in cattle in summer (48%) followed by rainy (30%) and inwinter seasons (22%), which contrasted to our observations that highest surgical cases at rainy season 34.38% followed by summer (33.97%) and winter (31.65%). The relation was significant in between surgical cause and season (χ^2 = 106.38)

Table no. 01: Prevalence of surgical case, breed,age& sex wise distribution of large ruminants

Surgical case	**Total case**	**Breed**		**Sex**		**Age**			**Surgery type**
		Cross-bred	**Indi-genous**	**Male**	**female**	**Calf (3 m ≤)**	**Yearling (> 3 & 12m ≤)**	**Adult (≥12 m)**	
Hernia	16.13% (91)	15.25% (86)	0.89% (5)	8.69% (49)	7.44% (42)	15.07% (85)	1.06% (6)	-	
Fracture	14.89%(84)	10.99% (62)	3.90% (22)	7.45% (42)	7.44% (42)	4.60% (26)	0.89% (5)	9.40% (53)	General 37.56%
Abscess	14.54% (82)	12.41% (70)	2.13% (12)	9.04% (51)	5.49% (31)	5.14% (29)	3.01% (17)	6.38% (36)	
Umbilical myasis	10.46%(57)	9.22% (52)	1.24% (7)	2.66% (15)	2.66% (15)	8.86% (50)	1.60% (9)	-	Congenital 28.71%
Atresia ani	6.38% (36)	5.67% (32)	0.71 4)	3.19% (18)	3.19% (18)	6.38% (36)	-	-	Orthopedics
Naval ill	5.85%(33)	5.67% (32)	0.18% (1)	2.84% (16)	3.01% (17)	5.85% (33)	-	-	19.92%
Others CG	4.07%(23)	3.72% (21)	0.35% (2)	1.06% (6)	3.01% (17)	4.07% (23)	-	-	
Injury	4.25%(22)	3.37% (19)	0.53% (3)	1.60% (9)	2.30% (13)	1.42% (8)	1.06% (6)	1.42% (8)	Gynecological 11.18%
ROP	3.37%(19)	3.19% (18)	0.18% (1)	-	3.37% (19)	-	-	3.37% (19)	
Pyometra	3.01%(17)	2.84% (16)	0.18% (1)	-	3.01% (17)	-	-	3.01% (17)	

UPF	2.52%(14)	2.30% (13)	0.18% (1)	2.13% (12)	0.35% (2)	-	-	2.48% (14)	Andrological 2.65%
Dystocia	1.77%(10)	1.77% (10)	-	-	1.77% (10)	-	0.53% (3)	1.24% (7)	
Mastitis	1.77%(10)	1.77% (10)	-	-	1.77% (10)	-	0.35% (2)	1.42% (8)	
Gangrene	1.98%(11)	1.42% (8)	0.53% (3)	0.89% (5)	1.06% (6)	-	0.53% (3)	1.42% (8)	
Dermoid cyst	1.24% (7)	1.06% (6)	0.18% (1)	0.89% (5)	0.35% (2)	1.24% (7)	-	-	
Prolapse	1.77% (10)	1.06% (6)	0.71% (4)	-	1.77% (10)	-	-	1.77% (10)	
ACM	1.06% (6)	1.06% (6)	-	0.35% (2)	0.71% (4)	0.89% (5)	0.18% (1)	-	
Fistula	0.89% (5)	0.8 (5)	-	0.71% (4)	0.18% (1)	-	0.35% (2)	0.53% (3)	
Hump sore	0.53% (3)	0.53% (3)	-	0.53% (3)	-	-	-	0.53% (3)	
Dehorning	0.53% (3)	0.53% (3)	-	0.35% (2)	0.18% (1)	0.53% (3)	-	-	
Others	3.37% (19)	2.66% (15)	0.71% (4)	0.53% (3)	2.84% (16)	1.24% (7)	0.71% (4)	1.42% (8)	
Total		87.41% (493)	12.59% (71)	43.26% (244)	56.74% (320)	55.32% (312)	10.29% (58)	34.39% (194)	

CG=Congenital disorders; ROP= Retention of placenta; UPF= Upper patellar fixation; ACM=Actinomyces

Table 2: Species, breed, age & sex wise distribution of surgical cases in small ruminants

Surgical cause	**species**	**Breed**		**Sex**		**Age**		**Surgical trait**
		Black bengal	**Jamunapari / Native**	**Male**	**Female**	**Kid**	**Adult**	
Castration	caprine	32.46%(136)	0.48%(2)	32.34%(138)	-	29.36%(123)	3.58%(15)	General
Myasis	Caprine (40) Ovine (4)	9.31%(39)	0.24% (1) 0.95%(4)	2.39% (10) 0.24%(1)	7.16%(30) 0.72%(3)	7.16%(30) 0.95%(4)	2.39% (10)	42.50%
Naval ill	caprine	9.07%(38)	0.24% (1)	6.68%(28)	2.63%(11)	1.91%(8)	7.38% (31)	
Abscess	caprine	7.16%(30)	0.24% (1)	2.86%(12)	4.53%(19)	1.67%(7)	5.73%(24)	Andrological 37.23%
Dystocia	caprine	5.73%(24)	0.48% (2)	-	5.97%(26)		5.97%(26)	
SC	caprine	5.25%(22)	0.24% (1)	1.91(8)	3.58%(15)	1.91%(8)	1.67%(7)	
ROP	caprine	4.53%(19)	-	-	4.53%(19)	-	4.53%(19)	
Urolithiasis	caprine	3.58%(15)	-	3.10%(13)	0.48%(2)		3.58%(15)	
Injury	caprine	3.34%(14)	-	1.43%(6)	1.91%(8)	1.19%(5)	2.15%(9)	
Gid disease	caprine	2.63%(11)	0.24% (1)	1.67%(7)	1.19%(5)		2.86%(12)	Gynocological 13.12%
Fracture	caprine	2.86%(12)	-	1.91%(8)	0.95%(4)	2.86%(12)	-	
CG	caprine	2.86%(12)	-	1.19%(5)	1.67%(7)	2.86%(12)	-	
Atresia ani	caprine	1.43%(6)	-	1.19%(5)	0.24% (1)	1.43%(6)		
Uterine prolapse	caprine	1.19%(5)	-	-	1.19%(5)	-	1.19%(5)	Congenital 7.15%
Hernia	caprine	0.95%(4)	-	0.95%(4)	-	0.95%(4)	-	

mastaitis	caprine	0.95%(4)	-	-	0.95%(4)	-	0.95%(4)
Amputation	caprine	0.95%(4)	0.24% (1)	0.72%(3)	0.48%(2)	-	1.19%(5)
Orchitis	caprine	0.72%(3)	-	0.72%(3)	-	-	0.72%(3)
Pyometra	caorine	0.48%(2)	-	-	0.48%(2)	-	0.48%(2)
Others	caprine	1.19%(5)	-	0.48%(2)	0.72%(3)	0.72%(3)	0.48%(2)
	total	96.66%(405)	3.34%(14)	60.38%(253)	39.62%(166)	52.98%(222)	47.01%(197)

*Native = ovine, Jamunapari = caprine; SC= Sub-cutaneous cyst;ROP= Retention of placenta; CG= Congenital problem

Age, sex, breed and case wise distribution of surgical cases of small ruminants

Among 1042 data, 419 (40.21%) were small ruminant (415 for goat&4 for sheep); the Black Bengal breed 405 (96.66%) showed more prevalent than others.Species, breed, age & sex wise distribution of surgical cases in small ruminants is presented in Table 2. Castration [32.94% (138)] was ranked top followed bymyasis (10.55%) naval ill (10.31%), abscess (7.44%),dystocia(6.24%), for and urolithiasis(5.49%). Male(60.38%)and female (39.62%) ratio was about 2:1 where 52.98% (222) were kid and 47.02% (197) was adult. According to surgical case classification 42.50% was attended with general surgery including parasitic cause; 37.23 % withandrological; 13.12% with gynecological and 7.15% for congenital disorders.

Temporal distributions of surgical cases are presented at Table 3. About 3.5%(15) surgical cases were referred from neighboring upazilla veterinary hospitals of Mymensingh region and others admitted to VTH, Bangladesh Agricultural University. During handling of different surgery of goat, 2% lidocaine HCl was commonly used for local anesthesia supplied by VTH, Bangladesh Agricultural University with payment and other appliances (suture, post-operative medication, saline etc) expenses were managed by owner form outside of VTH. Around 2% surgical cases were returned for secondary surgery due to complication of initial surgery.

Castration is the mostly attended surgical intervention which is understandable as most of the farmers want to fetch more income by castrating their male goats because castration help in improvement of meat quality which have a great demand at the local market and its management is easier and it was founded that they preferred to castrate their goat within 12 weeks which fully support the previous research (Khandoker et al. 2011) .The study revealed that she goat were significantly affected with surgical disorder observed whereas male responded as less. Analysis was revealed that myasis was more prevalent in female at rainy season which merges with Juyena et al.(2013). According to Islam et al. (2013); our study also revealed that females were prone to surgical cases and may be the genetic issues, geo-climetic situation and husbandry practices.The relations were significant in between surgical cause and species (χ^2 =34.419) surgical cause and breed (χ^2 = 107.89); surgical cause and sex (χ^2 =213.53) and surgical cause and age (χ^2 = 193.79)

Temporal distribution of surgical cases in large and small ruminants

Temporal distributions of surgical cases were presented in Table 3. Few studies like Sarker*et al.*, (2015);MN Islam et al., (2015) ;Banu*et al.* (2015) , samad et al. (2011) reported the highestoccurrence of diseases in cattle in summer (48%) followed by rainy (30%) and inwinter seasons (22%), which coincided to our observations that highest surgical cases at summer (41.87%) followed by winter (31.31%) and rainy season 26.82%. The relation was significant in between surgical cause and season (χ^2 = 91.051)

Table 3: Temporal distribution of surgical cases of different animals

Season	Cattle	Goat	Non ruminant
Rainy (Jul-Oct)	34.38%	26.82%	11.11%
Winter(Nov-Feb)	33.97%	31.31%	34.71%
Summer (Mar-Jun)	31.65%	41.87%	53.96%

We recommend for undertaking retrospective research focusing on the causes of most predominant surgical cases of animals on regular basis which will help to identify risk factors of diseases. Control measure should be initiated to reduce the burden of these diseases/disorders in the study area. This study generated information which is valuable not only for the clinicians, researchers, animal health practitioner and policy makers.

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Prevalence of infectious and non-infectious diseases in cattle population in Moulvibazar district of Bangladesh

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Abstract
Infectious and non-infectious diseases of cattle cause great economic losses of farmers as well as country every year by reducing growth, production and mortality of cattle population. The objective of this research work was to find out the prevalence of infectious and non-infectious diseases of cattle at Moulvibazar, Sylhet, Bangladesh. A total of 2285 clinical cases were diagnosed at District Veterinary Hospital in Moulvibazar, Bangladesh during January to June, 2016. Disease diagnosis was made on the basis of owner's statement, physical examination, clinical signs, gross pathology, and laboratory procedures. Data were analysed to determine disease prevalence in cattle with related to breed and sex. Diagnosed diseases were categorised as infectious diseases, parasitic disease, digestive disorder, respiratory disorder, reproductive disorder, metabolic disorder and other diseases. According to our result the highest prevalence was shown for parasitic diseases (49.32%) followed by digestive disorder (25.64%), other diseases (10.77%), Infectious Diseases (9.28%), respiratory disorders (2.32%), reproductive disorders (1.88%) and metabolic disorders (0.79%). The prevalence of diseases in male (53.57%) which includes infectious diseases (4.90%), parasitic diseases (30.55%), digestive disorders (12.30%),respiratory disorders (0.79%) and others (5.03%). Local bred of cattle were more susceptible to diseases as prevalence was 57.46% followed by Cross-bred (36.58%) and Red Chittagong (5.96%). The prevalence of diseases in male (53.57%) was higher than in female (46.43%). Our study on prevalence of infectious and non-infectious diseases of cattle in Moulvibazar district of Bangladesh provides necessary information to take proper strategies for control and prevention of diseases.

Keywords: Prevalence, Infectious, Non-infectious, Cattle, Bangladesh

INTRODUCTION
Bangladesh is a densely populated and agriculture based country in the world. Livestock sector plays very important role to fulfil the protein requirement as well promoting financial condition of people. There are about 23.785 million cattle, 1.471 million buffalo, 25.766 million goat, 3.335 million sheep, 268.393 million chicken and 52.240 million duck in Bangladesh (DLS, 2016). Contribution of Livestock in Gross Domestic Product (GDP) 1.66%, GDP growth rate of livestock 3.21% and share of livestock in agricultural GDP 14.21% (DLS, 2016). Malnutrition and diseases are the main cause of weak emaciation and non-satisfactory productive performance of the animals (Badruzzaman et al. 2015). Anthrax, foot and mouth disease, haemorrhagic septicaemia etc. are considered as endemic diseases in Bangladesh (Mondal et al. 2014). Huge economic loss in livestock sector occurs due parasitic infestation of cattle (Sardar et al. 2006). It is reported that reproductive disorders have great impact on economic losses to the dairy farmers in Bangladesh (Talukder et al. 2005). In Bangladesh, traditional management system and feeding habit of animals and geographical location act as favourable causes for incidence of different diseases described by Paul et al.(2011) and Lucky et al.(2016). Besides, variation in the age, breed and sex of animals, seasons and farming system greatly influence the incidence of diseases in livestock animals including cattle described by Alim et al.(2012), Sarker et al.(2011) and Sultana et al.(2008). Impact of climate change plays a major role to influence the disease occurrence in Bangladesh discussed by Chowdhury et al.(2016).

MATERIALS AND METHODS

Study area and study period
The study was carried out at District veterinary hospital, Moulvibazar, Bangladesh to determine the prevalence of infectious and non-infectious diseases in Moulvibazar. A total of 2285 clinical cases of cattle of different breed and sex were diagnosed during the period from January to June of 2016.

2.2 Diagnosis of diseases

The diseases were diagnosed by physical examination, clinical signs, gross pathology, and laboratory procedures. In the procedures of physical examination behaviour, physical condition, pulse, respiration, body temperature, posture, gait, salivation, enlargement of the abdomen, defecation, instability of locomotion of animals were observed by visual examination and were recorded. Disease history was taken into consideration while performing general physical examination. Examination of different parts and structure of the body of sick animals were performed by palpation, percussion, auscultation, needle puncture and movement of the affected animals. Bacterial, viral, and fungal diseases were diagnosed specifically on the basis of specific clinical signs and gross lesions (Jones et al. 1996).Mastitis was diagnosed on the basis of history and physical abnormalities of udder (Radostits et al. 2007). Parasitic infestations were diagnosed by faeces examination under microscope (Soulsby, 1986). Diagnosed diseases were categorised as infectious disease, parasitic disease, digestive disorder, respiratory disorder, metabolic disease and other diseases for statistical analysis.

2.3. Data Analysis

Table-1: Prevalence of diseases and disorders in cattle

Disease categories	Disease/Disorder	Affected Cattle No. (n=2285)	Percent Prevalence	Prevalence (%) by categories
Infectious disease	Foot and mouth disease	140	6.13	9.28(212)
	Black quarter	21	0.92	
	Mastitis	48	2.10	
	Hemorrhagic septicemia	1	0.04	
	Wart	2	0.09	
Parasitic disease	Babesiosis	6	0.26	49.32(1127)
	Anaplasmosis	9	0.39	
	Coccidiosis	26	1.14	
	Fasioliasis	477	20.88	
	Hump sore	41	1.80	
	Balantidiasis	23	1.00	
	Strongyloidiasis	58	2.54	
	Paramphistomiasis	194	8.49	
	Ectoparasite	293	12.82	
Digestive disorder	Non-specific diarrhea	175	7.66	25.64(586)
	Anorexia	110	4.81	
	Simple indigestion	88	3.85	
	Malnutrition	201	8.79	
	Colic	12	0.53	
Respiratory disorder	Pneumonia	50	2.19	2.32(53)
	Nasal granuloma	3	0.13	
Metabolic disorder	Milk fever	18	0.79	0.79(18)
Reproductive disorder	Placental retention	16	0.70	1.88(43)
	Uterine prolapse	4	0.17	
	Dystocia	8	0.35	
	Anestrus	15	0.66	
Others	Dog Bite	10	0.44	10.77(246)
	Arthritis	24	1.05	
	Fracture	13	0.57	
	Lacrimation	3	0.13	
	Non-specific fever	108	4.73	
	Wound	88	3.85	
Total		2285	100	100(2285)

RESULTS AND DISCUSSION

A total of 2285 different clinical cases were diagnosed and registered at District Veterinary Hospital, Moulvibazar during the study period of January to June, 2016. The diseases were categorized as infectious disease, parasitic disease, digestive disorder, respiratory disorder, reproductive disorder, metabolic disorder and other diseases. The highest prevalence was shown for parasitic diseases (49.32%) and the lowest prevalence was shown for metabolic disorder (0.79%) (Table-1). Among the other categories, prevalence of digestive disorder (25.64%) was significantly higher. Local breed was more susceptible to diseases compering with cross breed and Red Chittagong (Table-2). Males were more susceptible to diseases than females (table-3).

The prevalence of infectious diseases was 9.28% which consisted of foot and mouth disease (6.13%), black quarter (0.92%), mastitis (2.10%), hemorrhagic septicemia (0.04%) and wart (0.09%) (Table-1). Our result is similar to the result of Badruzzaman et al. (2015) who reported the prevalence of infectious diseases of cattle in Chittagong district of Bangladesh as 9.49%. Among the infectious diseases, highest prevalence was found in foot and mouth disease (6.13%) which was significantly lower than Sarker et al. (2011) who reported the prevalence of foot and mouth disease of cattle in Rajshahi district of Bangladesh as 25.07%. In our study prevalence of mastitis (2.10%) was slightly higher than the report of Hossain et al. (2016) that was 1.15%. The prevalence of infectious diseases were 4.51%, 3.76% and 1.01% respectively in the local, cross and Red Chittagong bred (Table-2). Males (4.90%) were more susceptible to infectious diseases than females (4.38%) (Table-3). Our results oppose to the result of Badruzzaman et al. (2015) who reported female (5.13%) were more susceptible than male (4.36%).

Highest prevalence (49.32%) was recorded in parasitic disease that constituted of babesiosis (0.26%), anaplasmosis (0.39%), coccidiosis (1.14%), fasioliasis (20.88%), hump sore (1.80%), balantidiasis (1.00%), strongyloidiasis (2.54%), paramphistomiasis (8.49%) and ectoparasite (12.82%) (Table-1). Our result of parasitic disease was higher than prevalence recorded (12.4%) in Mohammadpur, Magura (Karim et al. 2014). In parasitic disease category, highest prevalence was recorded in fascioliasis (20.88%) which was lower than the result of Ghosh et al. (2016) who reported as 25.88%. Among protozoan parasites, highest prevalence was found in coccidiosis (1.14%) followed by balantidiasis (1.00%), anaplasmosis (0.39%), babesiosis (0.26%). Mahmud et al. (2015) reported the prevalence of babesiosis in Sirajganj district of Bangladesh as 2.27%. Parasitic diseases were higher prevalent in the local bred (30.15%) than cross bred (16.49%) and Red Chittagong (2.67%) (Table-2). Prevalence of parasitic diseases in males (30.55%) was higher than females (18.77%) (Table-3). Azam et al. (2017) found the prevalence of parasitic diseases in male as 19% and in female as 19.26% at Joypurhat.

Prevalence of digestive disorder was 25.64% of which malnutrition was 8.79% followed by non-specific diarrhea (7.66%), anorexia (4.81%), simple indigestion (3.85%) and colic (0.53%) (Table-1). This result was significantly lower than the result recorded by Pallab et al. (2012) who described the prevalence of digestive disease as 47.05%. In digestive disease category, our result on prevalence of simple indigestion is slightly lower than the report of Hossain et al. (2016) who found the prevalence of simple indigestion as 4.81% in Moulvibazar. Prevalence of digestive disorder was found as 14.14%, 10.11% and 1.40% in local, cross and Red Chittagong bred respectively (Table-2). Digestive disorder was found comparatively higher in females (13.35%) than in males (12.30%) (Table-3). Kabir et al. (2010) also found the higher prevalence of digestive disorder in female than in male in Ulipur upazila, kurigram district, Bangladesh.

The prevalence of respiratory disorder was recorded as 2.32% which comprised of pneumonia (2.19%) and nasal granuloma (0.13%) (Table-1). Our study result on prevalence of respiratory disorder was lower than the prevalence recorded (5.5%) by Rahman et al. (2012). Among the respiratory disorder, we found the prevalence of pneumonia (2.19%) which was slightly lower than Rahman et al. (2012) (5.1%). Prevalence of respiratory disorder was calculated as 1.53%, 0.66% and 0.13% in local, cross and Red Chittagong bred respectively (Table-2). Females (1.53%) were more susceptible than males (0.79%) (Table-3).

Among the total clinical cases lowest prevalence was recorded for metabolic disorder (0.79%) (Table-1) that found only in case of females (Table-3). This result is lower than the record of Ullah et al. (2015) who

mentioned the prevalence of metabolic disorder in Chittagong as 4.24%. Cross bred (0.66%) was more susceptible than Red Chittagong (0.09%) and Local bred (0.04%) (Table-2).

Prevalence of reproductive disorder was 1.88% which included placental retention (0.70%), uterine prolapse (0.17%), dystocia (0.35%) and anestrus (0.66%) (Table-1). Among the reproductive disorder, highest prevalence was found in case of placental retention (0.70%) which was lower than the report of Juli et al. (2015) (0.73%) and Sarder et al. (2015) (4.10%). Prevalence of anestrous (0.66%), dystocia (0.35%) and uterine prolapsed (0.17%) were lower than Maruf et al. (2012) who reported anestrous (5.1%), dystocia (1.0%) and uterine prolapse (0.4%) at Chittagong. The prevalence of reproductive disorder was higher in Local among three breeds. The prevalence reproductive diseases were found as 1.05%, 0.74%, and 0.09% in Local, Cross-bred and Red Chittagong respectively (Table-2). Reproductive disease occurrence was reported only in females (Table-3).

In other diseases category, overall diseases prevalence was 10.77% which consisted of dog bite (0.44%), arthritis (1.05%), fracture (0.57%), lacrimation (0.13%), non-specific fever (4.73%), and wound (3.85%) (Table-1). Studied prevalence of dog bite (0.44%) agrees with the prevalence recorded at Dinajpur district of Bangladesh by Juli et al., (2015). In other category of disease prevalence was 6.04%, 4.16% and 0.57% found in Local, Cross-bred and Red Chittagong cattle. (Table-2). Local bred are mostly allowed for free grazing in the field so that dog bite mostly prevalent in Local bred. In this category female were more susceptible than male as the prevalence in female were 5.73% and prevalence in male were 5.03% were recorded. (Table-3).

Table-2: Breed wise comparison of clinical prevalence of diseases and disorders in male and female cattle

Categories	Local	Percent prevalence	Cross bred	Percent prevalence	Red Chittagong	Percent prevalence
Infectious disease	103	4.51	86	3.76	23	1.01
Parasitic disease	689	30.15	377	16.49	61	2.67
Digestive disorder	323	14.14	231	10.11	32	1.40
Respiratory disorder	35	1.53	15	0.66	3	0.13
Metabolic disorder	1	0.04	15	0.66	2	0.09
Reproductive disorder	24	1.05	17	0.74	2	0.09
Others	138	6.04	95	4.16	13	0.57
Total	1313	57.46	836	36.58	136	5.96

Table-3: Sex wise comparison of clinical prevalence of diseases and disorders in male and female cattle

Sl. no.	Disease category (n=2285)	Male		Female	
		No.	Percentage (%)	No.	Percentage (%)
01	Infectious disease	112	4.90	100	4.38
02	Parasitic disease	698	30.55	429	18.77
03	Digestive disorder	281	12.30	305	13.35

04	Metabolic disorder	0	0	18	0.79
05	Respiratory disorder	18	0.79	35	1.53
06	Reproductive disorder	0	0	43	1.88
07	Others	115	5.03	131	5.73
	Total	1224	53.57	1061	46.43

CONCLUSION

Infectious and non-infectious diseases are the major problem for animal health and production performance of livestock specially cattle. The study shows incidence of infectious diseases, parasitic disease, digestive disorder, respiratory disorder, reproductive disorder, metabolic disorder and other diseases in moulvibazar region. Study reveals high incidence of parasitic disease as well as digestive disorder. Regular deworming programme should be taken to minimize parasitic infestation. Our study on prevalence of infectious and non-infectious diseases of cattle in Moulvibazar district of Bangladesh provides necessary information to take proper strategies for control and prevention of diseases.

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Socioeconomic status of Buffalo farming communities of Jaintapur Upazilla of Sylhet, Bangladesh

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ABSTRACT

The study was conducted to investigate the socio-economic status of the buffalo farmers at different villages (Omanpur, Haripur and Fatehpur) of Jaintapur Upazilla under Sylhet district of Bangladesh. A total of 37 farmers were randomly selected from three villages. The data was collected through interviewing with pre-tested questionnaire. The investigation revealed that buffalo farmers are mostly the middle (30- 40 years) and old (>40 years) aged farmers. Majority (59.46%) of the buffalo farmers are illiterate but only 40.54% are educated. The major occupations of the selected farmers are agriculture (crop production, dairying and poultry rearing). The buffalo farmers basically have little or no relevant trainings, skills or experiences. They inherit buffalo rearing as their family profession and less than 10% of them are influenced by the neighboring buffalo farmers. Grazing is the most popular option for feed source for the buffaloes while concentrate feeding is least available. The average cost of one pair of buffalo is 75,000-200,000 BDT. The annual total cost of production is Tk 4,665; with a gross return is Tk 12,000 per buffalo. Considering all this parameters related to livelihood, it was clearly found that the socio-economic status of the buffalo farmers has been improved through buffalo rearing although the management practices need to be improved with scientific approaches.

Keywords: Socio-economic status, Buffalo, Jaintapur, Sylhet

INTRODUCTION

Buffaloes hold strategic place in ever expanding livestock economy of Bangladesh and serve three important purposes such as milk, meat and drought power supply (Cockrill, 1974). Farmers prefer to use buffaloes for draught purpose because of larger body size than the native cattle, higher draught power ability, long working life and docile temperament. Most of them are kept by medium and rich farmers for draught purpose. Ninety six percent of the world buffalo population is found in Asia (FAO, 2010), where only 0.6 percent buffaloes are found in the particular agro-ecological zones in Bangladesh (Faruque *et al.* 1990). In fact, buffaloes have better capacity of converting coarse feed stuffs into quality milk and meat. Though they can utilize roughage in the same ability as the cattle, their ability to process and communicate forages appears to be associated with relief of protein deficiencies rather than to extract energy (Kennedy, 1988). Buffaloes have a number of advantages over cattle in utilization of low quality roughages to produce more protein and to gain more body weight, more disease resistance power and outstanding draught capacity and longer life span (Pasha T N,Hayat Z.2012). Buffalo is the second largest source of milk supply in the world and milk production was 13% of the total world milk production in 2012. India and Pakistan produce 67% and 24% of the total world buffalo milk, respectively while Bangladesh has produced less than 1% of the world buffalo milk (FAOSTAT, 2012). In addition to the dairy buffaloes they are also used as an important source of meat from spent and surplus animals. In meat production, buffalo share 1.3% in the world, 24.4% in India and 26.9% in Pakistan (Suhail et al., 2009). Buffalo population in Bangladesh figures at 1.62 million and is contributing through the production of 3500 and 22400 metric ton meat and milk respectively per year (FAO, 2010; DLS,

2013). Buffalo have significant contribution to GDP through production of meat, milk and skin representing about 27.0, 23.0 and 28.0%, respectively to the total production from livestock sector in Bangladesh (FAO, 2010). Buffalo has some significant importance on livelihoods improvement. They are usually maintained on tree leaves, shrubs and bushes in the rural condition. Buffalo rearing increases livelihood status of the farmers especially for the farm women and development of this sector is the potential path to rural prosperity (Kalash et al., 2009; Sarker et al., 2013, Amin et al., 2015). In Bangladesh, very little work has been done so far on the socio-economic status of the buffalo farmers and the management practices of buffalos. Therefore, the present study was conducted to investigate the socio-economic status, income and the livelihood changes of the buffalo farmers and the management practices of buffalos in some selected areas of sylhet district.

MATERIAL AND METHODS

Area of study:

The study was conducted at Jaintapur upazilla of Sylhet district. Necessary informations were taken from Omanpur,Haripur and Fatehpur of Jaintapur upazilla.

Period of study:

The study was conducted on 02 September to 04 September, 2016.

Selection of farmers:

A total of 37 buffalo farmers were selected from three villages. Farmers of buffaloes were randomly selected. Therefore, in total 37 respondents were chosen from Jaintiapur upazila for collection of data for this investigation.

Collection of data and measurement of variables

The data was collected through personal interviewing. Interview schedule was carefully prepared based on the objectives of the study. The informations were recorded directly on the questionnaire. The informations were checked carefully before leaving the study areas in order to minimize errors. The selected variables in this study are as follows: educational status, occupational status, socio-economic status, breeding, feeding and housing condition, daily routine activities of farmers for buffalo rearing, annual cost of production and income from buffaloes and impact of income for livelihood improvement.

RESULTS AND DISCUSSION

Socio-economic status

Socio economic status means an individuals or groups position within a social structure. Socioeconomic status depends on a combination of variables, including occupation, educational status,income, wealth, place of residence etc. The socio-economic condition of the buffalo farmers at Jaintapur Upazilla is analyzed in this study.

Status of buffalo farmers

In the studied areas, buffalo rearing is mainly practiced by the middle and old aged (78%) farmers.

Table 1: Age categories of buffalo farmers

Category	Frequency	Percent
Young aged (>30)	8	21.62
Middle aged (30-40)	12	32.43
Old aged (<40)	17	45.96

More than 59% buffalo farmers are illiterate and 30 % have passed primary level but only 11% farmers have passed secondary level and there was no graduated farmer (Table 2). But in Bagerhat, only 18.3% farmers were illeterate (Sarkar et al. 2013).

Table 2: Education of buffalo farmers

Category	Frequency	Percent
Illiterate	22	59.46
Primary	11	29.73
Secondary	4	10.81

In the study areas, maximum of the selected farmers were engaged only in agriculture (agricultural crop farming, dairying and poultry rearing), few are involved in business or other occupations (Table 3).

Table 3: Occupation of buffalo farmers

Category	Frequency	Percent
Agriculture	32	86.49
Business	3	8.11
Other (Foreigner)	2	5.41

Training skill and source of capital of buffalo farmers

The training skill of farmers is presented in (Table 4). There were no farmers who got training on buffalo husbandry. Other study shows that, there were no trained farmers in Bagerhat (Sarker et al. 2013).

Table 4: Training for buffalo rearing

Category	Frequency	Percent
Training	0	0
No Training	37	100

The source of capital is presented in table 5. In the studied areas loan from banks or NGOs is not available. But in Bagerhat, there is availability of loan from bank or NGOs and 65% farmers took loan from bank or NGOs (Sarker et al. 2013).

Table 5: Source of capital

Category	Frequency	Percent
Own capital	37	100
Loan from Banks or NGOs	0	0

The duration of rearing buffaloes

The farmers have been rearing buffalo as their family profession and less than one third of the farmers are influenced by the neighboring buffalo farmers. More than 94% farmers have been rearing buffaloes for more than 2 years (Table 6)

Table 6: Duration of rearing

Category	Frequency	Percent
Less than 1 years	1	2.70
1-2 years	1	2.70
More than 2 years	35	94.59

Feeding of buffaloes

In the studied areas, buffalo farmers do not offer any concentrates. They are fully dependent on grazing and do not use cultivated fodders and tree leaves. Extensive feeding system is practiced in the studied areas. Intensive or semi-intensive feeding system is not practiced in the studied areas. The main advantages of buffalo over cattle are that the dry or growing buffaloes may utilize coarse feeds more efficiently than Zebu cattle (Singh et al. 1973). In Bagerhat 55% buffaloes are reared by semi-intensive feeding system (Sarker et al. 2013).

Table 7: Feeding system

Category	Frequency	Percent
Extensive	37	100
Semi-intensive	0	0
Intensive	0	0

Figure 2 : buffaloes are going to grazing land

Cost of the buffaloes

The cost of a pair of buffalo is presented figure 2. The price of the buffaloes varies according to the size and utility. The price of 2 buffaloes for draught purpose ranges from 75000-200000 BDT in the studied areas.

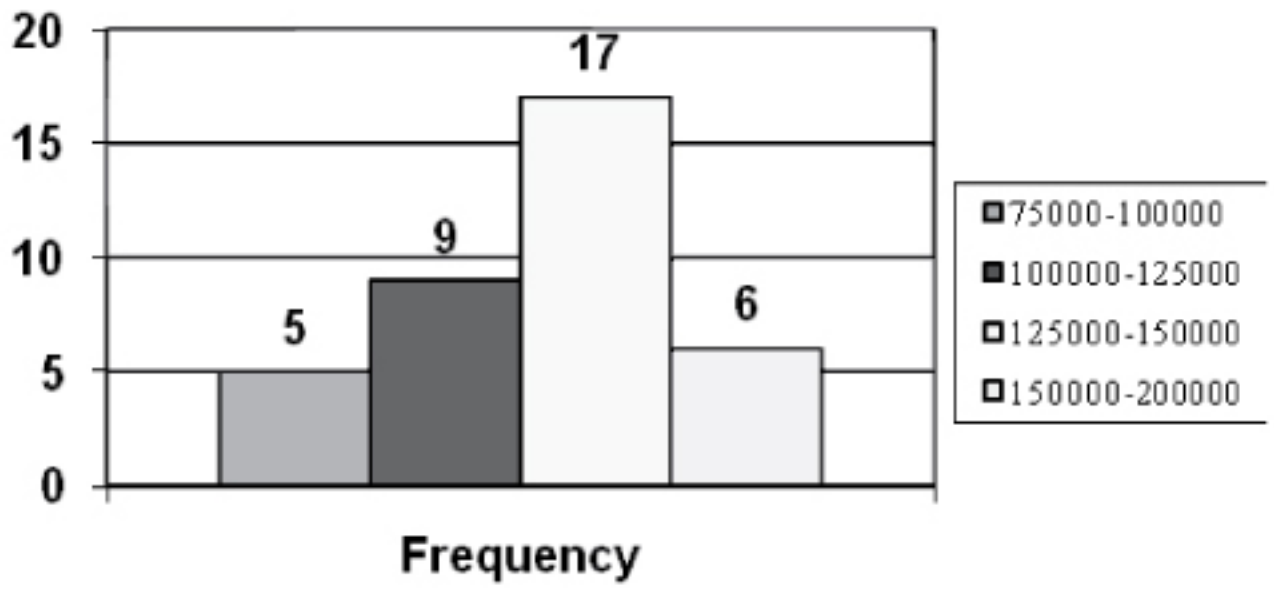

Figure 3: Price of buffalo (one pair)

The cost of feeding, breeding, housing and equipment and healthcare of buffaloes are presented in Table 8. To analyze the cost- return, it is necessary to describe the feed cost, breeding cost and cost of housing and equipment for buffalo rearing. The cost (per year) of housing and equipment was almost similar with feed cost. Farmers generally bred their buffaloes using their neighbor's buffalo bull and they sometimes artificially inseminate their buffaloes. For this reason, the breeding cost was low. On the other hand they never purchase feed for buffalo rearing. Farmers buy rice straw @3000tk/room (photograph-2). The major cost of healthcare

is medicinal cost followed by vaccination. Some NGO's of this area had several programs on vaccination, deworming and treatment. It was very much helpful to the buffalo farmers of this region. The average rearing cost of one buffalo was 4665 BDT.

Table 8: Different cost/buffalo in the studied areas

Category	Cost (Tk)
Average feed cost (year)	2000
Average cost of housing & equipment (year)	2000
Average cost of medicine & vaccine (year)	665
Total	4665

Figure 3: Farmers buy rice straw @3000tk/room

Cost of farmers family members

The average expenditure of farmers/year/head is presented in Table 9. Food cost was found highest among the total expenditure. The second highest expenditure was to maintain social status followed by the cost for clothing, health care, education and housing.

Table 9 : Average expenditure (BDT) per year per family in the studied area

Category	Average expenditure	Minimum	Maximum
Food	12000	5000	25000
Cloth	5571.43	1000	25000
House	2100	1000	6000
Education	2000	500	5000
Health Care	2428.75	1000	5000
MSS	3000	1000	6000

MSS = Maintaining social status (decoration, gift, furniture etc.)

Income of buffalo farmers

The yearly income of the farmers is presented in table 10. Most farmer's yearly income ranges from 150000 to 20000 (45.94%).

Table 10: Yearly income of the farmers

Yearly income (tk)	Frequency	Percentage
100000-150000	8	21.62
150000-200000	17	45.94

200000-250000	7	18.91
250000-300000	2	5.40
>300000	3	8.10

Total and net income from buffalo rearing

In this studied areas the farmers do not sell milk. The average income/year/ buffalo through draught was 12000 BDT but they can fulfill their need of milk.In Bagerhat, The average income per year per buffalo through draught was 11000 BDT and BDT 9700 by selling milk (Sarkar et al. 2013). As the farmers of Jaintapur upazilla do not sell milk the net income from buffalo rearing is not as high as the farmers of Bagerhat district.

Livelihood improvement of buffalo farmers

Buffalo rearing increased the livelihood status of the farmers especially for the farm women and the development of this sector is the potential path to rural prosperity

CONCLUSION

Though farmers prefer to use buffaloes for drought purpose, they also provides us with milk and meat. Buffalo plays an important role in family nutrition. Buffaloes are good converter of coarse feed stuff into quality milk and meat. In the studied areas most of the farmers are old aged (45.96%). Only 21.62% young farmers are involved in buffalo rearing. Most of the farmers are illeterate (59.46%) and earns their livelihood by other agricultural works (86.49%). There are no facilities for bank loan or necessary training for the farmers hindering the expansion of this industry in Bangladesh. Most of the farmers have been rearing buffaloes for more than two years (94.59%). As the farmers do not sell milk in the local market the net income from buffalo rearing is only 12000tk/year/buffalo. As the maintenance cost of buffalo is low, buffalo rearing in the study areas seems profitable with economic promise. Further attention is necessary to improve the buffalo farming through provision of funds and necessary training on buffalo production and diseases to the interested farmers.

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Value Chain Analysis and Comparative Advantage of Milk Production in Selected Areas of Bangladesh

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Abstract
Milk is considered as one of the best diets for human nutrition but gap between demand for and supply of milk is acute. Triggering these views, the objectives of this study were to estimate profitability of milk production, to determine value addition at different levels of milk marketing and to measure the comparative advantage of milk production. Study areas were selected from three districts namely Panchagarh, Chittagong and Sylhet. Selected samples were consisted of 300 farmers and 90 market actors. Field survey method was followed to collect primary data from January to March/2016. For getting the meaningful results, profitability analysis, marketing system, calculation of marketing margin and net margin of value chain actors and policy analysis matrix (PAM) were adopted.The study revealed that the production cost of milk for cross-bred cattle was estimated BDT 43,673/ ton whereasper ton net return was estimated BDT 2,543. Average net margin for milkmen, sweet seller and tea seller were calculated BDT 969, BDT 4,875, and BDT 6,194, respectively. Value addition among the milk value chain actors was also estimated. On average, milkmen added value 29%, sweet seller added 150% and tea seller added 175%. The NPCO value under import parity was found greater than one indicating polices of fresh milk production provided nominal protection for the producers. NPCI's value was found less than 1 of import parity price suggesting that the government policy are marginally reducing import cost and average market price of input just keeping the world price and government has been providing marginal support to the milk sector. The values of EPC were found to be greater than 1 implying that government polices provides positive incentives to the producers. The value of the DRC estimation revealed that Bangladesh had a comparative advantage for import substitution of fresh milk as DRC value was less than 1. The SCB is less than one meaning the benefit of government policy of protection is higher than the cost of protection. Estimated profitability is greater than 1 indicating private profit is higher than the social profit. The policy benefits are in favor of the producers.

Key words: Value chain, profitability, marketing margin, value addition, comparative advantage

Introduction

Milk is an ideal food and enriched with a high nutritional value. It contains all componentsthat are required by the human body in appropriate amount and in a very easily digestible way. Moreover, intake of milk increases the digestibility of other types of food. Livestock is the premier sources of milk and integral part in the economy of Bangladesh. Livestock creates employment generation 20 per cent directly and 50 per cent partially and livestock population in Bangladesh was 539.72 lakh where cattle are 236.36 lakh and buffaloes are 14.64 lakh (DLS, 2015). In fiscal year 2014-15, total milk production was 69.70 lakh metric tonns whereas demand for milk was 144.81 lakh metric tonns. Per capita milk availability was 122 ml/day and deficiency was 75.11 lakh metric tonns (DLS, 2015). Therefore, there is a huge gap between demand for milk and supply of milk. Sufficient milk production and its marketing needed proper attention to minimize the gap. Uddin, *et al.*(2011) in a study stated that number of dairy cattle per family decreased overtime. Irrespective of regions, per household average production of milk varied from 311 to 762 liters. In the same study, he stated that intake of milk did not show any consistent trend over time and the amount of intake was much higher than the national average. Rabbani, *et al.* (2004) performed a socioeconomic study on the participation of rural people in dairy enterprise and showed that large farmers raised the cross-bred cows and small farmers raised the indigenous cows.Kuddus (2006) performed a study to know the profitability of dairy farming, milk consumption pattern and marketing system of dairy owners and found from the study that net return of dairy milk in commercial region was significantly higher than that of other regions due to rearing of cross-bred

cows and feeding them high quality feed. It was observed the milk production in Bangladesh is increasing over the year i. e. in financial year 2003-04, production was 1.99 million tons whereas in 2012-13, production was 5.07 million tons (BBS, 2013).

Though the demand for milk and milk products are increasing due to rapid population growth and educated people are much conscious about nutrition. In fact, in most of the cities and towns, milk supply is scanty instead of its high demand. But research in this arena is limited. The present study will be able to find out the handicrafts of milk production and marketing, the comparative advantage of milk and suggests policy guidelines which will assist the researchers, academicians and planners.

Objectives: the study has the following specific objectives

i. To estimate profitability of milk production;
ii. To determine value addition at different levels of milk marketing;
iii. To measure the comparative advantage of milk production in Bangladesh; and
iv. To examine the policy implications arising from the findings.

Methodology

The methodology followed for the study was as follows:

Study areas were selected from three districts namely Panchagarh, Chittagong and Sylhet of Bangladesh representing normal milk available area, milk pocket area and milk deficit area in terms of native milk producton. The selected Upazilas were Tetulia and Debiganj under Panchagarh district, Anwara and Patiya under Chittagong district and Jaintiapur and Gowainghat under Sylhet district. The selected samples were consisted of 300 farmers and 90 market actors. The total sample size was 390 (Table 1& 2). In this study, we selected those farmers who reared dairy cattle for their livelihood and the traders who had supplied fresh milk and milk made products to the consumers.

Table 1: Distribution of sample farmers and traders in the study areas

Sample Types	Panchagarh		Chittagong		Sylhet		Total
	Tetulia	Debiganj	Anwara	Patiya	Jaintiapur	Gowainghat	
Farmers	50	50	50	50	50	50	300
Market actors	30		30		30		90
Total							**390**

Table 2: Sample distribution of market actors in the study areas

Market Actors	Panchagarh	Chittagong	Sylhet
Milkmen	11	19	6
Sweet seller	9	6	9
Tea seller	10	5	15
Sub-total	30	30	30
Total		**90**	

Simple random sampling technique was followed for selecting the respondents. Field survey method was followed to collect primary data from January/2016 to March/2016. Data were collected from respondents by using structured interview schedule and conducting FGD (Focus Group Discussion) for group information. The structured interview schedules were developed and field-tested for necessary rearrangement and modifications before starting data collection. Data were collected through direct interviews making personal visits to the house of selected farmers. Secondary data and information were collected and discussed for this research from different handouts, reports, publications, notifications, etc.

Profitability analysis

In this study, costs and returns analysis was done on total cost basis. The following equation was used to assess the profitability of the milk producers.

$$\prod i = \sum_{i=1}^{n} P_i Q_i - TC = \sum_{i=1}^{n} P_i Q_i - (VC + FC)$$

Where,

$\prod i$ = Profit from i^{th} dairy farm

Q_i = Quantity of the i^{th} milk production (Lit./lactation period)

P_i = Average price of i^{th} milk product (BDT/Lit.)

TC= Total cost (BDT/ton milk production)

FC= Fixed cost (BDT/tonmilk production)

i= 1, 2, 3,....., n

Per ton profitability of milk production from the view point of individual farmers was measured in terms of gross return and gross margin.

Marketing system

Network analysis mainly graphical technique was performed for this identification. First, market actors were identified. Second, volume of trade through each actor was measured. Third, a market chain was drawn.

Calculation of marketing margin and net margin of value chain actors

The marketing margin and net margin of different value chain actors were estimated by the following formulae:

$$GM_i = P_{R_i} - P_{P_i}$$

Where,

GM_i = Gross margin (BDT/Lit) for i^{th} intermediaries

P_{Ri} = Price received (BDT/Lit) by i^{th} intermediaries

P_{Pi} = Price paid (BDT/Lit) by i^{th} intermediaries

$$NM_i = GM_i - MC_i$$

Where,

NM_i= Net margin (BDT/Lit) for i^{th} intermediaries

MC_i = Marketing cost incurred (BDT/Lit) for i^{th} intermediaries

Estimation of value addition by value chain actors

$$Value\ addition(\%) = \frac{(Sales\ price - Purchased\ price)}{Purchased\ price} \times 100$$

Import parity price calculation

Import parity: Import parity price (border price) of milk at farm level is estimated using the following formulae

$$P_j = P_j^b + C_{jm} - C_{jd}$$

Where,

P_j= producer price of j^{th} importable commodity,

P_j^b= world price at port of entry (c.i.f),

C_{jm}= marketing margin from the port of entry to the wholesale market and

C_{jd}= Components of the marketing spread between the wholesale market and farm gate.

Policy analysis matrix (PAM)

Table 3: Policy analysis matrix (PAM)

Items	Revenue	Costs		Profit
		Tradable inputs	Domestic factors	
Private prices	A	B	C	D
Social prices	E	F	G	H
Divergences	I	J	K	L

Source: Monk and Pearson, 1989.

Private profits (D) = A-(B+C), Social profits (H) = E – (F+G), Output transfers (I) = A – E, Input transfers (J) = B – F, Factor transfers (K) = C – G, Net transfers (L) = D – H or I – J – K
NPCO=A/E and NPCI=B/F, EPC= (A-B)/ (E-F), DRC=G/ (E-F) and SCB= (F+G)/E, Profitability coefficient (PC) = (A-B-C)/ (E-F-G) or D/H.

Results and discussion

Value chain actors and the value chain map of milk marketing

Milk markets attempts to identify the actors involved in the milk value chain to construct value chain map and to estimate the value addition by market actors and milk processors. Value addition is primarily explained as the difference between total expenses involved in processing or buying of a specific goods and the total revenue accruing from its sales. Value addition activities are mainly concerned with the changes of utilities. When any product passes through distribution channels, it creates place, time, and form utilities. For this reasons, in this section, the study dealt with identifying the actors involved in value chain and their function of milk marketing.

Input suppliers: Milk value chain starts from input suppliers. Concentrate, green fodder, artificial insemination service providers, veterinary health care service from Upazila veterinary hospital etc. are the prime inputs for milk production. So, inputs providers play a vital role in the milk value chain as they supply those inputs to the farmers.

Dairy farmers: Dairy farmers are the production points from where the milk supply is started. Dairy farmers depend on input suppliers for producing milk to the market actors.

Milk processors: Milk processors are market actors who have permanent infrastructure with milk processing equipment. They have huge amount of operating capital. They processed milk and supply it to retail shop for retailing. Prime milk processors in Bangladesh are Milk Vita, PRAN, Arong, Fresh milk etc.

Sweet seller: Sweet sellers are milk processors. They made sweet meat from milk. Sweet seller, on average, bought from 20-100 liters of milk and processed for sale. Most of the time, it has seen that, they have permanent shop structure and sometimes not.

Tea seller: As a milk value chain actor, tea seller had a significant role. Tea seller bought milk and made tea with those milk. Besides, sometimes, they sold hot milk to the consumers and the consumer drunk milk instantly. Tea seller had permanent shop structure. On average, they purchased from 5to 10 liters of milk per day.

Milkmen: Milkmen were market actors who purchased milk from the dairy farmers and sold it to sweet seller, tea seller, and to the ultimate consumers. They had no permanent infrastructure for selling milk. Actually they were a vendor.

Consumers: Consumers were the last segment in the milk value chain actors. They purchased sometimes processed milk and raw milk from dairy farmers, milkmen and the retail shop.

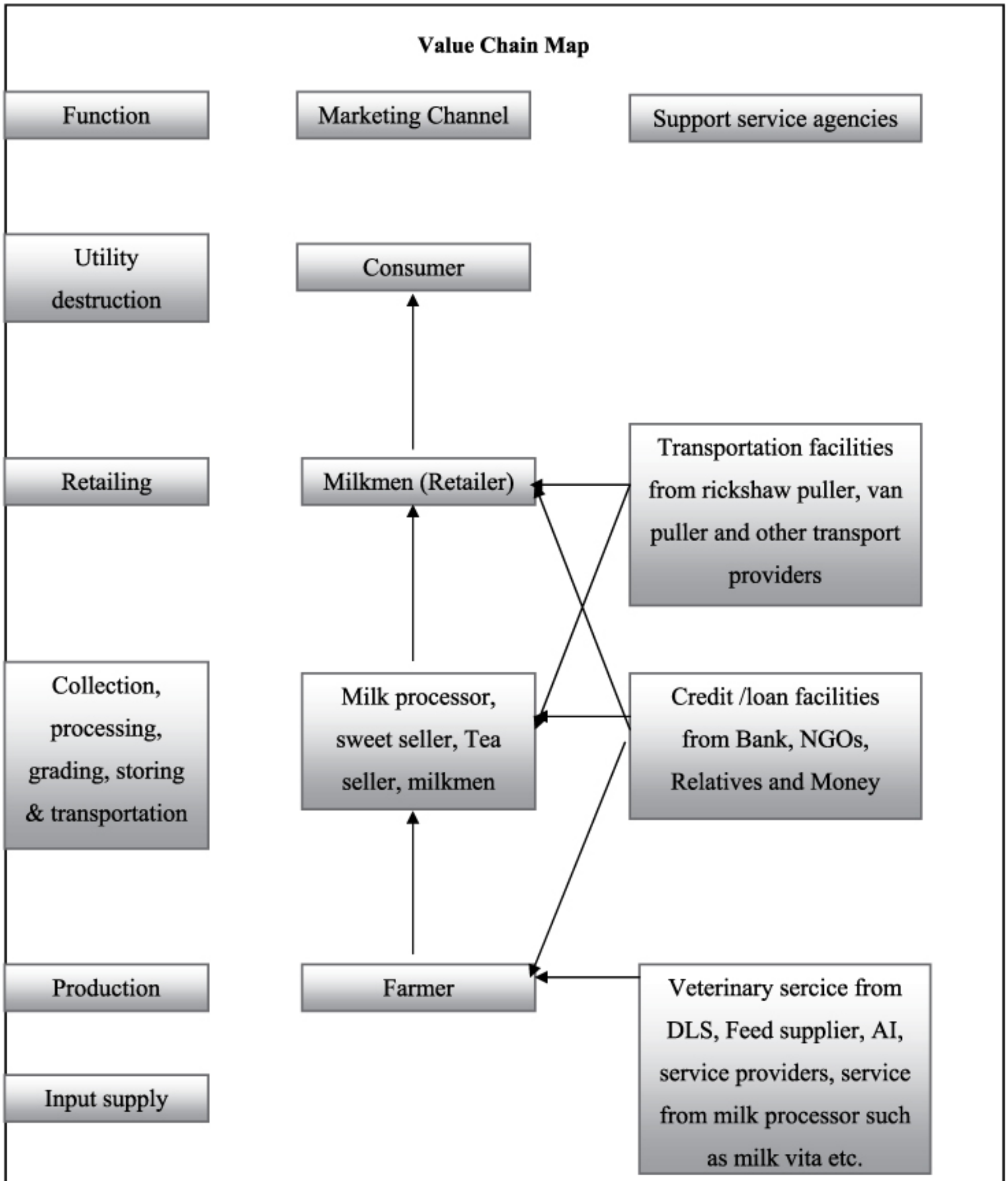

Fig. Value Chain Map of Milk

Value Chain Governance

Milk distribution channels: In the study area, the highest amount (39%) of milk was flowed from dairy farmers to consumers through retailer (milkmen) followed by 28% sweet seller, 26% directly flowed from dairy farmers to consumers, 5% tea seller and only 2% milk processors, respectively. Beyond dairy farmers

sweet seller and tea seller collected milk from milkmen also. Among 39% milk of milkmen, 1% flowed to tea seller, 9% to sweet seller and the remaining amount (29%) to the consumers.

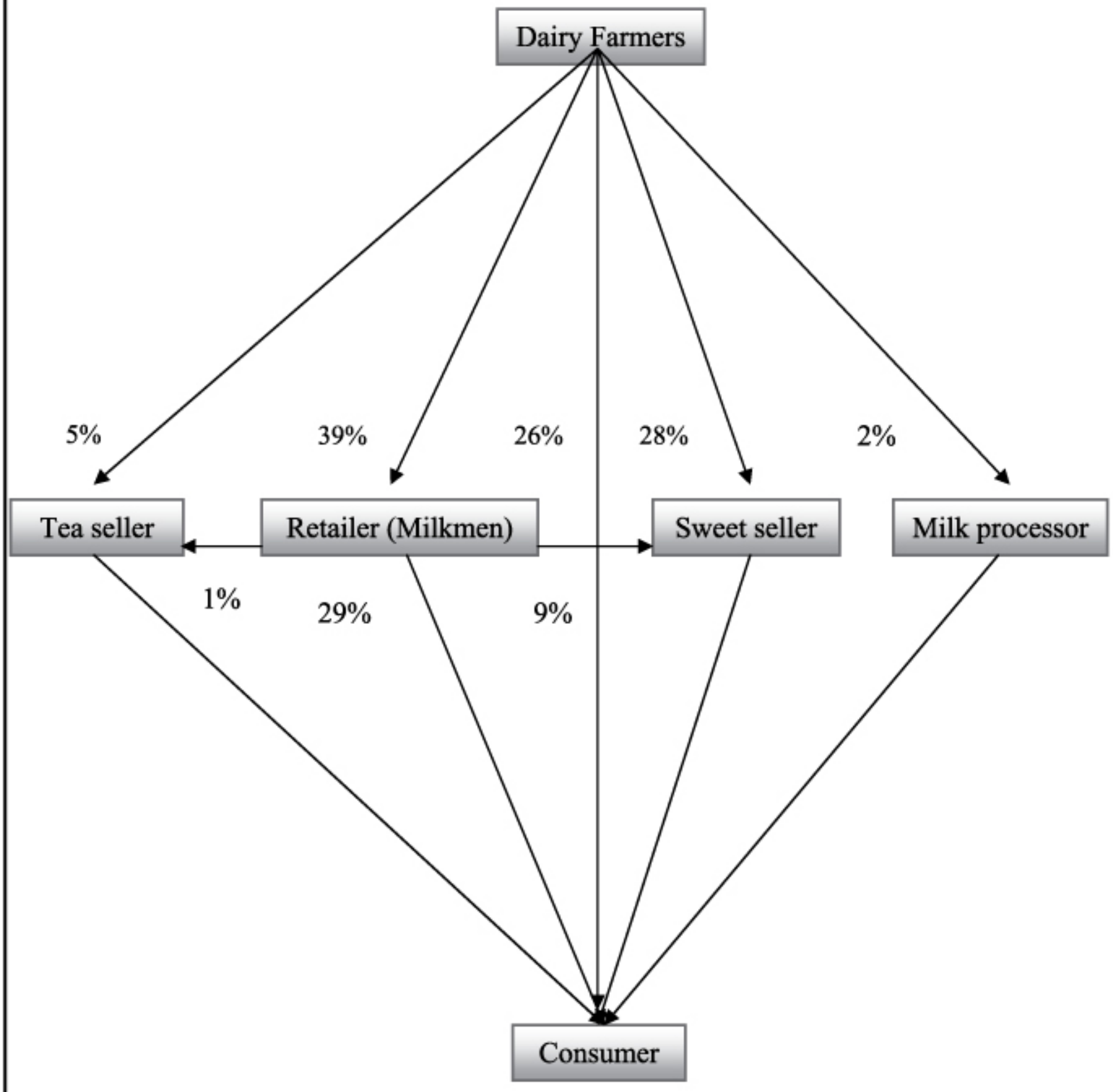

Fig. Milk Marketing Flow Chart

Marketing channels: Study found seven (7) marketing channels. Among these, channel IV was the leading followed by channel II, channel I, channel III and channel V, respectively. It was the prime cause for milk market volatility and price instability.

Channel I: Dairy farmer → Consumers
Channel II: Dairy farmer → Sweet seller → Consumers
Channel III: Dairy farmer → Tea seller → Consumers
Channel IV: Dairy farmer → Milkmen (Retailer) → Consumers
Channel V: Dairy farmer → Milk processor → Retailer (Shop) → Consumers
Channel VI: Dairy farmer → Milkmen (Retailer) → Tea seller → Consumers
Channel VII: Dairy farmer → Milkmen (Retailer) → Sweet seller → Consumers

Production cost of Milk (cross-bred): The study revealed that the production cost of milk for cross-bred cattle was estimated BDT 43,673/ton where variable cost was BDT 41,381/ton and fixed cost was BDT 2,291/ton. In variable cost items, feed cost covered BDT 23,529/ton that was 54% of the total cost. Human labourcost held the second highest cost, which was 39% of the total cost. Among total cost, variable and fixed cost was found 94.75% and 5.25%, respectively (Table 4).

Table 4: Cost of producing milk in Bangladesh (Cross-bred)

Cost		BDT/ton	
A. Variable cost		Fresh milk	Percentage (%)
Human labour		16953	38.82
Feed cost	Dry roughage	3816	8.74
	Green fodder	4002	9.16
	Concentrate	15711	35.97
Medicine & vitamin		854	1.96
Vaccination		45	0.10
Total variable cost		**41381**	**94.75**
B. Fixed cost			
Capital cost of housing		91	0.21
Capital cost of Animal before first calving		2200	5.04
Total fixed cost		**2291**	**5.25**
Total cost		**43673**	**100.00**

Source: Field survey2016 and author's calculation.

Profitability: It was found from that variable cost was incurred BDT 41,381 for per ton milk production by rearing cross-bred cattle where fixed cost was BDT 2,291. Per ton gross cost was estimated BDT 43,673 and gross return was estimated was BDT 46,206, respectively. Per ton net return was estimated BDT 2,543. The BCR (undiscounted) was calculated 1.06, if considered only milk production. When the study added the value (BDT 16,000)of a one year old calf, the BCR went up and it was 1.42 (Table 5).

Table 5: Profitability of producing fresh milk in Bangladesh (Cross-bred)

Costs & Returns	Fresh milk /ton
A. Variable cost	41381
B. Fixed cost	2291
C. Gross cost (A+B)	43673
D. Gross return	46206
E. Gross margin (D-A)	4825
F. Net return (D-C)	**2534**
G. BCR (Undiscounted)	**1.06**

Source: Field survey2016 and author's calculation.

Margins of Market Intermediaries: Average gross margin and net margin per 100 liters of milk for milkmen was estimated BDT 5,479 and BDT 969, respectively. In the case of sweet seller, average gross margin and net margin per 100 liters of milk (equivalent to 67 kg sweets) were estimated BDT 11,888 and BDT 4,875, respectively. Tea sellers` average gross margin and net margin was also estimated BDT 12,537 and BDT 6,194, respectively in the study areas (Table 6).

Table 6: Margins of market intermediaries in the study areas

BDT/100 liters

Areas	Actors	A. Gross margin	B. Purchase value	C. Marketing cost	D. Net margin
Panchagarh	Milkmen	4338	3577	157	604
	Sweet seller	10878	4202	1725	4951
	Tea seller	12624	4355	922	7347
Chittagong	Milkmen	6187	4332	494	1361
	Sweet seller	11840	4632	2245	4963
	Tea seller	13153	4873	1975	6305
Sylhet	Milkmen	5913	4827	144	942
	Sweet seller	12946	5496	2739	4711
	Tea seller	11833	4469	2435	4929
All Average	Milkmen	5479	4245	265	969
	Sweet seller	11888	4776	2236	4875
	Tea seller	12537	4567	1777	6194

Source: Field survey2016 and author's calculation.

Value addition: Value addition among the milk value chain actors in the selected areas was estimated in this study. It is observed that the all value chain actors i.e. milkmen, sweet seller and tea seller added different types of value. On an average, milkmen added value 29%, sweet seller 150% and tea seller 175% (Table 7).

Table 7: Value addition by market actors in the study areas

BDT/100 liters

Areas	Actors	Sales price	Purchase price	Value addition (%)
Panchagarh	Milkmen	4338	3577	21
	Sweet seller	10878	4202	159
	Tea seller	12624	4355	190
Chittagong	Milkmen	6187	4332	43
	Sweet seller	11840	4632	156
	Tea seller	13153	4873	170
Sylhet	Milkmen	5913	4827	22
	Sweet seller	12946	5496	136
	Tea seller	11833	4469	165
All average	Milkmen	5479	4245	29
	Sweet seller	11888	4777	150
	Tea seller	12537	4566	175

Source: Field survey2016 and author's calculation.

Policy Analysis Matrix (PAM)

To know the state of art of government policy incentives situation and to evaluate these government policies, the policy analysis matrix is very much helpful. In the Table, we see the tradable and non-tradable input costs at private price that account for BDT 23594 and BDT 36949 per lactation, respectively. On the other hand, at social price the subsequent costs are BDT 24232 and BDT 40004, respectively. The private profit per lactation period of fresh milk (raw milk) production is BDT 43094 which is greater than zero (0) which indicate the supernormal returns and possible to expand milk production in future, unless the per lactation milk production cannot be increased or substitutions are more profitable at private price. This also indicates that existing input and output prices, technologies, and government policies leads to the profitable milk production in Bangladesh. On the other hand, social profit of milk production is BDT 7108 per lactation which is also greater than zero. This value point out that milk production under free trade will be in favour of

producers compared to existing situations. Thus, Bangladesh has a static comparative advantage of domestic milk production for import substitution and it uses scarce resources efficiently.

The Table also shows different policy transfer or divergences such as output, input, factor and net policy transfers. It is evident that output transfer (difference between private revenue and social revenue) is 32292. The value is positive which indicates that government protective policies affect positively to the producer incentives. The input transfer (difference between private and social price of tradable inputs) is -638 which is also negative. The negative value illustrates the domestic producer buy the imported inputs less than the world price. Thus the government has implemented input subsidy policy to the livestock sector to decrease cost of production. Therefore, producer receives input subsidies for milk production in Bangladesh. The factor transfer (difference between private and social price of non-tradable inputs) is -3055 which is negative. The negative value shows the opportunity costs of non-tradable inputs are higher than their market prices. On the other hand, the net policy transfers (difference between private and social profit or social revenue minus social cost of tradable and not tradable inputs) is 35986 which is positive. This positive value means milk producer could earn less profit (or high loss) without government intervention. That means under free trade, producer will make less profit in contrast to the existing policy situation. It can be concluded that milk producers earn high profit under current government policy orientation (Table 8).

Table 8: Policy analysis matrix for fresh milk (cross-bred) per lactation period in Bangladesh

Items	Revenue	Costs		Profit
		Tradable inputs	Domestic factors	
Private prices	103638	23594	36949	43094
Social prices	71345	24232	40004	7108
Divergences	32293	-638	-3055	35986

Source: Own estimation

In the study NPCO value under import parity were found to be greater than one (>1) for fresh milk (cross-bred). This indicates that polices of fresh milk provide nominal protection for the producers.

NPCI's values was found to be less than 1 (<1) for fresh milk of import parity price suggesting that the government policy are marginally reducing import cost and average market price of input just keeping the world price. NPCI values of less than 1(<1) clearly indicate that government has been providing marginal support to the milk sector.

In addition, the study also estimated EPC (Effective protection coefficient) which is better indicator of effective incentive than the then NPC, as it finds the impact of production on inputs and outputs, and depicts the degree of protection according to the value addition process in the production activity. The values of EPC were found to be greater than 1 (EPC>1) for fresh milk (cross-bred), implying that government polices provides positive incentives to the produces.

DRC calculation has been done on import parity prices. This depends actually on the tradability status on commodity. The value of the DRC estimation revealed that Bangladesh had a comparative advantage for import substitution of fresh milk as on DRC values were less than 1 (<1). In other words, government policy could save foreign exchange by producing fresh milk domestically and efficiently, it causes import subsidies. This is because the opportunity cost of domestic resources and non- traded inputs use in producing milk is less than (<) foreign exchange saved.Alam, J. *et al*. (2007) conducted a study on profitability and comparative advantage of producing livestock and poultry products in Bangladesh and their international competitiveness, found the DRC for fresh milk was 1.11.The SCB (social cost benefit) is less than one, it indicates that the benefit of government policy of protection is the higher than the cost of protection.Estimated profitability is greater than 1 (>1) indicates that the private profit is higher than the social profit. The policy benefits are in favour of producers (Table 9).

Table 9: Different indictors of protection and comparative advantage

Items	Unit	Value
NPCO = Nominal Protection co-efficient (subsidies to output)	Ratio	1.45
NPCI = Nominal Protection co-efficient (subsidies to inputs level)	Ratio	0.97
EPC = Effective protection co-efficient	Ratio	1.70
DRC = Domestic Resource Cost	Ratio	0.84
SCB = Social Cost Benefit	Ratio	0.90
PC = Profitability co-efficient	Ratio	1.20

Source: Own estimation

Conclusions and Recommendations

The production cost of milk for cross-bred cattle was estimated BDT 43,673/ ton whereasper ton net return was estimated BDT 2,543. The private profit per lactation period of fresh milk production is BDT 43094.5 which is greater than zero (0) indicates the supernormal returns and possible expansion of milk production in future, unless the per lactation milk production cannot be increased or substitutions are more profitable at private price. This also indicates that existing input and output prices, technologies, and government policies leads to the profitable milk production in Bangladesh.

Rearing milch cow, no doubt is a profitable enterprise now-a-days and the country has comparative advantage over milk importation. Government should increase subsidy on tradeable and non-tradeable inputs to encourage milk production domestically. GOs-NGOs along with other organizations should come forward to invest more for R&D and to boost up livestock sector in developing high yielding milk breeds and pay attention for milk market stabilization through establishing mini milk processing plant throughout the country which would be a sustainable development approach in milk sector.

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Passive prevalence of bovine viral diarrhoea virus by detection of antibody in bulk tank milk samples

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Abstract
In view of the scarcity of information regarding Bovine viral diarrhoea virus (BVDV) infection in dairy cattle in Bangladesh, this study was designed to evaluate the status of BVDV infection in dairy herds of different districts of Bangladesh. The present research use the commercial ELISA kit (IDEXX BVDV total Ab) for the detection of specific antibodies (BVDV-Ab ELISA) in bulk tank milk (BTM) samples. A total of 460 bulk tank milk (BTM) samples were collected from 352 dairy farms from different areas of Bangladesh. The BTM samples were centrifuged for 10 minutes at 3000 g at 4°C and the skimmed milk was collected below the fat layer and was used for the ELISA. ELISA was conducted and the results were interpreted following manufacturer's instructions. The ELISA result showed that the average degree of prevalence of BVDV was 88.04% in 460 samples. Prevalence of BVDV was highest (92.1%) at Sirajgonj followed by Pabna (89.4%) and Mymensingh (78%). Since no vaccination is being practiced in Bangladesh, the high prevalence of BVDV antibodies in BTM samples of research areas indicating BVDV contaminated environment in Bangladesh which may be sequel of naturally occurring either vertical or horizontal BVDV infection.

Key words: BVDV, BTM, ELISA, antibody

INTRODUCTION

Bovine viral diarrhoea (BVD) is a wide spread viral infection affecting mainly dairy cattle but also other ruminants such as sheep and goats worldwide. It is a significant economic viral disease of livestock industry worldwide due to its high prevalence, persistence and clinical consequences (Moennig *et al*., 2005; Greiser-Wilke *et al.,* 2003). It is caused by a single stranded, enveloped RNA virus belonging to the genus Pestivirus under the family Flaviviridae (Fenner, 2011). Two antigenically distinct genotypes of BVDV exist, types 1 and 2, with further subdivisions discernable by genetic analysis (Vilcek*et al*., 2001). Seroprevalence of BVDV in cattle varies between different regions of the world from 12 to 89%. The first evidence of BVDV infection in the Indian subcontinent was reported in 1982 in Orissa (Nayak*et al*., 1982). Phylogenetic analysis conducted on serum samples collected in 2000 and 2002 showed that all the cattle BVDV isolates originating from India belonged to BVDV-1. In 2011, BVDV-2 was also isolated from Indian cattle (Behera*et al*., 2011). The first evidence of BVD infection in cattle of Bangladesh was reported by Samad (1999) testing cattle sera from 2 cattle farms and one veterinary hospital. Very recently (Chowdhury *et al*., 2015)conducted a seroprevalence in southern part of Bangladesh and reported 51.1% cattle were positive for BVDV antibodies.HoBi like pestivirus has recently been identified in Bangladesh (Haider *et al*., 2014). Although BVD-3 has been reported in Bangladesh but the other two types BVD-1 and BVD-2 have not confirmed yet but both the virus are circulating in India. Live cattle trading between Bangladesh and India are a continuous process. It is not impossible that the other two types of BVD (BVD-1 and BVD-2) reported in India has been circulating in Bangladesh long time without being detected.

In addition, veterinarian of Bangladesh often complains the existence of BVDV in the cattle of Bangladesh on the basis of clinical symptoms. Confirmatory diagnosis of the disease has not been done in most cases. So, it is very essential to investigate the status of BVD in Bangladesh and develop a control strategy to control the disease in Bangladesh. BVDV can be detected frequently from serum sample by serum neutralization test, Antigen ELISA, indirect ELISA and RT-PCR. RT-PCR can also be used for bulk tank milk (BTM) samples to detect any persistently infected (PI) cows contributing to the tank (Lanyon *et al*., 2014; Bownlie, 1985).

Viral RNA may also be detected using RT-PCR for clinical specimens or in situ hybridization on fresh or fixed tissues. Now-a-days, it is the most accurate and modern technology for the detection of BVDV in which virus can be detected by specific band production. Genotype is generally determined by PCR with subsequent nucleic acid sequencing (Merck & Co, 2008).

Farmer's expectation is that this would be done at the lowest possible cost. One such option is testing the BTM samples. The presence of single persistently infected (PI) animals in a herd is enough to mount significant levels of neutralizing antibodies, which can be detected in milk of the herd mates having contact with the virus shedder. The aim of the present study was to determine passive prevalence of BVDV infection in dairy herds based on BTM testing in some selected districts of Bangladesh.

Materials and Methods

For this study Bulk Tank Milk (BTM) samples were collected from cows of Mymensingh, Pabna and Sirajgonj districts of Bangladesh and the laboratory test was conducted in Molecular Microbiology laboratory , Department of Microbiology and Hygiene, Bangladesh Agricultural University. The study was done during the period of January 2016 to June 2017.

Collection of Samples

A total 460 BTM samples were collected by using 1.5 ml sterile eppendorf tubes for detection of antibody against BVDV from different districts (Mymensingh, Pabna and Sirajgonj) of Bangladesh. During the collection of sample one important thing was considered that the number of milk samples in each BTM was not less than 5 cows in a given herd.

Transportation of Samples

BTM samples were transferred to the department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh-2202 maintaining 4°C in ice box by ice bag.

Processing of Samples

The BTM samples were centrifuged for 10 minutes at 3000 rpm at 4°C by using centrifuge machine and the skimmed milk was collected below the fat layer which was used for the ELISA.

Storage of Samples

The skimmed milk samples were stored at -20°C until tested.

Preparation of wash solution

The wash concentrate (10X) was brought to 18-26°C and mixed to ensure dissolution of any precipitated salts. The wash concentrate was diluted 1 to 10 with distilled/ deionized water before use.

ELISA Test Procedure

The collected milk samples were analyzed by indirect ELISA using Bovine Viral Diarrhea Virus antibody testing test kit (IDEXX Switzerland AG, Stationsstrasse 12, CH-3097 Liebefeld-Bern, Switzerland) containing BVDV antigen coated plates.

The test procedure in brief was:

1. The frozen milk samples were thawed.
2. Antigen coated plate was obtained and the sample position were recorded.
3. 100 µl of the sample diluents was added only into the wells for negative control and positive control.
4. 25 µl negative control (NC) was added into duplicate wells.
5. 25 µl positive control (PC) was added into duplicate wells.
6. 100 µl undiluted milk samples (from underneath the fat layer) were dispensed into remaining wells of the plate.
7. The content of the microwells was mixed by gently tapping the plate or using a microplate shaker.
8. The plate was incubated for overnight (12-18 hours) at 2-8°C. Plates were sealed or covered with aluminium foil and incubated in a humid chamber.

9. After the incubation period, the solution was removed and each well was washed with approximately 300 µl of wash solution 5 times. Plate drying between plate washings and prior to the addition of the next reagent was avoided. Each plate was tapped onto absorbent material after the final wash to remove any residual washed fluid.
10. 100 µl of conjugate was dispensed into each well.
11. Incubation was done for 30 minutes (±2 min) at 18-26°C.
12. After the incubation period, the solution was removed and each well was washed with approximately 300 µl of wash solution 5 times. Plate drying between plate washings and prior to the addition of the next reagent was avoided. Each plate was tapped onto absorbent material after the final wash to remove any residual washed fluid.
13. 100 µl of TMB Substrate was dispensed into each well.
14. Incubation was done for 10 minutes (±1 min) at 18-26°C.
15. After the incubation period, 100 µl of Stop Solution N.3 was dispensed into each well.
16. The absorbance of the samples and controls was measured and recorded at 450nm filter using a 96 well plate reader.
17. Negative control mean, positive control mean, S/P ratio (sample value related to positive control value) were calculated according to the following formula:

Calculation

I. Negative control mean $(\overline{NCX}) = \frac{NC1\ A\ (450) + NC2\ A\ (450)}{2}$

II. Positive control mean $(\overline{PCX}) = \frac{PC1\ A\ (450) + PC2\ A\ (450)}{2}$

III. S/P ratio $= \frac{Sample\ A\ (450) - \overline{NCX}}{\overline{PCX} - \overline{NCX}}$

Interpretation of result

The result was interpreted by S/P ratio of each sample. Such as:

a. Samples with S/P ratio less than 0.20 were considered as negative.
b. Samples with S/P ratio greater than or equal to 0.20 were considered as positive.
c.

Determination of S/P ratio from sample absorbance

Negative control OD (optical density) from NC1 A and NC2 A were 0.0891 and 0.0793 while positive control OD (optical density) from PC1 A and PC2 A were 0.9177 and 0.9085.

In ELISA result, the highest S/P ratio 1.024 and the lowest S/P ratio 0.0021 were recorded.

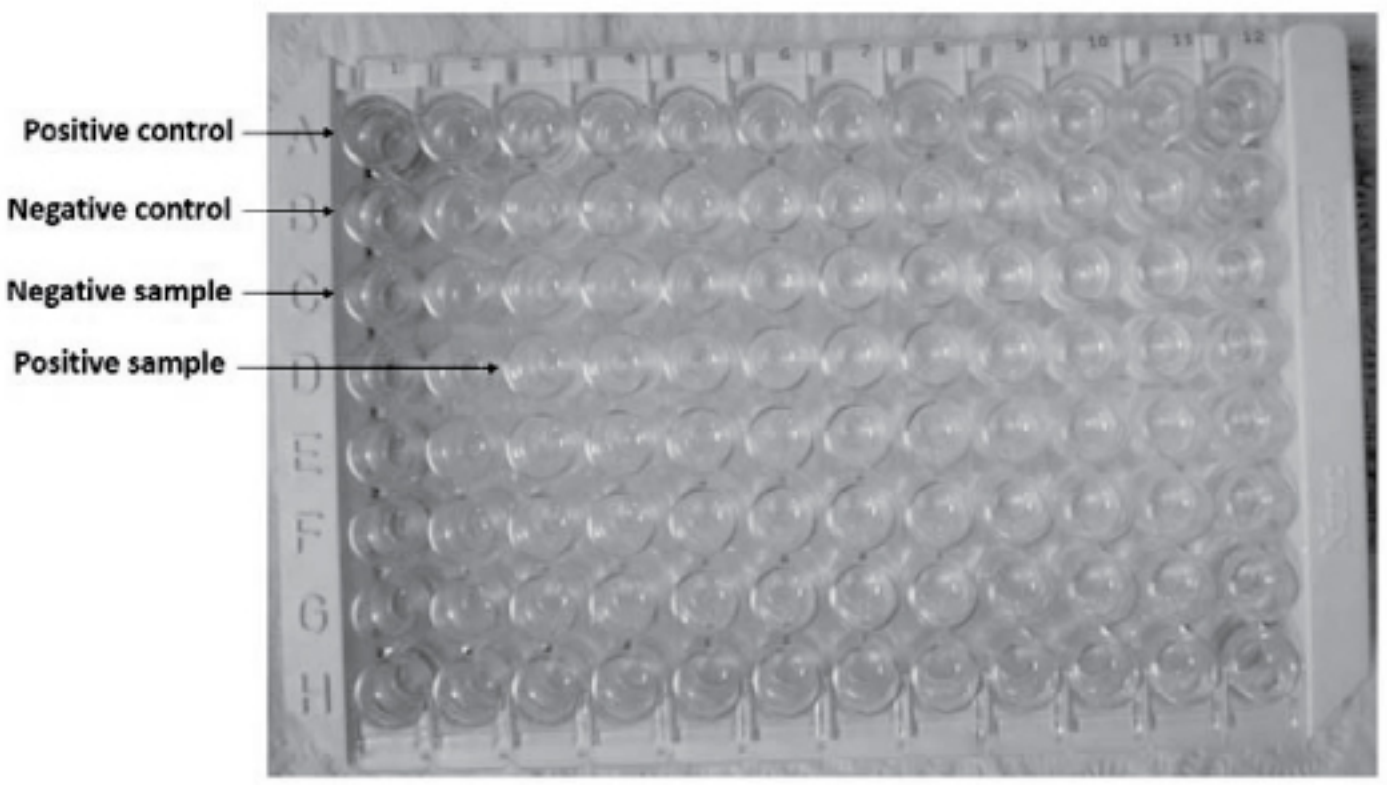

Figure1. ELISA Plate showing the color reaction (After adding the stop solution)

Table 1: ELISA Plate (S/P ratio)

	1	2	3	4	5	6	7	8	9	10	11	12
A	**0.9177**	**0.9085**	0.4049	0.3437	0.3912	0.45	0.2561	0.7589	0.4348	0.4178	0.2705	0.4859
B	**0.0891**	**0.0793**	0.3023	0.5633	0.2245	0.3552	0.3461	0.5914	0.4333	0.6504	0.6243	0.8175
C	0.16	0.3088	0.5073	0.39498	0.6035	0.4508	0.4701	0.6941	0.3959	0.3242	0.5765	0.4589
D	0.1539	0.2041	0.4347	0.4669	0.3905	0.0738	0.4645	0.4927	0.2201	0.3528	0.3666	0.4476
E	0.2456	0.5326	0.209	0.435	0.4475	0.3345	0.6275	0.5276	0.1732	0.6506	0.2127	0.2199
F	0.1668	0.566	0.5296	0.3624	0.5083	0.5974	0.4174	0.4894	0.5786	0.5019	0.5151	0.4838
G	0.3312	0.242	0.4797	0.4403	0.4495	0.477	0.3851	0.3646	1.024	0.6684	0.5429	0.4676
H	0.2378	0.3227	0.4407	0.1167	0.6617	0.4099	0.1518	0.382	0.6863	0.6567	0.3646	0.4859

Results

According to districts, the prevalence of antibody against BVDV is described in the Figure 2 and table 2. It was noticed that higher number of herds were found to be positive in Sirajgonj and Pabna districts than Mymensingh district.

In Mymensingh district, out of 100 samples from different herd of Bangladesh agricultural university area, Mymensinghsadar and Tarakanda 78 sample (78%) were found positive. In Pabna districts, out of 170 samples from different herds of Bera, Sathia, Faridpur and Bhanguraupazillas 152 samples (89.4%) were found positive. In Sirajgonj district, out of 190 samples from different herds of Shahajadpur, Ullapara and Belkuchiupazillas 175 samples (92.1%) were found positive.

In overall prevalence analysis, a total out of 460 milk samples, 405 (88.04%) samples showed BVDV positive with a range 88.01% to 88.07% at 95% confidence interval. Among these, Cattle of Sirajgonj district having the highest position with 92.1% followed by Pabna (89.4%), and Mymensingh (78%).

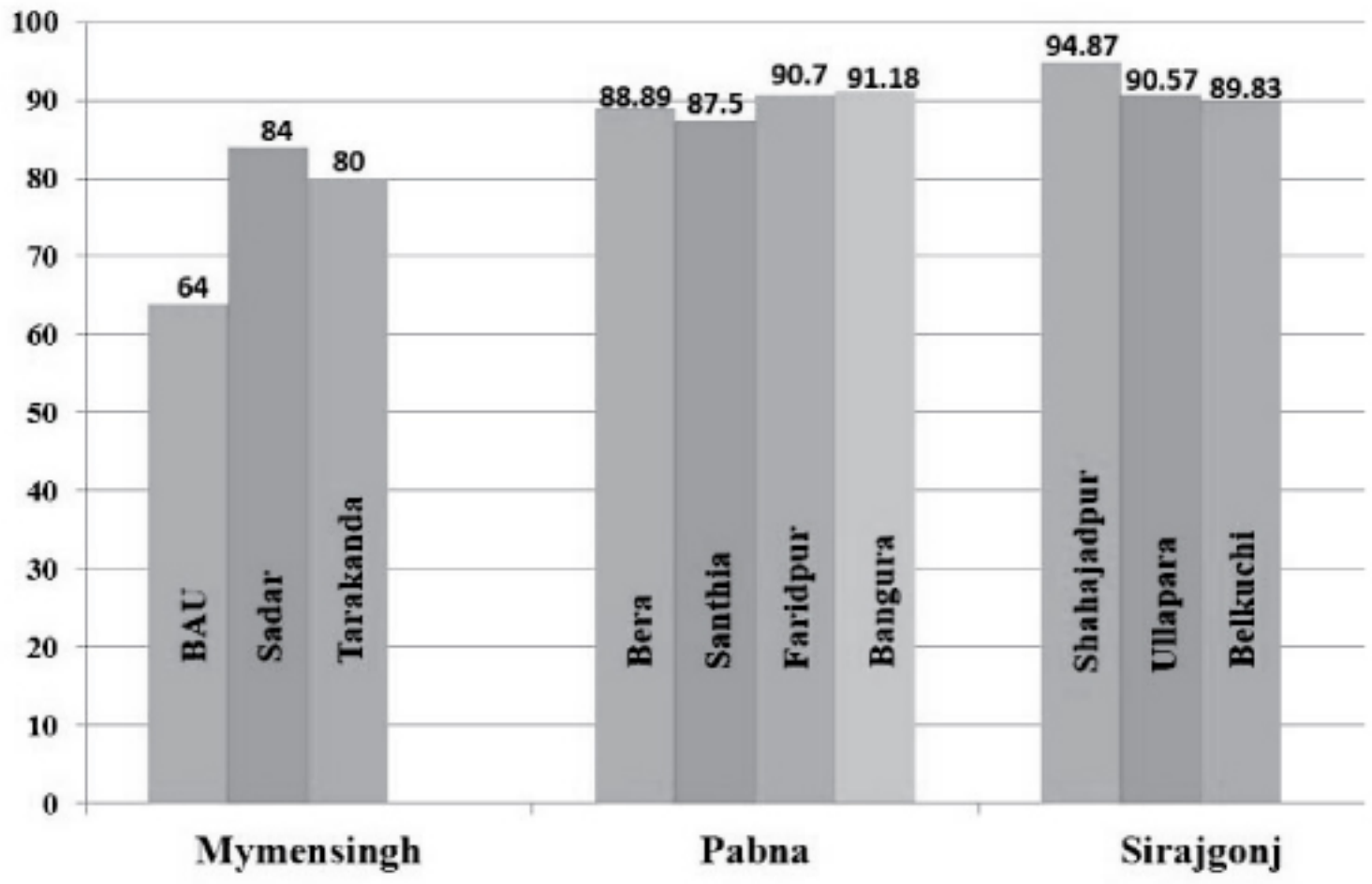

Figure 2. Data presented in bar diagram showing the prevalence of BVDV in cattle of different districts

Discussion

A very few research works have been conducted on BVDV in Bangladesh, we know very little about its epidemiology and most of the farmers of our country are not aware of the disease. They neither vaccinate their animals nor take any preventive measures against this disease.

The study was aimed at the investigation of the prevalence of BVDV antibody in selected areas of Bangladesh. For this, a total of 460 BTM samples were collected from three selected areas, such as Mymensingh, Pabna and Sirajgonj districts of Bangladesh and were subjected to indirect ELISA. The result of this tests are presented as sample to positive (S/P) ratio as were instructed by ELISA kit. The result showed that ubiquitous and infects all the cattle farms of selected areas of Bangladesh with an average prevalence of 88.04%. Among the three selected areas Sirajgonj district had the highest prevalence (92.1%) than those of Pabna (89.4%) followed by Mymensingh (78%).

Natural infection of cattle with HoBi-like viruses or BVDV-3 has been reported since mid 2000s in Brazil, Italy, Thailand and more recently in Bangladesh (Kampa*et al*., 2009; Haider*et al*., 2014). However, there is no information on prevalence of HoBi-like virus in India, a country with the highest cattle population in the world, whereas BVDV-1 has been reported to be widely prevalent and BVDV-2 sporadically (Behera*et al*., 2011).Pabna and Sirajgonj is one of the livestock rich areas regarded as milk producing area in Bangladesh where BVDV might have predisposed for cattle through both vertical and horizontal transmission, because BVDV is transmitted via both horizontally and vertically *(*Brownlie *et al., 1987;*Anne- France *et al*., 2004; Niskanen*et al*, 2003). . This might be a plausible explanation for such a large number of milk samples being positive. If the hypothesis is so, the reason for comparatively lower prevalence in cattle of Mymensingh region has favorable argument.

In present investigation, BVDV antibody was detected in 405 (88.04%) of 460 milk samples. The reports on BVDV prevalence from many parts of the world (Houe, 1999;*Moennig et al., 2005;* Almeida *et al*., 2013; Grousi*et al*., 2008) are highly in agreement with our observation. Recently, several researchers from Asian and European countries have observed high prevalence rate in their cattle herd *(*Kampa *et al., 2009;* Almeida *et al*., 2013; Sasha *et al*., 2014;Foddai*et al*., 2014; Riona*et al*., 2015; Gonzalez *et al*., 2014; *Lanyon et al. 2014;* Santman-Berends*et al*., 2015) indicating BVDV to be an emerging pathogen worldwide. A study which was performed by Houe (1993) showed that there are many possible means of transmission of infection and direct contact with animals persistently infected (PI) with bovine viral diarrhea virus (BVDV) is probably the most important method of transmission of infection. Virus can also be transmitted via body secretions (saliva, nasal secretion, milk, semen etc.) and contaminated fomites (Brownlie*et al*., 1987; Silva *et al*., 1995). Semen of breeding bulls which are used for AI should be tested regularly due to presence of virus genome in semen (Silva *et al*., 1995).

Nevertheless, it should not be forgotten that the number of milk samples were not equal in all events of parameters used in the experiment. This remained a weakness of performing the study of prevalence, which is to be remembered while undertaking such investigation in future. The other parameter such as gender, age as well as breed of individual cattle needs further study.

Conclusion

In this investigation, the average degree of prevalence of BVDV was 88.04%. Prevalence was highest (92.1%) at Sirajgonj followed by Pabna (89.4%) and Mymensingh (78%). Dairy herds in Pabna and Sirajgonj have high levels of antibodies against BVD in BTM samples. Since no vaccination is being practiced in Bangladesh, the high prevalence of BVDV antibodies in cattle indicates the widespread of BVDV infection in Bangladeshi cattle. In view of the present findings, an attempt can be made to isolate, identify and characterize the type of BVDV circulating in Bangladesh. In addition, identification of persistently infected (PI) calves in the herd and their removal might be an important tool for controlling the BVDV infection in this country as the PI calves shed virus in the environment throughout their life.

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Disease

Pro-inflammatory cytokines as indicators of health status around calving in dairy cows

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Abstract:

During transition period the functionality of immune system is considered impaired and this allows the development of many infectious and metabolic diseases in dairy cows. Pro-inflammatory cytokines (PIC) are crucial regulators of immune functions and mediate the acute phase response. The objective of this study is to evaluate whether calving affect per se the release of PIC and to verify the relationship of PIC release and subsequent inflammometabolic variations and the occurrence of clinical problems. Blood samples were collected from 12 Holstein Friesian cows from -7 to 7 days relative to calving and analyzed for plasma interleukin-1 beta (IL1β), interleukin-6 (IL6) and tumor necrosis factor alpha (TNF-α) concentration. Feed intake (DMI) and health status was daily monitored and cows were grouped into healthy (7) and sick (5) with clinical diseases like ketosis, milk fever, mastitis, diarrhea etc in the first week of lactation. A wide metabolic profile was also measured. No significant effect of calving has been observed on the release of PIC, when considered all cows together. The sick vs. healthy animals had significant ($P<0.05$) and tendency of high concentration of TNF-α from -7 to 0.5 days from calving. Sick animals showed the tendency of higher concentration of IL6 from -1 day and reach significant ($P<0.05$) level at 1 day from calving and also showed the tendency of higher IL1β concentration from -2 to 3 days from calving. Moreover, sick vs. healthy cows showed lower DMI and a more severe acute phase response after calving, as supported by the significant ($P<0.05$) higher value of positive acute phase proteins (haptoglobin and ceruloplasmin) and the lower values of negative acute phase proteins (e.g. albumin) and the higher concentration of zinc (index of metallothionein). Results suggest that higher levels of PIC the week before parturition can indicate the health status. Thus, elevated PIC concentrations precede the birth complications and clinical onset of typical diseases in early lactation, and can be used as indicators of these health problems at the end of the gestation.

Key words: Pro-inflammatory cytokines; Peripartum; Post partum, Dairy cows.

Introduction

The periparturient period is widely recognized as a critical time for the health and productivity of dairy cows. The incidence of several metabolic and infectious diseases, such as mastitis, metritis, milk fever, displaced abomasum, ketosis, acidosis, and lameness, is elevated during this period (Drackley, 1999). Total disease incidence after parturition accounts for a substantial proportion of all morbidity on many dairies (Ingvartsen, 2006). It is well documented that cows suffering from one transition disorder are at greater risk for contracting others, including such seemingly unrelated conditions as mastitis and ketosis (Goff, 2006). Thus the multitude of disorders that dairy cows face during the transition to lactation is a perennial source of concern for dairy producers, nutritionists, and veterinarians.

The immune system is depressed in cows around the time of parturition (Goff et al. 2006) and immune functionality is considered as an important factor that may favour the development of different infectious and metabolic diseases in dairy cows. After stimulation by various factors the immune cells produce mediator like cytokines that induce inflammation (Bertoni et al., 2015; Medzhitov, 2008). The PIC (e.g. tumor necrosis factor-alpha (**TNF-α**), interleukin-1beta (**IL-1β**), interleukin-6 (**IL-6**), and interferon-gamma (**INF-γ**)) (Koj, 1998) are released mainly by macrophages and stimulate the liver to synthesize positive acute phase proteins (**pos-APP**), such as haptoglobin, serum amyloid A and ceruloplasmin (Gruys et al., 2005). Concomitantly, PIC impair synthesis of negative acute phase proteins (**neg-APP**), some of them are important for normal

liver metabolism (Bionaz et al., 2007). Indeed, the low neg-APP concentrations (e.g. albumin, lipoproteins, retinol binding protein) have been associated with decreased liver function, reproductive performance, milk yield and increased disease incidences (Bionaz et al., 2007; Bertoni et al., 2008). Moreover, PIC have a role in worsening the anorexic status(Johnson, 1997),condition which often occurring around parturition (Drackley, 1999; Trevisi et al., 2012). Perusal of available data revealed the information regarding the pre-partum level of pro-inflammatory cytokines in relation to the occurance of only postpartum reproductive disease (Ishikawa et al. 2004; Islam et al. 2013a and 2013b). However, Jahan et al., (2013) and Trevisi et al, (2015) reported the highest concentrations of IL-1β, IL-6 in late pregnancy (35 day before calving) has been related with the worst condition in term of health status, metabolic and inflammatory conditions and performances in early lactation. Hence the present study was planned to evaluate whether calving affect per se the release of PIC and to verify the relationship of PIC release and subsequent inflammometabolic variations and the occurrence of clinical problems, which may help in timely management of the cows for the health problems.

Materials and Methods

This study complied with Italian laws on animal experimentation and ethics. The experiment was carried out in the dairy farm of Istituto di Zootecnica, Catholic University of the Sacred Heart, Piacenza, Italy.

Animals and diets

Twelve multiparous Holstein dairy cows in around calving (7 days before and after parturition) were included in this study. Cows were housed in an artificially lighted and ventilated tie-stall. Climatic conditions were maintained under constant settings: environmental temperature around 20°C, relative humidity between 60% to 70%, photoperiod with 14 h of light (from 5:00 to 19:00 h) and 10 h of dark. Cows were individually fed and forages were offered ad libitum: forages every 12 h and concentrates by an auto-feeder every 12 or 3 h in dry and lactating cows, respectively.

On average, dry cows received 9–12 kg of grass hay, 8–10 kg of maize-silage, 1–2 kg of concentrate per day. After calving, the daily diet included 2 kg of grass hay and 3 kg of alfalfa hay, while concentrate and maize-silage were gradually increased (on day 30 of lactation, on average cows received 11–13 kg of concentrate, 18–20 kg of maize-silage). The average composition of diets consumed in the last month of pregnancy and in the first month of lactation has been reported in table 1.

Table 1. Ingredients and main chemical and nutritional traits of the diets fed todry (last week of pregnancy) and lactating (first week of pregnancy) dairy cows

Components, % DM[1]	Dry cows	Lactating cows
Grass hay	75.0	11.9
Alfalfa hay	–	11.3
Corn silage	15.3	23.0
Soybean meal	8.8	–
Minerals and vitamins supplementation[1]	0.9	0.8
Commercial concentrate	–	53.0
Chemical composition of total diet		
[2]NEL, Mcal/kg DM	1.26	1.57
[3]CP, % DM	11.9	17.6
[4]NDF, % DM	48.4	34.6
Starch + sugar, % DM	10.6	29.4
Ether extract, % DM	1.6	4.70

[1]DM, dry matter; [2]NEL, net energy lactation; [3]CP, crude protein; [4]NDF, neutral detergent fibre; mineral and vitamin supplement provided at each cow every day: 150000 U vitamin A; 20000 U vitamin D; 100 mg vitamin K;750 U vitamin E; 0.5 mg vitamin B12; 500 mg vitamin PP; 2 mg Co; 20 mg I; 350 mg Mn; 80 mg Cu; 3 mg Se; 800 mg Zn); 15 g yeast (Yea- Sacc,$^{102.6}$, Alltech Italia S.r l., Bologna, Italy).

Sample collection

Blood samples were collected from each cow in the morning before feeding on -7, -2, -1, 0, 1, 2, 3, 7 days from parturition (DFP). For the day of calving (day 0), the samples were collected within 3 hours before the labour (day -0.1; the cows were closely monitored for the behavioural signs and the body temperature to estimate the expected the time of calving); immediately after parturition (day 0); 4 hours after parturition (day0.1); 12 hours after parturition (day 0.5). All the blood samples were collected from the jugular vein into evacuated tubes containing Li-heparin as an anticoagulant (Vacuette, Kremsmünster, Austria) and immediately placed in ice water until arrival in the laboratory. Within one hour of collection, small amount of blood was used for packed cell volume determination (Centrifugette 4203, ALC International Srl, CologneMonzese, Italy); the remainder was centrifuged at 3,500 × g for 15 min at 4°C and the plasma was frozen in several aliquots at −20°C until analyses.

DMI and health problems

During the experiment, the animal's health conditions were checked every day by general inspections. All cows were treated at drying-off with a dry-cow intramammary preparation (penethamatehydroiodide, bentamin penicillin, framycetin sulphate; Mamyzin®, Boehringer Ingelheim Italy S.p.A.) and intramuscular treatments with tylosin (Eli Lilly, Sesto Fiorentino, Italy) (Trevisi et al., 2010). During the trial clinical inspections (rumination activity; fecal consistency; uterine discharge; presence of injuries, lameness or joint swellings; milk yield and presence of milk clots) were carried out daily on all cows, whereas rectal temperature was measured every morning at feeding time (8.00). All cows were routinely vaccinated against bovine viral diarrhea, infectious bovine rhinotracheitis, parainfluenza type 3 and bovine respiratory syncytial viruses by a live attenuated vaccine (CattleMaster 4, Pfizer Animal Health, Exton, PA, USA) every 6 months except in the 4 weeks before and after calving.

The DMI was measured daily by weighing differences of the supplied feed and the orts. The feed dry matter content was routinely assessed during the trial by the sampling of hay and concentrate at every lot change and maize-silage at every 4 weeks from the bunker silo.

Blood analysis

Blood samples were analysed to assess the following metabolites: i) inflammatory response indices: pos-APP (haptoglobin, ceruloplasmin) and neg-APP (albumin, cholesterol as lipoprotein index); ii) liver indices: total bilirubin, aspartate amino-tranferase (**GOT**), γ-glutamiltransferase (**GGT**), alkaline phosphatase (**ALP**), paraoxonase (**PON**); iii) energy metabolism indices: glucose, NEFA, BHBA; iv) protein metabolism indices: urea, creatinine; v) oxidative stress response index: total reactive oxygen metabolites (**ROMs**); vi) minerals (Ca, P, Mg, Na, K, Cl, Zn); vii) vitamins: retinol (index of its carrier protein), tocopherol, β-carotene; viii) other parameters (total proteins, globulins).

Methods used for the determination of these parameters were previously described by Bertoni et al. (2008). Briefly, glucose, total protein, albumin, total cholesterol, total bilirubin, creatinine, urea, Ca, P, Mg, GOT, GGT and ALP were detected at 37°C by a clinical auto-analyzer (ILAB 600, Instrumentation Laboratory, Lexington, MA, USA) using commercial kits purchased by Instrumentation Laboratory (IL Test). Globulins were calculated as the difference between total protein and albumin. Electrolytes (Na+, K+, and Cl−) were detected by the potentiometer method (Ion Selective Electrode connected to ILAB 600). Zn and NEFA were determined by commercial kits (Wako Chemicals GmbH, Neuss, Germany). ROMs were measured using a method patented by Diacron International S.r.l. (Grosseto, Italy). BHBA was determined by commercial kit Ranbut (Randox Laboratories Ltd., Crumlin, Co. Antrium, UK).

Haptoglobin, and ceruloplasmin were analysed using methods proposed by Bertoni et al. (2008) adapted to ILAB 600 conditions. Plasma PON activity was assessed by adapting the method of Ferré et al. (2002) to the ILAB 600. Plasma retinol, α-tocopherol and β-carotene were extracted with hexane and analysed by reverse-phase HPLC using Spherisorb ODS-2.3µm, in a 150 × 4.6 mm column (Alltech, Deerfield, IL, USA); a UV detector set at 325 nm (for retinol) or 290 nm (for α-tocopherol); 460 nm (for β-carotene) and 80:20 methanol: tetrahydrofurane as the mobile phase.

Cytokine Assay

The concentrations of IL-1β, IL-6 and TNF-α were analysed by commercially available ELISA (Enzyme-Linked ImmunoSorbent Assay) kit screening set specific for bovine. For IL-1β and IL-6 were used the Elisa Screening Set (Pierce, Thermo Scientific, Meridian Rd., Rockford, U.S.A.) and for TNF-α was used the Bovine TNF-alpha DuoSet ELISA (DY2279, R&D Systems, Inc; Minneapolis, Stati Uniti). All the samples were analysed in duplicate. For the IL-1β, IL-6 and TNF-α, the intra assay coefficients of variation ranged between 3.5 and 5 and inter assay coefficients of variation ranged between 13 and 17%.

Data Handling and Groups

To investigate the effect of plasma concentration of cytokines on health status, blood parameters and DMIthe 12 cows were retrospectively divided in two groups: healthy (7 cows) with no clinical signs of disease, and sick (5 cows) with clinical diseases like ketosis, milk fever, mastitis, diarrhea etc in the first week of lactation.

Statistical Analyses

Statistical analyses were performed with SAS software (SAS Inst. Inc., Cary, NC; release 8.0). These data were subjected to ANOVA using the REPEATED statement in the MIXED procedure of SAS. The statistical model for plasma parameters included the fixed effect of groups (Healthy and Sick), day from parturition =DFP (-7, -2, -1, -0.1, 0, 0.1, 0.5, 1, 2, 3, 7and 28) and interaction between groups and DFP. For all the parameters the random variable was cow within the groups. Each parameter was tested for normal distribution using UNIVARIATE procedure of SAS and normalized when necessary, using either natural logarithmic (IL-1β, IL-6, glucose, cholesterol, urea, magnesium, GOT, GGT, bilirubin, haptoglobin, tocopherol, somatic cell count) or cubic (calcium) conversion, to reach the proper kurtosis and skewness values. Each parameter was subjected to 4 covariance structures: first order autoregressive, compound symmetry, spatial power, toeplitz, and the best covariance structure was retained (Littell et al., 1998).

Result

Pro-inflammatory cytokines

The plasma concentration of TNF-α, IL-1β and IL-6 from -7 DFP to 7 DFP in all cows around calving and mean values of plasma concentrations of TNF-α, IL-1β and IL-6 in healthy and sick cows from -7 DFP to 7 DFP are shown in Figure 1 and Table 2 respectively.The calving time did not affect the plasma concentrations of TNF-α, IL-1β and IL-6 besides a short lasting pick in the sample collected after the calving (within 24 hours following) when considered all cows together (Figure 1).

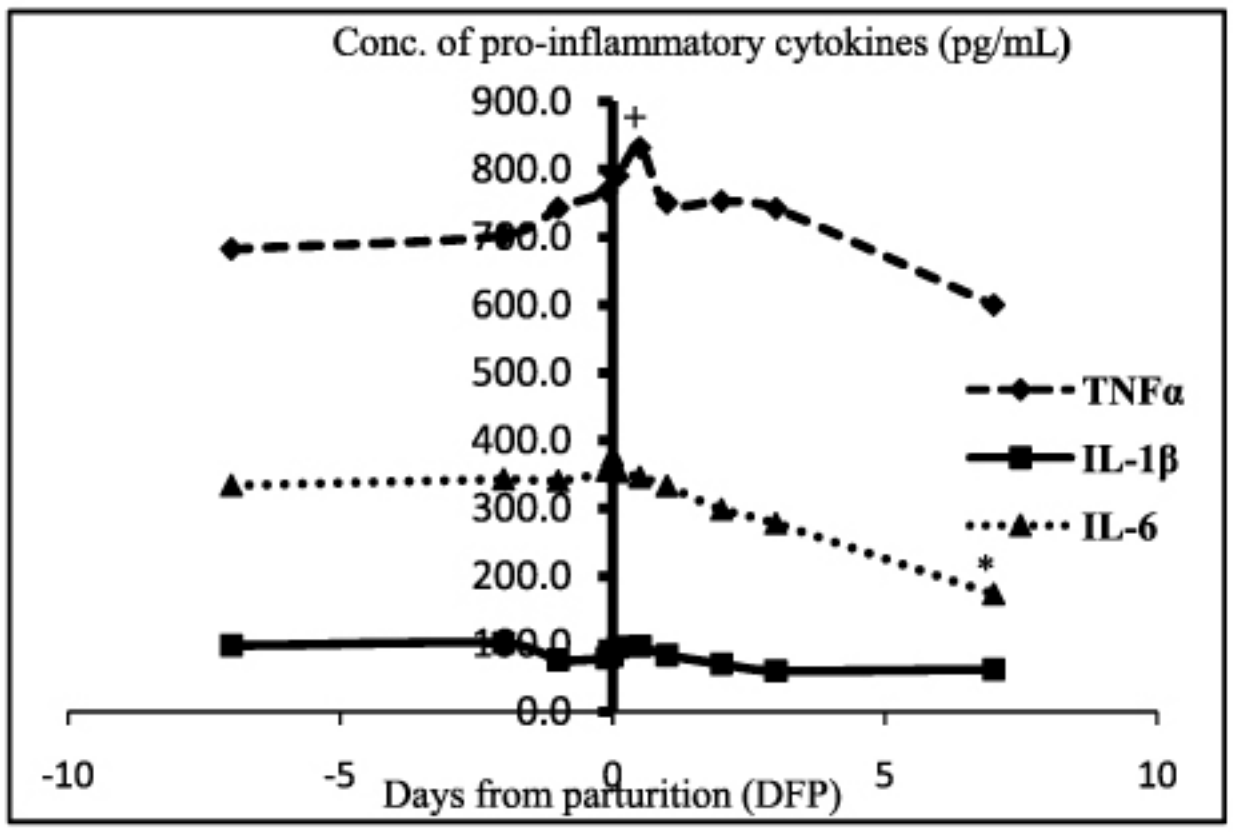

Figure 1. Plasma concentrations of TNF-α, IL-1β and IL-6 in all cows around calving. Statistically significant differences between each day from parturition (DFP) and -7 DFP is shown by *= ($P < 0.05$) and += ($P < 0.1$).

Grouping the animals according to health status after calving the sick vs. healthy animals showed significant (P<0.05) and tendency of high concentration of TNF-α from -7 to 0.5 days from calving. Sick animals showed the tendency of higher concentration of IL6 from -1 day and reach significant (P<0.05) level at 1 day from calving and also showed the tendency of higher IL-1β concentration from -2 to 3 days from calving (Table 2).

Table 2. Mean values of plasma concentrations of TNF-α, IL-1β and IL-6 in healthy and sick cows from -7 DFP to 7 DFP.

Parameters	Group	[a]DFP											SEM	Significance level		
		-7	-2	-1	-0.1	0	0.1	0.5	1	2	3	7		Problem	DFP	Problem*DFP
		*	*	*	+			+								
[b]TNF-α	healthy	452.7	500.7	518.6	577.0	642.9	635.3	658.7	594.6	587.0	599.5	484.7	246.03	0.0977	0.0082	0.745
	sick	1004.1	984.4	1057.2	1034.0	1006.9	1007.3	1074.3	968.3	985.4	941.6	761.2				
			+	+				+	+		+					
[c]IL-1β	healthy	67.3	50.9	36.8	40.6	43.4	56.9	36.0	35.7	40.2	33.8	39.5	66.90	0.129	0.0398	0.7354
	sick	140.4	171.9	132.8	133.5	137.6	149.1	181.7	150.5	113.3	97.5	94.8				
				*	+	+	+	*	*							
[d]IL-6[d]	healthy	263.9	268.2	250.8	270.8	288.2	269.3	253.7	237.7	227.5	216.67	143.6	113.17	0.0861	0.0057	0.9472
	sick	431.6	447.3	466.2	475.4	501.7	470.8	475.6	466.1	397.7	363.2	215.1				

For each parameter, a significant difference between groups at the same day from parturition is shown by *= (P < 0.05) and += (P < 0.1).
[a]DFP= Days from parturition, [b]Interleukin-1 beta =IL1β, [c]Interleukin-6 =IL6 [d]Tumor necrosis factor alpha =TNF-α

Inflammometabolic profiles and DMI

The mean values of plasma concentrations of ceruloplasmin, haptoglobin, albumin and zinc in healthy and sick cows from -7 DFP to 7 DFP are shown in Table 3.The pos-APP (haptoglobin and ceruloplasmin) showed significant (P<0.05) higher value and the lower values of negative acute phase proteins (e.g. albumin) and the higher concentration of zinc (index of metallothionein) after calving in sick cows compared to healthy one. The sick cows also showed lower DMI.

Table 3. Mean values of plasma concentrations of ceruloplasmin, haptoglobin, albumin and zinc in healthy and sick cows from -7 DFP to 7 DFP.

Parameters	Group	DFP[a]											SEM	Significance level		
		-7	-2	-1	-0.1	0	0.1	0.5	1	2	3	7		Problem	DFP	PROB*DFP
			+	*					+	*	*	**				
Ceruloplasmin, µmol/L	healthy	2.62	2.49	2.47	2.58	2.70	2.74	2.78	2.88	3.05	3.13	3.31	**0.2942**	0.0557	<.0001	0.3386
	sick	2.95	3.02	3.11	3.06	3.11	3.12	3.19	3.42	3.79	3.83	4.18				
											*					
Haptoglobin, g/L	healthy	0.16	0.18	0.13	0.20	0.17	0.19	0.35	0.60	0.72	0.52	0.48	**0.1697**	0.5994	<.0001	0.3123
	sick	0.17	0.26	0.25	0.20	0.18	0.19	0.30	0.50	0.74	0.90	0.65				
					+	+	+	*		*	*	**				
Albumin, g/L	healthy	36.6	36.4	36.0	36.8	37.3	36.7	36.8	36.0	36.1	35.5	36.3	**1.3305**	0.0306	0.2605	0.7218
	sick	35.5	34.7	34.8	34.5	35.1	34.5	33.9	34.1	33.2	32.2	32.5				
									*	*		+				
Zn,µmol/L	healthy	12.35	12.13	11.64	11.12	10.65	8.72	8.30	9.26	10.69	11.79	13.48	**1.3911**	0.0712	<.0001	0.7731
	sick	12.48	10.72	10.18	9.06	9.56	7.41	6.46	6.48	7.75	10.33	11.13				
	healthy									*	**					
DMI[b], Kg/day	sick		13.10	11.75	10.54		10.06		11.66	13.52	14.45	16.43	**1.1237**	0.0300	<.0001	0.5435
			12.6	11.2	9.8		10.1		10.3	11.1	11.4	14.94				

For each blood parameter, a significant difference between groups at the same day from parturition is shown by is shown by *= ($P < 0.05$) and += ($P < 0.1$).

[a]DFP: Days from parturition [b]Feed intake= DMI

Discussion

Immune alterations and inflammatory conditions are signaled in the peripartum period, and often without clinical symptoms. Understanding the cause of this phenomenon is very relevant to improve the health and welfare of dairy cows. Reports on the changes of PIC concentrations, during the transition period in dairy cows are very few and until now it was erroneously assumed that their changes in plasma were very quick and difficult to detect. Though Jahan et al., (2013), Trevisi et al. (2015) and Jahan et al., (2015) depicted the changes of IL-1β and IL-6 concentrations during the transition period. In this context to observe the condition around calving we measured the changes of TNF-α, IL-1β and IL-6 one week before to one week after calving and tried to correlated this value with health status of cows after calving.

Effect of calving on plasma concentration of PIC:

The concentration of PIC showed no significant increase around calving besides a short lasting increase in the sample collected after the calving (within 24 hours following). This increase was not statistically different in comparison to previous days, but it is in agreement with the results of Trevisi et al. (2015) who observed the same having the study period -35 to 28 DFP and suggested a proper evaluation of the calving effects on PIC production in dairy cows with more frequent samplings around parturition. Hebisch et al. (2004) also measured IL-1β and IL-6 in women during pregnancy, delivery and early puerperium. Moreover, our data also partially agree with Johnson et al. (2013), who observed an increase of the IL-1β expression in the leukocytes collected from heifers within 24 h after calving.

Relationship of plasma concentration of PIC, health status and DMI:

Several studies were focused to identify inflammatory andoxidative stress markers able to predict clinical events in periparturient dairy cows (Ishikawa et al., 2004; Islam et al., 2013a, 2013b), but the results often do not agree. Our results on post-partum clinical problems agree with Trevisi et al. (2015) and Jahan et al. (2013) who observed the cows with the highest concentrations of IL-1β in dry period showed the worst condition in term of health status, metabolic and inflammatory conditions and performances in early lactation. Our results on post-partum clinical problems are partly in agreement with Ishikawa et al. (2004), who showed the high concentrations of IL-6 in pre-partum were able to predict post-partum reproductive disorders. Our result also partially agree with Kasimanickam et al. 2013 who found postpartum dairy cows diagnosed with metritis, clinical endometritis, or subclinical endometritis had higher serum concentrations of TNF-α, IL-1β and IL-6 1 week before calving to 7 week postpartum compared to normal cows. In contrast, Islam et al. (2013a) recently suggested that low concentrations of IL-1β at 15 d prior or at calving might be used to identify the cows susceptible to develop post-partum reproductive diseases. In another experiment, Islam et al. (2013b) claimed that increased concentration of the IL-10 (an anti-inflammatory cytokine) at 15 d pre-partum could be a good predictor of retained placenta and metritis. Unfortunately, the data of Islam et al. (2013a, 2013b) are difficult to compare with our results because in these studies no inflammatory or metabolic markers in plasma were investigated. In agreement with our study El-Deeb et al. (2017) found a significant increase in the levels of IL-1β, IL-6, TNF-α dairy cows affected with postpartum ketosis compared to the normal cow and suggested the PIC and APP (haptoglobin, Serum Amyloid A, Fibrinogen, ceruloplasmin, α1- acid glycoprotein) as promising biomarkers for ketosis in dairy cows in the postparturient period.The sick cows, which have high concentration of PIC before calving, also showed lower DMI. More consistent appear researches, which have investigated concentrations of PIC and the feed intake. PIC have a profound behavioral, neuroendocrine, and metabolic effect and act in the brain to reduce food intake in laboratory animal species (Kasimanickam et al. 2013; Plata-Salamán, 2000; Johnson, 1997). Our result agreed with Trevisi et al. (2015) who also found cows with the higher concentration of pro-inflammatory cytokines before calving showed a lower DMI for the whole period and a higher frequency of mastitis. In chicks, a series of experiments demonstrated that a variety of inflammatory agents reduced feed intake and efficiency of gain, increased body temperature and plasma corticosterone (Klasing et al., 1987). In cows with induced ketosis (Loor et al., 2007) the increased expression of IL-6 may have been causally involved in the reduced DMI. In overall these data agree with our research.

Relationship of acute phase reactants, PIC and health status

Sick cows showed a more severe acute phase response after calving, as supported by the significant higher value of positive acute phase proteins (haptoglobin and ceruloplasmin) and the lower values of negative acute phase proteins (e.g. albumin) and the higher concentration of zinc (index of metallothionein) indicate more severe inflammation in these cows after calving which agree with the results of (Bionaz et al., 2007; Bertoni et al., 2008; Abdul-Aziz, 2008). As a result of the inflammation, proinflammatory cytokines may release and stimulate the release of acute phase proteins from macrophages or other organs.

Conclusion and Implications

The present study demonstrates no significant effect of calving on the release of PIC, when considered all cows together. Cows having higher levels of PIC at the week before parturition show clinical diseases and more severe acute phase response after calving. Therefore, PIC in accordance with acute phase proteins could be used as promising indicator of postpartum diseases in dairy cows at the end of the gestation. Measurement of the changes of the PIC concentration may assist in early detection and treatment of sick cows.

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Pathological investigation of trachea and lungs of buffaloes in Gazipur district

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Abstract

Pathological conditions of trachea and lungs of slaughtered buffaloes were investigated in the present study. A total of 50 slaughtered buffaloes were examined individually and trachea (80) and lungs (80) were collected for histopathology from the slaughterhouses of Gazipur City Corporation, Gazipur, Bangladesh, during the period from July 2016 to March 2017. Among them 9 trachea and 30 lungs were found to be apparently abnormal in naked eye. Grossly, tracheal and lungs lesions were 11.25% and 37.5% in buffaloes, respectively. The gross tracheal lesions were petechial haemorrhages on the mucosa of trachea and haemorrhage and congestion in lungs (16.25%), hard nodule formation (7.5%), cyst in lungs (6.25%), emphysematous lung (5%) and thickened pleura (2.5%). In histopathology, congestion (5%) and mild tracheitis (8.75%) were recorded as tracheal lesions. Histopathological examination of lungs noticed a number of conditions or lesions such as chronic interstitial pneumonia (3.75%), edema fluid (3.75%), broncho-pneumonia (3.75%), bronchitis (2.5%), bronchiolitis (2.5%), pneumonia-congested stage (8.75%), hydatid cyst (6.25%), fibrinous pneumonia (3.75%), healed nodule (7.5%), purulent broncho-pneumonia (1.25%), fibrino-purulent pneumonia (1.25%), subacute fibrinous pneumonia (1.25%), pulmonary adenomatosis (1.25%), pleuritis (2.5%) and hemosiderosis (3.75%). For morphological descriptions, the lesions were categorized into various types; however a single section showed intermixed of various lesions.

Keywords: Pathology, trachea, lungs, buffaloes

Introduction

In recent years, the buffalo commonly known as an "Asian Animal" has attracted global concern. The buffalo is the dairy, draught and meat animal of Asia. In Bangladesh buffalo has been addressed and remained neglected species despite their important role in the national economy (2016). The domesticated buffaloes are mainly found in the coastal areas of Asia mainly India, Malaysia, Singapore, Philippines, Thailand, Indonesia, Southern-China and other countries of the Far East. Total world buffalo populations are 138.37 million among which in India (75 million), China (20 million), Pakistan (13 million), Thailand (6 million) (Banerjee, 1998) and Bangladesh (14.71 lakh) (BBS, 2016). The diseases of the respiratory system, either acute or chronic, cause debility and death leading to great economic loss. This loss can be minimized by prevention and treatment of the diseases, affecting the respiratory system, after proper diagnosis. In Bangladesh, few works have been performed on some aspects of buffalo diseases (Dewan *et al.,* 1979; Hossain and Baki, 1987; Hossain, 1990; Hossain *et al.,* 1991) but no systemic works have been performed on buffalo respiratory system. Therefore, the present investigation was undertaken to study the pathological lesions of trachea and lungs of buffaloes after slaughter.

Materials and Methods

Collection of samples

A total of 160 (trachea = 80, lungs = 80) samples were collected from buffaloes after slaughter from two slaughterhouses of Gazipur City Corporation, Gazipur, Bangladesh, during the period from July 2016 to

March 2017. After slaughter, gross tissue changes were observed and recorded carefully by observation in naked eye. Besides, samples were collected in 10% buffered neutral formalin for histopathology. All the samples were transferred to Histopathology Laboratory and Bacteriology Laboratory of the Department of Pathobiology, Bangabandhu Sheihk Mujibur Rahman Agricultural University, Gazipur for histopathology.

Histopathology

The tissue samples were trimmed properly and fixed for 24 h more. To remove the fixative, the tissues were kept in running tap water for overnight. The tissues were dehydrated in ascending grades of alcohol. The tissues were cleared by two changes in chloroform. The tissues were embedded with molten paraffin wax. Paraffin blocks containing tissue pieces were made using templates. The tissues were sectioned with a rotary microtome at 5µm thickness. Then the sections were allowed to spread on warm water bath (45^0C) and taken on oil- and grease- free glass slide. A small amount of gelatin was added to the water bath for better adhesion of the sections to the slide. The slides containing sections were air dried and kept in cool place until staining. The sectioned tissues were deparaffinized in three changes of xyline. Then the tissues were rehydrated through descending grades of alcohol. The sections were stained with Harris hematoxylin and eosin. Finally the sections were mounted with cover slip using DPX and dried (Luna, 1968).The tissues were examined under microscope and photomicrograph was taken using compound microscope and photomicrographic camera (Olympus PM-C 35 Model).

Results and Discussion

A total of 80 buffaloes respiratory tract (trachea and lung) were examined and of them 09 trachea (out of 80) and 30 lungs (out of 80) were found to be apparently abnormal in naked eye. The occurrence of tracheal petechial haemorrhages was recorded 11.25% (Table 1) and histopathologically, the occurrence of different tracheal lesions diagnosed under microscope is shown in Table 2. Overall tracheal lesions were 13.75% of which congestion was 5% and mild tracheitis was 8.75%. The similar tracheal haemorrhage was described by Radostits *et al.* (2002) and Jubb *et al.* (1997) but some other lesions like tracheal edema, obstruction of trachea, pustular lesion were also described by Radostits *et al.* (2002).

The gross lesions lungs were 37.5% in buffaloes (Table 1). Grossly, five types of lung lesions were recorded such as haemorrhages and congestion in lungs (16.25%), hard nodule formation (7.5%), cyst in lungs (6.25%), emphysematous lungs (5%) and thickened pleura (2.5%) (Table1). The overall gross lesions of lung recorded were 37.5%. The similar lesions were also described by many authors (Hussain, 1992; Jones *et al.,* 1997; Jubb *et al.*, 1997; Radostits *et al.,* 2002). Some other gross lesions like black coloured lungs, pus in lungs were recorded by Jubb *et al.* (1997) and Radostits *et al.* (2002), but no such lesions were recorded in the present investigation.

Histopathologically, different types of pneumonic lesions and some other lesions were found in the lungs (Table 2). Chronic interstitial pneumonia was 3.75% which was characterized by the thickened alveolar septae due to accumulation of inflammatory cells and proliferation of fibrous connective tissue. Inflammatory cells were macrophages containing hemosiderin pigments and lymphocytes. Exudates and hemorrhage was present within the alveoli (Fig. 1). Edema fluid was found in 3.75% cases and it was characterized by presence of pink color edematous fluid in between lung alveoli and inflammatory cells mainly neutrophils. Broncho-pneumonia was 3.75% and its features were the presence of exudates and infiltration of inflammatory cells in the bronchiolar wall as well as in the lumen of bronchiole. There was also presence of exudates within the alveoli and showed slight necrosis of the alveoli (Fig. 2). Bronchitis was 2.5% in cases and it was characterized by the presence of exudates in most of the lumen of the bronchus. Infiltration of inflammatory cells was found in and around the bronchial wall. There was presence of haemorrhage in some alveoli and congestion of blood vessels. Bronchiolitis which was characterized by the infiltration of reactive cells in and around the bronchiolar wall and necrotic lesions in mucosal epithelia was recorded in 2.5% cases. In other areas of lung tissue, there was presence of haemorrhage in the alveoli and congestion of blood vessels. Congested stage of pneumonia was 8.75% and it was characterized by the presence of excessive hemorrhage within the alveoli and inters alveolar septa associated with leukocytic infiltration. Haemorrhage and leukocytic infiltration were also found in the lumen of the bronchus. The wall of the bronchus also showed the inflammatory changes and mild necrosis in the mucosal layer. In some places, only pink colour

edema fluid was present in the lumen of the alveoli. Hydatid cyst was noticed in 6.25% cases while fibrinous pneumonia, healed nodule, purulent broncho-pneumonia, fibrino-purulent pneumonia, subacute fibrinous pneumonia, pulmonary adenomatosis, pleuritis and hemosiderosis were also recorded here in 3.75%, 7.5%, 1.25%, 1.25%, 1.25%, 1.25%, 2.5% and 3.75% cases, respectively. Hydatid cyst was characterized by the cyst wall was composed of proliferation of fibrous connective tissue with infiltration of mononuclear cells and few eosinophils (Fig. 3). In fibrinous pneumonia, there were presences of collagen fiber associated with few reactive cells. In addition, reactive cells were also found in the lumen of bronchiole and some in alveoli and inter-alveolar septa. There was diffuse alveolar necrosis and edema in the inter-alveolar septa and sloughing of bronchial mucosa. Healed nodule was characterized by the nodular areas showed excessive proliferation of fibrous connective tissue indicating the feature of healing. In addition, multifocal nodules, characterized by the presence of lymphocytes, macrophages and giant cells were found (Fig. 4). Purulent broncho-pneumonia was characterized by the presence of inflammatory cells consisted of mainly neutrophils within the lumen of bronchiole. Adjacent to the bronchioles, there were focal infiltration of large number of neutrophils and few lymphocytes. On the other part of the lung tissue, there were the presence of plasma cells, macrophages and lymphocytes (Fig. 5). Presences of neutrophil, collagen fiber, macrophage, edema fluid and hemosiderin pigment were found in fibrino-purulent pneumonia. Subacute fibrinous pneumonia was characterized by the presence of immature collagen fiber associated with few reactive cells. The glandular pattern of growth due to pneumocyte type-II cells proliferation was noticed in pulmonary adenomatosis. The presence of edema fluid and inflammatory cells indicated pleuritis while hemosiderosis was characterized by the presence of yellow coloured hemosiderin pigment in the macrophages and RE cells (Fig.6). For morphological descriptions, the lesions were categorized into various types; however a single section showed intermixed of various lesions. The overall histopathological lesions of lungs were recorded 53.75%. Like the present investigation, similar lesions have been described by other authors (Haridy *et al.,* 2006; Manandhar *et al.,* 2006; Jones *et al.,* 1997; Jubb *et al.*, 1997; Radostits *et al.,* 2002; Gogolewski *et al.,* 1987; Gupta *et al.,* 1978; Verma and Kalra, 1974; Rathore and Singh, 1970; Dwivedi, 1968).

Table 1. Gross lesions of trachea and lungs of buffaloes

Organs	Findings	Disorder/conditions (n=80)	
		No.	%
Trachea	Petechial hemorrhages in trachea	09	11.25
Lungs	Haemorrhages congestion in lungs	13	16.25
	Hard nodule formation	6	7.5
	Presence of cyst in lungs	5	6.25
	Emphysematous lungs	4	5
	Thickened pleura	2	2.5
	Total	30	37.5

Table 2. Histopathological lesions of trachea and lungs of buffaloes

Organs	Findings	Disorder/conditions (n=80)	
Trachea		No.	%
	Congestion	04	5
	Mild tracheitis	07	8.75
	Total	11	13.75
Lungs	Chronic interstitial pneumonia	03	3.75
	Edema fluid	03	3.75
	Broncho pneumonia	03	3.75
	Bronchitis	02	2.5
	Bronchiolitis	02	2.5
	Pneumonia congested stage	07	8.75
	Hydatid cyst	05	6.25
	Fibrinous pneumonia	03	3.75
	Healed nodule	06	7.5
	Purulent bronchopneumonia	01	1.25
	Fibrino-purulent pneumonia	01	1.25
	Subacute fibrinous pneumonia	01	1.25
	Pulmonary adenomatosis	01	1.25
	Pleuritis	02	2.5
	Hemosiderosis	03	3.75
	Total	43	53.75

n= Total number of samples examined.

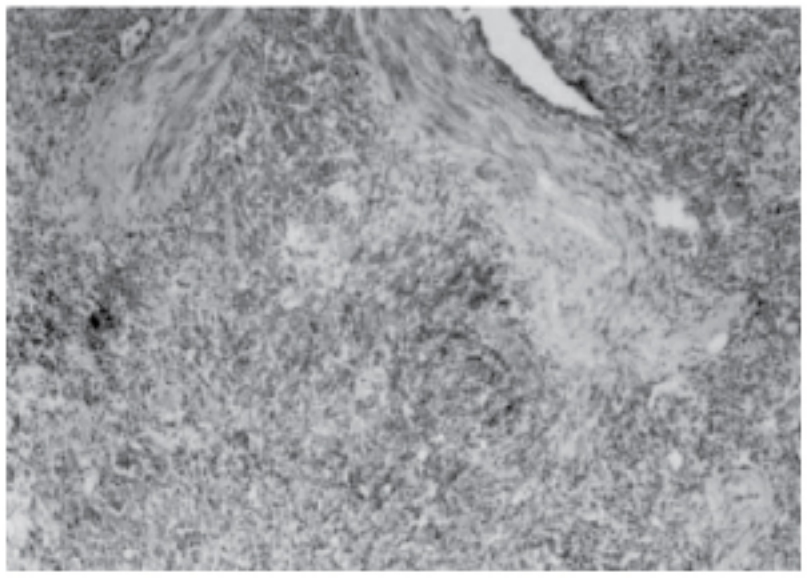

Figure 1. Section of lung showing chronic interstitial pneumonia: Note the inflammatory cells are macrophages containing hemosiderin pigment and lymphocyte (H & E, × 83)

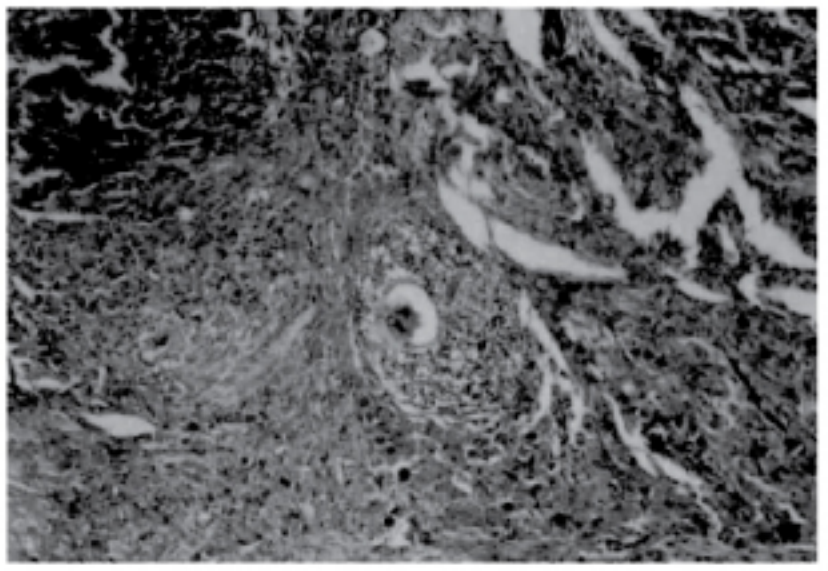

Figure 2. Sections of lung showing broncho-pneumonia: Note the inflammatory cells present in and around the bronchial wall and exudates present within the alveoli (H & E, × 330)

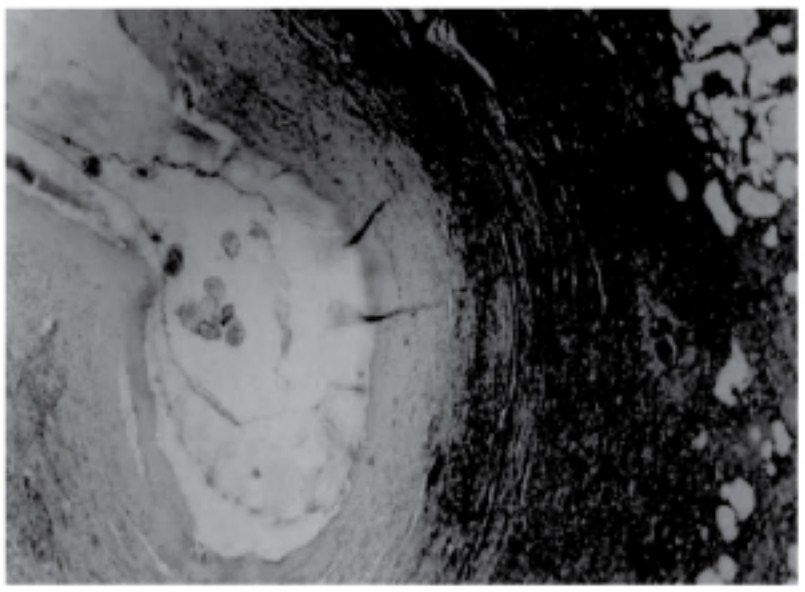

Figure 3. Section of the cyst wall in lung showing infiltration of inflammatory cells and proliferation of fibrous connective tissue around the cystic wall (H & E, × 330)

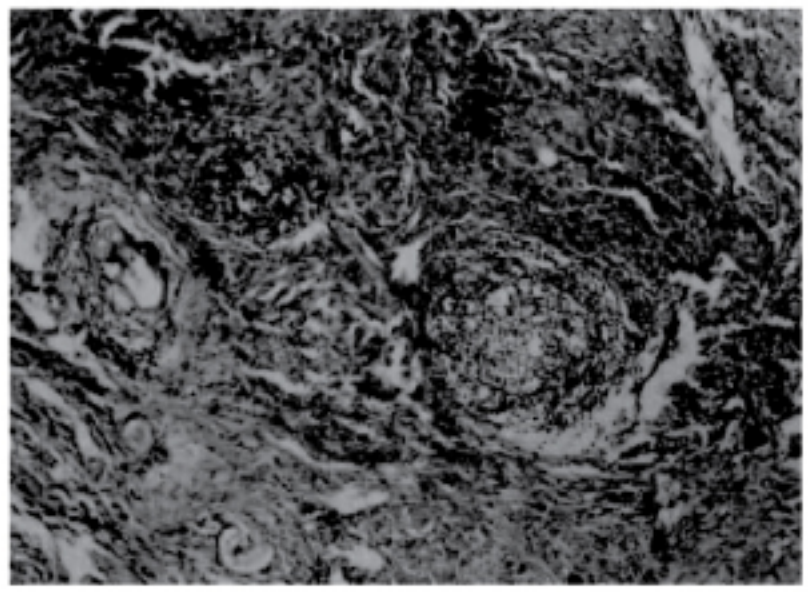

Figure 4. Section of lung showing healed nodule: Note the excessive proliferation of fibrous connective tissue with the presence of multifocal nodules characterized by the presence of lymphocytes, macrophages and giant cells (H & E, × 83)

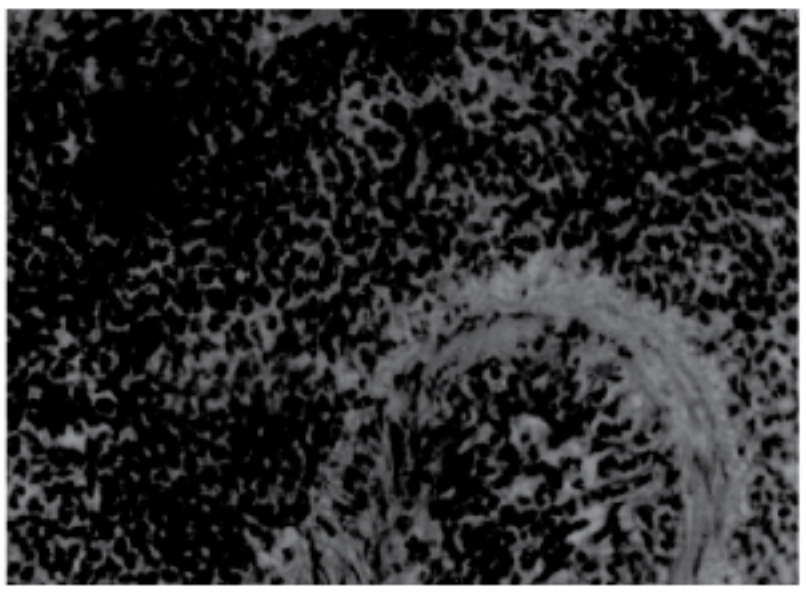

Figure 5. Section of lung showing purulent bronchopneumonia: characterized by the presence of inflammatory cells mainly neutrophils within the lumen of bronchiole. Adjacent to the bronchioles, presence of neutrophils and lymphocytes. On the other part of the lung tissue, there is the presence of plasma cells, macrophages and lymphocytes (H & E, × 330)

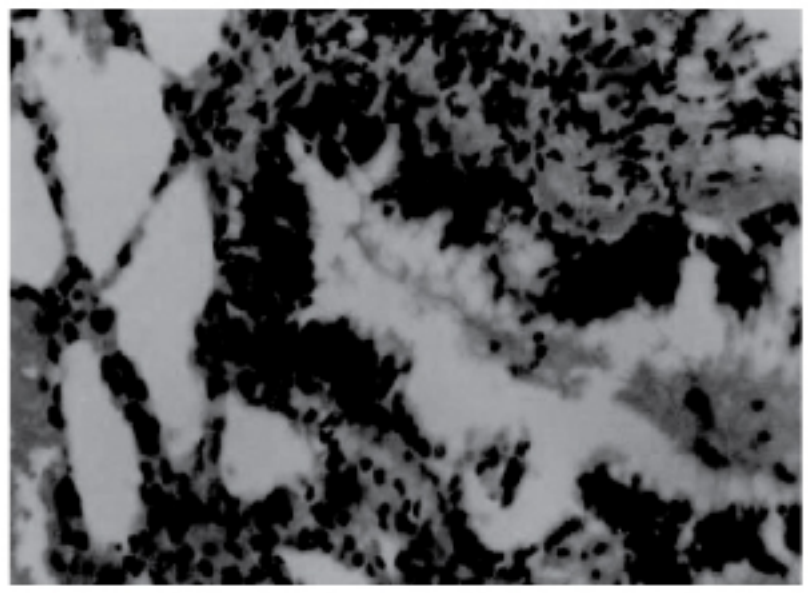

Figure 6. Section of lung showing hemosiderosis: characterized by the presence of yellow coloured hemosiderin pigments in the macrophages and RE cells (H & E, × 83)

Conclusion

Though the buffalo is an important part of livestock in Bangladesh, there is no documented research studies so far that investigated the scopes and opportunities of buffalo production in Bangladesh. The findings of this investigation will help the veterinarian to know the nature and type of common pathological lesions of respiratory system of buffaloes.

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Investigations on Retained Placenta in Dairy Cows and observation of its prevention with common drugs

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Abstract

The study was undertaken to investigate the incidence retention of placenta of dairy cows in relation to breed, age, body weight, body condition score, parity, farm type, farm size, housing system, floor type, feed quality, educational qualification and farming experience of farmer, breeding system, time of parturition and dystocia. A total of 1205 dairy cows from 9 (nine) upazilas and four metro thana of Rajshahi district were selected for the study. The effect of some prophylactic drugs on retention of placenta was evaluated on 100 dairy cows at late stage of pregnancy from 23 selected farms over a period of one year. The data were collected directly from individual dairy cow owner by using questionnaires from Rajshahi district and from official record book of cattle improvement and dairy farm of Rajabarihat at Rajshahi and from private dairy farm. The prophylactic measure applied with the history of retained placenta. The treatment was done in differently: in Group I (n=20) Oxin® (oxitocin synth. 10 iu/ml) @ 5 ml/ cow (i.m.); Group II (n=20) Metherspan® (methyleargometrine meleate 0.200 mg/ml) @ 5 ml/ cow (i.m.); Group III (n=20) Dinoprost® (dinoprost as trometamol 5 mg/ml) @ 5 ml/ cow (i.m.); Group IV (n=20) Utoklin® (herbal patent drugs) @ 450 ml/ cow (orally); Group V (n=20) placenta expelled spontaneously within 6 to 12 hours (control). The overall incidence of retained placenta was 13.4%. The highest incidence of retained placenta were observed in cross breed (10.7%) cows particularly in L ×SL genotype (4.6%), <3 years of age (4.5%). Recovery rate from retained placenta was always better in treatment groups. It was two to five times better then the control group. The recovery rate from retained placenta among prophylactic measure of dairy cows at the time of parturition is according to about 2, 3, 5 and 2 times better than the Control group.

Key words: Retained Placenta, Dairy Cows and Common drugs.

Introduction

Under normal circumstances, in dairy cows the placenta should be expelled within half an hour to 12 hours of birth. The reduced uterine contraction (the reduced force to push it out) 12 hours after calving, results in retained placenta. According to Kay (1978) retention of placenta in cows may be defined as failure of the fetal placenta (tufts) to separate from maternal placenta (crypts), and retention for more than 12 hours after parturition. The incidence of retention of placenta is much more common in dairy breeds than in beef breeds. Among the dairy breeds much common in crossbred than their pure breed counterparts (1986). The incidence of retained fetal membrane appears to be varying from area to area and from year to year (Grunert, 1986; Paisley *et al.,* 1986; Younguist and Threlfall, 2007). A higher incidence rate was observed in younger cows than older one. Aged cows showed a higher incidence (Sarder, 2008) of retained fetal membrane than 4, 5, 6 and 7 years old (Ali *et al*., 1997). The incidence of retention of placenta in cattle ranged between 5.2 and 23.5% (Roberts, 1986; Majeed *et al.,* 1989). Its incidence can be as high as 12% even in normal delivery (Dewan and Rabman, 1987). Shamsuddin *et al.* (1988) recorded about 63% of the retained placenta in Governoment Dairy Farm, Savar. Bangladesh has an increasing demand of livestock products like milk, meat and eggs. Bangladesh government has also given priority on the development of dairy sector at farmers' level to increase the supply of milk from small dairy farms. Dairy cows should have a maximum of 60 to 90 days open and should get one calf per cow per year. Retention of placenta prolongs days open which results in decrease milk production fertility, market value of the animals (Ahmed *et al*., 1999; Semacan and Sevinc, 2005) and increase calving interval, culling rate, treatment and labor cost (Kelton *et al*., 1998; Ahmed *et al*., 2004). The health condition in affected animal deteriorate. There are many factors influencing the incidence

of retained fetal membranes like abortion, dystocia, multiple birth, poor body condition score, age, nutritional deficiencies, hormonal imbalance (Grunert, 1986; Alam and Dobson, 1986) season of the year and premature birth. Inadequacy of oxytocin and calcium are important agents associated with retention of placenta Majeed *et al*., 2009). Various prophylactic and therapeutic approaches have been postulated by many workers like no treatment, hormonal treatment, chemotherapeutic and manual removal (Majeed *et al*., 2009). Use of ecbolic, like oxytocin, helps in inducing myometrial contraction. The study was aimed to Know the efficacy of prophylactic drugs viz; oxytocin, methyl ergometrine maleate, prostaglandin analogue, and herbal patent medicine on the prevention of retention of placenta.

Materials and Methods

Cows were selected from 50 dairy farms in Bangladesh from June 2010 to June 2011 having same management condition. A Total of 100 Holstein- Friesian cross-bred pregnant cows chosen from 50 dairy farms aged between 3-10 years and parity 1-7. The prophylactic measure or treatment was applied just after delivery of calf. The treatment was done according to the following schedule: Group I (n=20) Oxin® (oxitocin synth. 10 iu/ml) @ 5 ml/ cow (i.m.); Group II (n=20) Metherspan® (methyleargometrine meleate .0200 mg/ml) @ 5 ml/ cow (i.m.); Group III (n=20) Dinoprost® ($PGF_2\alpha$ contain dinoprost as trometamol 5 mg/ml) @ 5 ml/ cow (i.m.); Group IV (n=20) Utoklin® (herbal patent drugs) @ 450 ml/ cow (orally); Group V (n=20) placenta expelled spontaneously within 6 to 12 hours (control). The information was recorded carefully in specially prepared cards. Cows with RP were divided into subgroups based on age, Group 1: < 3years aged, Group 2: 3years to <5 years, Group 3: 5years to <7 years, Group 4: >7years type.

Statistical analysis: The collected data were compiled and tabulated Microsoft Excel for statistical analysis. Calculation of incidence for each of parameter and Student's t test were done using computer package SPSS/PC+ program.

Results and discussions

The overall incidence of the RP in the Holstein Friesian cross bred dairy cows was 11% but in control group it was 30%. These incidences indicated the RP in cows is quite high. It requires further study to find out proper solution to this problem. The findings is lower than that reported by Majeed, 1979, Islam *et al*., 2012 & 2013 and Majeed *et al*., 1989. There was reduced cases of 10%, 5%, 5% and 5% in different treatment groups, such as Uteoklin, Oxytocin, Metherspan and Dinoprost treatment, respectively (table 1). The results were in agreement with Islam & Sarder, 2013 and Majeed *et al*., 2009. Effect of expulsion time of retained placenta after several prophylactic treatments of Holstein Friesian cross bred dairy cows were observed in Table 3 and Figure 1. All the logistic regression coefficients were found to be positive and statistically insignificant except Dinoprost ($P<0.05$) and Metherspan ($P<0.10$). The odds ratios of Oxytocin, Metherspan, Dinoprost, Uteoklin are 2.154, 3.051($P<0.10$), 4.846 ($P<0.05$) and 2.154, respectively which means recovery rate from retained placenta of dairy cows at the time of parturition was 2, 3, 5 and 2 times better than the Control group. Hormone therapies had been widely used in the treatment of retained placenta (Roberts, 1986). Among the treatments, the third group $PGF_2\alpha$ (Dinoprost) showed the best results and 89% efficacy for the expulsion of RP and 4.85 times more likely than control group. $PGF_2\alpha$ increases uterine contractility (Watson, 1984) and dilatation of the cervix, which enhances placental expulsion. Similar finding was reported by Majeed *et al.,* 1991, it could be concluded that using of $PGF_2\alpha$ was more effective for treatment of RP than other treatment regimen. The second group methyl ergometrine maleate (Metherspan) showed second highest (84%) response among groups and 3.051 times more likely than control group. Ergonomic or a similar drug developed from ergot may be more beneficial than oxytocin in cases of atonic myometrium because of its prolonged oxytocic effect (Roberts, 1986). The first group oxytocin (Oxin) and fourth group patent herbal drug (Utoklin) obtained similar result and with an efficacy of 79%. It has been suggested that oxytocin plays a role in dropping of placenta; through a stimulation effect on phagocytosis by uterine leucocytes. Exogenous oxytocin used to prevent the occurrence of retained placenta immediately postpartum (Roberts, 1986; Azad, 2010 and Miller *et al*., 1984). Uteoklin and Oxytocin showed 2.154 times more effects than control group. A higher incidence rate was observed in younger cows than older one (table 2). This might be due to shorter gestation periods (Noakes, 2003 and Youngquist & Threlfall, 2007). Cows aged more than 7 years showed a higher incidence

(44.45%) of RP than others aged group. This is in agreement with Islam *et al*., 2012 & 2013 and Islam & Sarder 2013 but disagreement with Majeed *et al*., 1989 and Majeed 1979.

Table 1. Effect of various factors on the incidence of RP in Holstein Friesian cross bred dairy cows

Prophylactic treatment group	No of RP	Incidence (%)
Control	6	30
Patent herbal drug (Utoklin)	2	10
Oxytocin	1	5
Ergometrine maleate (Metherspan)	1	5
$PGF_2\alpha$ (Dinoprost)	1	5
Total	11	11

Table 2. Effect of age on the incidence of RP of dairy cows

Category	**Aged group**				**Total**
RP	**<3 years**	**3years to <5 years**	**5years to <7 years**	**>7years**	
Number (N)	2	1	2	4	9
Percentage (%)	22.22	11.11	22.22	44.45	100

Table 3. The Logistic Regression analysis effect on different prophylactic treatment and Index of placental expulsion of Holstein Friesian cross bred dairy cows

Independent Variable	**B**	**S.E**	**Exp (B)**
Treatment Group			
Oxytocin	0.767	0.730	2.154
Metherspan	1.116	0.782	3.051**
Dinoprost	1.578	0.881	4.846*
Utoklin	0.767	0.730	2.154
Control$^{(r)}$	-	-	1.000
Constant	0.619	0.488	1.857
Chi-square (calculated)	11.647*		
-2 Log likelihood	89.361		
df	4		
Cox & Snell R Square	0.234		
Chi-square (tabulated)	**9.488**		

r = Reference Category; B = Logistic Regression Coefficient; and Exp (B) = Odds Ratio
'*' = Significant at 5% level, '**' = Significant at 10% level

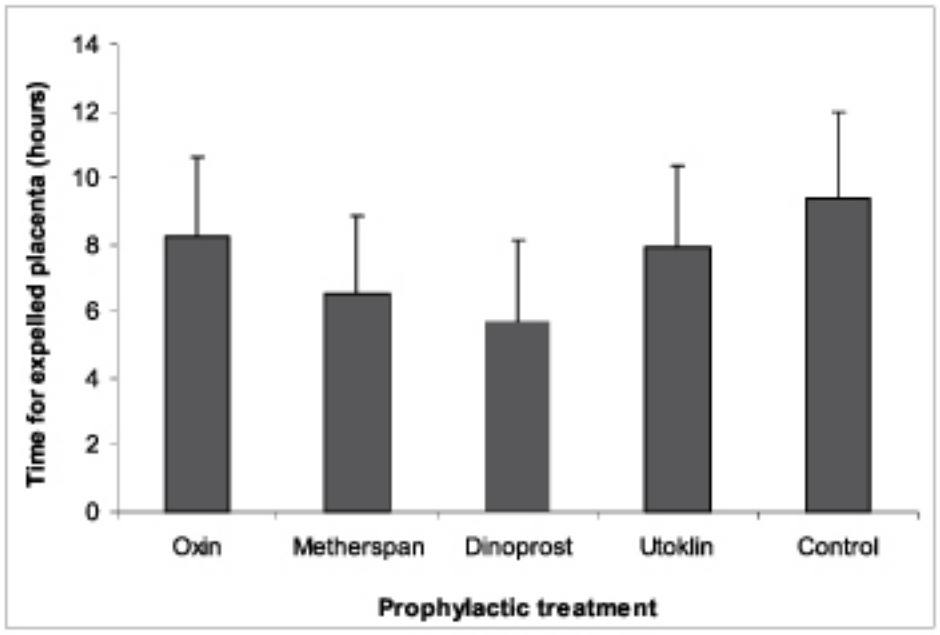

Figure 1. Expulsion time of retained placenta after several prophylactic treatments of Holstein Friesian cross bred dairy cows

Conclusion

Prostaglandin $F_2\alpha$ (Dinoprost) is more effective to treat retention of placenta than Methyl ergometrine maleate (Metherspan). Oxytocin (oxin) and patent herbal medicine (uteoklin) have similar effect when compared with control group. Higher incidence of RP was observed in > 7years and <3years groups than that of other aged group cows.

Author's contribution

It was the MS thesis experiment of first author. Other authors contributed equally. All authors read and approved the final manuscript.

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Prevalence and risk factors of brucellosis in cattle

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Abstract
Brucellosis is considered to be the most widespread zoonosis throughout the world and is caused by different species of the genus *Brucella*. Brucellosis causes a great economic loss to the livestock industries through abortion, infertility, birth of weak and dead offspring, increased calving interval and reduction of milk yield.It is endemic in Bangladesh. The present research was carried out to estimate the prevalence and to identify the risk factors of brucellosis along with determination of genetic diversity in Bangladesh. Out of 1043 samples collected, 28 milk samples (2.68 %) and 23 serum samples (2.21 %) were positive by MRT and RBT respectively. The highest prevalence was 3.05% in the Holstein Friesian cross using MRT, while 2.49% in the Holstein Friesian cross cows using RBT. Age-wise prevalence of brucellosis by MRT and RBT were 2.06% and 1.23% respectively in 1-4 years age group. On the other hand prevalence of brucellosis based on MRT and RBT were significantly higher (2.88% and 2.50%) in > 5 years age group ($p \leq 0.01$) than those of other age groups. Based on parity, significantly higher prevalence 3.07% and 2.58% of MRT and RBT were obtained respectively in parity 3-5 in comparison to other parity group ($p \leq 0.01$). It is, however, obvious that although the MRT is 1st-line screening tests for brucellosis in cows in some countries, their lack of specificity is of concern. Therefore, the requirement for other confirmatory tests that are more specific were used for the diagnosis of the disease, especially in Bangladesh.

Key words: cattle, MRT, RBT, prevalence, risk factors, brucellosis

Introduction
Brucellosis is a disease of domestic and wild animals that can be transmitted to human. Brucellosis is a recognized public health problem with worldwide distribution and one of the major causes of morbidity. Brucellosis causes a great economic loss to the livestock industries through abortion, infertility, birth of weak dead offspring, increased calving interval and reduction of milk yield and it is endemic in Bangladesh (Rahman*et al*., 2014). Brucellosis is caused by small non motile, gram negative and intracellular coccobacilli belonging to the genus Brucella. *Brucella spp*. is facultative intracellular bacteria causing chronic disease which may persist for the whole live of the affected organism. In animals brucellosis affects reproduction, fertility and reduces newborns survival and also milk production. The mortality in adult animals are insignificant (Sewel and Blocklesby, 1990).In humans the symptoms of brucellosis are weakness, joint and muscle pain, headache, undulant fever, hepatomegaly, splenomegaly, night sweats and chills marked asthenia and anorexia (Huge-Jones, 2000). Despite preventive and control measures that exist in developing countries, there is still a high potential for transmission and spread of *Brucellaspp*.via animals and their products and bye-products imported from these countries (Acha et al.,1987). Prevalence of brucellosis in cattle might constitute a significant hurdle for the development of livestock in Bangladesh. So, early and accurate diagnosis is important for effective control measure against brucellosis.The MRT and RBT are widely used for screening of brucellosis exclusively in eradication programs (Sarker et al, 2014). The RBT is a simple agglutination technique. Because this test does not need special laboratory facilities and is simple and easy to perform, it is used to screen sera for antibodies to *Brucella* (Cadmas et al, 2008).These tests were chosen because they are less cumbersome to perform on a large scale and/or require no special equipment and expertise compared with other commonly used assays such as CFT and ELISA. Therefore, the present study was doneto know the prevalence of brucellosis in cattle some selected areas of Bangladesh.

Materials and methods

The study area included Mymensingh, Jamalpur, Rangpur,Kurigram, Bagerhat and Gaibandha district and the Government owned Central Cattle Breeding and Dairy Farm (CCBDF),Savar, Dhaka, of Bangladesh. There are two types of animal population in this research work. In first population: the total livestock population is 1043.These areas were chosen because of the cattle population is high. Because this is a poor area and people are very much reared with livestock from the beginning of their agriculture.They are more benefited reared with livestock with respect to other crop production.That is why they reared the livestock. CCBDF is the largest farm in Bangladesh with major objectives to produce crossbred heifers and bulls for distribution to farmers, to collect semen from tested bulls to continue support the national artificial insemination program and to supply milk to the public. During the last 28 years, this farm maintains on average a herd of about 3500 cattle. Holstein Friesian, Sahiwal breeds and crossbred cattle are mainly used for semen production. The system of animal management is intensive and only artificial insemination is used for reproduction and disease control purpose.The common breeds are indigenous and their crosses with Holstein Friesian, Sahiwal breeds and cross breed.In case of male animal collected orchitis testis from buck and bull.

Rose Bengal Test (RBT)

The RBT was performed according to the procedure as described by Uddin*et al*(2007). The test serum samples and *Brucellaabortus* antigen (William James House, Cowley Rd. Cambridge,CB4 OWX,UK) were kept 1 hour in room temperature before beginning the test. 30 µl of each serum to be tested was placed on a glass plate circled approximately 2 cm in diameter. Then the vial of antigens was shaked gently and 30 µl of antigen put beside each of the sera. The antigen and serum was mixed on the plate with a stirrer and spread over the area enclosed by the circle. Then the plate was placed on a mechanical rotator at 80-100 rpm for 4 minutes and the reading was taken immediately. Any agglutination or precipitation was considered as positive, whereas no reaction indicated the absence of Brucella antibody in the sera(Fig.1).

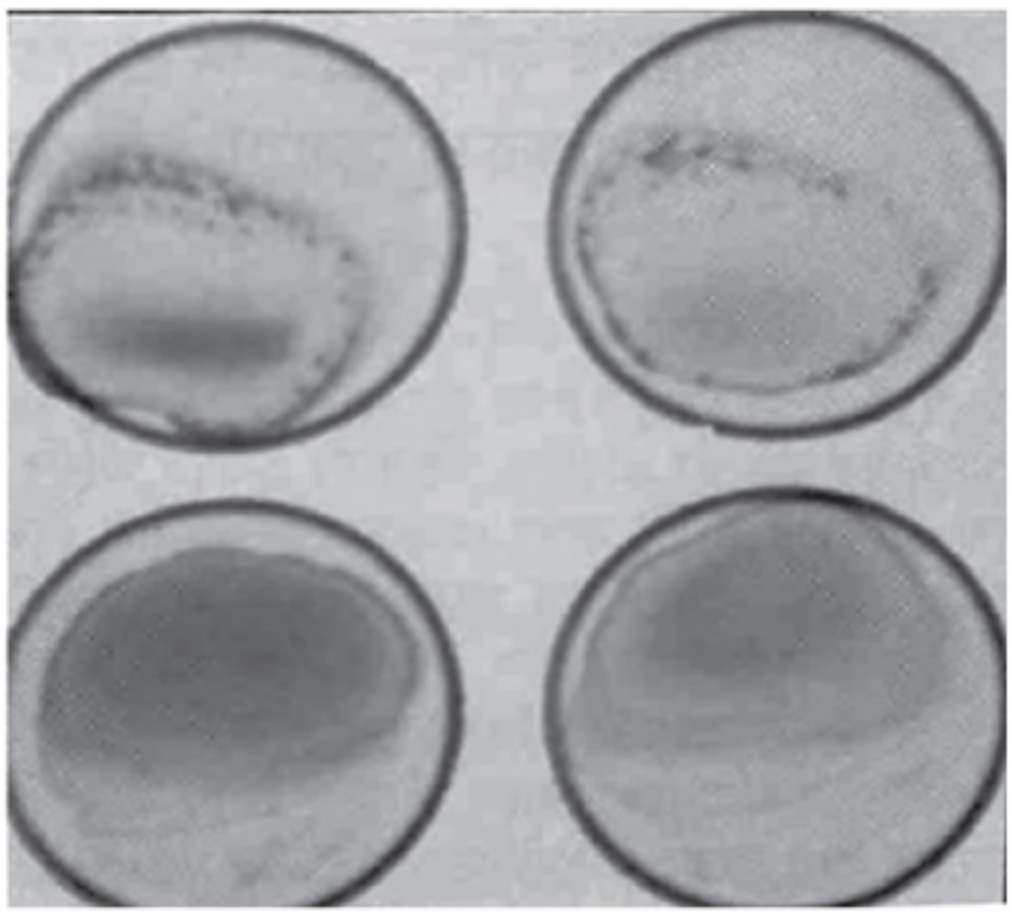

Fig 1: Rose Bengal Test(RBT).Upper row positive and lower row negative.

Milk Ring Test (MRT)

Milk Ring test was performed following the manufacturer's instruction. In brief, antigen was kept at room temperature (18-23°c) for 1 hour before starting the test. After proper mixing, 1.0 ml of milk sample and 50 µl of MRT antigen was added in each tube. The milk and MRT reagent was mixed with vortex and incubated for 1 hour at +37°c and then between +2°c to +8 °c for 18 to 20 hours. The result was read as positive if the ring of cream was equally or more colored than the underling milk and as negative if the ring of cream less colored than the underlying milk (Fig. 2).

Statistical analysis: The Chi-square test (λx^2) was performed to find out the relationship between the prevalence of brucellosis and demographic variables of cows.

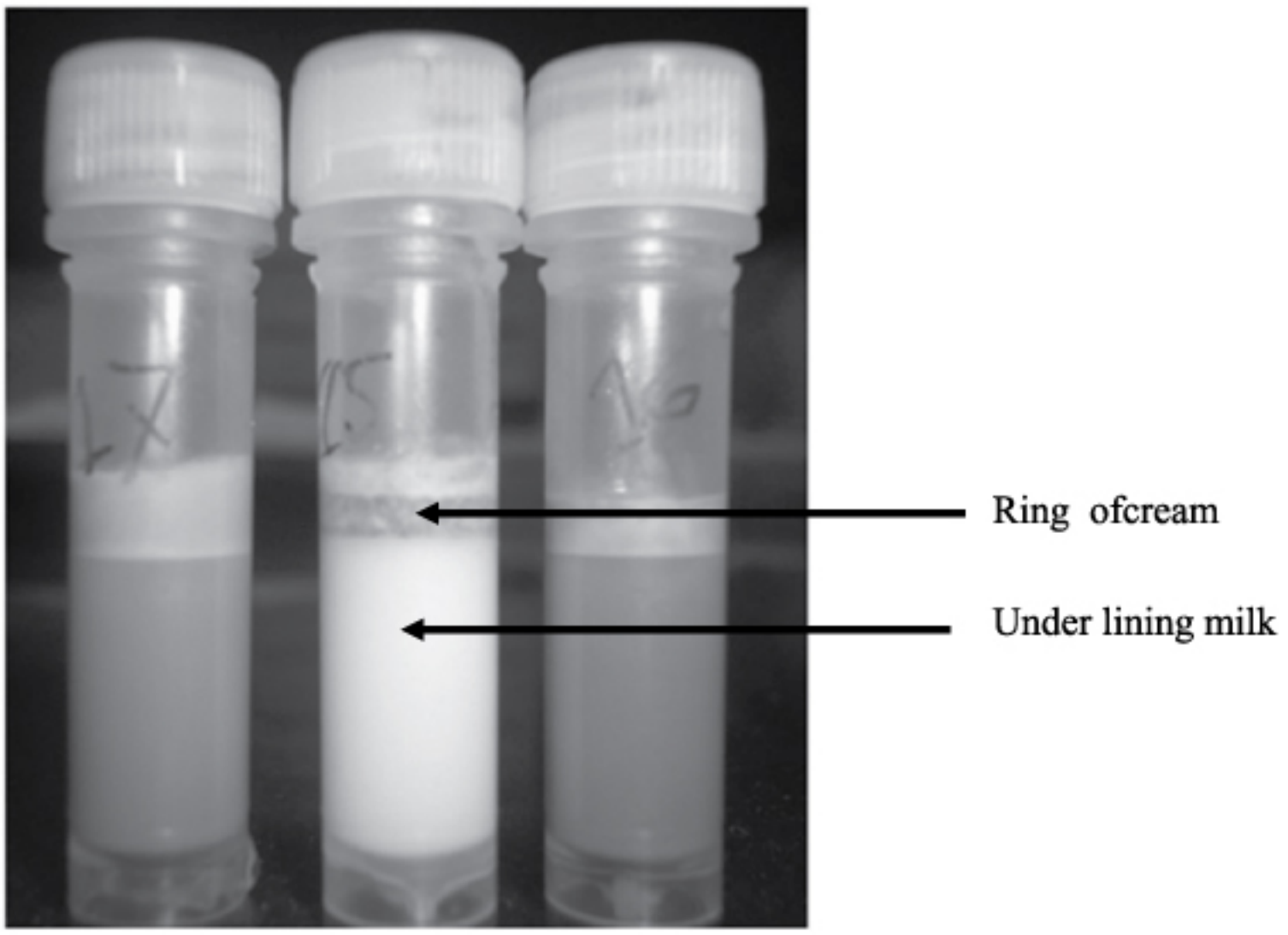

Fig 2: Milk Ring Test. Milk in the middle tube indicates positive result showing ring of cream more colored than underlying milk;Milk in the two corner tubes indicate negative result shows ring of cream less colored than underlying milk.

Results

Out of 1043 samples 28 milk samples (2.68 %) and 23 serum samples (2.21 %) were positive milk and serum screened according to MRT and RBT respectively (Table 1). The highest positive rate of 3.05% was obtained among the Holstein Friesian cross of cows using MRT, while the highest positive rate of 2.49% obtained among the Holstein Friesian cows using RBT (Table 1).Age-wiseprevalence of brucellosis based on MRT and RBT were 2.06% and 1.23% respectively in 1-4 years age group.Onthe other hand prevalence of brucellosis based on MRT and RBT were significantly higher(2.88% and 2.50%) in > 5 years age group ($p \leq 0.01$) than those of other age group (Table 2). Based on parity, significantly higher prevalence 3.07% and 2.58% of MRT and RBT were obtained respectively in parity 3-5 in comparison to other parity group ($p \leq 0.01$)(Table 2).

Table 1.Breed wise prevalence of brucellosis based on MRT and RBT in cattle.

Breeds of cattle	No.of cows tested	MRT positive	Prevalence on MRT %	Odds ratio (95% CI)	P-value	RBT positive	Prevalence on RBT %	Odds ratio (95% CI)	P-value
Holstein Friesian cross	722	22	3.05	1.65 (0.66-4.11)	0.277	18	2.49	1.62 (0.59-4.39)	0.342
Sahiwal cross	321	6	1.87	Reference	-	5	1.56	Reference	-
Total	1043	28	2.68			23	2.21		

Table 2.Age and parity-wise prevalence of brucellosis in cattle based on MRT and RBT.

Parameter		No. of cows tested	MRT positive	Prevalence on MRT %	RBT positive	Odds ratio (95% CI)	P-value	Prevalence on RBT %	Odds ratio (95% CI)	P-value
Age	1-4 years	243	5	2.06	3	Reference	-	1.23	Reference	-
	>5 years	800	23	2.88	20	1.41 (0.53-3.75	0.489	2.50	2.05 (0.60-6.96)	0.239
Parity	1-2	228	3	1.32	2	Reference	-	0.88	-	-
	3-5	815	25	3.07	21	2.37 (0.71-7.93	0.148	2.58	2.99 (0.69-12.84	0.122

Discussion

The overall prevalence of brucellosis based on MRT and RBT was recorded as 2.68% and 2.21% respectively. The MRT is generally used for screening; other test are required for confirmatory diagnosis. This is an agreement with Morgan (1967) who stated that the test should be used in conjunction with the established tests and not instead of them. The higher proportion of positives by MRT might result from false positives which could be due to many causes including mastitis, colostrum, collection at the end of lactation period or a hormonal disorder (Bercovich and Moerman, 1979). samples were collected Carefully from dairy cows excluding mastitis infected and recently delivered cows to avoid false positive reactions. It has been shown that different serological tests used for the diagnosis of brucellosis vary considerably in their ability to detect antibodies of a particular immunoglobulin class (OIE 2009). Infected animals may or may not produce all antibody isotypes in detectable quantities (FAO 2004). Vaccination against brucellosis is not carried out in Bangladesh, so that our result shouldreflect natural infection. In general the MRT have been shown in other studies to have high sensitivity but lower specificity. The MRT is not normally used on individual animals because of false positives (less specificity). Based on outcome of the study, it is suggested that although MRT and RBT are generally useful for screening for brucellosis especially in developing countries where other tests are cumbersome to perform on a large scale and require special equipment and expertise, but these tests still have limitations where vaccination or medical records are not available. As a result of these limitations other confirmatory tests like ELISA, CFT, SAT shouldbe carried out in conjunction with MRT and RBT due to confirm the brucellosis status of cattle in Central Cattle Breeding Station and Dairy Farm, Savar, Dhaka and different Upazilas of Gaibandha, Rangpur, Jamalpur and Mymensingh district. The ELISA is an available assay for use on milk and serum and is very useful where large number of samples require testing. Milk ELISA is used on pooled samples which is more cost effective than testing individual animals. Although these tests may be very expensive they are needed to confirm the brucellosis status of cattle in our study area in order to safeguard the public and in particularly that of the people directly involved in the meat inspection, milk collectors and meat-milk processing. The older animal supposed to be more infected because of more contact with infectious agents and sometimes become more susceptible from malnutrition during pregnancy. It appears that the prevalence of infection in animals more than 5 years of age compared to younger animals and higher prevalence of brucellosis among older cows might be related to maturity with the advancing age. Seropositivity to be considered due to natural infection occurred because vaccination in cows has never been practiced in Bangladesh. The prevalence and severity of disease may vary with the breed, geographic location, types of diagnostic tests, husbandry and environmental factor as well as the biovar of the organism.

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Genetics

Candidate genes for milk production traits of Red Chittagong Cattle

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Abstract

In order to achieve sustainable genetic improvement, it is necessary to have a population with genetic variation and a tool for selection. Identification of genes that are in quantitative trait loci (QTL) can provide the more accurate markers for marker-assisted selection (MAS) in livestock population. The study was carried out on *Stearoyl-CoA desaturase* (*SCD,) diacylglycerolacyltransferase-1* (*DGAT1*) and *ATP-binding cassette G2 (ABCG2)* genes which are responsible for variation in milk production traits (milk yield, fat yield, protein yield and SNF yield) in cattle. These genes were used as candidate genes in Red Chittagong Cattle (RCC) breed of Bangladesh Livestock Research Institute (BLRI) herd for detection of single nucleotide polymorphism (SNP) causing variation in milk production traits. Focusing on the effects of SNPs on milk production traits, phenotypic variation within RCC breed was identified and categorized based on milk production traits. Average lactation yield varied from 527 to 1436 kg (n=29). About 18% of lactating cows showed an average of >1000 kg milk production per lactation. Average fat percent ranged from 4.71 to 6.25 (n=15). Eighteen (18) set of primer were designed where 8 set from *DGAT1,* 6 set from *SCD* and 4 set from *ABCG2* gene for sequencing of *SCD, DGAT1* and *ABCG2* genes. Pooled DNA from 50 RCC cows and 5 RCC bulls were used in sequencing. In sequence analysis the *SCD, DGAT1* and *ABCG2* alleles found fixed in RCC. This study provides an indirect evidence that RCC breed has fixed alleles with respect to *SCD, DGAT1* and *ABCG2* genes reported to be responsible for higher milk fat yield, higher fat and protein percent. However, further study needed to unlock genetic reason behind these phenotypic variations using other genes involved in milk production and metabolism pathways.

Key words: Red Chittagong Cattle, *SCD, DGAT1* and *ABCG2* genes

INTRODUCTION

The primary goal of researchers in the development of dairy industry is to increase milk production without reducing the quality of milk. However, the traits of interest are generally sex-limited, dairy cattle have a lengthy generation interval, it is time-consuming and expensive to perform progeny tests, and many have low heritability. In order to achieve sustainable genetic improvement, it is necessary to have a population with genetic variation and a tool for selection. The most efficient method of genetic selection is with the use of gene-assisted selection, which involves the identification of functional single nucleotide polymorphisms (SNPs) responsible for changes in phenotypes. Identification of genes that are in quantitative trait loci (QTL) can provide the more accurate markers for marker-assisted selection (MAS) in livestock population. Therefore, MAS is thought to be very useful in the dairy industry (Ruane *et al.,* 1996, Boichard *et al.,* 2006). However, potential SNPs in some candidate genes have been reported for their association with milk production traits. Candidate genes underlying this variation may be found in milk production and metabolism pathways, which are under the control of multiple genes. SNPs in the *diacylglycerolacyltransferase 1* (*DGAT1*), *stearoyl-CoA desaturase 1*(*SCD1*), *ATP-binding cassette G2 (ABCG2), fatty acid synthase* (*FASN*), *oxidized low-density lipoprotein receptor 1 (OLR1), prolactin (PRL), signal transducer activator of transcription 5A (STAT5A)* and *growth hormone receptor (GHR)* genes have been shown to affect the composition of bovine milk in different cattle populations (Schennink *et al.,* 2009; Sun *et al.,* 2009; Alim *et al.,* 2012). This study was undertaken to detect SNPs in *DGAT1, SCD* and *ABCG2* genes against observed phenotypic variation in milk production traits of RCC, and to use of potential SNPs responsible for higher milk production in marker assisted selection (MAS).

Materials and Methods

Phenotypic data recording on milk production traits of RCC

RCC herd of Bangladesh Livestock Research Institute consists of about 50 cows were at different lactation stages and dry cows. Phenotypic data of milk yield and milk traits were collected from 29 lactating cows under a period of two years. Phenotypic data on milk yield collected from daily milk yield record book and milk traits (fat yield, protein yield, SNF yield and lactose yield) were recorded from milk sample analysis using LACTOSTAR (Milk component analyzer, Model 3510) in every 15 days' interval. The milk yield and milk sample analysis data were used in Microsoft Office Excel 2007 program to analyze and identify phenotypic variation in RCC for association study with *DGAT1, SCD* and *ABCG2* alleles.

Identification and Genotyping of the Polymorphisms and Statistical Analysis

DNA samples were isolated from collected blood samples of 29 lactating, 21 dry cows and 5 breeding bulls using a commercial kit (QIAGEN DNA Mini Kit) following manufacturer instruction. Total 55 DNA samples were pooled to produce one sample and to perform DNA amplification of *DGAT1, SCD* and *ABCG2* genes. Eighteen set of primers (Table 1) of *DGAT1, SCD* and *ABCG2* genes were used to amplify all exons (some cases specific exons and introns) and their partial flanking intronic sequences to identify potential SNPs. Eight pair of primer were designed based on the reference sequence of the bovine *DGAT1 gene* (Gene Bank Accession No. AC000171, NCBI), 6 pair of primers from SCD were collected from Alim *et al.*, (2012) and 4 pair of primers from *ABCG2* genes were collected from Tantia *et al.* (2006) and Cohen *et al.* (2005). Primers from *DGAT1* gene were designed using Primer3 (v.0.4.0.) (http://frodo.wi.mit.edu/) and Net Primer (www.PremierBiosoft.com) web based program and all the primers were synthesized by Invitrogen Life technologies, Beijing, China. PCR amplifications of *DGAT1, SCD* and *ABCG2* genes were performed using 18 sets of primers and pooled DNA from 50 (lactating and dry) cows and 5 breeding bulls with a programmable thermal cycler. PCR reaction mixture contained 0.2mM dNTPs, 2mM $MgCl_2$, and 0.05U Taq DNA polymerase in final concentration of 20µl volume. The amplification condition was: 5 min at 94ºC for initial denaturing followed by 30 cycles at 94ºC for 30 s; annealing at Tm (ºC) for 30 s, 72ºC for 30s; a final extension at 72ºC for 10 min for all primers. Amplification was confirmed by gel electrophoresis of the PCR products in 2% agarose gel followed by visualization under UV (Figure 1).

Table 1. PCR primers for sequencing and SNPs detection

Gene	Exons/introns	Primer no.	Sequence (5' to 3')	Length (bp)
SCD	1	P1	L- GTTGGCAACGAATAAAAGAGG	21
			R- CGCGGTGATCTCAACTCTTC	20
	2	P2	L- GGACCGGGTCTATGCCTATC	20
			R- CCATCCAGCCTCTCAGGAC	19
	3	P3	L- GTTCCCTGGGACTCCTAAGC	20
			R- CCGGAACTTAACCACAAGGA	20
	4	P4	L- GGCAACTCCATGACTTCTCC	20
			R- CATGACCGTCCTAGGTCAAC	20
	5	P5	L- CCCATTCGCTCTTGTTCTGT	20
			R- CGTGGTCTTGCTGTGGACT	19
	6	P6	L- GCCTCTGAGGGGATCTATTTG	21
			R- AGGCAGAGTTGTTGGCTTTC	20
DGAT1	1	P7	L-GGACTACAAAGGTATGCGCG	20
			R-CCGTATCAGGGGTCAAAGGT	20
	2	P8	L-CTTCCGTCTTGCATCACCAG	20
			R-ACAGAGCTCCATTCACCACA	20
	3	P9	L-TCGCAGATCTTAAGCAACGC	20
			R-TTGAGGCAAGTCAGGGGAG	19
	4	P10	L- TTTCGTGGCCTTCCTGAGAG	20
			R-TAGGTCAGGTTGTCGGGGTA	20

	5	P11	L-CTACCCCGACAACCTGACCTA	21
			R- GGGCTTCATGGAGTTCTGGAT	21
	6	P12	L- TCCCCAACCACCTCATCTG	19
			R- GCGGAGGCCAGAAACACT	18
	7	P13	L- CCCCGCAGACACTTCTACAA	20
			R- CAAAGCAGTCCAACACCCAC	20
	8	P14	L-CACCATCCTCTTCCTCAAG	19
			R-AAGGAAGCAAGCGGACAG	18
	1	P15	L- TAAAGGCAGGAGTAATAAAG	20
			R- TAACACCAAACTAACCGAAG	20
	2	P16	L- CAGGGCTGTTGGTAAATCTCA	21
ABCG2			R- GCACGGTGACAGATAAGGAGA	21
	3	P17	L- GGCGTCTGGCTTCAATACTTG	21
			R- ACAGGTGACAGATAAGGAGAACAT	24
	4	P18	L- GTATTCACGAGACTGTCAGGG	21
			R- GGCTTTATTCTGGCTGTTTCC	21

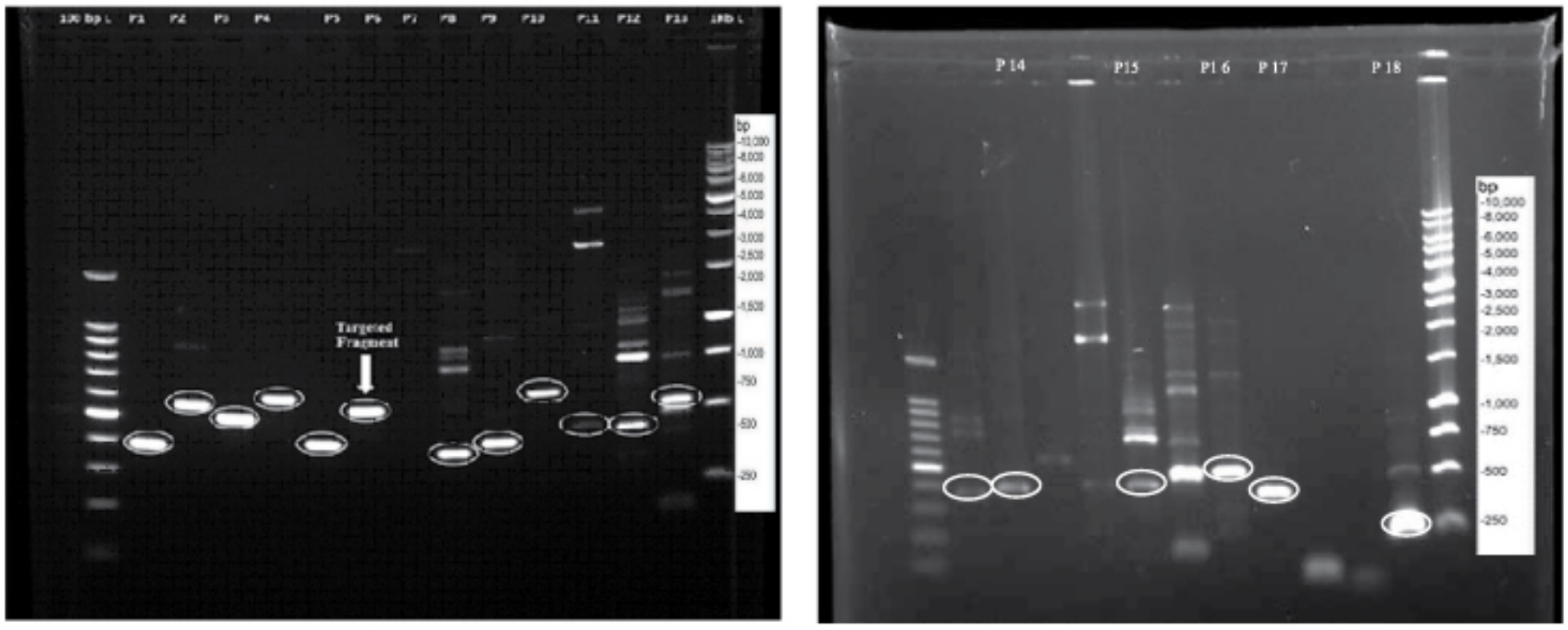

Figure 1. PCR amplification of SCD, DGAT1 and ABCG2 Genes

The targeted DNA fragments (Figure 1) of DGAT1, SCD and ABCG2 genes were cut from agarose gel for DNA isolation. Isolated DNA fragments from agarose gel were used in PCR amplification to get purified PCR products. The purified PCR products were sequenced using Genetic Analyzer ABI 3730 in 1st BASE Laboratory, Malaysia. BioEdit Sequence Alignment Editor, version 7.0.9.0 (Hall 1999) and ClustalW multiple sequence alignment programs (Hall 1999) were used to align both forward and reverse sequences and to determine the presence of SNPs.

Results and Discussion

Phenotypic variation within RCC breed in BLRI herd condition had been identified on the basis of milk production traits. So far lactation yield varied from 527 to 1436 kg with an average of 838±179(29) kg. Lactation length varied from 142 to 305 days with an average of 219±35 days. There were small variation in milk fat, protein, lactose and SNF (Table 2) of RCC. RCC were categorized according to its milk yield (Graph 1). About 18% of lactating cows showed an average of >1000 kg milk per lactation and 20% of cows showed >500 kg milk per lactation (Table 3).

Table 2. Phenotypic variation in milk yield, lactation length, fat, protein, lactose and SNF of RCC cows

Parameter	Milk yield (kg)	Lactation length (days)	Fat %	Protein %	Lactose %	SNF %
Mean±SD (n)	838±179(29)	219±35(29)	5.26±0.42 (15)	4.05±0.07 (15)	5.82±0.10 (15)	10.75±0.17 (15)
Maximum	1436	305	6.25	4.13	5.95	10.96
Minimum	527	142	4.71	3.86	5.57	10.27

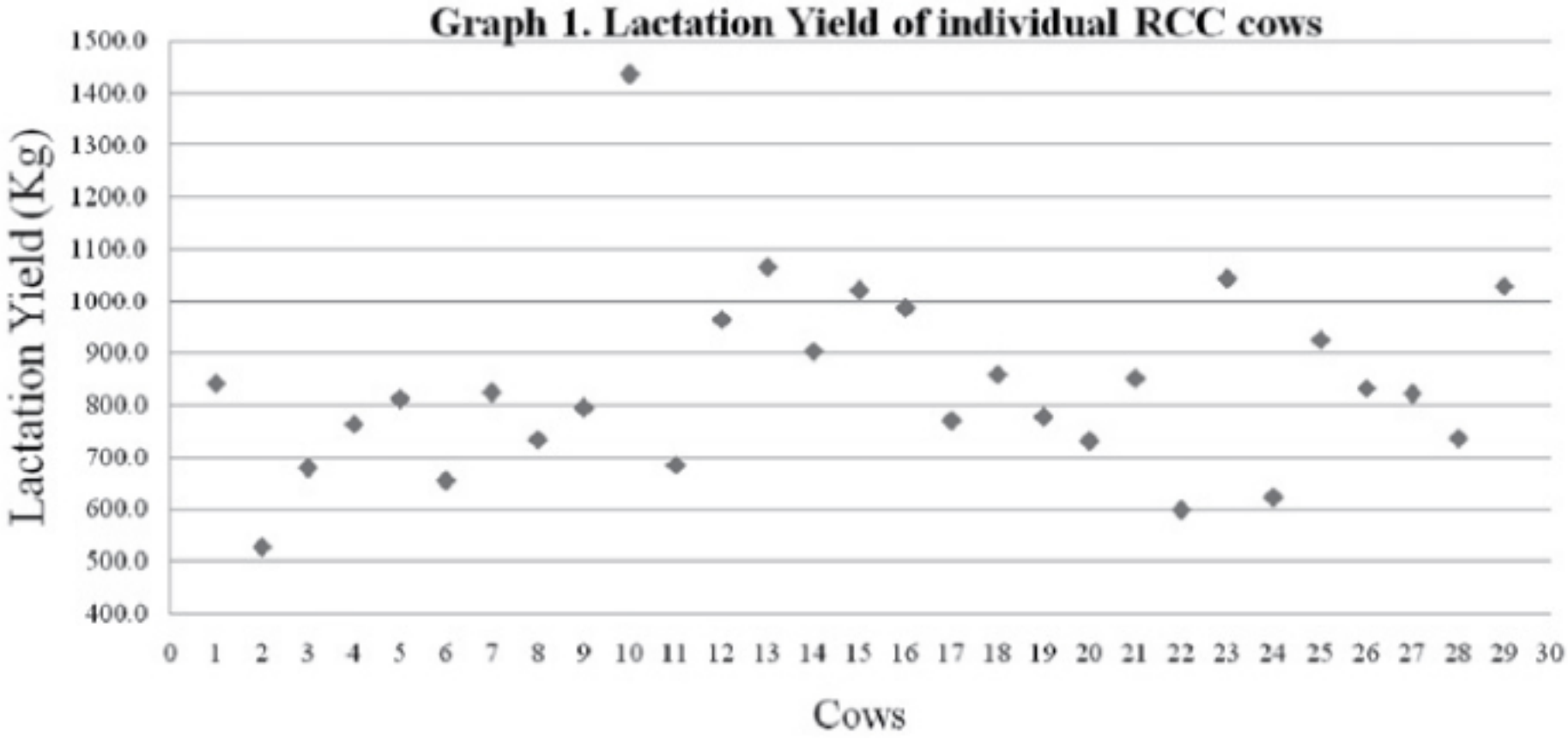

Table 3. Phenotypic variations in lactation yield

Lactation yield (kg)	No. of cows	% Cows
>500	6	20
>700	18	62
>1000	5	18
Total recorded cows	29	100

The *SCD, DGAT1* and *ABCG2* allele found fixed in RCC from sequencing results. This study provides indirect evidence that RCC breed has fixed alleles with respect to *SCD, DGAT1* and *ABCG2* genes reported to be responsible for higher milk fat yield, higher fat and protein percent. The *DGAT1* and *ABCG2* alleles were also found as fixed in Indian Cattle (*Bos indicus*) by Tantia *et al.*(2006). Cohen *et al.*(2005) reported that an increase in frequency of $ABCG2^{Y}$ allele with selection for higher milk fat and protein percentage in the Israeli Holstein population. The phenotypic variations in milk production traits, which, found within RCC breed at BLRI herd were not due to effect of *SCD, DGAT1* and *ABCG2* genes. Besides these 3 genes, there are many other genes involved in milk production and metabolism pathways like *FASN, CSN3, PPARGC1A, IGF1, OLR1, PRL, STAT5A, GHR* (Mele *et al.* 2007; Moioli *et al.* 2007; Schennink *et al.* 2008; Schennink *et al.* 2009; Sun *et al.* 2009), which might have effect on the studied phenotypic variation.

Conclusion and Implications

The RCC has within breed phenotypic variation in milk yield, but the studied genes *SCD, DGAT1* and *ABCG2* were fixed in RCC, which are indirectly responsible for higher milk fat yield, higher fat and protein percent. There is considerable genetic variation in milk yield in RCC which can be utilized to increase the milk yield without having an adverse effect on fat and protein percent. However, further study needed to unlock genetic reason behind these phenotypic variations using other genes involved in milk production and metabolism pathways.

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Genotyping of cattle based on lactoprotein genes: Kappa-casein and Alpha-lactalbumin

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Abstract
Milk productivity of indigenous cattle in our country is low. Different traits of milk including milk yield, protein content, etc. are regulated by milk protein genes. Kappa-casein (κ-CN) and alpha-lactalbumin (α-LA) are two major casein and whey protein of milk, respectively. There is lack of report on composition of different milk protein genes of cattle available in Bangladesh and hence the study was taken. A total of 50 blood samples previously collected from indigenous and crossbred cattle of Shibpur, Narsingdi were used in this study. DNA was extracted from blood samples followed by PCR with gene specific primers. For genotyping PCR products of κ-CN gene was digested with *Hind*III and *Hae*III restriction enzyme while the PCR products of α-LA gene was digested with *Bsp1286I*. The products were run in 4% gel and genotyped based on digestion pattern. Overall, from all the tested samples we could amplify specific DNA viz. 935 and 429 bp for κ-CN and α-LA genes, respectively. For κ-CN gene 30 out of 50 samples were analyzed for genotyping. Of these 66.67% (20/30) samples were genotyped as homozygous AA, 30% (9/30) as heterozygous AB and only 3.33% (1/30) as homozygous BB. For this geneAA genotype was found dominant. Allele frequency for A was 82%. AA genotype was higher in indigenous (0.366) than cross bred (0.30) animals. Homozygous BB genotype was not found in crossbred animals. For α-LA gene 50 samples were genotyped and found that all the animals were belonging to genotype AA (100%). In combination 66.67% samples were found to contain AA genotype which was reported as potential for higher milk production. Though the selection of animal depends on many factors, these criteria may be considered for animal selection along with other criteria for better milk production.

Key words: Cattle, Genotypes, Casein, Lactalbumin, PCR, RFLP

INTRODUCTION

In an agriculture based country like Bangladesh, livestock plays a major part in economy. More than half of the total population of our country live in the rural area and their livelihood is based on cultivation and farming. In developing countries, livestock is rapidly expanding as an agricultural subsector and accounting for approximately 40 percent of the gross value of agricultural production (FAO 2003; Thornton 2010). Cattle is one of the most important species of livestock in Bangladesh performing multifarious functions such as provisions of food, nutrition, income, savings, foreign currency earning (by exporting hides and skin, bone and other products), draft power, manure, fuel, transport, social and cultural functions. The densities of livestock in Bangladesh is quite higher in the world, 145 large ruminants/km^2 compared with India, Ethiopia and Brazil which have 90, 30 and 20 ruminants/km^2 respectively (Uddin et al. 2010). In spite of this good density of cattle population in Bangladesh, the productivity of the species is far below the world average. Low milk yield of the local livestock species may be the resultant effect of inferior breed, low genetic potentialities, poor nutrition, high disease susceptibility, lack of standard management practices and late age at puberty(Khan et al. 2014; Leng 1991).It has been reported that milk protein genes have the ability to regulate different milk production traits of cattle including milk yield, milk protein content, etc. (Deb et al. 2014; McLean et al. 1984; Mir et al. 2014; Singh et al. 2015). For all six major milk protein genes, there are autosomal and codominant alleles, which are called genetic variants. If those polymorphic sites in the corresponding genes causing amino acid changes in proteins are considered, genetic variants of milk proteins reported so far are as follows(Caroli et al. 2008; Farrell et al. 2004):

1) AlphaS1-casein: A, B, C, D, E, F, G, H
2) AlphaS2-casein: A, B, C, D
3) Beta-casein: A1, A2, A3, B, C, D, E, F, G, H1, H2, I
4) Kappa-casein (κ-CN)): A, A1, B, B2, C, D, E, F1, F2, G1, G2, H, I, J
5) Beta-lactoglobulin: A, B, C, D, E, F, G, H, I, J, W
6) Alpha-lactalbumin (α-LA): A, B, C

Kappa-casein (κ-CN) is one of the major casein proteins and alpha-lactalbumin (α-LA) is a major whey protein of milk. There is no report about κ-CN and α-LA gene variants of cattle in Bangladesh. Therefore present study was undertaken to detect κ-CN and α-LA genes in cattle, to characterize these two gene variants using molecular techniques and to determine the genetic frequencies of these two genes in cattle.

Materials and Methods

Specimen and laboratory analysis

For genotyping of cattle based on their milk protein genes κ-CN and alpha-lactalbumin, various laboratory works has been performed. Fifty blood samples those are randomly collected previously from Shibpur upazila, Narsingdi were taken from the repository of Animal Biotechnology Division (ABD) of National Institute of Biotechnology (NIB). The samples originated from both male and female animals and both from indigenous and crossbred animals. Distributions of the samples are presented in Table 1.Molecularanalysis was performed at ABD of NIB.

Table 1.Distribution of samples used in the study collected previously from Shibpur Upazila, Narsingdi

Animal	Local	Cross	Total
Male	06	03	09
Female	19	22	41
Total	**25**	**25**	**50**

DNA extraction and quantification

DNA was extracted from the retrieved blood samples using phenol chloroform method also known as organic method. Extracted DNA was analyzed in NanoDrop spectrophotometer (NanoDrop 2000/2000c) to measure the DNA concentration as well as purity.

PCR-RFLP of kappa-casein gene

A 935bp segment of κ-CN gene was amplified using specific forward (**5'**-AGCGCTGTGAGAAAGATG -**3'**) and reverse primer (**5'**- GTGCAACAACACTGGTAT-**3'**) reported by Soria *et al.,* (2003). The PCR reaction using this primer set was performed in 25µL reaction scale. The reaction mixture consisted of12.5µL of 2X master mix (GeneAmp® Fast PCR Master Mix, Applied Biosystems), 0.2µL Taq DNA polymerase, 0.5µL forward primer(10 pmole), 0.5µL reverse primer, approximately 50ng of extracted DNA and molecular grade water to make final volume of 25µL.The samples were subjected to heating at 94˚C for 5 min; followed by 32 cycles of 94˚C for 1min, 61˚C for 1min and 72˚C for 1min; next a final extension at 72˚C for 10min (Özgen 2009). After the PCR reaction is completed, a portion of the product was subjected to agarose gel electrophoresis. Visualization of 935bp segment of DNA in gel indicated successful amplification of target DNA.

Then the remaining PCR products were subjected to digestion with the restriction endonuclease *Hind*III and *Hae*III (NEB, USA).The digestion reaction (10µl) consisted of 1.5µl nuclease free water, 1µl compatible 10X buffer, specific restriction enzyme 0.5µl and PCR product 7µl. The reaction mixture was incubated at 37˚C water bath for 1hour. Upon digestion the products were electrophoresed in 4% agarose gel containing ethidium bromide. All of the bands in agarose gel were visualized in Compact Digimage System, UVDI series

(Major Science, USA) gel documentation apparatus. Expected fragment pattern from restriction digestion of 935bp PCR product of κ-CN gene with these two restriction enzymes are as follows(Soria et al. 2003).

Table 2. Expected banding pattern from restriction digestion of 935bp PCR product of Kappa-casein gene

Kappa-casein genotypes	Restriction enzymes	
	***Hind*III**	***Hae*III**
AA	935	641+294
AB	935+520+415	641+294
BB	520+415	641+294

PCR-RFLP of Alpha-lactalbumin (α-LA) gene

A 429bp fragments of α-LA gene was amplified using specific forward (5'-GATCAGTCCTGGGTGGTCATT-3') and reverse (5'- CAGTGGGTACCCATCCTAAGT-3') primer reported earlier (Voelker and Bleck, 1997). The composition of PCR reaction mixture was similar to κ-CN gene. Thermal cycling conditions were 96˚C for denaturation for 5min,followed by 30 cycles of 94˚C for 1min, 54˚C for 1min and 72 ˚C for 1min and final extension was at 72˚C for 3min (Voelker et al. 1997).Similar to the κ-CN gene, the PCR products were digested with the restriction endonuclease*Bsp1286I* (NEB, USA) after visualizing a 429bp band in agarose gel. Digestion reaction mixture was as mentioned for κ-CN gene. Expected banding pattern from restriction digestion of 429bp PCR product α-LA gene with *Bsp1286I* restriction enzyme are as follows (Voelker et al. 1997).

Table 3. Expected banding pattern from restriction digestion of429bp PCR product of α-LA gene

α-LA genotypes	Restriction enzyme (B*sp1286I*)
AA	429
AB	429+260+169
BB	260+169

Results

The core purposes of this study were to detect lactoprotein genes: κ-CN and α-LA from cattle and their genotyping based on genetic variation in these two genes. To achieve this, 50 blood samples were retrieved from repository of ABD followed by DNA extraction, PCR and RFLP analysis using specific restriction enzyme. Results are presented below under several sub-headings.

Amplification of Kappa-casein gene

For κ-CN gene amplification and genotyping a total of 30 out of 50 samples were tested. With reported primers we were able to amplify 935bp fragments from κ-CN gene of 100% (30/30) tested samples. A representative image of gel is shown in Figure 1. From the figure it is very clear that the bands are very specific.

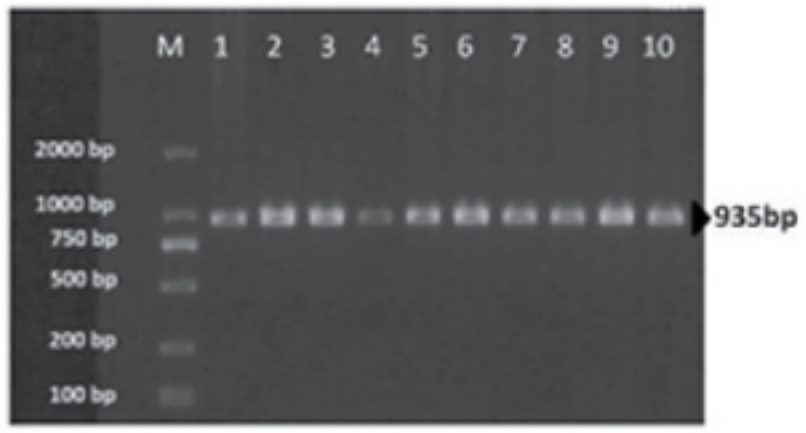

Figure 1. Amplification of fragment of kappa-casein gene from genomic DNA of cattle. Lane M: Marker, Lane 1-10: test samples. Amplified 935bp products are marked.

RFLP based genotyping of samples

PCR products (n=30) of κ-CN gene were digested with *Hind*III and *Hae*III restriction enzymes. A representative image in shown in Figure 2. PCR products digested with *Hind*III (upper

panel) could cut few samples and yielded three bands. On the other hand *Hae*III digested almost all tested samples (lower panel). Based on fragment length obtained through digestion about 66.67% (20/30) samples were genotyped as AA, 30.0% (9/30) were genotyped as AB and 3.33% (1/30) were genotyped as BB. For κ-CN gene genotype AA was found dominant.

For κ-CN gene, it has been found that, frequency of A allele was about 82% (20+4.5/30) and frequency of B allele was about 18% (1+4.5/30). Out of 66.67% AA genotype, 36.67% (11/30) are indigenous and rests 30% (9/30) are crossbreed cattle. Out of 30% AB genotype, 13% (4/30) are indigenous and 17% (5/30) are crossbreed cattle. 3% of BB genotyped cattle are indigenous (1/30). The table 4 summarizes the genotypes and allele frequencies among of the analyzed indigenous and crossbred cattle samples.

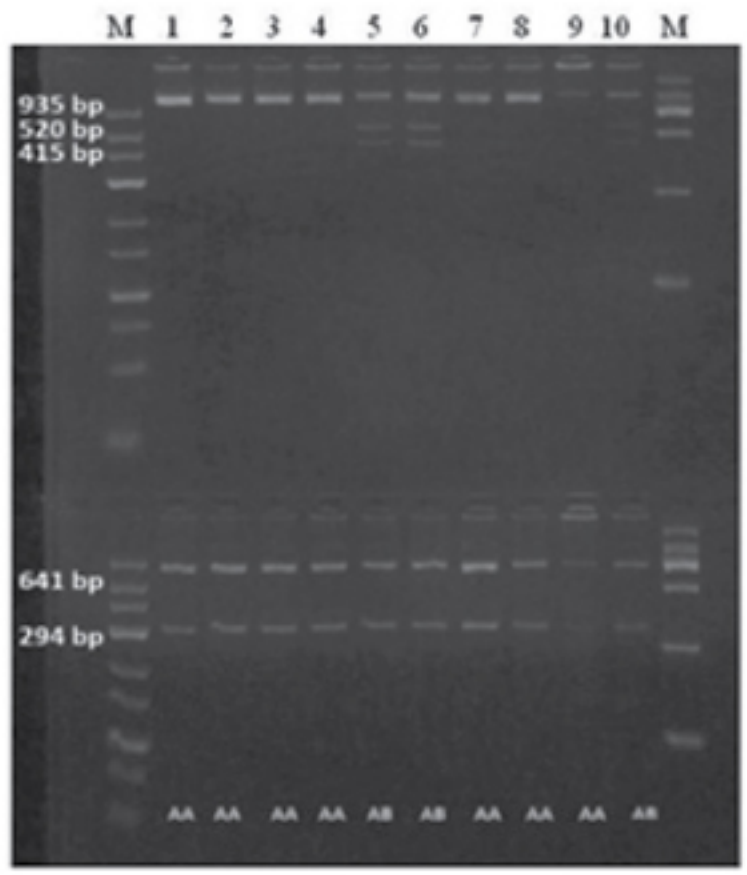

Figure 2. Fragment length analysis of kappa-casein gene. PCR products of 935bp were digested with *Hind*III (upper panel) and *Hae*III (lower panel) and DNA fragments were visualized in 4% agarose gel. Lane M: marker; Lane 1-10: tested samples. Genotype of each sample is indicated as AA, AB and BB based on digestion pattern.

Table 4. Genotype and allele frequencies of Kappa-casein gene in cattle

No. of sample	Genotype	Indigenous cattle	Crossbred cattle	Overall	Allele frequency	
					A	B
30	AA	0.366 n = 11	0.30 n = 9	0.666 n=20	0.82	0.18
	AB	0.13 n =04	0.17 n =05	0.30 n=09		
	BB	0.03 n =01	- n = 00	0.03 n=01		

Amplification of Alpha-lactalbumin gene

A total of 50 samples were tested for α-LA gene. With reported primers we were able to amplify 429bp fragments from α-LA gene of 100% (50/50) tested samples. A representative image of gel is shown in Figure 3. From the figure it is very clear that the bands are very specific.

RFLP based genotyping of samples

PCR products (n=50) of α-LA gene were digested with *Bsp1286I* restriction enzyme. A representative image in shown in Figure 4.Out of 50 samples, 100.0% (50/50) were genotyped as AA. Thus for Shibpur, Narsingdi region AA genotype of α-LA gene is found to be dominant.

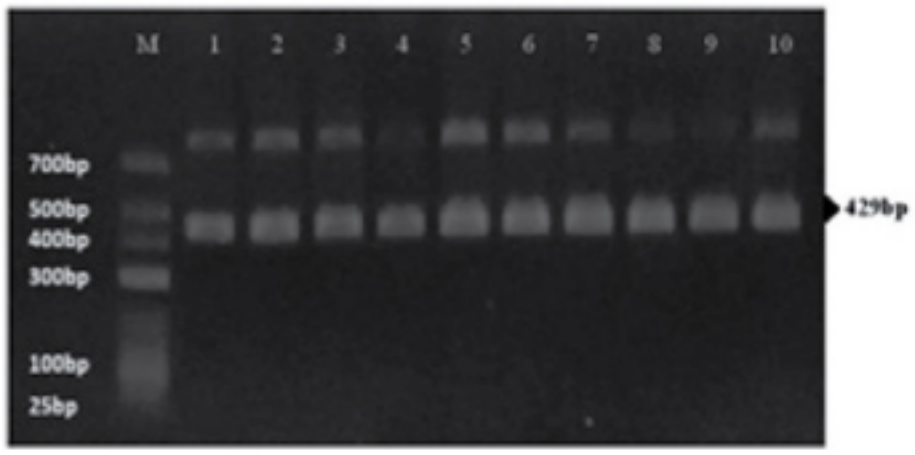

Figure 3. Amplification of DNA fragments of alpha-lactalbumin gene (429bp) from the genomic DNA of cattle. Lane M: Marker; Lane 1-10: Test samples. Amplified product 429bp marked.

For α-LA gene, it has been found that, frequency of A allele is about 100% (50/50) Out of 100% AA sample, 50% (25/50) are indigenous and rest 50% (25/50) are crossbreed cattle. Table 5 summarizes the genotypes and allele frequencies among of the analyzed indigenous and crossbred cattle samples.

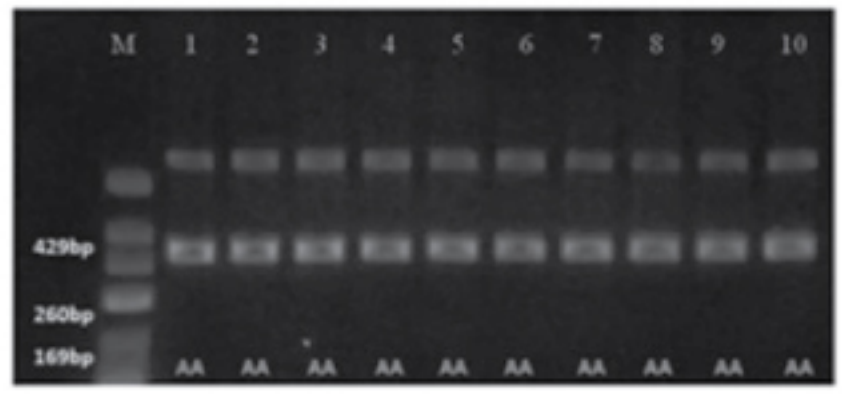

Figure 4. Fragment length analysis of alpha-lactalbumin gene. PCR products of 429bp were digested with *Bsp1286I*and DNA fragments were visualized in 4% agarose gel. Lane M: marker; Lane 1-10: tested samples. Genotype of each sample is indicated as AA based on digestion pattern.

Table 5. Genotype and allele frequencies of Alpha-lactalbumin gene in cattle

No. of sample	Genotype	Indigenous cattle	Crossbred cattle	Overall	Allele frequency	
					A	B
50	AA	0.50 n = 25	0.50 n = 25	1 n=50	1.00	0.00
	AB	n = 0	n = 0	n=0		
	BB	n =0	n = 0	n=0		

Discussion

PCR-RFLP method was used to genotype cattle based on milk protein genes. This is a well- known and most applied molecular tool for characterizing different gene and genotyping (Hristov and Radoslavov 2015; Kumar et al. 2005; Soria et al. 2003). Electrophoretic analysis of the PCR-RFLP products revealed that three genotypes viz. AA, AB and BB of κ-CN gene is available among the tested indigenous animals while only AA and AB genotypes were found in crossbred animals. The frequencies of A and B allele in those animals were A (0.82) and B (0.18) (Table 4). It has been reported that, BB form of κ-CN is responsible for higher yield in cheese making as well as milk and milk protein yield(McLean et al. 1984). The cheese production can be increased by 10% if milk from cows of genotype BB of κ-CN is used(Marziali and Ng-Kwai-Hang 1986). κ-CNA allele was predominantly observed in the studied cattle populations. This allele is presumably associated with high milk yield(Morkūnienė et al. 2016). In some other study in different region showed that A allele has been found predominant (Bech and Kristiansen 1990; Graml et al. 1986; Khatkar et al. 2004; Ng-Kwai-Hang et al. 1990; Rachagani et al. 2006). All these studies showed an individual effect of κ-casein gene on milk yield and milk fat yield.

For α-LA gene a specific forward and a specific reverse primer were used to amplify 429bp product. After PCR amplification, 429bp long fragment of α-LA gene was obtained and it was digested with *Bsp1286I* restriction enzyme. α-LA genotypes of individuals were determined by analyzing sizes of gathered α-LA gene fragments visualized by agarose gel electrophoresis (Figure 4). α-LA A variant has 429bp pairs long fragments as it does not digest with *Bsp1286I* where α-LA B variant has 260 and 169 base pairs long fragments when digested with *Bsp1286I*. Table 3 summarizes the sizes of expected α-LA gene fragments in base pairs after restriction with *Bsp1286I* restriction enzyme, for the specified α-LA genotypes. We could not find any other genotype for this gene except AA. The frequency of A allele for α-LA gene remains 1.00. Unexpectedly two bands had been visualized in the agarose gel electrophoresis of the α-lactalbumin PCR product. Among those two, one band is our desired one (429bp) and another one is larger.In order to solve the

underlying problem the bands of 429bp PCR product were cut from the gel. Then purified and again amplified with specific primers. But the problem persists.These PCR products were subjected to restriction digestion with *Bsp1286I* repeatedly. However, these products remained undigested. Therefore they are genotyped as AA. Cows with the A variant of the gene had higher predicted transmitting ability (PTA) for milk yield, protein yield, protein dollars, fat yield, and fat dollars; the B allele was associated with higher PTA for percentage of protein and fat (Voelker et al. 1997). AA genotype was observed with the highest frequency in both local and crossbreed cattle. Different groups studied with different breeds of cattle and reported various genotype of α-LA gene. Our result matches with the results of the group worked with Jersey breed (100% allele frequency of A). Both A and B allele has been reported from Holstein, Brown Swiss, Ayrshire Guernsey Cow (Voelker et al. 1997).The frequencies of AA genotype in local and crossbred animals were 0.50 and 0.50, respectively (Table 5). The frequency of B allele remains 0 though this work has been done with several samples from only one area.

Conclusion

Present study focuses on lactoproteins of milk and genotyping animals based on κ-CA and α-LA variants. We could genotyped fifty samples using PCR-RFLP. We found AA, AB and BB genotype of cattle for κ-CA and only AA genotype for α-LA. As the work had been conducted with a small number of samples from one area of the country, hence, it does not reflect the cattle genotype for other area in the country. More work should be done with samples from different areas. Moreover analysis of other milk protein genesneeded to be done. It would be of great interest if genotypes are linked with phenotypes. Plenty of phenotypic data will be necessary for this purpose.

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Differentiation of meat origin by polymerase chain reaction-restriction fragment length polymorphism using 12S rRNA gene

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Abstract

The verification of authenticity of meat and meat products is relevant for economical, religious or public health reasons. Forty six meat samples (10 samples of each like beef, buffen, chevon, and mutton; six samples of pork) were investigated for the differentiation of meat origin. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the mitochondrial 12S rRNA gene was performed for the differentiation of meat origin. PCR amplification yielded a 456-bp fragment in each of these species. PCR products from beef and buffen were treated with *Alu*I and *Hha*I restriction enzymes. Beef DNA was digested into two fragments of 359 and 97bp with *Alu*I but not buffen. On the other hand *Hha*I digested buffen DNA into 247 and 209bp fragments but not beef. Amplicons of chevon and mutton were digested with *Alu*I and yielded 246 and 210 bp fragments in both cases. For differentiation of these two products, *Apo*I was used to digest and yielded 329 and 127bp fragments in case of mutton but not chevon. Further, these two PCR products were also digested with *BspT*I and yielded 323 and 133 bp fragments in case of chevon but not mutton. Hence, mutton or chevon can be differentiated with either *Apo*I or *BspT*I. PCR product from pork was digested with *Alu*I, *Hha*I, *Apo*I and *BspT*I restriction enzymes. Only *Alu*I can cut the PCR product into two fragments of about 125 and 240bp but rest three restriction enzymes did not. For consistent results further study is necessary in case of pork. This method may be used for differentiation of origin of meat or species of animal cattle, buffalo, goat and sheep.

Key words: Meat origin, Mitochondrial 12S rRNA, PCR-RFLP.

INTRODUCTION

Large ruminants, cattle and buffalo and small ruminants, sheep and goat constitute the major portion of livestock. The domestic production of meat was 7.15 million tons in the fiscal year 2016-2017 against the demand of 7.14 million tons (DLS, 2016-2017). To date, although there are several meat types available for human consumption, beef, buffalo meat (buffen), goat meat (chevon) and sheep meat (mutton) account for the largest share within the total meat consumption. A part of these meats can be used as fresh meat for immediate consumption while some can be processed in different ways into a wide variety of processed meat products. Meat and meat products include nutrients that humans require for growth, physiological functions, health, etc. However, owing to the increasing price, meat producers may be tempted to commit fraud. Adulteration of meat may take the form of substitution of one cheaper meat with a costly expected species (Yman and Emanuelsson, 1998), for example buffalo meat (buffen) mixed with beef, which is a very old and common practice in low developing countries of the world (Munira et al., 2016). However, goat meat or chevon is the most popular red meat in Bangladesh. On the other hand, sheep meats (mutton) are generally not popular to the consumers of Bangladesh due to its characteristic odour. Although a considerable number of sheep populations are available in Bangladesh, but it is hard to find butcher shop or slaughterhouse who sells sheep meat. Furthermore, in the perspective of some religions, i.e. Islam and Judaism, pork and diet or foods containing pork or pork derivatives are serious matters, and for some reasons, pork is highly restricted from their diet. Additionally, consumer satisfaction, certain social and religious concerns and possible health hazards associated with particular type of meat warrant honest labeling of the source of meat and meat products. Therefore, precise differentiation of origin of meat has become a vital element in food quality

control procedures. Protection of consumers and producers from mislabeled meat products, fraudulent actions were always critical concerns that enforce legal authorities as well as many researchers to develop different techniques based on measuring either DNA or protein (Matsunaga et al., 1999; Calvo et al., 2001; Herman, 2001; Myers et al., 2003; Peter et al., 2004; Aida et al., 2005). Consequently, present study was taken to identify meat species using molecular technique PCR-RFLP.

Materials and Methods

Collection of meat samples

In this study a total of forty six (46) meat samples of cattle, buffalo, goat, sheep and pig were collected from different regions of the country. Ten meat samples from each species (cattle, buffalo, goat, and sheep) and six meat samples from pig encompassed this study.

Isolation of genomic DNA from individual meat samples

About 0.5 gm of meat samples were minced and subsequently subjected to DNA extraction. Minced sample was taken in to microcentrifuge tube and 600µl of TNES buffer and 35µl of Proteinase-K (20 mg/ml) was added and mixed by inverting the tube several times. The samples were incubated overnight at 50ºC. About 166.7µl of 6M NaCl, 225µL of chloroform was added and shacked for 10 minutes followed by centrifugation at 15,000 rpm for 10 minutes. The supernatant was removed in to a new microcentrifuge tube and equal volume (~ 800µl) of ice cold 100% ethanol was added, gently mixed by inverting the tube and centrifuged at 15,000 rpm for 15 minutes at 4ºC. The supernatant was poured off and DNA pellet was washed with 700µl of 100% ethanol. Then 200µL TE buffer was added and incubated at 37°C for overnight. The mixture was homogenized by pipetting about 30 times and stored at -20°C for future use.

Polymerase Chain reaction

Polymerase chain reaction was carried out in 25µL volume containing approximately 2.5µL of 5µmol/L each primer, 2.5µL of 10x PCR buffer (50mmol/L KCl, 10mmol/L Tris-HCl [pH8.0], 0.1% Triton X-100), 1.8µL of 25mmol/L $MgCl_2$, 2.5µL of 2mmol/L of each dNTP, 2.0µL of 50 ng/µL genomic DNA, 1.0 U Taq DNA polymerase (Promega, Madison, WI, USA) & the rest was ddH_2O. Primer sequences (F: 5′-CAAACTGGGATTAGATACCCCACTAT-3′ and R: 5′-GAGGGTGACGGGCGGTGTGT-3′) used in this reported earlier (Kocher et al., 1989). Thermal condition was as follows: initial denaturation at 94°C for 5 minutes followed by 30 cycles of denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds and extension at 72°C for 1 minute (Girish et al., 2005 and Kocher et al., 1989). A final extension was performed at 72°C for 10 minutes. Thermal cycling was performed using GeneAtlas (Model: G02, Japan).The PCR products were analyzed by electrophoresis in 1% agarose gel with ethidium bromide staining.

Preparation of meat mixture and isolation of DNA

Meat mixtures were prepared by mixing the meat samples of two different meat species. For this purpose equal volume of beef and buffalo (50:50) and chevon and mutton (50:50) was mixed. After mincing the mixed meat samples DNA was extracted as mentioned above.

Restriction fragment length polymorphism

PCR amplicons of the mitochondrial 12S rRNA gene were subjected to restriction enzyme digestion with restriction enzymes reported earlier (Kocher er al., 1989 and Girish et al., 2005). The restriction enzyme digestion mixture of 10µl contain nuclease free water 3.5µl, 10X Buffer Tango 1µl, enzyme 0.5µl (suitable enzyme) and the sample (PCR products obtained using 12SrRNA primer) 5µl. Digested product was visualized by electrophoresis in 2% agarose gel along with 100 bp or 50 bp DNA ladder.

Results and Discussion

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of 12S rRNA gene was performed to determine and differentiate the species of meat. A total 46 meat samples of cattle,

buffalo, goat, sheep and pig were collected. DNA was extracted from the sample followed by amplification with universal primer targeting 12S rRNA gene. Then the PCR amplicons were digested with *Alu*I, *Hha*I, *Apo*I, and *BspT*I restriction enzymes resulting in a pattern that could identify and differentiate each of the above species. Similar study was conducted in case of meat mixture to identify and differentiate meat species. A 456bp of DNA fragment was amplified from all 46 samples A representative image is shown in Figure 1.

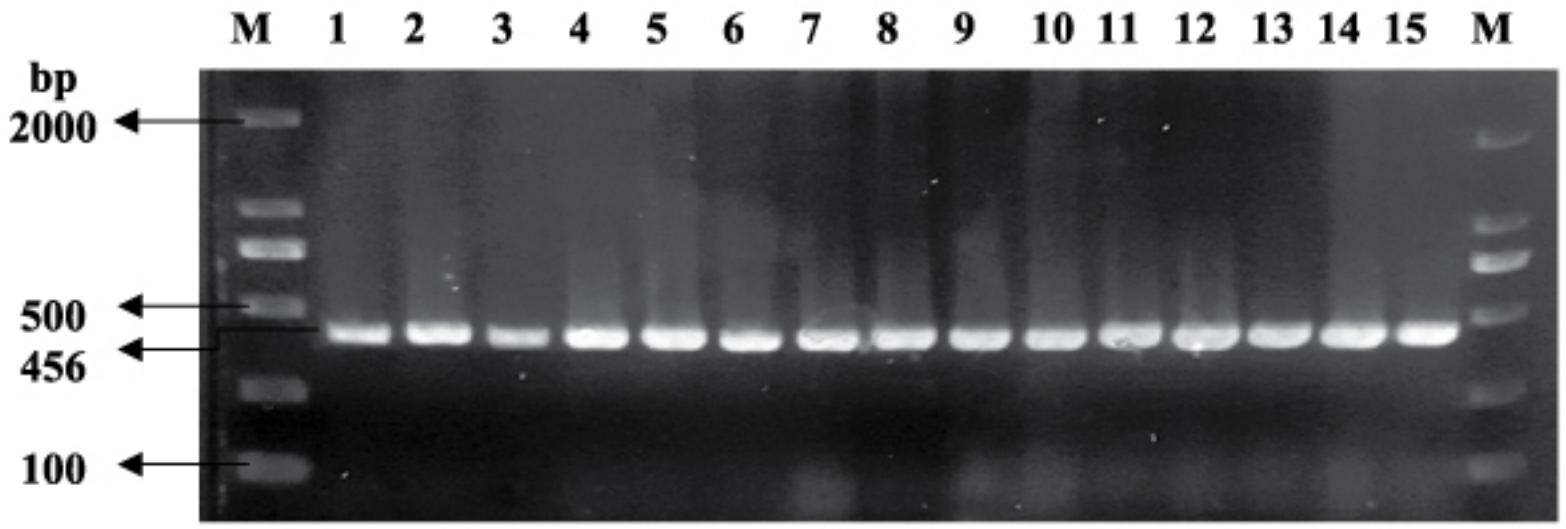

Figure 1: Amplification of 456 bp DNA fragments from different meat samples using mitochondrial 12S rRNA gene. Lane M: 100bp DNA ladder; Lane 1-3: Beef samples; Lane 4-6: Buffalo meat samples; Lane 7-9: Chevon; Lane 10-12: Mutton; Lane 13-15: Pork

Restriction enzyme digestion

PCR amplicons were subjected to digestion with different restriction enzymes. Based on restriction pattern the samples were categorized. Results of restriction digestion are presented below.

Restriction enzyme digestion of PCR products obtained from Beef and Buffen

PCR amplicons obtained from beef DNA and buffen DNA were digested with *Alu*I and *Hha*I restriction enzyme, respectively. *Alu*I cut the 456 bp products of beef into two fragments of 359 and 97 bp but not buffen while *Hha*I yielded 247 and 209 bp from buffen but not from beef. Representative gel images are shown in Figure 2 and Figure 3

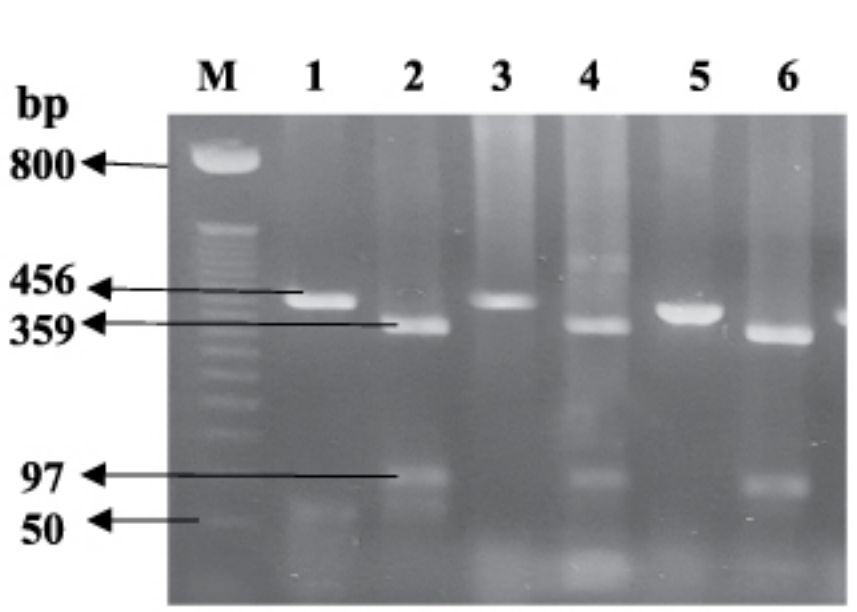

Figure 2: RFLP of mitochondrial 12SrRNA gene. AluI digested the beef sample and yielded fragments 359 and 97 bp.

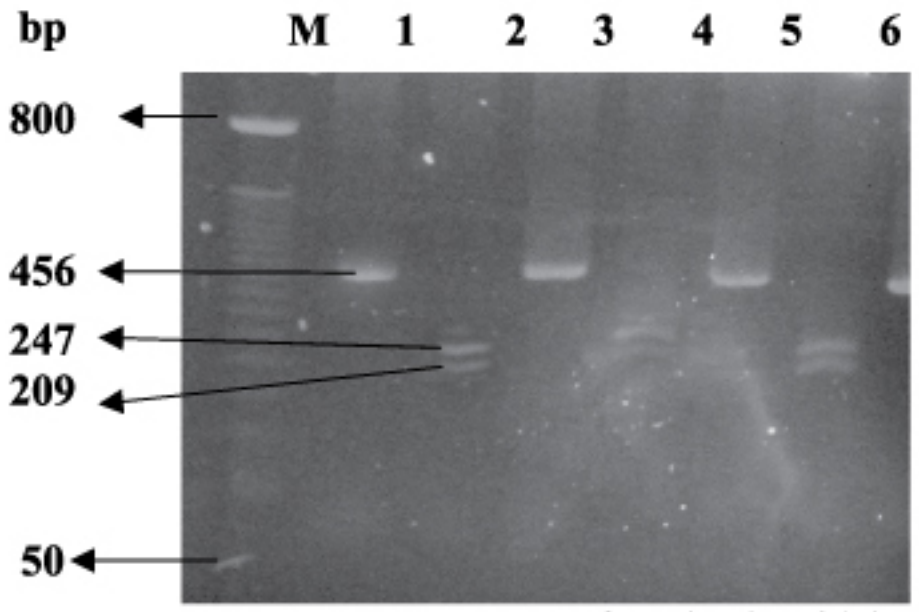

Figure 3: RFLP of mitochondrial 12SrRNA gene. HhaI digested the buffalo sample and yielded fragments 247 and 209 bp.

Restriction enzyme digestion of Chevon and Mutton

PCR amplicons obtained from chevon DNA and mutton DNA were digested with *Alu*I and *Apo*I respectively. *Alu*I cut the 456 bp products into two fragments of 246 and 210bp in both chevon and mutton. Digestion with *Apo*I restriction enzyme yielded 329 and 127 from sheep DNA. *Apo*I could not digest DNA from goat meat. Representative gel images are shown in Figure 4, 5 and 7. However, PCR amplicons from goat meat were digested with *BspT*I that yielded fragments of 323 and 133 bp (Figure 6). Hence, chevon and mutton were differentiated with either *Apo*I or *BspT*I.

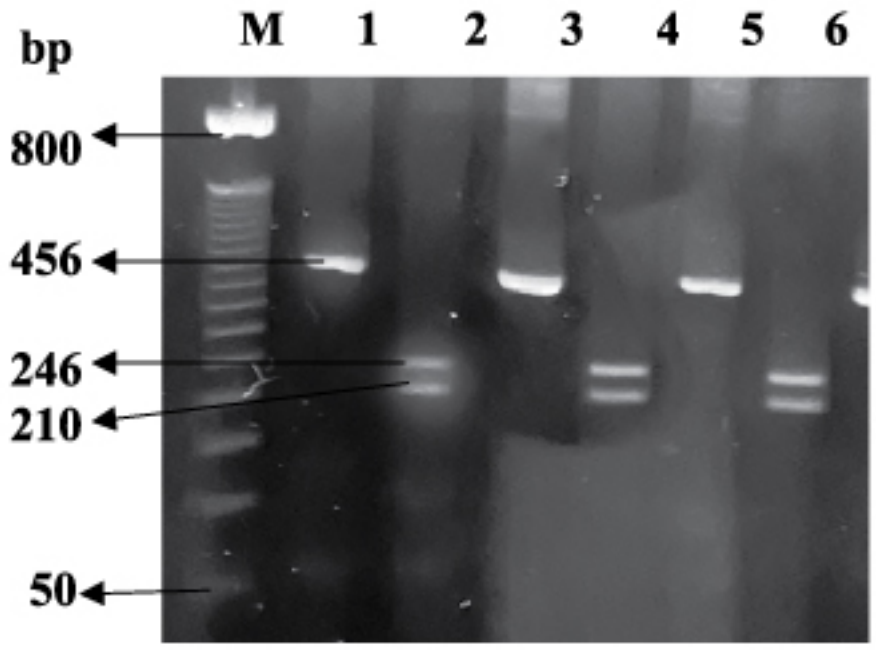

Figure 4: RFLP of mitochondrial 12SrRNA gene. AluI digested the goat (chevon) DAN and yielded 246 and 210bp fragments

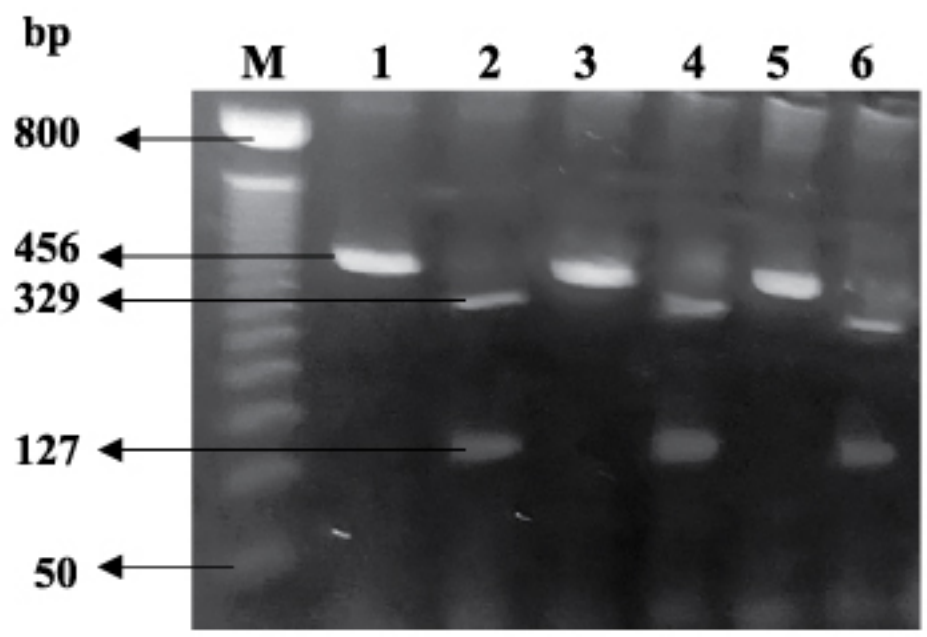

Figure 5: RFLP of mitochondrial 12SrRNA gene. ApoI digested the sheep (mutton) DNA and yielded 329 and 127 bp fragments.

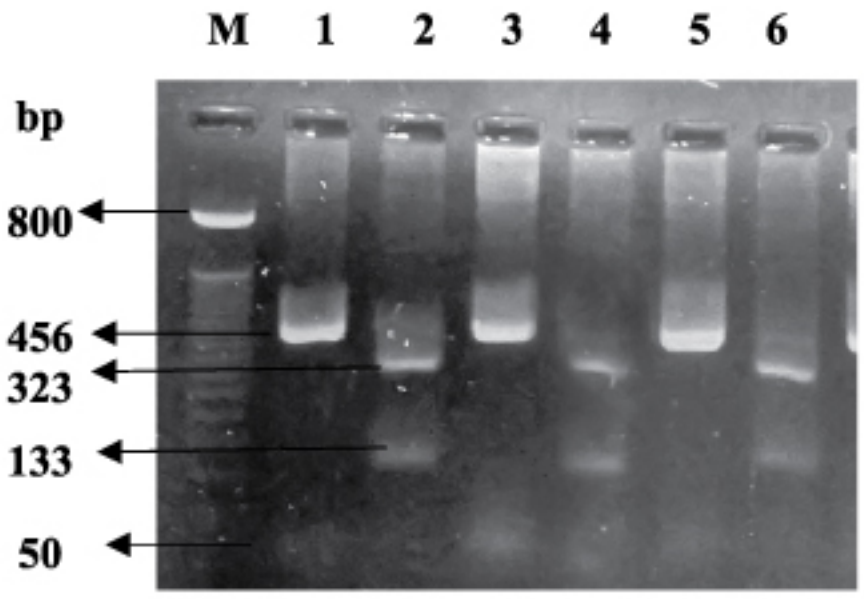

Figure 6. RFLP of mitochondrial 12S rRNA gene. BspTI digested the goat meat (chevon) sample and yielded fragments 323and 133 bp.

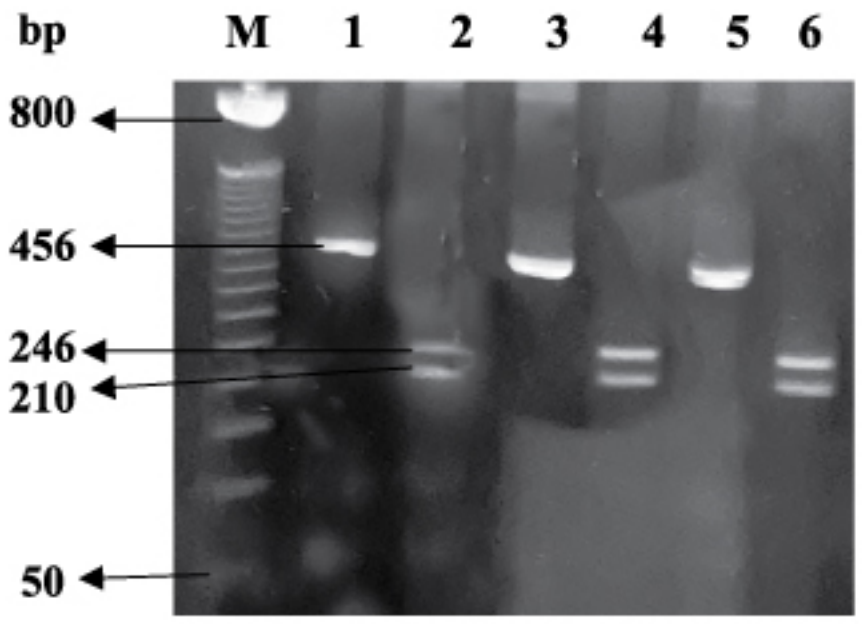

Figure 7. RFLP of mitochondrial 12S rRNA gene. AluI digested the sheep meat (mutton) sample and yielded fragments 246 and 210 bp.

3.5 Restriction digestion of Pork

PCR amplicons obtained from pork DNA were digested with *Alu*I, *Hha*I, *BspT*I and *Apo*I enzymes. *Alu*I digested pork DNA and generated two fragments of 240 and 125bp (Figure 8, Lane 1-6; left) but rest 3 enzymes could not cut pork DNA.

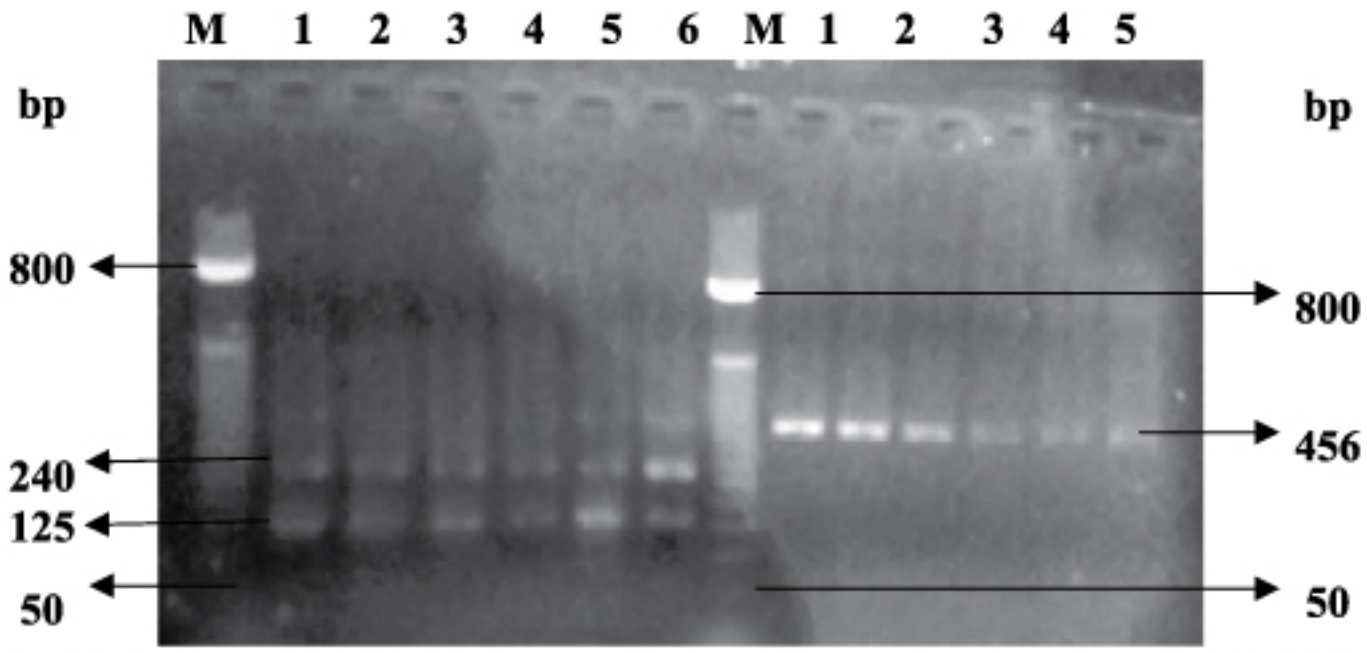

Figure 8: RFLP of mitochondrial 12S rRNA gene. PCR amplicons of pork digested with AluI, HhaI, BspTI, ApoI. AluI generated fragments of 240 and 125 bp (Lane: 1-6; left). HhaI, BspTI, ApoI could not digest (Lane:

Restriction enzyme digestion of mixed meat

PCR amplicons derived from DNA of mixed meat samples were digested with *Alu*I and *Hha*I. Three types of bands were found in both cases. PCR amlplicons were digested with *Alu*I that yielded fragments of 456, 359 and 97 bp while 456, 247 and 209 bp fragments were found in case of *Hha*I digestion. On the other hand, the rest of PCR amplicons of mixed meats were also digested with *BspT*I and *Apo*I. In both cases three types of DNA fragments were generated. *BspT*I generated fragments of 456, 323 and 133 bp and *Apo*I generated 456, 329 and 127 bp fragments. A representative gel image is shown in Figure 9.

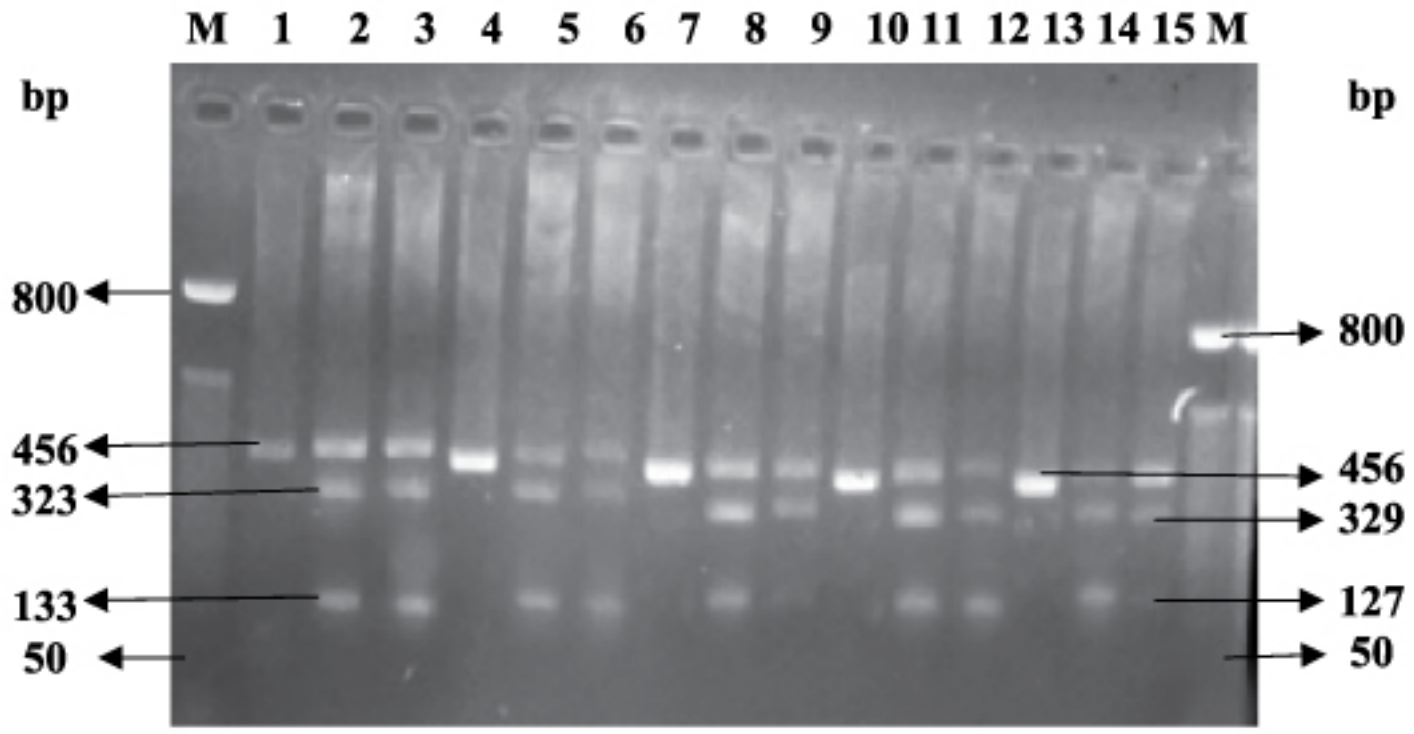

Figure 9. RFLP of PCR products from mitochondrial 12S rRNA gene of meat mixtures. Lane M: 50bp DNA ladder. Lane-1, 4, 7, 10, 13: Undigested PCR product of mixed meat samples; Lane-2, 5, 8, 11, and 14: BspTI digested PCR products into fragments of 456, 323 and 133bp; Lane-3, 6, 9, 12 and 15: ApoI digested PCR product yielded fragments of 456, 329 and 127 bp.

Differentiation of meat species is often necessary in view of the consumer preferences and for regulatory purposes. Mitochondrial DNA sequence is highly conserved in different animal species enabling design universal primers for amplification of 12S rRNA gene (Kocher et al., 1989). We employed PCR-RFLP analysis targeting mt12S rRNA gene in the present investigation for differentiation of meat species and found the technique to be effective in accurate differentiation of beef, buffen, chevon, mutton. We also used pork samples identification and differentiation. In a study Meyer et al. (1995) amplified the 359-bp fragment of the cytochrome b gene using PCR followed by restriction digestion with *Alu*I, RsaI, TaqI and HinfI for identify pig, cattle, wild boar, buffalo, sheep, goat, horse, chicken and turkey meat. In another study Partis et al., (2000) generated DNA fingerprints for 22 animal species by amplifying 359bp regions within the cytochrome b gene and digesting the amplified product with HaeIII and HinfI. All species could be discriminated except

kangaroo and buffalo. PCR amplicons of beef were digested with *Alu*I enzyme and generated fragments of 359 and 97 bp. This enzyme also produced 246 and 210 bp fragments from both chevon and mutton. *Alu*I could not digest buffalo DNA, as per expectation. *Hha*I, *Apo*I and *BspT*I were specific only to buffalo meat, mutton and chevon generating 247 and 209 bp, 329 and 127 bp and 323 and 133 bp fragments, respectively. Pork was subjected to digestion with four enzymes (*Alu*I, *Hha*I, *BspT*I and *Apo*I). Only *Alu*I digested the pork sample and yielded fragments 240 and 125 bp. But the rest of three enzymes (*Hha*I, *Apo*I and *BspT*I) could not digest PCR products. Consequently, meat origin of individual samples were identified and differentiated. The results obtained in the present study are in agreement with those of Girish et al., (2005) who employed PCR-RFLP of mt 12S rRNA gene and found the technique to be effective in differentiation of beef, buffalo meat, goat meat and sheep meat. Thus PCR-RFLP analysis of mt12S rRNA gene is a technique of potential v*Alu*e in identification and differentiation of meat species.

Conclusion

Differentiation of meat origin of different species is considered important due to religious belief, social, forensic and public health reasons. The present study analyzed 46 meat samples for their differentiation of individuality of origin such as cattle meat (beef), buffalo meat (buffen), sheep meat (mutton), goat meat (chevon) and pig meat (pork) as well as their mixed meat samples. Universal primers 12S rRNA gene was used in all five species, viz., cattle, buffalo, sheep, goat and pig. We could amplify a 456bp fragment of mitochondrial 12S rRNA gene in all five species. Also we could differentiate individual meat samples as well as meat from mixture. From the the present study it may be concluded that the technique may be useful for identification of meat and differentiation of origin of meat in a mixture of meat.

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Intrafollicular transfer of abattoir-derived immature or in vitro-matured oocytes results in blastocysts' gene expression profiles comparable to fully in vivo-produced blastocysts

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Abstract
Since the inception of the in vitro embryo production in cattle, a plenty of efforts has been employed aiming to improve the competence of the embryos as like as fully in vivo-produced embryos. However, the quality of in vitro-produced embryos is still far from its in vivo counterparts. Intrafollicular oocyte transfer (IFOT) has emerged as a viable alternative option. Analyses of the molecular mechanisms and pathways involved in IFOT-generated embryos provide valuable insight into the technique for further improvement. Global gene expression profiles of bovine IFOT embryos either produced from immatureor in vitro-matured oocytes along with fully in vivo- or in vitro-developed embryos were investigated by using Affymetrix Bovine Gene 1.0 ST Array. In comparison with fully in vivo-produced embryos, embryos produced through IFOT of immature or in vitro-matured oocytes had less differentially expressed genes (122 and 144, respectively) while in fully in vitro-produced embryos had 1053 genes. The biological processes involved in IFOT embryos differed depending on the stages of transferred oocytes. Only genes associated with chaperone-mediated protein folding are over-represented in fully in vivo produced embryos in comparison with embryos produced from immature oocytes. On the other hand, genes associated with different metabolic processes are over-represented in fully in vivo produced embryos in comparison with embryos derived from in vitro-matured oocytes. Importantly, biological processes related to transport of unfolded or misfolded proteins from endoplasmic reticulum to cytosol are over-represented in the embryos derived from IFOT of immature oocytes in comparison with its in vitro-matured counterparts that may play a vital role in preimplantation embryo development. Transcriptome profiles of IFOT embryos derived either from immature or in vitro-matured oocytes showed very closer resemblance to that of fully in vivo-derived embryos. In contrast, embryos produced from fully in vitro condition showed altered gene expression profiles.

Keywords: IFOT embryos, In vivo embryos, Immature oocytes, Mature oocytes, Microarray

Introduction

Since the inception of the in vitro embryo production, a plenty of efforts has been employed for further improvement of the technique. Although a tremendous improvement has already been achieved, the in vitro-produced embryos are still lagging behind in comparison to the in vivo-derived embryos. The in vitro culture condition is thought to be one of the important hindering factors affecting the early embryo development. This is, in fact, extensively investigated in bovine blastocysts by transferring of 4- and 16-cells embryos in alternative culture conditions (in vitro vs. in vivo or vice versa) at the time of embryonic genome activation (Gad et al., 2012). The authors reported that in vitro culture condition critically alters the transcriptome profiles of the blastocysts. Supplementation of serum in in vitro culture media is considered to be associated with altered transcriptome profiles as well as epigenomics that may result in fetal overgrowth in ruminants, commonly known as large offspring syndrome (Chen et al., 2013). Therefore, many laboratories are nowadays underscoring in producing embryos without supplementation of serum in the culture media. In this regards, Heras et al. (2016) compared the whole transcriptome profiles by RNA-seq where they reported that the transcriptome profiles of blastocysts produced in FBS-free culture condition were more closer to in vivo-derived blastocysts. However, the embryos produced in serum-free or fatty acid-free conditions have a quite lower developmental rate than that of serum or fatty acid containing medium (Wydooghe et al., 2014).

In order to minimize the limitations of in vitro culture environment, the essential roles of oviduct have been investigated in many laboratories. Accordingly, sheep oviducts (Gutierrez-Adan et al., 1996), and bovine oviducts (Cordova et al., 2014) have widely been used for the development of bovine embryos. Furthermore, in order to take an advantage of real life situation, in vitro-produced early embryos have been cultured up to the blastocyst stages in the fallopian tubes through endoscopically transferring of embryos directly into the live cows. These studies explicitly reported that culturing of in vitro-fertilized bovine zygotes into the fallopian tubes resulted in blastocysts with greater cryotolerance capability. However, intrafallopian transfer of bovine embryos is a perplexing task which requires a high level of skill and also a small incision in the flank region. Consequently, we have recently adopted a robust technique;intrafollicular oocyte transfer (IFOT) where the in vitro-matured slaughterhouse-derived oocytes were directly transferred into the preovulatory follicle through ultrasonographical guidance and subsequently fertilization was taken place in the fallopian tube after artificial insemination (Kassens et al., 2015). This new technique allows us collection of large number of blastocysts (up to 12) from the same donor. As expected, the transfer of IFOT-derived embryos resulted in viable pregnancies that culminated into birth of healthy calves(Kassens et al., 2015). The pregnancy rates, however, were lower in IFOT-derived embryos in comparison with fully in vivo-derived embryos that warrant further investigation of the differences in molecular mechanisms and pathways between IFOT versus fully in vivo-derived embryos. In the present study, we additionally generated embryos by directly transferring of slaughterhouse-derived immature oocytes into the dominant follicles with the help of ultrasonographical guidance. Furthermore, Spricigo and coworkers (2016) also reported viable pregnancies in cows following transfer of embryos derived from IFOT of immature oocytes. Surprisingly, the research group did not find any significant difference between IFOT- (immature oocytes) and in vitro-derived blastocyst's quality in terms of total cell number and apoptotic cell ratio. Hence, the detailed transcriptome profiles, molecular mechanisms and pathways associated with IFOT-derived embryos are of utmost importance before being their potential widespread application.

Therefore, the aim of the present study was to investigate the detailed molecular mechanisms and pathways involved in embryos that are generated following IFOT of slaughterhouse-derived in immature or vitro-matured oocytes in comparison with fully in vivo- or in vitro-generated embryos. Our results possibly for first time open up the molecular mechanisms and pathways (almost the similar of mother in selection of competent oocytes) for further improvement of embryos that may help in modulating the IFOT-derived embryos and consequently can be used as a model for human assisted reproduction.

Materials and methods

In vitro oocyte maturation for IFOT

In vitro oocyte maturation was performed as described by Kassens et al. (2015). In brief, bovine ovaries were obtained from a local slaughterhouse and brought to the lab in physiological saline (0.9% NaCl) at 30°C within 3 h of slaughter of cows. Cumulus oocyte complexes (COCs) were aspirated from small follicles (2–8 mm), and COCs with a homogenous, evenly granulated ooplasm, with oocytes surrounded by at least three layers of cumulus cells, were transferred to modified Tissue Culture Medium 199 (TCM; Sigma) supplemented with 4.4 mM HEPES, 33.9 mM NaHCO3, 2 mM pyruvate, 2.9 mM calcium lactate, 55 μg ml^{-1} gentamycin and 12% heat-inactivated ECS. After washing three times, COCs were cultured in groups of 50 in 400 μl of modified TCM supplemented with 10 μg ml^{-1} FSH (FSH-p) at 39°C in a humidified atmosphere with 5% CO_2 in air.

Intrafollicular oocyte transfer and embryo production

The detailed procedures are available in our previous paper (Kassens et al., 2015). In brief, intrafollicular in vitro-matured oocyte transfer (IFMOT) was performed in synchronized Simmental heifers. Immediately prior to the IFMOT procedure, 16 h after the final GnRH injection, heifers were artificially inseminated using fresh semen (50 million spermatozoa per dose) from the same Red Holstein Friesian bull used for superovulation as well as for in vitro embryo production. IFMOT was performed 16–22 h following the final GnRH injection. In contrary, intrafollicular immature oocyte transfer (IFIOT) was performed into the dominant follicles following the final GnRH injection. To coincide with the time of IFMOT, immature oocytes were allowed at

least 16-22 h for intrafollicular maturation. The system to transfer the oocytes into the preovulatory follicles or dominant follicles consisted of an OPU device. For transferring to the follicle, oocytes were loaded into the tip of the OPU system represented by a disposable single-lumen needle (0.45 × 22 mm; G26; Sterican). A maximum volume of 200 µl of PBS containing 50 oocytes was aspirated. Afterwards, by observing the preovulatory follicle (for IFMOT) or dominant follicle (for IFIOT) on the monitor, the needle was pushed carefully through the vaginal wall and peritoneum.

IVF and embryo production

In vitro oocyte maturation was performed for 22-24 h as mentioned earlierKassens et al. (2015). IVF was performed in Fert-TALP medium supplemented with 20 µM penicillamine, 10 µM hypotaurine, 2 µM epinephrine, 6 mg/ml BSA-*FFA*, 50 µg/ml gentamycin, and 1 µg/ml heparin. For IVF, semen of the same red Holstein Friesian was used for the generation of IFOT and in vivo embryos as 2×10^6 sperm/ml. Following 18 h of co-incubation, the presumptive zygotes were vortexedand washed three times. Then the embryo culture was performed in groups of 50 with 5% CO_2 in air at 39°C for up to 7 days in 400 µl of SOFaa supplemented with 0.4% BSA-*FFA* overlaid with mineral oil.

Superovulation and in vivo embryo production

The detailed protocol for superovulation has been described by Kassens et al. (2015). In brief, Simmental heifers were synchronization by administration of 500 µg of cloprostenol, i.m. (Estrumate, Essex Tierarznei, Germany) 11 days apart. Forty eight hours after each of the cloprostenol injections, cows received 0.02 mg of GnRH (Receptal; Intervet). Superovulation treatment was started after 12 days of the last GnRH administration. Accordingly, cows received the first of 8 consecutive injections of FSH during the course of 4 days in decreasing dosages (in total, 300–400 mg of FSH; Stimufol, University of Liege). At 60 and 72 h after the initial administration of FSH, cows received two doses of cloprostenol. Finally, 48 h after the first of the two cloprostenol injections, ovulation was induced by 0.02 mg of GnRH and immediately after GnRH injection AI was performed (3 times).

Embryo flushing

The detailed procedures are described in Kassens et al. (2015). Briefly, seven days after the IFOT procedure or superovulation treatment (day of heat for IFOT or day of AI for superovulation was defined as Day 0), embryos were flushed from the uterus of recipient cows. An embryo-flushing catheter (CH15; Wörrlein) was fixed in the uterine horns. Afterwards, the embryos were flushed out by washing each uterine horn with 500 ml of PBS. In this regards, the uterus was flushed using a catheter connected with an embryo filter (Immuno Systems Inc.). Immediately after flushing, embryos were washed twice in PBS and calculated after subtracting the recipient's own embryo. In order to do so the embryo with the best quality was subtracted for each IFOT recipient to calculate accurate quantities of embryos flushed out in excess. The subtracted embryos were thought to be derived from the respective recipient's oocytes. Therefore, it can be assumed that the chances of false positive inclusion of recipient oocyte-derived embryos as of IFOT-derived embryos were very low.

Embryo storage and RNA extraction

The blastocysts were washed three times in RNase-free PBS (Phosphate buffered saline; Ambion), placed in pools of 5 blastocysts in 2 µL of lysis buffer having RNase inhibitor and immediately stored at −80°C. In each group, 15-20 blastocysts were used to yield the required amount of total RNA for Affymetrix Array without prior amplification biases. The total RNA was extracted using the Single Cell RNA Purification Kit (Norgen, P/N 51800) according to the manufacturer's instructions. On-column DNA digestion was done by using RNase-free DNase (Qiagen GmbH, Hilden, Germany). RNA was eluted in 18 µl of elution buffer and the initial quantity and purity were measured by Agilent 2100 Bioanalyzer (Agilent Technologies, CA) using RNA 6000 Pico Kit.

Microarray target preparation and hybridization

About 15 ng total RNA samples from each of the biological replicates were processed with the GeneChip® WT Pico Reagent Kit (Affymetrix Inc., CA) in order to prepare the target probes of 12 microarrays according to the manufacturer's instructions. In brief, the total RNA was subjected to synthesize the first-strand cDNA containing a T7 promoter sequence at the 5′ end followed by synthesis of double-strand cDNA using DNA polymerase with the presence of RNase H. Then, the double-stranded cDNA was subjected to in vitro transcription by T7 RNA polymerase for synthesis of the antisense RNA. The cRNA preparation was then purified using purification beads to improve its stability. From 20 µg of purified cRNA, the sense-strand cDNA (ss-cDNA) was synthesized by reverse transcription. The ss-cDNA contained dUTP at a fixed ratio relative to dTTP and the remaining cRNA was degraded by RNase H. Following purification and quantification, 5.5 µg of ss-cDNA in a 46 µl volume was fragmented by uracil-DNA glycosylase (UDG) and apyrimidinic endonuclease 1 (APE 1) at the unnatural dUTP residues and breaks the DNA strand. The fragmented ss-cDNA was then labeled with TdT using the biotin-linked labeling reagent. The hybridization of microarray probes followed by washing and staining was performed with the GeneChip® Hybridization, Wash and Stain Kit (Affymetrix Inc.). For hybridization, about 130 µl of cocktail containing at least 3.5 µg of biotinylatedss-cDNA probes were injected into the GeneChip® Bovine Gene 1.0 ST Array (P/N 901921, Affymetrix Inc.) and incubated for 16 h in a hybridization oven (Gene-Chip® Hybridization oven 640; Affymetrix Inc.) at 45°C with 60 rpm. The hybridized chips were stained and washed in a fluid station and scanned by AffymetrixGeneChip® scanner 3000 7G. The AffymetrixGeneChip® Command Console™ (AGCC) software was used to evaluate the array images and to export the spot intensity data in the .CEL file format.

Microarray data analysis

Pre-processing of microarray raw dataset was performed using the Affymetrix Expression Console software (v1.0). The RMA (Robust Multi-array Average) based background correction and quantile normalization of microarray data were performed at transcript level. For quality control, some quality plots of the raw intensity data were checked before and after the normalization. In total 12 arrays were used for further analyses. After normalization, 24415 transcript probes were analyzed by TAC (v.3.0) and subjected for downstream analyses.

Hierarchical clustering and functional annotation of genes

Hierarchical clustering was performed with RMA normalized transcripts differentially expressed among the target groups (FDR corrected p-value < 0.05, FC > 2) using the Affymetrix TAC software.Ensembl gene IDs of the differentially expressed genes (Bonferroni corrected p-value < 0.01, absolute FC > 2) were further analyzed with the ClueGO v2.3.3 plugin of the Cytoscape 3.5.0 software (Heras et al., 2016). The Cytoscape plugin ClueGO was used to export the significantly enriched gene ontologies (levels 3-5) related to biological process, cellular component, and molecular function. Furthermore, a KEGG pathway of gene ontologies was also conducted.

Results and discussion

Gene expression deviations in comparison with in vivo derived embryos

Using Affymetrix Transcription Analysis Console software (TAC, v.3.0), we have identified the differential expressed genes (DEGs) among the blastocyst groups. Irrespective of False Discovery Ratio (FDR) corrected p-value, TCA found the greatest variation of DEGs in fully in vitro-developed embryos followed by IFOT embryos produced either from immature or in vitro-matured oocytes in comparison with fully in vivo-developed embryos (Table 1). The objective was to determine whether the gene expression profiles of the embryos produced from IFOT were more resemblance to in vivo-derived embryos. A comparison of DEGs among the embryo groups has been shown in Venn diagram (Fig. 1). The number of DEGs in the embryos derived from IFOT of immature and in vitro-matured oocytes was almost 8 times fewer than the embryos derived from fully in vitro condition (122, 144, and 1053, respectively) in comparison with fully in vivo-developed embryos. Interestingly, while the unique DEGs in fully in vitro-developed embryos were 913, the unique DEGs were sharply decreased in embryos derived from IFOT of in vitro-matured and immature

oocytes (28 and 57, respectively) in comparison with fully in vivo-developed embryos. Remarkably, very few DEGs were upregulated in all embryo groups in comparison with in vivo-derived embryos. For example, only 66 out of 1053 DEGs were upregulated in fully in vitro-developed embryos. Similarly, only 9 (out of 144) and 19 (out of 122) DEGs were upregulated in embryos derived from IFOT of in-vitro matured and immature oocytes. These results indicated that transfer of either immature or in vitro-matured oocytes resulted in embryos' gene expression profiles more similar to in vivo-developed embryos than that of in vitro-developed embryos.

Table 1. Results of microarray analysis of transcripts differentially expressed among the groups compared.

Comparison		**FDR p value (None)**	**FDR (p < 0.1)**	**FDR (p < 0.05)**	**FDR (p < 0.01)**
Vitro vs. Vivo	Up	77	66	38	4
	Down	1000	987	848	162
	Total	**1077**	**1053**	**886**	**166**
Mature vs. Vivo	Up	17	9	4	0
	Down	155	135	95	0
	Total	**172**	**144**	**99**	**0**
Immature vs. Vivo	Up	24	19	6	0
	Down	132	103	25	0
	Total	**156**	**122**	**31**	**0**
Mature vs. Vitro	Up	213	146	59	0
	Down	29	15	6	0
	Total	**242**	**161**	**65**	**0**
Immature vs. Vitro	Up	866	532	204	0
	Down	293	160	61	0
	Total	**1159**	**692**	**265**	**0**
Immature vs. Mature	Up	169	28	5	0
	Down	113	24	1	0
	Total	**282**	**52**	**6**	**0**

Number of differentially expressed up- or down-regulated genes in different groups with different FDR *p*-values, ranging from 0 - <0.01 and an absolute fold change (|FC|) >2.

A hierarchical clustering was performed to identify the similar or more closer gene expression profiles of embryos produced in three different experimental conditions in comparison with fully in vivo-produced embryos (Fig. 2). In general, two major hierarchical clusters were formed, separating fully in vitro developed embryos from the embryos developed in fully in vivo and IFOT of in vitro-matured and immature oocytes. Furthermore, hierarchical clustering firstly clusters the differential expressed genes of embryos derived from IFOT of in vitro-matured oocytes with the differentially expressed genes of fully in vivo-derived embryos that were again clustered with embryo derived from IFOT of immature oocytes and thereby completely separating the embryos derived from fully in-vitro condition. The similarity of gene expression profiles of IFOT embryos with fully in vivo-derived embryos could be the culture condition at the time EGA that had a great influence on gene expression profiles (Gad et al., 2012).

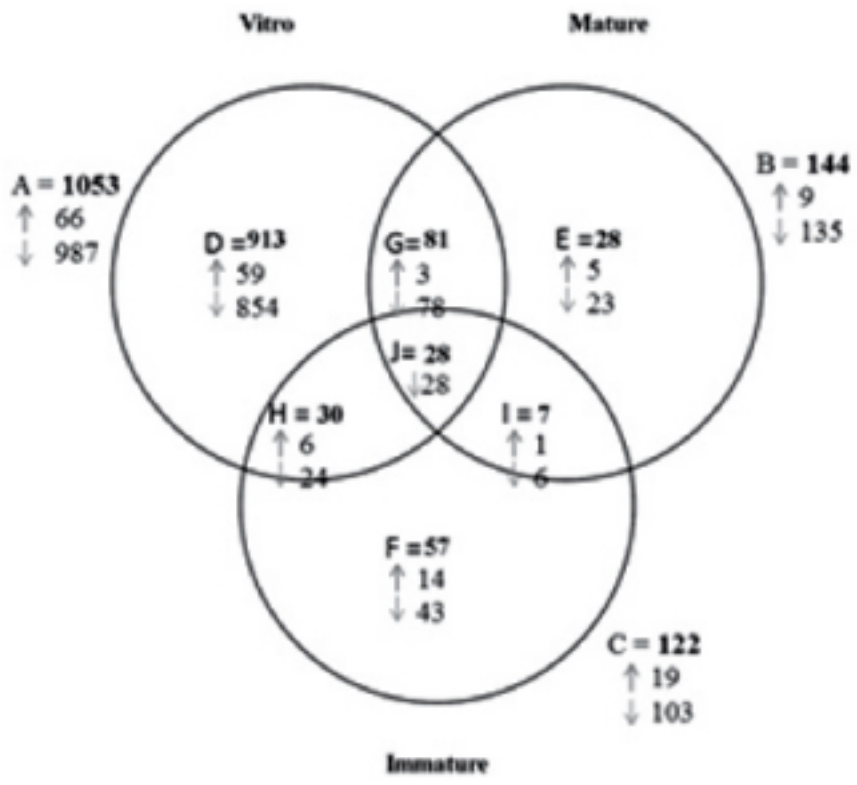

Fig. 1. Venn diagram shows the number of unique and common genes among embryos of Vitro, Mature and Immature groups. (A) whole DEGs in fully in vitro developed embryos in comparison with fully in vivo developed embryos, (B) whole DEGs in the embryos derived from in vitro-matured oocytes in comparison with fully in vivo developed embryos, (C) whole DEGs in the embryos derived from immature oocytes in comparison with fully in vivo developed embryos, (D) unique DEGs in Vitro group, (E) unique DEGs in Mature group, (F) unique DEGs in Immature group, (G) common DEGs between Vitro and Mature groups, (H) common DEGs between Vitro and Immature groups, (I) common DEGs between Mature and Immature groups, and (J) common DEGs among Vitro, Mature and Immature groups. Up- and down-regulated genes are presented by red and green arrows, respectively with Fold change >2, $p< 0.05$ and FDR < 0.1.

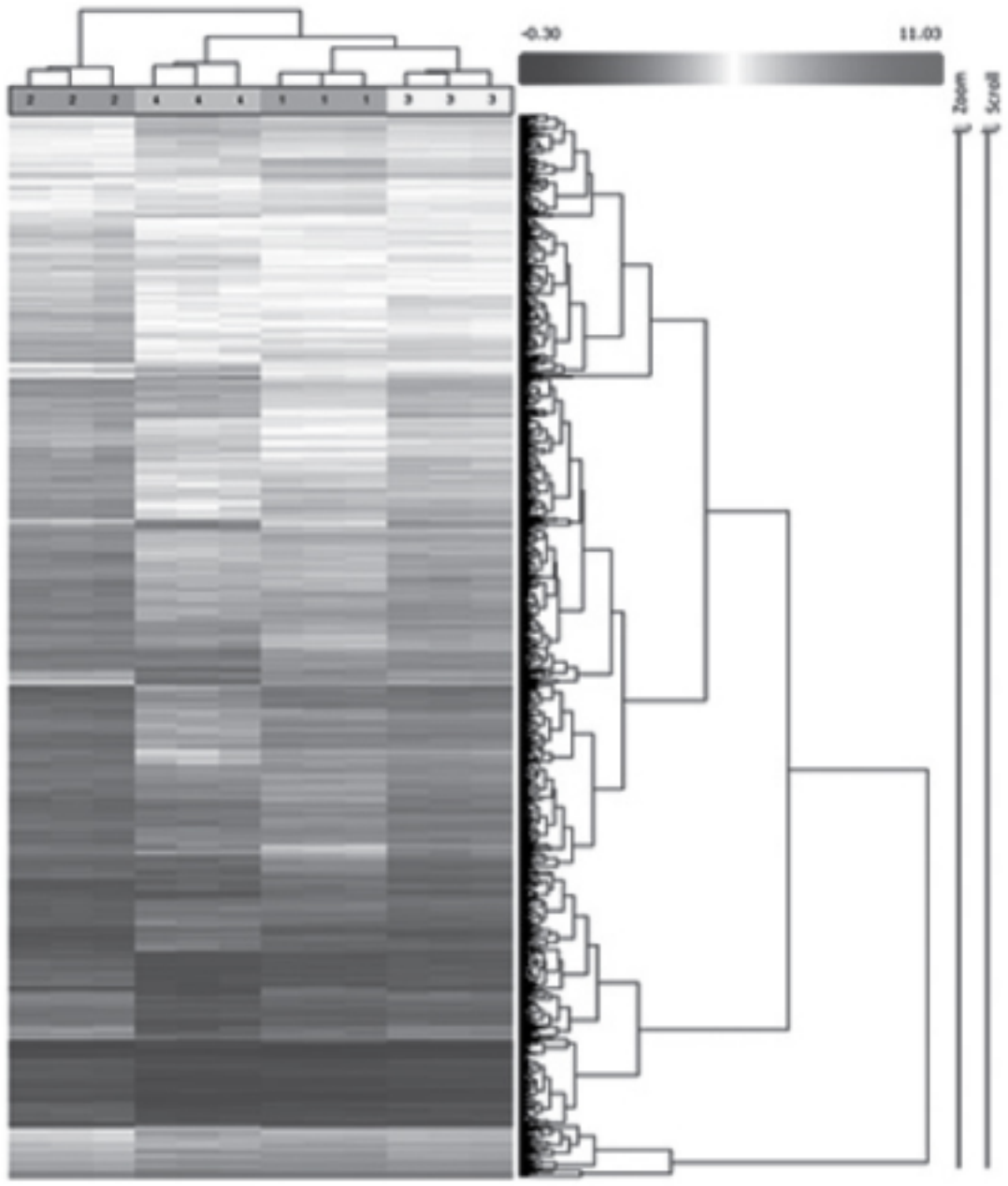

Fig. 2.Hierarchical clustering of the differentially expressed genes among different blastocyst groups. Groups 1, 2, 3, and 4 represent Vivo, Vitro, Mature, and Immature, respectively. The color spectrum, ranging from blue through white to red represents low to high expression. Two main clustered are formed, Vivo, Immature, and Mature in one cluster and Vitro in other cluster.

Functional classification and pathway analysis

Gene ontology (GO) classification of all DEGs between fully in vitro and in vivo-produced embryos showed that 33 biological processes, including "mitotic nuclear division", "cellular metabolic process", "proteolysis involved in cellular catabolic process", and "protein localization"; 60 cellular components, such as "membrane-bounded organelle", "nuclear lumen", "bounding membrane of organelle", and "extracellular exosome"; 9 molecular functions, including "ubiquitin-like protein ligase activity", "RNA binding", and "enzyme binding" were over-represented in the in vivo developed embryos. Moreover, 4 KEGG pathways, including "RNA transport", "proteasome", "cell cycle", and "ubiquitin-mediated proteolysis" were also over-represented in the in vivo derived embryos (Fig. 3).

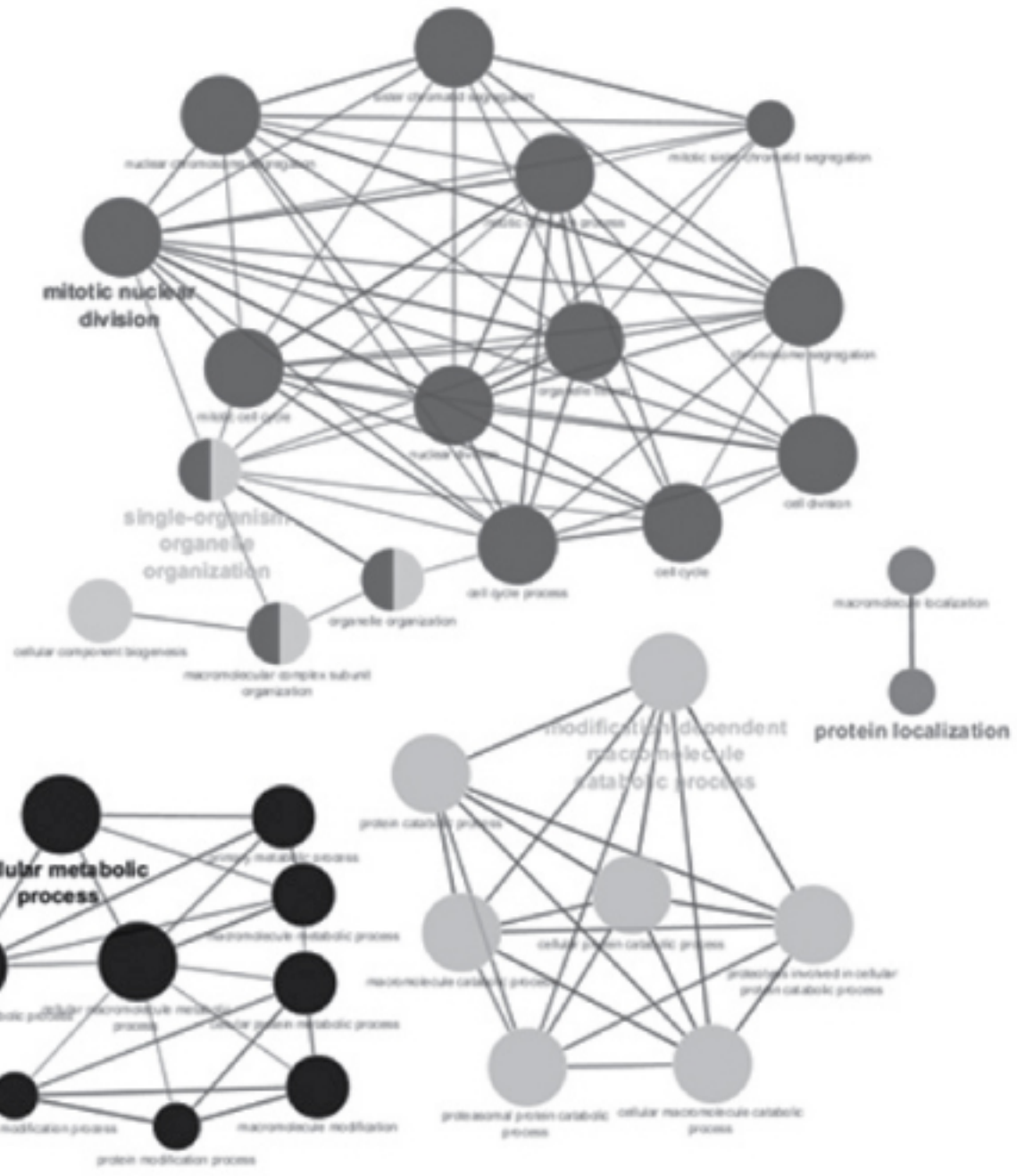

Fig. 3. GO biological processes enriched in genes differentially expressed in fully in vitro vs. in vivo. Enriched GO biological processes with Bonferroni corrected *p*-value < 0.01, genes per term/pathway ≥5, GO term levels 3–8, in the genes differentially expressed (FDR corrected *p*- value < 0.1, | FC | ≥2). The analysis was performed with the ClueGO 2.3.3 plugin of the Cytoscape 3.5.0. The size of the nodules represents their significance. The color of the nodes represents the contribution of GO biological processes where purple nodules had higher contribution followed by blue, sky blue, green, and green gray.

The vast majority of the genes involved in the GO terms and KEGG pathways were upregulated in the in vivo embryos in comparison with fully in vitro embryos, indicating that in vitro culture condition has an adverse effect on the functional pathways. For example, over-expression of MAPK family genes (MAPK1, MAPK1IP1L, JKAMP) involved in the several biological processes of the in vivo developed embryos indicates that embryos utilize MAPK pathways to have signals from the external environment in order to prepare for adaptation to the changing environment (reviewed in Zhang et al., 2007). In agreement, we previously showed that heat stress induced its effect through the activation of MAPK14 pathway in sperm cells (Rahman et al., 2014).

While we compared IFMOT embryos with that of in vivo embryos, only 4 biological processes, such as "isoprenoid metabolic process", "isoprenoid biosynthetic process", "sterol metabolic process", and "secondary alcohol metabolic process" , and only one molecular process namely "oxidoreductase activity, acting on CH-CH group of donors, NAD or NADP as acceptor" were over-represented in the fully in vivo embryos. Additionally, five KEGG pathways, including "steroid biosynthesis", "biosynthesis of unsaturated fatty acid", and "terpenoid backbone biosynthesis" were over-represented in the fully in vivo embryos (Fig. 4). Isoprenoids (also known as terpenoids) are found in all living being that may have many important biological functions. For example, quinones (a product of isoprenoid) act as part of the electron transport chain, sterols as cell membrane components, carotenoids as essential biopigments, dolichol as glycoprotein biosynthesis, etc. (reviewed in Chang et al., 2013). Therefore, the biological processes associated with isoprenoid metabolic and biosynthesis are considered of highly importance for better embryo quality. On the other hand, when we compared IFIOT embryos with fully in vivo developed embryos, we observed only one biological process namely "chaperone-mediated protein folding", three cellular components, such as "NADH dehydrogenase complex", "respiratory chain complex I", and "mitochondrial respiratory chain complex I" were over-represented in the fully in vivo embryos. Moreover, only one KEGG pathway namely "Ferroptosis" was observed in the fully in vivo embryos.

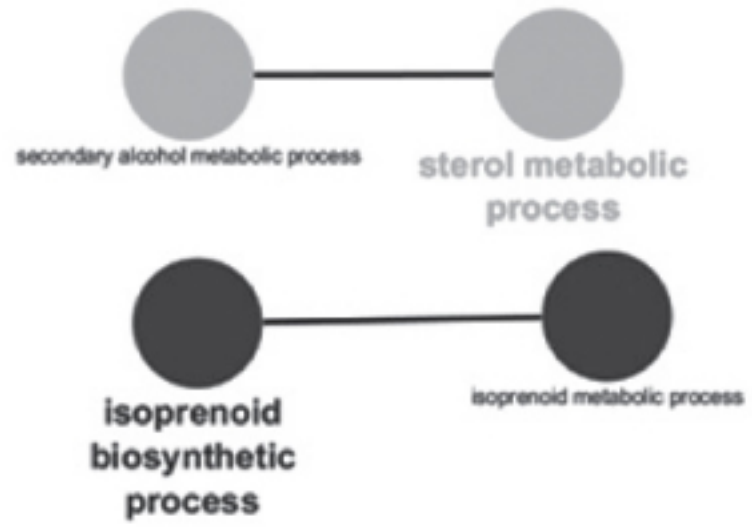

Fig. 4. GO biological processes enriched in genes differentially expressed in in vitro-matured vs. in vivo. Enriched GO biological processes with Bonferroni corrected *p*-value < 0.01, genes per term/pathway ≥5, GO term levels 3–8, in the genes differentially expressed (FDR corrected *p*-value < 0.1, | FC | ≥2). The analysis was performed with the ClueGO 2.3.3 plugin of the Cytoscape 3.5.0. The size of the nodules represents their significance. The color of the nodes represents the contribution of GO biological processes where green and green gray had almost similar contribution.

The biological process "chaperone-mediated protein folding" is of utmost importance for promoting and maintaining the native conformation of cellular proteins (reviewed in Young et al., 2004). In contrast, the expression of a recently recognized regulated cell death pathway, "Ferroptosis" indicates that the embryos were in stress to some extent since ferroptosis is distinguished morphologically by the presence of smaller than normal mitochondria (Xie et al., 2015). This might be due to use of superovulatory hormones that would be an interesting area for further research. Superovulatory hormones are known to alter gene expression (GRB-10, IGF2 and IGR2R) in the oocytes (Mundim *et al*., 2009). Thus, it is clear that only very few biological processes and pathways are differed in the embryos derived either from IFOT of in vitro-matured or immature oocytes in comparison with in vivo derived embryos. Hence, we can infer that IFOT embryos had little or no sign of external stress, rather they possess defensive mechanisms. Modulation of IFMOT embryos for "isoprenoid biosynthesis" biological pathways and IFIOT embryos for only "chaperone-mediated protein folding" pathway would be an excellent area of research for further improvement of IFOT embryos.

Keeping in mind of minimal effect of in vitro-maturation of oocytes and considering the difficulty in transferring of embryos into the fallopian tube, we stepped further in our previous study where we transferred in vitro-matured oocytes into the preovulatory follicles (Kassens et al., 2015). Follicles possess a large stockpile of mRNA, proteins, hormones, etc. in order to nurture growing oocytes. Besides, ROS scavenging enzymes, such as superoxide dismutase, catalase (Slupphaung et al., 2003), and peroxiredoxin (Leyens et al., 2004) are also present in the follicle in order to protect the oocytes from oxidative damage. In our previous study, we have transferred in vitro-matured oocytes aiming to natural ovulation and fertilization in the ampullary-isthmus junction as happened in natural condition in the cows. Although the ratio of extra embryos collection including morulla (flushing at Day 7) was lower (5.0%), the technique worked well which culminated into born of five healthy claves. In the present study, we have additionally transferred abattoir-derived immature oocytes directly into the dominant follicles targeting to maturation in the follicular environments followed by ovulation and fertilization as usual. As like as our previous study, this technique also yielded comparable ratio of extra embryos collection (8.6%) and transferring to the recipients yielded into born of two healthy calves (Michael et al., 2017). Furthermore, Spricigo and colleagues (2016) also reported a good ratio of extra embryos (12.9%) following transfer of abattoir-derived immature oocytes. The discrepancies between the studies might be due to number of transferred immature oocytes where Spricigo and coworkers transferred 25 immature oocytes/dominant follicles while we transferred 50 immature oocytes. Since our previous study proved feasible with transferring of 50-60 in vitro-matured oocytes, we wanted to be consistent with the similar number of immature oocytes, aiming to compare of both techniques. Moreover, according to our previous experience the embryo recovery rates were varies with the respect of individual recipient heifers but not the number of transferred oocytes. Moreover, our main objective of this study was to compare gene expression profiles of the embryos derived either from IFMOT or IFIOT in comparison with fully in vivo embryos. According to our hypothesis, we observed a comparable number of DEGs between the embryos originated either from IFMOT or IFIOT (144 vs. 122; FDR corrected $p < 0.1$, $|FC| > 2$) in comparison with fully in vivo derived embryos. On the other hand, the number of DEGs in fully in vitro embryo group was 7-8 times higher (1053) than IFOT embryos in comparison with fully in vivo embryo

group. Remarkably, among the DEGs some transcription factors, including NFE2L2, POU5F1, ROCK2, FOXN2, TGIF1, HSF2, and KLF family (KLF3, KLF6, KLF7 and KLF10) were upregulated in the fully in vivo embryos in comparison with fully in vitro embryos. Consequently, NFE2L2-mediated antioxidant genes (SOD1, and CAT) were also upregulated in fully in vivo developed embryos. On the other hand, these antioxidant genes were downregulated in the in vitro developed embryos. This is in fact indicating that in vitro developed embryos were in stressed condition during EGA (Gad *et al*., 2012). Surprisingly, the above mentioned transcription factors were not differentially expressed in the embryos derived either from IFMOT or IFIOT groups, indicating the embryos were not in stressed conditions during EGA. However, the antioxidant gene (SOD1) was found to be upregulated in the in vivo embryos compared to embryos derived from IFMOT, indicating that IFMOT embryos are less competent to overcome stress. In contrast, SOD1 did not differentially express between the embryos derived from IFIOT and in vivo condition, indicating IFIOT embryos are of equal competent as of in vivo embryos. Therefore, it can be postulated that embryos derived from IFIOT followed by IFMOT were very close resemblance to fully in vivo derived embryos whereas fully in vitro-produced were very far. To the best of our knowledge, this is the first study where we studied the whole transcriptome profiles of the embryos derived from IFOT of immature or in vitro-matured oocytes.

Conclusions

IFOT embryos derived from immature or in vitro-matured oocytes showed gene expression patterns that were nearly similar to that of fully in vivo-derived embryos. To the best of our knowledge, this is the first evidence where we show comparable gene expression profiles of IFOT embryos as of fully in vivo produced embryos. The most important feature of IFOT embryos is that they are produced without using any superovulatory hormones. The results of this study suggest that intrafollicular transfer of abattoir-derived immature or in vitro-matured oocytes could be a viable alternative to fully in vivo derived embryos for assisted reproduction in cattle.

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Characterization of sperm attributes separated by modified swim up method using hTF in crossbred cattle (Brahman X) and indigenous ram in Bangladesh.

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Abstract:

Swim up separation with centrifugation had found a useful technique to harvest quality spermatozoa in the field of ART's (Assisted Reproductive Technologies), but centrifugation could damage sperm functionality. However, with the aim of better quality semen production, we tried to separate sperm in both cross breed cattle (Brahman X; n=02) and indigenous sheep (n=03) using modified swim up procedure without centrifugation to minimize sperm damage. In the swim up procedure using m-hTF (modified human tubular fluid), supernatants were collected in every 15 minutes interval for four times and kept separately. All semen samples were tested using phase contrast microscopy, CASA (Computer assisted semen analyzer; IVOS II), viability test using CFDA/PI, membrane integrity using HOST (Hypo-osmotic swelling test) before and after separation. Results indicate that, swim up separation produces a better quality semen sample in terms of sperms motility and kinematics parameters both in cattle and sheep. However, the differences observed in cattle was not significant ($p > 0.05$), whereasin sheep was significant ($p<0.05$).better quality semen attributes were found in Fraction 1 and followed by Fraction 2, Fraction 3 and Fraction 4 in comparison with fresh semen sample. In both species, when we considered the viability and plasma membrane integrity of spermatozoa, quality of swim up separated spermatozoa gradually decreased ($p<0.05$) with the increasing of fraction numbers. In conclusion, swim up techniques using m-hTFcanbe an effective tool for production of high quality semen in cross breed cattle and indigenous ram but emphasis should be given on further improvement of viability and functional integrity by modifying the technique.

Key words: Cattle, Ram, semen, swim up, hTF.

Introduction:

On the basis of swimming ability of spermatozoa from the ejaculate into the overlying medium had found a useful method for separating a subpopulation of highly motile spermatozoa (Berger *et al.,* 1985; Lopata*et al.*, 1976 andTanphaichitr*et al.,* 1987). However, the standard swim up procedure involves centrifugation steps that induce a decline in motility of spermatozoa(Berger *et al.,* 1985). To harvest better quality sperms sample, a modified swim-up technique, without centrifugation, was developed by Alvarez *et al.* (1993) for separation of motile human spermatozoa. This technique does not affect the motility of spermatozoa. In our study we followed the technique mentioned above by making little modification in incubation time and volume of swim up medium used.

After invention of computer-assisted semen analysis (CASA) system, detailed information about an individual sperm's motility can be easily evaluated (Mortimer 1997). Moreover, structural changes in the sperm plasma membrane can be evaluated using specific fluorochrome probes, such as the bisbenzimide stain, Hoechst 33258 (H33258), SYBR-14, propidium iodide (PI), ethidiumhomodimer (EthD), 6-carboxyfluorescein diacetate (CFDA) and Yo-Pro-1 (Garner *et al.,* 1986; Garner and Johnson, 1995; Johnson *et al.,* 2000; Silva and Gadella, 2006; Fraser *et al.*, 2011b andPartyka*et al.*, 2012). The osmotic resistance test (ORT) and hypo-osmotic swelling (HOS) test have also been used to assess the functional integrity of the sperm plasma membrane (Jeyendran*et al.*, 1984; Schilling and Vengust, 1987).A fertile ejaculate must meet certain semen parameter quality standards, such as: progressive motility, normal morphology, active energy metabolism, structural integrity and functionality of the membrane along with sperm kinetics.

In order to be applied on a larger scale, the method of sperm separation has to be quick, relatively

inexpensive, repeatable and easily performed. The aim of the present research is to evaluate the efficiency of swim up technique to be used as semen separation tool considering sperm kinetics and fertilizing ability.

Materials and methods

The experiments were carried out in crossbred cattle (Brahman X) (Experiment 1) and in indigenous rams (Experiment 2). Similar procedures were followed for both species.

Animals:

This study was performed on 02crossbred (Brahman X)bull sand 03 indigenous rams used in experimental artificial insemination (AI)services. The animals were housed in the Research Animal Farm of the Department of Surgery and Obstetrics, Bangladesh Agricultural University, Mymensingh.

Semen collection and subjective evaluation:

Semen was collected using artificial vagina (AV) methodand evaluated using traditional and advanced technologies. Volume was read from the graded collection tube soon after collection, concentration was determined using Accucell® photometer (IMV Technologie,L'Aigle, France), motility parameters were evaluatedby computer assisted semen analyzer (IVOS II, Hamilton-Thorne Bioscience, Beverly, MA, USA) and morphology was evaluatedusing phase contrast microscopy (Olympus, CH2, Tokyo, Japan @magnification: X1000) after staining with Farelly stain (Farelly®, Minitube GmbH, 84184 Tiefenbach, Germany). Seven ejaculates for each animalwere collected, evaluated and used in swim up separation techniques.

Sperm motility evaluation:

Before and after swim up separation, semen was evaluated for motilityparameters using a CASA IVOS II (Hamilton-Thorne Bioscience, Beverly, MA, USA). The chamber used in all experimentswas Leja 4 chamber slide (Leja®,Nieuw-Vennep, The Netherlands). The parameters considered in this study were total motility (TM, %), progressivemotility (PM, %), average path velocity (VAP,µm/s), straight line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), amplitude of lateral head displacement(ALH, µm), beat cross frequency (BCF,Hz), straightness (STR, as VSL/VAP, %), and linearity(LIN, as VSL/VCL, %). The software was set using the manufacturer'srecommendation, with a slight adjustment to allow theclear identification of all spermatozoa. After each scan, theplayback facility performed a quality control of the correctacquirement of the field. Spermatozoa with a VAP 80µm/s and STR 75% were considered progressive. CASA settings were set either for bovine or ovine depending on the semen sample as per manufacturer's recommendation.

Viability evaluation:

5 µl of PI (Propidium Iodine) and5 µl of 6-CFDA (6-Carboxy fluorescein di acetate) was added to the 10 µl of semen sample, and then 5 µl of formaldehyde was added into sample (Marti *et al.,* 2008). Stained sample was then incubated at 37^{0}C for 30 min in dark. After incubation time samples were evaluated using fluorescence microscope (Olympus® BX51, Japan). Viable sperm% (CFDA+PI-, green), moribund sperm% (CFDA+ PI+, partial green and red) and dead% (CF-PI+, red) was recorded. Point to be noted that both moribund and dead spermatozoa were considered as nonviable spermatozoa.

HOS (Hypo-osmotic swelling) test:

The plasma membrane functional integrity of spermatozoawas evaluated by using hypo-osmotic swelling test (HOST) (Jeyendran*et al.,* 1984). To get maximum reactive spermatozoa for osmolarity, by osmolar solution (100 mOsm/ L) with sodium citrate and fructose was used in this study. A 1.0m1 of hypo-osmotic solution was mixed with 0.1ml of semen sample and was incubated at 37°C for 1 hour. After incubation, a total of 200 spermatozoa were examined under phase contrast microscope (400X) and percentage of spermatozoa positive to HOS test (with bent tail) was recorded.

Dextran-Swim up method:

The swim-up procedure was performed according to the method of Alvarez *et al.* (1993) with several modifications. A 4 steps procedure was carried out. Aliquots of 0.5 ml of unprocessed semen were pipetted into round-bottomed, 15 mm diameter tubes under 0.5 ml of dextran solution (30 mg/ml) and then overlaid

with 3.0 ml of modified m-hTF containing 5 mg/ml BSA. The tubes were kept at 37°C in a vertical position for 10 min. After the incubation period, 1 ml supernatant was collected,kept in a separate glass tube and replaced with 1 ml modified m-hTF containing 5 mg/ml BSA. The incubation sequence was then repeated 3 times so that 4 supernatants were obtained. The 4 top layers obtained from the consecutive swim-up (Fraction 1, 2, 3 and 4) were then considered for evaluation.

Statistical analysis:

In this study, all data are reported as mean ± standard deviation (mean±sd). Differences in sperm kinetic values, viability and plasma membrane functional integrity among the groups (Fresh vs Fraction 1, Fraction 2, Fraction 3 and Fraction 4) due to Swim up separation were tested in both experiment with a univariate general linear model followed by a Tukey's and Duncan's post-hoc test. Pearson's correlation was also evaluated among few variables. Animalswere considered as random variables. Statistical analyses were performed using SPSS 17.0 (SPSS Inc. Chicago, IL, USA). Differences were considered significant when $P< 0.05$.

Results:

The research workwas done in two experiments; Experiment 1 in crossbred cattle (Brahman X) bulls and Experiment 2 in indigenous rams. All the procedures followed in the experiments were similar. In case of CASA analysis, bull's semen was analyzed using bovine setting and ram's semen was analyzed using ovine setting.

Experiment 1: Effect of swim up separation on sperm quality in crossbred cattle (Brahman X) bull:

Sperm quality in terms of CASA parameters:

Four supernatants were collected with 10 (15 in M&M)minutes interval from each fresh semen sample, and fractions were named as first fraction, second fraction, third fraction and fourth fraction. Semen quality in terms of motility%, progressive motility% and sperm kinetics were monitored for each fraction to study the effect of swim up method and results are shown in Table 1. Semen quality parameters of fresh semen were used as control group.

Following swim up separation, more active spermatozoawerefound in separated semen sample in comparison to fresh semen. In fraction 3, more motile spermatozoas including better progressiveness were harvested but straightness is little bit lower than fraction 4. Other sperm kinetics parameters were comparatively better in fraction 3 than other fractions including fresh sample. Point to be noted that all the differences were found statistically not significant ($p>0.05$).

Table 1: Comparison of motility and sperm kinetics of fresh and different fractions of semen in crossbred cattle (BrahmanX) bulls.

Parameters	*Fresh*	*Fraction1*	*Fraction2*	*Fraction3*	*Fraction4*
Total motility%	53.77±10.28	67.28±1.66	64.73±6.03	75.47±7.01	69.52±3.37
Progressive motility %	29.17±9.67	41.21±6.84	43.17±8.80	49.10±8.20	47.47±9.01
VAP	105.63±20.91	103.89±2.55	108.39±2.96	110.03±3.56	103.91±2.00
VSL	81.75±23.32	75.97±4.20	83.37±6.09	82.99±4.21	87.95±4.65
VCL	204.08±32.94	197.29±13.35	187.44±16.02	195.95±16.66	174.94±14.29
BCF	24.08±4.8	20.75±1.45	21.98±1.63	20.66±1.78	29.00±3.84
ALH	10.50±0.35	10.59±1.10	10.51±1.05	11.76±1.12	8.61±1.19
STR	72.43±7.65	73.38±5.20	76.79±4.52	75.49±3.50	84.61±3.94
LIN	38.47±5.35	39.57±5.37	46.35±7.33	43.76±5.66	51.88±6.54

There are no significant differences among parameters before and after separation ($p>0.05$).

Sperm quality in terms of viability and plasma membrane functional integrity:

Swim up separation could separate more motile sperms but when we considered viability and plasma

membrane functional integrity were foundhighly significant($p<0.05$)(Figure 1) ($p<0.05$).Among separated fractions, more viable and intact membrane sperm could be observed in the Fraction 3.

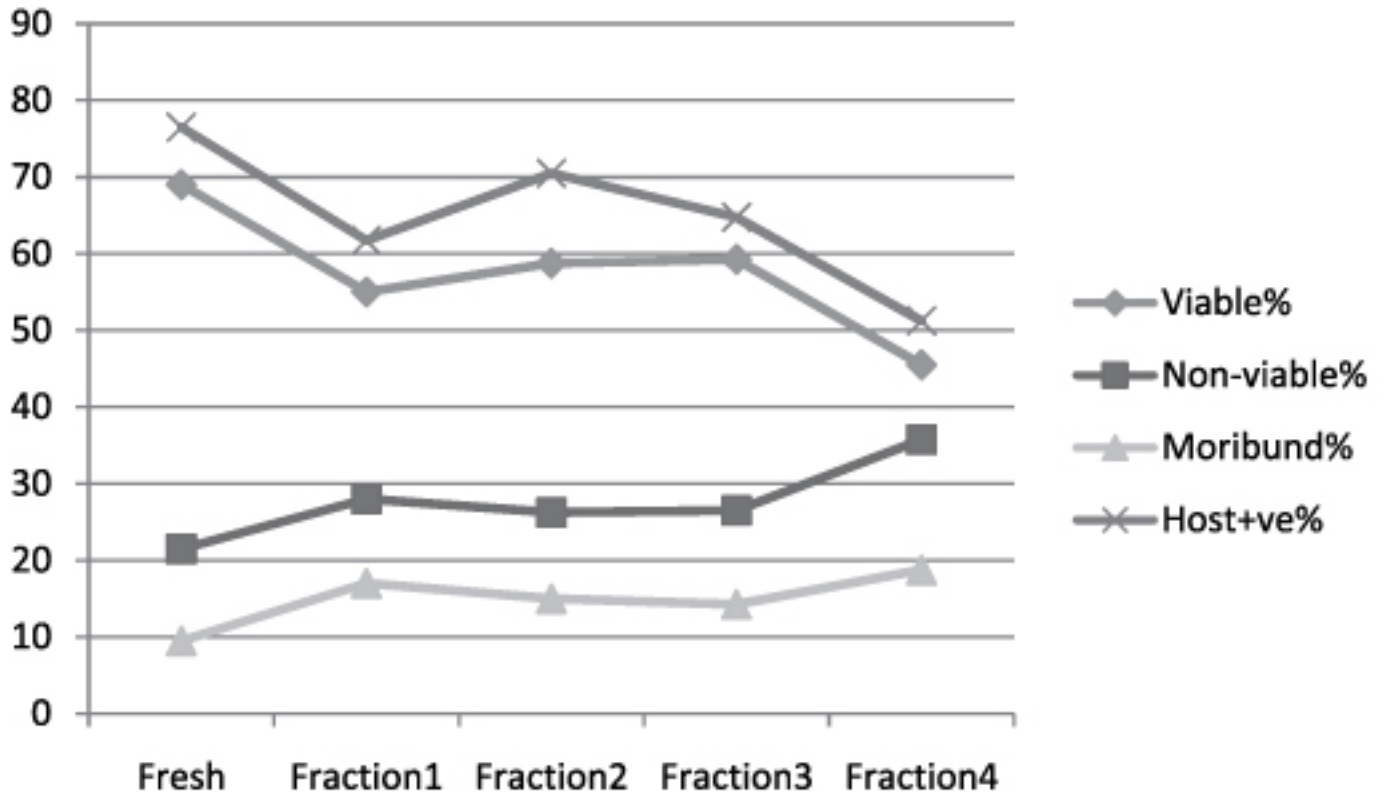

Figure 1: Comparison of viability and functional integrity of spermatozoa among fresh and different fraction of crossbred cattle (BrahmanX) semen.

Correlation among quality parameters of separated sperm:

A Pearson's correlation test was done to establish a relationship among viability, functional integrity and motility percentage of separated sperm. According to the Table 3, a positive relationship was observed among these parameters but the differences were not statistically significant ($p>0.05$).

Table 3: Relationship among viability, functional integrity and motility percentage of swim up separated semen of crossbred (Brahman X) bull.

Parameters	Values	HOST +ve%	Motility%
Viability%	r	0.944	0.560
	p	0.056	0.440
HOST+ve%	r	-	0.678
	p	-	0.322

Experiment 2: Effect of swim up separation techniques on sperm quality in indigenous ram's semen:

Sperm quality in terms of CASA parameters:

In indigenous rams, following swim up separation, more motile spermatozoa were found in Fraction 1 in comparison to fresh semen ($p<0.05$). Among the fractions, more progressive number of spermatozoa was found in the 2nd and 3rd Fractions but differences were not significant (Table 4).

Sperm quality in terms of viability and plasma membrane functional integrity:

Swim up separation could produce more active sperms but the process could affect viability and plasma membrane functional integrity. A statistically high significant percentage of viable and intact membrane spermatozoa were observed (Figure 2) in fresh sample than separated samples ($p<0.01$).

Table 4: Comparison of sperm kinetics of fresh and different fractions of semen in indigenous ram.

Parameters	*Fresh*	*Fraction1*	*Fraction2*	*Fraction3*	*Fraction4*
Total motility%	68.59±5.13*	74.77±2.14*	70.06±3.54	69.42±3.53	64.97±2.54
Progressive motility %	28.99±4.21*	39.86±2.49*	41.31±4.43	41.44±3.14	32.56±3.40
VAP	71.05±3.19	90.34±4.17	88.58±6.68	86.77±4.65	76.06±2.01

VSL	58.76±4.58	72.46±3.13	72.94±3.92	71.07±2.75	59.95±2.78
VCL	131.45±14.07	153.57±10.37	151.02±16.84	125.33±3.52	139.36±21.72
BCF	24.30±1.73	29.39±1.30	29.46±1.15	31.91±1.08	30.43±1.00
ALH	6.53±0.45	8.21±0.42	6.89±0.21	5.72±0.24	5.47±0.20
STR	82.63±2.87	80.22±0.63	82.46±2.14	82.00±3.03	78.84±3.42
LIN	45.38±7.99	47.45±4.99	48.63±4.06	56.77±3.49	43.78±6.25

*indicates significant differences within a single row ($p<0.05$).

Among separated fractions, more non-viable and moribund sperms wereobserved in the Fraction 4 ($p<0.01$).

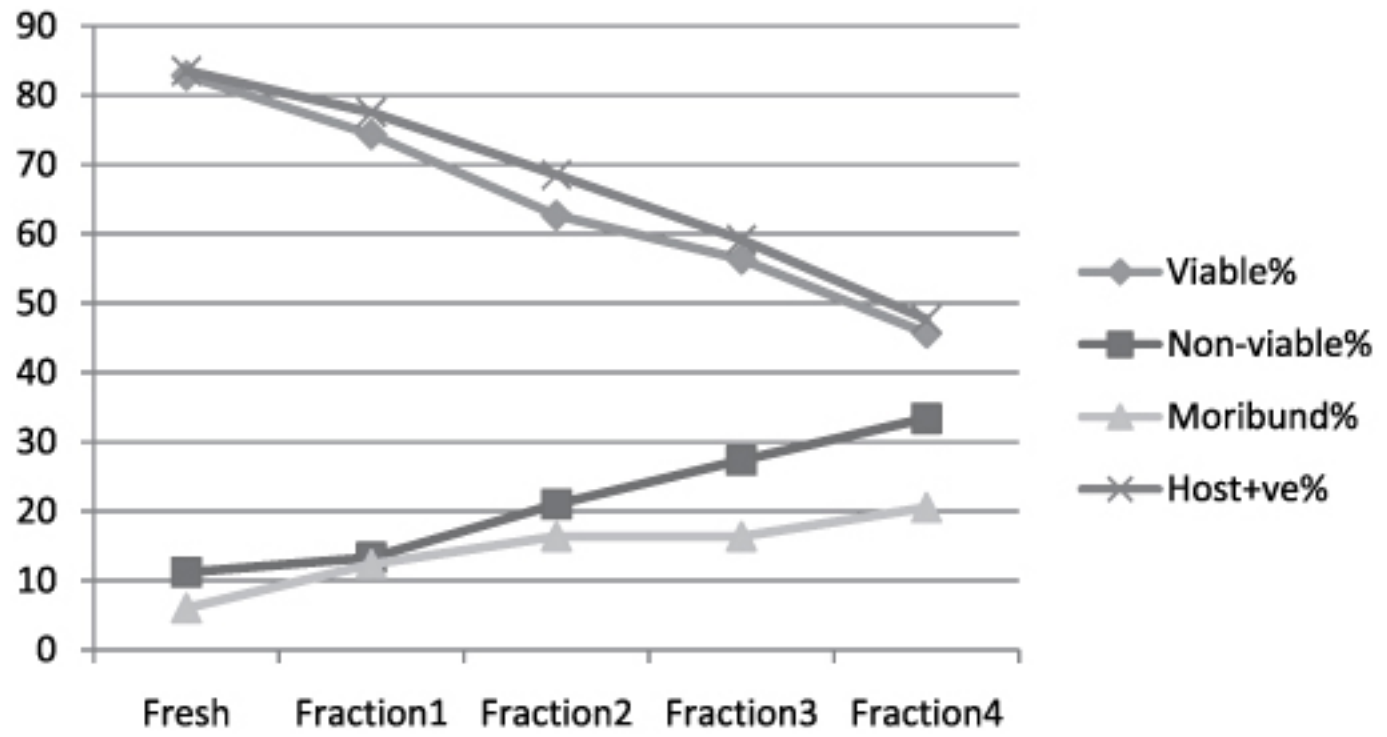

Figure 2: Comparison of viability and functional integrity of spermatozoa among fresh and different fractions of semen in indigenous ram.

Correlation among quality parameters of separated sperm in indigenous rams:

In case of ram semen, Pearson's correlation analysis was performed to understand the relation among the three quality parameters of separated sperm. Correlation is shown in Table 6. Results showed a significant ($P< 0.01$) positive relation among viability%, motility% and membrane functional integrity%. Surprisingly, a tendency of negative correlation was observed between functional integrity% and motility%, ($p>0.05$)

Table 6: Correlation among viability, functional integrity and motility percentage of swim up separated semen of indigenous ram.

Parameters	Values	HOST +ve%	Motility%
Viability%	r	0.265**	0.817**
	p	0.002	0.000
HOST+ve%	r		-0.107
	p		0.228

**indicates significant differences at 1% level ($p<0.01$).

Discussion:

The ultimate goal of semen separation is to find out the way to harvest high quality spermatozoa with better fertility potentials. Different methods have been evaluated throughout the last decades, but only few methods have been adopted for practical work. In the present study, we attempted to separate high quality spermatozoa by dextran swim up method using modified human tubal fluid (m-hTF) and the quality of spermatozoa was assessed by both subjective and objective technologies, likely, phase contrast microscope, CASA, photometer, fluorescence probe and osmotic tonacity test.

The dextran swim-up method reported here was developed to remove seminal plasma from semen without centrifugation as well as to separate a subpopulation of motile and highly viable spermatozoa (Garcia-Lopez*et*

al., 1996). Swim-up technique based on the swimming abilities of spermatozoa could produce higher percentage of motile sperm with intact plasma membrane integrity(Zavos, 1992).

In the present study, the percentage of recovered motile sperms in the swim-up sample in crossbred cattle (Brahman X) increased in comparison to fresh sample (67.28±1.66, 64.73±6.03, 75.47±7.01 and 69.52±3.37vs53.77±10.28) including improved progressive motility % (29.17±9.67 vs 41.21±6.84, 43.17±8.80, 43.17±8.80, 49.10±8.20 and 47.47±9.01), for all the semen samples assayed, slightly higher than the one reported by Alvarez et al. (2) for human semen by the dextran swim-up method. Similar result was found in indigenous rams in our study (total motility% fresh vs fraction 1, fraction 2, fraction 3 and fraction 4; 68.59±5.13vs 74.77±2.14, 70.06±3.54, 69.42±3.53 and 64.97±2.54), with progressive motility% (28.99±4.21 vs 39.86±2.49, 41.31±4.43, 41.44±3.14 and 32.56±3.40).The mean values of sperm kinetics parameters evaluated by CASA found better in swim up samples, in Fraction 3 for crossbred cattle, and in Fraction 1 for indigenous rams, in comparison to fresh samples. This result is with partial agreement of earlier reports (Hallap*et al.*, 2004; Mehmood*et al.*, 2009 and Matas*et al.,* 2011). In our study, more active spermatozoa of indigenous rams are found in the Fraction 1, where as in crossbred cattle it found in the Fraction 3. This might be due to difference in sperm inherent motility characteristics. Moreover, ovine sperm head are comparatively smaller in size than that of bovine.

Some studiesshowedthat, spermatozoa showing goodmotility have serious membrane damage (Valcarcel*et al.,*1994). Thus, sperm viability (cell membrane integrity) as well as membrane function also appears to be important factors in determining the potential fertilizing capacity of a sperm sample (Jeyendranb *et al.,* 1992). In fact, an intact and functionally active membrane is essential for the spermatozoon to sustain metabolism, undergo capacitation and acrosome reaction (AR) and, further, attach to and penetrate the oocyte'szonapellucida (ZP) (Lopata*et al.,* 1976 and Check *et al.,* 1991). In both experiments of our study, more viable and functionally active sperms were found in the fresh samples. After swim up separation, viability and plasma membrane intactness might be affected. However, the values recorded in the present study were more or less similar to the result reported by Correa and Zavos (1996). In contrast, Shamsuddin and Rodriguez-Martinez (1994) reported a significant increase in membrane intactness after swim up in comparison to fresh sample. The loss of viability and membrane intactness could be resulted from osmolarity of swim up medium. Similar declaration was also made by Ashworth *et al.,* (1994).

The assessment of structural and functional integrity of the sperm membrane along with motility parameters would be a more accurate predictor of high quality sperm with fertility potentials (Ashworth *et al.,* 1994; Check *et al.,* 1991; Check and Check, 1991 andValcarcel*et al.,* 1994). So, sperm separation protocols need to be directed towards improving membrane quality.

In both species, a positive correlation was found among motility%, viability and HOST+ve% after sperm separation, but only exception is there that a negative relation was observed between HOST+ve% and motility% in ram's semen, though it was not significant ($p \geq 0.05$). In crossbred cattle, similar result was also found by other researchers (Sharma *et al.,* 2012; Zubair*et al.*, 2013).

Conclusion:

Dextran swim up method can bea useful tool to harvest high quality spermatozoa. The results of this study provide the incentive for improving sperm selection procedures by swim-up methods. However furtherresearch is necessary with the concentration on viability and plasma membrane functional integrity of spermatozoa.

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Improvement of Fertility of Cows Using Prostaglandin F2α Analogue

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Abstract

The present research was carried to study the estrus response and conception rate in dairy cows' consequence the double injection of prostaglandin in relation to different manufacturing drugs, genotypes, age, body weight, body condition and lactation status in the private dairy farm under Rajshahi district. Estrus synchronization was performed by single and double injection of $PGF_2\alpha$ (Ovuprost® / Prostanol® 2 ml/cow) in purposively selected 240 dairy cows and a preset questionnaire was answered by the farmers. The selected anoestrus cattle were administered injection on 60 days postpartum, 18 months of heifer of crossbred and 30 months in case of indigenous on Day 1 followed by 2nd injection on 11th day and then insemination was carried out at observed oestrus. The data were compiled, tabulated and analyzed by using SPSS statistical program and result obtained by DMRT or χ^2 test. The conception rate at 1st PGF2α injection, 2nd injection, fixed time AI and control groups were observed 57.83%, 65.62%, 38.00% and 58.33%, respectively. The oestrus synchronization and conception rate (39.02% & 65.62%) at day 11 had statistically higher ($P<0.05$) than others. The oestrus synchronization (66.66%) by ovuprost® and conception (61.42%) after artificial insemination was better effect than prostenol® (50.00% & 60.00%) and control (20% & 58.33%). The highest results of synchronization obtained in L×F crossbred (35%) than L×SL crossbred (30%) and local breed (30%) at day 1 were as day 11 it was in L×F crossbred (42.30%), L×SL crossbred (39.25%) and local breed (35.71%), respectively. The 4 to <8 years, 250 to <350 kg body weight, good body condition and non lactating cows were observed good effects of fertility of cows. Finally, it may be concluded that double injection of Prostaglandin has reduced the calving interval to service period would even truly reduce calving interval in dairy cows of Bangladesh.

Key words: Conception rate, estrus response, dairy cows, prostaglandin, Artificial insemination .

Introduction

Cattle plays an important role in Agricultural operation provides valuable food of animal origin like milk, meat, milk products; industrial raw materials like skin and manures, socio-economic relevance security income generation and human nutrition and stability to farming system. Moreover, cattle hold the top position in livestock sector in Bangladesh. Increased milk production demands led to a decline in the reproductive performance of dairy cows due to a prolonged intercalving period. Several factors, such as longer time period from parturition to first estrus, poor estrus expression or detection, improper timing of artificial insemination, and reduced pregnancy rate at first AI, have contributed to a longer intercalving period. About one thirds of total cows are culled in many developed countries due to fertility problems (Talukder *et al.,* 2005). There are a lot of causes of subfertility which are complicated. Basically many factors are concerned in successful reproduction. Economy of dairy farming largely depends on a good conception rate after insemination or natural service. The twelve-months calving interval is advantageous for maximum milk yield per cow per year with good economic return (Opsomer *et al.,* 1996). One of the major constraints of profitable dairy farming is low conception rate (Alam and Ghosh, 1994; Mehedi, 2002, Sarder, 2008). It was observed that the loss in gross margin (milk sell over feed cost) tended to be increased when calving interval exceeded twelve months. Oestrus synchronization programmes are used in dairy cattle mainly involving the use of the luteolytic agent's prostaglandin F2α analogue or their synthetic analogues. Prostaglandin F2α analogue is able to synchronized oestrus by inducing the regression of the corpus luteum (CL). Because prostaglandin is only effective in dioestrus cows, a double prostaglandin protocol applied 11 to 14 days apart seems to be capable of bringing most cows to oestrus. The rational use of hormones is a major concern of cattle reproduction. $PGF_2\alpha$ is a potent luteolytic agent in cows which has been used successfully for induction of oestrus in cows that fail to show oestrus signs. In Bangladesh, anoestrus problems is one of the most important issues

confronting the dairy industry but the extent and causes are not being properly identified. Anoestrus is the major causes of reduced fertility in cattle that results in failure to produce or delay in producing the annual life calf (Faruq, 2001). There is a need involving the recent reproductive technologies for the proper diagnosis and amelioration of various reproductive problems encountered in cattle. The present study was undertaken to improve the fertility of cows using Prostaglandin $F_2\alpha$ analogue for identifying the effective commercial drugs (PGF2α analogue) against anoestrus non pregnant dairy cows as well as their risk factors.

Materials and Methods

The study was carried out in private dairy farms under Rajshahi district during the period from January 2013 to December 2014.

Selection of Animals: Different breeds of dairy cows, heifers and up to 6 parities cows were considered and selected for the present study. A total of 240 dairy cows were selected from Rajshahi district.

Housing and feeding management: The cows were dram stall feeding system and supplied average 2.5 kg concentrate and approximately 5 kg roughage at dry matter (DM) basis. All cows were almost under same housing system and had straw and / or tin made shed.

Data collection procedure: Pretested questionnaire were developed for recording data about anoestrus dairy cows and thorough gynaecological examination was done. The cows were classified according their breed, age, dairy status, body weight, body condition and their management system. After selection, cows were classified according to their genetic composition such as: Group-I: Local × Friesian (n=80); Group-II: Local× Sahiwal (n=80) Group-III: Local (n=80); Age of these cows were determined from birth register and examined by teeth and cornual ring reading. After confirmation of age of these cows, they were classified as follows –

Group I: <4 yrs (n=80), Group II: 4-<8 yrs (n=96), Group III: >8 yrs (n=64); The body weights of dairy cows were calculated according to the Shaeffer's formula adopted by McNitt (1983).

$$\text{Body weight (kg.)} = \frac{\{\text{Length (inch)}\} \times \{(\text{Heart Girth (inch)})^2\}}{300 \times 2.2}$$

The selected cows were again classified according to their body weight such as: Group-1: <250kg body weight (n=60); Group=2: 250 to 350 kg (n=130) and Group=3: >350 kg (n=40); the selected cows were classified according to milking status- Group-I: Lactating (n=60); Group-II: None lactating (n=130); and Group–III: Heifer (n=40). The body condition was divided into grossly three groups-Group-I: Excellent (n=60); group II: Good (n=120) and group III: Poor (n=60). Anoestrus cattle were selected as and when the cows fail to mate even if it had attained 2.5 years old in case of crossbred heifer and 36th months of age in case of local heifer. But in case of cows of 60 days postpartum who failed to show oestrus in due period as mentioned before.

Experimental animals: Two hundreds and forty cows that failed to show oestrus by their 60 days postpartum, in crossbred cattle of 2.5 years old and in local heifer of 3 years age were selected. The cows were divided into two equal groups (A and B: n=120/group) after considering breed, age, body weight, lactating status, body condition, management system and farm sizes.

Treatment

The cows (n=120) in group A were receive intramuscular injection of 2 ml prostaglandin $F_2\alpha$ (Ovuprost® each ml containing 25 mg of Cloprostenol, BOMAC, New Zealand/ Prostenol Inj. each ml containing Cloprostenol Sodium BP 250 µg, Techno Drugs Ltd, Bangladesh) at day 60 postpartum. The same treatment was given in crossbred local heifer of 2.5 and 3 years respectively. Eight to eleven days later, a second injection of $PGF_2\alpha$ was given to those cows which did not show oestrus in response to the first injection. The cows (n=120) in group B was not given any treatment and served as control.

Hormone therapy: Different types of $PGF_2\alpha$ have been used as mentioned below-
Prostaglandin $F_2\alpha$ like Ovuprost® each ml containing 25 mg of Cloprostenol, BOMAC, New Zealand and Prostenol® each ml containing Cloprostenol sodium 250 µg, Techno Drugs Ltd, Bangladesh/ Dinoprost® containing 25 mg of Strometamol, Techno, Bangladesh etc.

Oestrus detection: All the animals were monitored for the manifestation of oestrus signs three times daily i.e at 6 am, 2.00 pm and 10.00 pm for six days after each injection.

Artificial insemination: The animals under the group were observed by the farmers closely. After confirmation of oestrus they informed AI technician and inseminated the animals accordingly. Animal found oestrus in the morning was inseminated in the evening and vice versa.

Pregnancy diagnosis: Palpation of the uterine contents rectally is probably the most commonly used method for pregnancy diagnosis. Pregnancy diagnosis was conducted as early as 90 days after service. From day 120, the vibration in the uterine artery, fremitus was felt. Diameter of the uterine artery increases from about 3-4 mm in a non-pregnant cow to about 10-15 mm in late pregnancy. The uterine position, diameter and structure of fetus were also felt.

Statistical Analysis: The raw data were sorted and computed and statistically analyzed to calculate the effect prostaglandins on the oestrous cycle in relation to commercial drugs, breed, age, body weight, body condition and lactation status. Data pertaining to the induction of oestrus has been analyzed by using unpaired t-test (Steel and Torrie, 1982). Oestrus response by 1st PGF and 2nd PGF and conception rate were expressed in percentage. The data were analyzed by one-way ANOVA using SPSS computer package programme version 11.5.

Results and Discussion

The pregnancy rate determined through rectal palpation for induced, timed AI and spontaneous oestrus is presented in Table-1. It was observed that the oestrus signs showed for the induced oestrus by PG after 1 and 2nd injection were 58.3% while 20% cows found in spontaneous oestrus. But 1st PG injection induces only 31.7% cows were in heat and in case of 2nd injection was 39.0%. The pregnancy rate was 61.4% in induced oestrus and 58.3% in case of control group. The fixed time AI pregnancy rate was found only 38.0%.

Table 1. Effect of prostaglandins analogue on oestrus and pregnancy rate after Artificial Insemination.

Groups	No. of cows heat shows after used of $PGF_2\alpha$	% of cows heat after used of PGF	Artificial Insemination of cow		Fixed timed AI at 72 hours and 96 hours after 2nd $PGF_2\alpha$	
			No. of cows conceived	Pregnancy rate	No. of cows AI	No. of cows conceived and %
Treatment 1st day used $PGF_2\alpha$ n=120	38	31.7%[b]	22	57.8[b]	-	-
11th day used $PGF_2\alpha$ n=82	32	39.0[a]	21	65.6[a]	50	19 (38.0%)
Total	70	58.3%	43	61.4		
Control (No treatment)	24	20%	14	58.3	-	-

The use of double injection of $PGF_2\alpha$ at 12 days apart were effective in getting pregnancy rates and comparable with natural oestrus (Rao and Venkataramaiah, 1985). Effect of prostaglandins analogue on oestrus and pregnancy rate after Artificial Insemination are shown in Table 1. The oestrus and pregnancy rate at 2nd injection was higher ($P<0.05$) than 1st injection. Raoyos (1995) reported that pregnancy rate for hormone treatment after AI in cows to be 70% compared to 50% in control cows which was partially agreed. Synchronization of estrus and correspondence conception was varying due to semen, timing of AI, health status of cows, skillness of AI technician, environment and others factors.

The hormonal influence in oestrus detection and insemination of cows were given in Table 2. The oestrus synchronization by ovuprost® and pregnancy after artificial insemination after 1st and 2nd injection were 35.0 % & 57.8% and 66.7% & 61.4% but when used prostenol® these were 25% & 60% and 33% & 60%, respectively. Fixed time AI conception was 38% in case of ovuprost® and 36.7% in case of prostenol® used. The effect of prostaglandin had statistically significant and the higher value was in day 11th day 11 come in heat, similar effect in conception of cows. Braganca et al. (2004) conducted a study synchronization and artificial insemination (AI) programme. They used prostaglandin analogue and gonadotropin releasing hormone (GnRH) for oestrus synchronization. The oestrus (40.2% and 50.0%, respectively) and conception rate (54.5% and 54.8%, respectively) did not differ between groups. However, the pregnancy rate of the first AI was higher (42.7%, $P<0.05$) in hormone treated heifers than in artificial insemination programme (27.3%). The results are almost similar with the result of present study.

Table 2. Efficacy of ovuprost (PGF2α; Ovuprost each ml containing 25 mg of Cloprostenol, BOMAC, New Zealand) and Prostenol (Cloprostenol Sodium, Techno, Bangladesh) on oestrus and conception rate of cows.

Groups		No. of cows heat shows after used of $PGF_2\alpha$	% of cows heat after used of PGF	Artificial Insemination of cow		Fixed timed AI at 72 hours and 96 hours after 2nd PGF2 α	
				No. of cows conceived	conception %	No. of cows AI	No. of cows conceived (%)
Treatment	**1st day n=60**	21	35.0	13	57.8	-	-
Ovuprost	**11th day n=39**	19	48.7	12	63.1	20	8 (38.0%)
	Total=	40	66.7	25	61.4	-	-
Prostenol	**1st day n=60**	15	25.0	9	60.0	-	-
	11th day n=45	15	33.0	9	60.0	30	11 (36.7%)
	Total=	30	50.0	18	60.0	-	-
	Overall	70	58.3	43	61.4	50	19 (38.0%)
Control (No treatment)		24	20%	14	58.3	-	-

Effect of genotypes on fertility of cows using the prostaglandin was observed in Table 3. The highest percentage of heat showed after synchronization was observed in L×F crossbred (35%) and lowest in local breed (30%) at day 1 and day 11 it was in L×F crossbred (42.3%) and Local breed (35.7%). The conception

rate of L×F, L×SL and L breed were 57.1%, 50% & 58.3 at day 1; 72.7%, 45.5% & 70% at day 11; 40%, 29.4% & 44.4% in fixed time AI and 62.5%, 42.9% & 66.7%, respectively. The L×F crossbred cows statistically higher significant effect in estrus synchronization but in pregnancy the local one were highly significant (P>0.05) than others two groups.

35.0	30.0	30.0

Table 3. Effect of genotypes on fertility of cows using the prostaglandin.

Items	Genotypes			Total
	L×F	L×SL	L	
Treatment groups	n=40	n=40	n=40	n=120
No. of cows shows heat after using $PGF_2\alpha$ Day 1	14	12	12	38
% of cows shows heat	35.0	30.0	30.0	31.7
No. of cows AI after heat	14	12	12	38
No. of cows conceived	8	6	7	22
Conception rate	57.1	50.0	58.3	55.0
No. of cows shows heat after 2nd $PGF_2\alpha$ Day11	11	11	10	32
% of cows show heat	42.3	39.3	35.7	39.0
No. of cows AI	11	11	10	32
No. of cows conceived	8	5	8	21
Conception rate	72.7	45.5	70.0	65.6
No. of cows fixed time AI at 72hours and 96 hours	15	17	18	50
No. of cows conceived after fixed time AI	6	5	8	19
Conception rate	40.0	29.4	44.4	38.0
Control group	n=40	n=40	n=40	n=120
No. of cows shows heat selection to 14 days	8	7	9	24
% of cows shows heat naturally	20.0	17.5	22.5	20.0
No. of cows AI	8	7	9	24
No. of cows conceived	5	3	6	14
% of conception	62.5	42.8	66.7	58.3

Effects of age on fertility of cows using prostaglandin have been shown in Table 4. Different group of age were significant effect on estrus and pregnancy status. Day 1, Day 11 and control group estrus status at <4 years, 4 to <8 years and >8 years of aged cows were 25%, 37.5%, 31.25%; 30%, 46.66%, 40.9% and 20%, 20.8% & 18.8%. The pregnancy rate of different ages group had significant effect and at <4 years, 4 to 8 years and >8 years were 50%, 61.1%, 60%; 55.6%, 71.4%, 66.7%; 39.1%, 43.8%, 30.8 and 50%, 70% & 50% at day 1; day 11; fixed timed AI and control group, respectively. Different author show various age effect and Oliveira *et al.* (2001).The effects of body weight on fertility of cows using the prostaglandin are stated in Table 5. The percentages of heat show and their corresponding conception rate at day 1, day 11 and control group were 30.0% & 55.6%, 33.3% & 57.1%, 16.7% & 60.0% at, 250kg body weight of cows; 31.4% & 59.1%, 41.7% & 70%, 22.9% & 22.9% at 250 to <350 kg body weight and 35% & 57.1%, 38.5% & 60%, 15.0% & 66.7% at >350 kg body weight. The effect of calving and heifer cow on fertility of cows using the prostaglandin is shown in Table 6. Similarly the calving and heifer cows observed heat and their corresponding conception rate at day 1, day 11 and control group were 30.0% & 55.6%, 33.3% & 64.3%, 18.3% & 54.5% at, 250kg lactating cows; 36.7% & 63.6%, 47.4% & 77.8%, 23.3% & 71.4% at non lactating and 30% & 55.6%, 42.9% & 55.6%, 20.0 % & 50.0% at heifer group of cows. Alam and Ghosh (1993) reported parity is found to exert an effect on the onset of post-partum ovarian cyclicity in dairy cattle.

Table 4. Effect of age on fertility of cows using the prostaglandin.

Items	Age groups			Total
	< 4 years	4 to < 8 years	> 8 years	
Treatment groups	n=40	n=48	n=32	n=120
No. of cows shows heat after using $PGF_2\alpha$ day 1	10	18	10	38
% of cows shows heat	25.0	37.5	31.3	31.7
No. of cows AI after heat	10	18	10	38
No. of cows conceived	5	11	6	22
Conception rate	50.0	61.1	60.0	55.0
No. of cows shows heat after 2nd $PGFF_2\alpha$ at day11	9	14	9	32
% of cows show heat	30.0	46.7	40.9	39.0
No. of cows AI	9	14	9	32
No. of cows conceived	5	10	6	21
Conception rate	55.6	71.4	66.7	65.6
No. of cows fixed time AI at 72hours and 96 hours	21	16	13	50
No. of cows conceived after fixed time AI	7	7	5	19
Conception percentage	39.1	43.7	30.7	38.0
Control group	n=40	n=48	n=32	
No. of cows shows heat selection to 14 days	8	10	6	24
% of cows shows heat naturally	20.0	20.8	18.8	20.0
No. of cows AI	8	10	6	24
No. of cows conceived	4	7	3	14
% of conception	50.0	70.0	50.0	58.3

The effects of fertility of cows on body condition and management with the use of prostaglandin is summarized Table 6. The proportion of heat observed and their corresponding conception rate at day 1, day 11 and control group were 36.7% & 63.6%, 52.6% & 70.0%, 23.3 %& 71.4% at, excellent body condition; 33.3% & 60%, 40.0% & 68.7%, 20% & 58.3% at good body condition and 23.3% & 42.8%, 34.8% & 37.5%, 16.7% & 40.0% at poor body condition cows. The conception rate at AI was 44.4%, 41.7% and 33.3% at three consecutive body weight groups of cows (Table 5) after using induce oestrus. The conception rate was highest in excellent body condition than others two groups. Youngquist (1988) stated that when artificial insemination is used, the most common cause (about 90%) of anestrus is failure to observe oestrus, while 10% due to an abnormality that suspended the oestrous cycle. It becomes difficult to identify the accurate time of insemination. In practice, some of the animals would have been inseminated at wrong time (Alam and Ghosh 1994 and Shamsuddin *et al.,* 2001). Oliveira *et al.* (2001). Diskin *et al.* (1980) stated that a better order to obtain more precise control of the oestrous cycle allowing one AI at a predetermined time giving high pregnancy rates without recourse to detection of oestrus. Alam *et al.* (1993) reported that PGF2α is poorly effective up to Day 5 of the cycle. They stated that synchronization of oestrus has been established as an effective practice in reproductive management. Choudhury *et al.* (1998) reported that there is a high risk of failure of oestrus induction after PGF2α analogue treatment in cows where the luteal activity is not known before initiation of treatments. Watts and Faquey (1985) found lower oestrus induction efficiency with PGF2α in early dioestrus than in late dioestrus. Kerr *et al.* (1991) reported that primarily there are two applicable methods developed fro oestrus synchronization using only PGF2α or it's analogue. The cows treated with PGF2α should be observed for one hour at every occasion at 12 hours intervals for 5 days. Following each injection of prostaglandin preparation insemination of the animal can be done at fixed times i.e 72 and 96

hours after the last injection. Rhodes *et al.* (2003) reported that in year round calving dairy herd, 11-38% cows are reported as anoestrus by 50 or 60 days post calving.

Table 5. Effect of body weight on fertility of cows using the prostaglandin.

Items	Body weight groups			Total
	< 250 kgs	250 to < 350 kgs	>350 kgs	
Treatment groups	n=30	n=70	n=20	n=120
No. of cows shows heat after using $PGF_2\alpha$ day 1	9	22	7	38
% of cows shows heat	30.0	31.4	35.0	31.7
No. of cows AI after heat	9	22	7	38
No. of cows conceived	5	13	4	22
Conception rate	55.6	59.1	57.1	55.0
No. of cows shows heat after 2^{nd} $PGFF_2\alpha$ at day11	7	20	5	32
% of cows show heat	33.3	41.7	38.5	39.0
No. of cows AI	7	20	5	32
No. of cows conceived	4	14	3	21
Conception rate	57.1	70.0	60.0	65.6
No. of cows fixed time AI at 72hours and 96 hours	14	28	8	50
No. of cows conceived after fixed time AI	4	12	3	19
Conception percentage	28.6	42.9	37.5	38.0
Control group				
No. of cows shows heat selection to 14 days	5	16	3	24
% of cows shows heat naturally	16.7	22.7	15.0	20.0
No. of cows AI	5	16	3	24
No. of cows conceived	3	9	2	14
% of conception	60.0	56.3	66.7	58.7

Table 6. Effect of calving and heifer on fertility of cows using the prostaglandin.

Items	Cows			Total
	Lactating	Non-lactating	Heifer	
Treatment groups	n=30	n=70	n=20	n=120
No. of cows shows heat after using $PGF_2\alpha$ day 1	18	11	9	38
% of cows shows heat	30.0	36.7	30.0	31.7
No. of cows AI after heat	18	11	9	38
No. of cows conceived	10	7	5	22
Conception rate	55.6	63.6	55.6	55.0
No. of cows shows heat after 2^{nd} $PGFF_2\alpha$ at day11	14	9	9	32
% of cows show heat	33.3	47.4	42.9	39.0
No. of cows AI	14	9	9	32
No. of cows conceived	9	7	5	21
Conception rate	64.3	77.8	55.6	65.6
No. of cows fixed time AI at 72hours and 96 hours	28	10	12	50
No. of cows conceived after fixed time	10	5	4	19

AI				
Conception percentage	35.7	50.0	33.3	38.0
Control group				
No. of cows shows heat selection to 14 days	11	7	6	24
% of cows shows heat naturally	18.3	23.3	20.0	20.0
No. of cows AI	11	7	6	24
No. of cows conceived	6	5	3	14
% of conception	54.6	71.4	50.0	58.3

Conclusions

Oestrus synchronization and best pregnancy rate was obtained after double injection of PGF2α at 11 days apart. Ovuprost® induces better oestrus synchronization than prostenol®. The use of prostaglandin for synchronization of cattle had good effect on L×F crossbred, 4 to <8 years aged, 250 to <350 kg body weight, non-lactating, good body condition, better management. It is concluded that double injection of Prostaglandin reduces the calving to conception which would eventually reduce calving interval in cows in Bangladesh.

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Existence of A1 and A2 allele of Beta-casein gene in cattle in Bangladesh

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Abstract

Milk is a most nutritious, perishable and nature's almost complete food. It contains all the nutrients required for normal functioning of our body system. However, consumption of dairy products reported to play a role in the development ischemic heart disease, insulin dependent diabetes, atherosclerosis, schizophrenia, infant sudden death syndrome, gastrointestinal dysfunction and innume/inflammation related disorders, etc. β-casomorphine-7 (BCM-7) derived from digestion of A1 milk in human intestine, has opioid and cytomodulatory properties, is attributed as accountable agent. BCM-7 can potentially affect numerous opioid receptors in the cardiovascular, respiratory, nervous system and immune system in human. It is reported that the populations consume A2 milk have a lower incidence of cardiovascular disease and type-1 diabetes. Classification of A2 or A1 milk is based on β-casein gene variants. A2 β-casein is recognized as the original β-casein protein because it existed before a mutation caused the appearance of A1 β-casein in European cattle (*Bos taurus*) a few thousand years ago. The difference between A2 and A1 β-casein variants is a single nucleotide CCT to CAT which alter amino acid P at proline to histidine at 67th position of β-casein peptide chin. Present research focuses to determine genetic composition of cattle in Bangladesh in terms of A1/A2 β-casein. A total of 539 blood samples collected from different regions of the country were analyzed by allele specific PCR. Of these, 275 samples were derived from local and 264 from crossbred animal. Among the samples 136 samples were obtained from male and 403 from female animals. The ages of sampled animals ranges from 0.5 to 10 years. Of the tested samples, 26.7% (144/539) samples were found homozygous A2A2 (A2 milk producer), 65.5% (330/497) heterozygous A1A2 (Mixed A1A2 milk producer) and 7.8% (42/539) homozygous A1A1 allelic (A1 milk producer). Homozygous A1A1 genotype was found in 3.3% local (18/539) as well as 4.5% crossbred (24/539) animals. A1A2 allele was found higher in local (34.0%, 183/539) and crossbred (31.5%, 170/539) animals. It is evident that A1 (gene frequency 0.40) and A2 (gene frequency (0.60) allele of β -casein gene is existed in dairy animals of Bangladesh.

Keywords: Beta-casein gene, A1/A2 milk, Cattle, Bangladesh

Introduction

Bovine milk is a source of lipids, amino acids, vitamins and minerals, immunoglobulins, hormones, growth factors, cytokines, nucleotides, peptides, polyamines, enzymes and other bioactive peptides dispersed in water necessary for growth and development of body systems (Haug *et al.,* 2007). Among milk proteins the major groups are caseins (beta-lactoglobulin) and whey (alpha-lactalbumin) proteins (Zohreh *et al.,* 2015). Casein accounts for around 80% of the total milk protein and casein genes are located on chromosome 6q31-33 (Sulimova *et al.,* 2007). They are encoded by members of a multi-gene family (Ivano *et al.,* 2009). Bovine beta-casein cDNA consist of a 672 nucleotide coding region, flanked by 60 nucleotide 5′ and 358 nucleotide 3′-noncoding regions. Among the four bovine milk caseins, the beta-casein is 25-30% of total milk proteins (Keating *et al.,* 2008). Beta-casein is the most polymorphic milk protein gene with ~13 variants viz. A1, A2, A3, B, C, D, E, F, H1, H2, I and G. Mostly observed forms of beta-casein in dairy cattle breeds are A1 and A2 (Kaminski *et al.,* 2007). A2 beta-casein is recognized as the original beta-casein protein because it existed before a mutation caused the appearance of A1 beta-casein in European cattle (*Bos taurus*) a few thousand years ago and later transmitted to across the globe due to modernization of farming. The mutation at 67th codon CCT (in A2) to CAT (in A1) beta-casein results in substitution of proline that is present in A2 variant with histidine at position 67th (P67H) of the beta-casein chain in A1 variant (Jaiswal *et al.,* 2014; Sharma *et*

al., 2013). Alteration from proline to histidine in the A1 variant of beta-casein preferentially yields the beta-casomorphine-7 (BCM-7) after enzymatic hydrolysis of proteins with gastrointestinal enzymes called pepsin and trypsin (Korhonen and Pihlanto, 2006). BCM-7 is a strong opioid can potentially affect numerous opioid receptors in the cardiovascular, respiratory, nervous system and immune system in human (Bell et al, 2006; Truswell, 2005). The affinity of bovine BCM-7 to opioid receptors is approximately 10 times greater than that of human BCM-7 and it requires a 10-fold greater naloxone concentration to prevent receptor binding (Herrera-Marschitz et al., 1989). BCM-7, being an oxidant is reported to be associated with milk intolerance (Keith, 2007) and play a role in development or aggravation of symptoms of some human diseases such as human ischemic heart disease (Laugesen and Elliott, 2003; McLachlan, 2001), insulin dependent diabetes (Laugesen and Elliott, 2003; Elliott *et al.,* 1999), atherosclerosis (Tailford *et al.,* 2003), and infant sudden death syndrome (Sun *et al.,* 2003), autism (Kawashti et al., 2006; Reichelt et al., 2003.; Cade et al., 2000; Reichelt et al., 1990; Dohan et al., 1988), Schizophrenia (Niebuhr et al., 2011; Severance et al., 2011 and 2010; Reichelt et al., 1995). BCM-7 also contributes in the gastro-intestinal dysfunction and immune or inflammation related disorders (Barnett et al., 2014; Ho et al., 2014, Ul Haq et al., 2014; Haq et al., 2013; Holmer-Jensen et al., 2011; Andiran et al., 2003; Daher et al., 2001; Defilippi et al., 1995). Additionally, recent studies reported that consumption of milk containing A1 beta-casein was associated with increased gastrointestinal inflammation, worsening of post-dairy digestive discomfort (PD3) symptoms, delayed gastro-intestinal transit, and decreased cognitive processing speed and accuracy in Chinese Han people (Jianqin et al., 2016). Genetic characterization of beta-casein gene for the majority of *Bos taurus* breeds indicated that they are carrier of A1 beta-casein variants in most of the dairy breeds except few taurine breeds like Jersey and Gurensy having A2 beta-casein variant (Kaminski et al. 2007). Mishra et al. (2009) reported that A2 variant is predominant in zebu cattle breed. In this context, consumption of milk containing A1 variant could be a huge risk factor for health. Besides, A2 variant have positive relationship with milk performance traits especially protein and milk yield while A1 variant made opposite results (Olenski *et al.,* 2010; Ikonen *et al.,* 2001 and 1999). So due to the opposite functions of beta-casein variants of A1 and A2 on human health, and quality and quantity of milk, the purpose of the present study was to determine the A1 or A2 variant, estimate possible genotypes and gene frequency in the cattle available in Bangladesh. There is no report on the frequency of bovine beta-casein gene variants in the bovine population of Bangladesh.

Materials and Methods

Sampling location and blood sample collection:

A total of 539 blood samples along with necessary information were collected from seven different locations of the country. These includes Shibpur, Narshingdhi; Noakhali Sadar; Shahzadpur, Sirajganj; Babuganj, Barisal; Atghoria, Pabna; Raninagar, Naogaon and Thakurgaon Sadar (Figure 1). Distribution of samples including information on sex and breed of cattle, etc. are presented in Table 1.

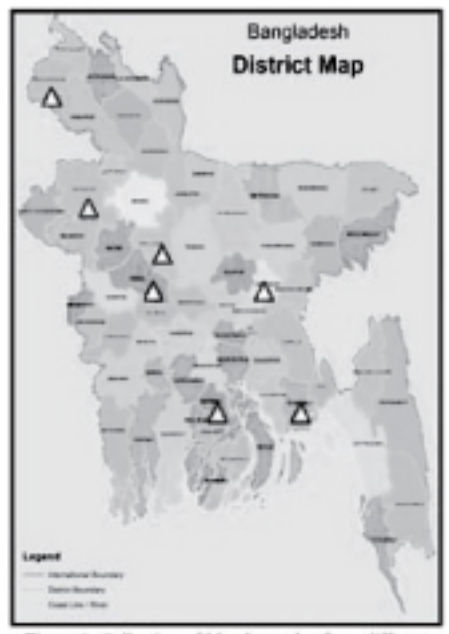

Figure 1. Collection of blood samples from different regions of the country. Sampling locations are marked by open faced red triangle

Table 1. Distribution of samples collected from seven different areas of the country (n= 539)

Location	Local	Cross	Total
Shibpur, Narshingdhi (n=100)			
Male	8	4	12
Female	44	44	88
Sub-total	**52**	**48**	**100**
Noakhali Sadar (n=10)			
Male	4	0	4
Female	6	0	6
Sub-total	**10**	**0**	**10**

Shahzadpur, Sirajganj (n=98)			
Male	0	25	25
Female	0	73	73
Sub-total	**0**	**98**	**98**
Babuganj, Barisal (n=87)			
Male	20	3	23
Female	59	5	64
Sub-total	**79**	**8**	**87**
Atghoria, Pabna (n=100)			
Male	12	22	34
Female	13	53	66
Sub-total	**25**	**75**	**100**
Raninagar, Naogaon (n=94)			
Male	12	8	20
Female	50	24	74
Sub-total	**62**	**32**	**94**
Thakurgaon Sadar (n=50)			
Male	17	1	18
Female	30	2	32
Sub-total	**47**	**3**	**50**
Grand total	**275**	**264**	**539**

DNA extraction and quantification:

DNA was extracted using the protocol used in the Animal Biotechnology Division. Briefly, 400µl of blood was mixed with 700µl water, vortexed, and centrifuges at 12000 rpm for 10min. After discarding supernatants 200µl of lysis buffer and 2µl of proteinase K was added and mixed by inverting the tube several times and then incubated at 58°C for 4 hours. Upon incubation, 100µl of 4.5M Nacl was added and mixed by inverting the tube. Then about 225µl chloroform was added and mixed by shaking for 10 min. The mixture was then centrifuged at 12000 rpm for 10 min and about 200µl upper phase was transferred into a new tube. Then 200µl of propanol was added and mixed by inversion of the tube and centrifuged at 12000 rpm for 15min. The supernatant was discarded and 500µl of 70% ethanol was added and incubated at room temperature for 15min. Upon incubation the mixture was centrifuged at 12000rpm for 15 min and then decants the alcohol. About 100µl of TE buffer was added and incubated at 56oC for 5 hours and then mixed by pipetting. Finally obtained DNA was stored at -20°C until use. The concentration and purity of DNA was assessed with a Nanodrop spectrophotometer (Nanodrop 2000/200c).

Primer: Primers used in this study are presented in Table 2 along with expected PCR product size and reference.

Table 2. Sequences of primers used in this study

Primer name	Sequence 5'-3'	Expected Band size	Reference
A-IGBhF	CTT CCC TGG GCC CAT CCA	244bp	Ganguly et al., 2013
C -IGBpF	CTT CCC TGG GCC CAT CCC		
IGBR	AGA CTG GAG CAG AGG CAG AG		

Allele specific polymerase chain reaction (PCR):

The PCR reaction was performed in 25µL reaction scale. The reaction consisted of 12.5µL of 2x master mix (Tris-HCl 20mM, dNTPs 400 mM), $MgCl_2$ 3mM, Taq DNA polymerase 0.1 U/µL), 1µL forward primer, 1µL

reverse primer, 8.5µL molecular grade water and approximately 50ng of extracted DNA with final volume of 25µL. Thermal conditions were: initial denaturation at 94°C for 5 min; followed by 35 cycles of 94°C for 1 min; 64°C for 1 min and 72°C for 1 min; thereafter a final extension at 72°C for 5 min. PCR products were visualized and evaluated after electrophoresis in ethidium bromide stained 2% agarose gel. In the PCR assay allele specific forward primer (A-IGBhF for detection of A1 variant or C-IGBpF for detection of A2 variant of casein gene) and a common reverse primer was used (Ganguly et al., 2013).

Results and Discussion

General overview: Due to the functional importance of bovine milk protein polymorphisms, their correct discrimination is of great interest both from a scientific and practical point of view. To analyze the beta casein gene variants, a total of 539 blood samples along with some relevant information viz., age, breed, sex, etc. of the individual animal were collected from seven different regions of Bangladesh. Among these 275 samples were derived from local cattle and 264 samples from crossbred animals (Table 1). The differentiation of local and crossbred animal was based on eye estimation and/or farmers response. The ages of sampled animals were ranges from 0.5 to 10 years. Among the samples 136 samples obtained from male and 403 from female animals (Table 1).

Beta-casein gene amplification and genotype frequency:
Allele-specific polymerase chain reaction (AS-PCR) was used to analyze the samples. Allele either A2 or A1 specific forward and a common reverse primer were used to amplify 244bp product spanning the target site of the chromosome 6. We could successfully amplify the specific gene product (Figure 2). Electrophoretic analysis of the AS-PCR products revealed that three genotypes A1A1, A1A2 and A2A2 of beta-casein gene are prevalent among the tested animals (Table 3). Of the tested samples, 26.7% (144/539) samples were found homozygous A2A2 (A2 milk producer), 65.5% (330/497) heterozygous A1A2 (Mixed A1A2 milk producer) and 7.8% (42/539) homozygous A1A1 allelic (A1 milk producer). Homozygous A1A1 genotype was found in both local and crossbred animals. A1A2 genotype was found higher in local and crossbred animals. From the study it is evident that A1 (gene frequency 0.40) and A2 (gene frequency (0.60) allele of beta-casein gene is existed in dairy animals of Bangladesh (Table 3).

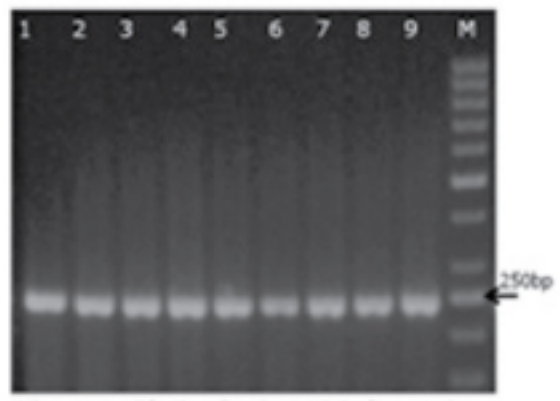

Figure 2. Amplification of portion (244bp) of beta-casein gene of bovine using allele specific primer. Lane 1-9: Test sample; Lane M: DNA marker

Table 3: Beta-casein genotype and gene frequencies of tested samples obtained from cattle of seven different regions of the country

Breed	No. of sample tested	Genotype frequency			Gene frequency	
		A2A2	A1A2	A1A1	A1	A2
Local	270	0.26 (n=69)	0.68 (n=183)	0.06 (n=18)	0.40	0.60
Cross	269	0.28 (n=75)	0.63 (n=170)	0.09 (n=24)	0.40	0.60

We have also sequenced representative samples for confirmation of genotypes. Figure 3 represents the different genotypes. Crossbred animals mostly originated either from crossing of Holstein Friesian and local (HF X L) or from Jersey and local (JX L) animals. It is reported that generally the frequency of A1 allele in different exotic breeds varies between 0.01-0.06 (Guernsey), 0.09-0.22 (Jersey), 0.31-0.66 (Holstein), 0.43-0.72 (Ayrshire) and 0.71 (Danish Red) (Kaminski *et al.,* 2007). AS-PCR was found incredibly useful in distinguishing A1A1, A1A2 and A2A2 genotypes. Recently, it is reported that Polish Holstein Friesian has comparable A1 (0.35) and A2 (0.65) allele frequencies (Olenski et al., 2010). The predominance of

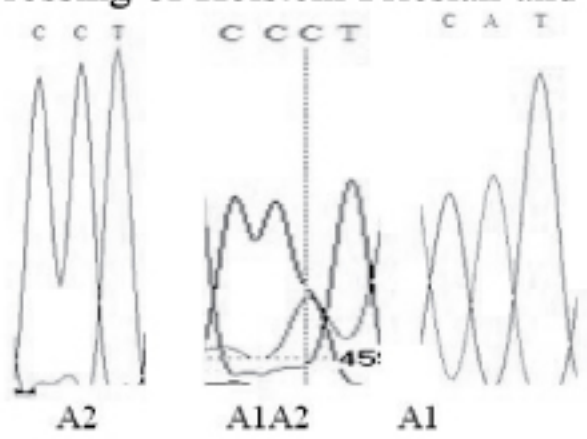

Figure 3. Different genotype of A1 and A2

CSN2 A2 allele (0.764) detected Caroli *et al.* (2008) in population of Carora cattle. Manga *et al.,* (2006) presented lower frequency of allele A1 in population of Czech Spotted and Czech Holstein breed. The higher frequency of allele A1 was reported by Bech *et al.* (1990) for Black-and-White breed, and Ehrmann *et al.,* (1997) for Red-and-White breed. Ikonen *et al.* (1997) reported slight superiority of allele A1 for Ayrshire breed and Hanusová *et al.* (2010) for Holstein bulls. Our finding regarding higher frequency of A2 allele than A1 allele is complying with the findings reported mentioned above. Milk has a diverse composition of nutrients for human (Islam *et al.,* 2014). The importance to detect the genetic polymorphism for milk protein beta-casein genes in dairy cattle population is due to their association with a range of illnesses by preferentially releasing an exorphin (exogenous origin with morphine-like activity) called BMC-7 upon digestion. BCM-7 (Try-Pro-Phe-Pro-Gly-Pro-Ile) was first isolated as a peptide having morphine-like activity in 1979 (Brantl *et al.,* 1979). The release of BCM-7 during hydrolysis depends on the beta-casein variant containing a histidine residue at 67^{th} position. It can potentially affect numerous opioid receptors in the nervous, endocrine and immune system. It is also known to oxidizing LDL (low density lipoprotein) which is believed to be vital in formation of arterial plaque. BCM-7 has cytomodulatory properties too (Meisel, 2001). Sampled indigenous and crossbreed cattle exhibit a superiority of allele A2 which does not produce BCM-7, and thus is safe for human consumption. However, A1A1 allele also exists in the sampled population that might encode A1 beta casein which is harmful for human health.

Conclusion

We have lucratively genotyped 539 blood samples obtained from local and crossbred bovine animals from seven different regions of the country. Three genotypes viz. A1A1, A1A2 and A2A2 were detected with high frequency of A1A2 genotype. We could find A1A1 genotype though lower frequency.

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High yielding beef production in Bangladesh: Comparative growth performance of F_1 progeny of different crossbred beef cattle

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Abstract

Bangladesh has a great demand of beef but it has been facing demand and supply mismatches due to insufficient production and supply of beef, low carcass yield of native cattle and recent no-cattle export policy of a long bordered neighboring country. Thus, the present work was undertaken with an objective to develop market beef cattle of average 150.0 kg carcass weight with an average FCR of ≈ 6.50 at 2 years of age under on farm feeding and management conditions. Aiming at developing breeding bulls the native dams of BCB-1 (BLRI Cattle Breed 1) were inseminated with the imported frozen semen of Brahman, Simmental, Charolais and Limousine. The crossbred progeny of different assorted F_1 genotypes are being selected and their production and breeding performance are being evaluated and compared with BCB-1 (Control). Irrespective of sex all crossbred progeny performed better than BCB-1 in terms of live weights and average daily gains. Among the crossbreds, Charolais×BCB-1 had the highest ($p<0.001$) birth weight (26.9±6.8 kg) followed by Brahman×BCB-1 (23.9±2.5), Simmental×BCB-1 (22.1±2.7), Limousin×BCB-1 (21.7±5.6) and BCB-1×BCB-1 (17.8±3.7), and the genotype differences were highly significant ($p<0.001$). Charolais cross grew faster followed by Simmental, Limousin, Brahman crosses and purebred BCB-1. At 18 months of age Charolais×BCB-1 had the highest body weight (417.5±62.0) followed by Simmental (396.0±54.5), Limousin (380.6±19.0), Brahman (312.7±38.5) crosses and purebred BCB-1 (267.1±32.5). Purebred BCB-1 had the lowest daily DM intake and showed the highest feed conversion efficiency in all ages compare to other crosses. Calf mortality was found as 3.92%. In conclusion, Charolais×BCB-1 is performing as the best among the five genotypes in terms of growth up to 18 month of age.

Key words: Growth performance, crossbred, beef cattle, F_1 progeny

Introduction

Livestock sector plays a sustainable role in the national economy of Bangladesh. But the per capita intake of meat is only 8.6 kg in Bangladesh against 42.1 kg and 32.2 kg for world and developing countries, respectively (Huque, 2012). In addition to these, quality beef is not so available in the country, as most beef comes from culled animals, dairy male calves, and spent dairy cows. Young animals with a moderate body condition score (BCS) are occasionally slaughtered as well (e.g., during Eid-ul-Azha-a religious Muslim festival). To meet this gap, meat production of the country must be increased many folds. Conventional beef production system coupled with intensive beef farming may help increasing beef production. According to last Agricultural Census 2008, Bangladesh had very high density of cattle which was about 188 head/km^2 (Huque and Khan, 2017). So, Bangladesh has little opportunity in increasing cattle population (horizontal development) instead of increasing productivity (vertical development) of cattle. Vertical development should be the keys for unlocking potentialities of beef cattle in this country. Thus, Bangladesh has taken an opportunity for boosting its bovine industry through on-going program like cattle fattening and crossbreeding for dairy and beef cattle production. Our country has native cattle which grow at a slower rate than most of the temperate fast growing beef breeds. Brahman crosses are being produced to support increased productivity

of cattle, but strategic approach for breed development that needs screening of multiple genotypes is ignored. On the other hand, the dam line, which used in Brahman crossbred production, was not from similar breed which caused difficulties in evaluation of heterosis effect. In the recent studies of BLRI and DLS reported that Brahman crossbred bulls showed the higher FCR (12.1) compared to native BCB-1 (9.5) or Red Chittagong Cattle (9.9) (Roy *et al*., 2013; Rashid *et al.,* 2014). Simmental, Charolais, Limousine and American Brahman are worldwide recognized beef cattle breed and BCB-1 is a native cattle developed through selective breeding of native cattle named 'Pabna' evolved through admixture of Hariana, Tharparker and Sahiwal genetic materials (Bhuiyan *et al.,* 2007). The coat color and body size of BCB-1 bulls are attractive which have a great market value as a meat type cattle. Considering the facts, the present work was undertaken to evaluate comparative growth performance of different crossbred exotic beef genotypes and thus, market beef cattle of average 150.0 kg carcass weight with an average FCR of ≈ 6.50 under on farm feeding and management conditions will be produced. Here, three exotic beef breeds i.e. Simmental, Charolais and Limousine along with American Brahman were used as sire line with a single dam line BCB-1for the production of crossbred progeny.

Materials and Methods

Selection of BCB-1 dams and collection of exotic beef sires semen

The BCB-1 cows and heifers were selected from BCB-1 herd of BLRI based on their body weight, individual and pedigree performance and reproductive health for artificial insemination (AI) with exotic beef breeds semen to produce crossbred beef cattle. Frozen semen of purebred Simmental (Black and red), Charolais (white and red) and Limousin (light wheat to darker red) were imported from Australia. In addition, 20 doses of frozen semen of purebred American Brahman sires were collected from DLS for this study. The production performance of the selected exotic beef breeds were reviewed and documented from secondary data sources.

Artificial insemination (AI) and production of crossbred beef progeny

AI was performed following the natural estrus of the selected BCB-1 dams. Before insemination all selected dams were checked for brucellosis and ovarian condition. The BCB-1 dams were divided into 4 groups, thus 15-20 BCB-1 dams were allocated for crossing with one beef breed. Four exotic beef breeds were considered as treatment groups and BCB-1 was considered as control group for comparison of the production performance of their progeny. Under this study control BCB-1 were collected from BCB-1 herd of BLRI which produced by natural services in the farm. After 2 months of AI, pregnancy was tested by rectal palpation to ascertain the conception. Artificial insemination was performed following standard procedure for the production of crossbred progeny. Not more than 2 AI services were allowed for single conception and subsequent calculation of service per conception.

Table 1 showed the number of crossbred and purebred progeny produced and studied under this breeding program. Table 1 indicated that a total number of 49 progeny were produced up to June 2017 and out of that 10 were purebred BCB-1 and the 39 were assorted F_1 progeny of BCB-1.

Table 1. Number of F_1 progeny of different crossbreds and BCB-1

Genotype of calves	Sex		Total
	Male	Female	
Limousine×BCB-1	6	4	**10**
Simmental×BCB-1	2	7	**9**
Charolais×BCB-1	4	4	**8**
Brahman×BCB-1	7	5	**12**
BCB-1×BCB-1	5	5	**10**
Total	**24**	**25**	**49**

Performance recording of F_1 progeny

Birth weight, weaning weight, yearling weight, average daily gains (ADGs) at different physiological stages, calf mortality and disease incidence were recorded throughout the year. From birth (0) to 1 year of age every 15 days interval and after that every 1 month interval body weight of F_1 progeny (male & female) were recorded.

Feeding and management system for crossbred beef progeny

All pregnant (> 6 months of gestation period) dams were in pre-natal care, and all calves were raised in a single plane of nutrition and management. Total milk and feed intake, FCR, disease incidence and calf mortality of BCB-1 and its assorted genotypes were recorded. Calves were supplied whole milk according to body weight described in Table 2. The concentrate was supplied 50% of their DM requirement and rest of DM requirement was fulfilled by roughage (green grass or silage). The proximate composition of the supplied feed was stated in Table 3.

Table 2. The milk feeding schedule of produced calves from birth to weaning

Calf Age (Days)	Milk supplied percentage (%) of Body Wt. (Kg)
Birth to 30	10
31 to 45	8
46 to 60	6
61 to 75	4
75 to 90	2
91 to 97	Gradual decreases from 2 % to 0 % (Weaning)

Table 3. Chemical composition of feed supplied to the calves

Feed	% DM	% CP	% Ash
Concentrate	88-89	16-18	10-11
German Grass	15-16	14-16	10-12
Napier Grass	14-16	9-10	-

Statistical analysis

The progeny performance data used in the present study were from five different genotypes and sample sizes were unbalanced due to insemination of dam following natural estrus. Hence, it fulfilled the characteristics of a non-orthogonal factorial design and all recorded data were analyzed by General Linear Model (GLM) procedure of SPSS (17.0). Duncan's multiple range test (DMRT) by Kramer (1957) was used for mean comparisons.

Results and Discussion

Table 4 and 5 revealed that all crossbred progeny performed better than BCB-1 in terms of live weight and ADG. The effect of genotype on live weight at different ages was highly significant ($p<0.001$). Among the crossbred progeny Charolais×BCB-1 had the highest cumulative birth weight (26.91±1.56 kg) followed by Brahman×BCB-1 (23.91±1.27 kg), Simmental×BCB-1 (22.13±1.47 kg) and Limousine×BCB-1 (21.74±1.39 kg) (Table 4). Similarly, the birth weight Charolais crossbreds showed the highest live weight at weaning, 6 months, yearling and 18 months of age compared to other crosses (Table 4). In ADG, genotype also had highly significant effect ($p<0.001$) (Table 5). The Charolais cross gained the highest daily live weight up to

18th month of age except in 0 to 3 month. In 0 to 3 months of age, Simmental cross performed the best (0.58±0.04 kg/d) followed by Charolais (0.56±0.04kg/d), Limousine (0.51±0.03kg/d) and Brahman crosses (0.50±0.03kg/d). The purebred BCB-1 always showed the lowest daily weight gain (Table 5). The figure 1 and 2 showed the growth curves of male and female F_1 progeny, respectively.

Table 4. Effects of genotypes on live weight at different ages

Live weight (kg)	Genotype Mean±SE(n)					Significance
	Limousine× BCB-1	Simmental × BCB-1	Charolais× BCB-1	Brahman× BCB-1	BCB-1× BCB-1	
Birth weight	21.74±1.39 (10)	22.13±1.47 (9)	26.91±1.56 (8)	23.91±1.27 (12)	17.84±1.39 (10)	*** (P = 0.002)
Weaning weight (3months)	66.84±3.47 (10)	75.82±3.66 (9)	77.01±3.88 (8)	68.66±3.16 (12)	49.08±3.47 (10)	*** (P= 0.000)
At 6 months	129.15±5.12 (9)	141.66±5.12 (9)	145.00±5.43 (8)	127.25±4.43 (12)	94.01±4.85 (10)	*** (P= 0.000)
At yearling (12 months)	255.571±9.45 (7)	279.42±9.45 (7)	281.00±10.20 (6)	240.71±9.45 (7)	187.90±7.90 (10)	*** (P= 0.000)
At 18 months	380.57±15.55 (7)	396.00±15.55 (7)	417.50±20.57 (4)	312.66±23.75 (3)	267.10±13.01 (10)	*** (P= 0.000)

Table 5. Effects of genotypes on average daily body weight gains (ADG) at different ages

ADG (kg/d)	Genotype Mean±SE(n)					Significance
	Limousine× BCB-1	Simmental× BCB-1	Charolais× BCB-1	Brahman× BCB-1	BCB-1× BCB-1	
0-3 months	0.51±0.03(10)	0.58±0.04(9)	0.56±0.04(8)	0.50±0.03(12)	0.35±0.03(10)	*** (P= 0.001)
3-6 months	0.67±0.03(9)	0.72±0.03(9)	0.73±0.03(8)	0.65±0.02(12)	0.50±0.03(10)	*** (P= 0.000)
6-12 months	0.70±0.04(7)	0.74±0.04(7)	0.75±0.04(6)	0.64±0.04(7)	0.53±0.03(10)	*** (P= 0.001)
12-18 months	0.69±0.04(7)	0.64±0.04(7)	0.71±0.06(4)	0.47±0.07(3)	0.43±0.04(10)	*** (P= 0.001)

Figure 1. Growth curve of F_1 male progeny

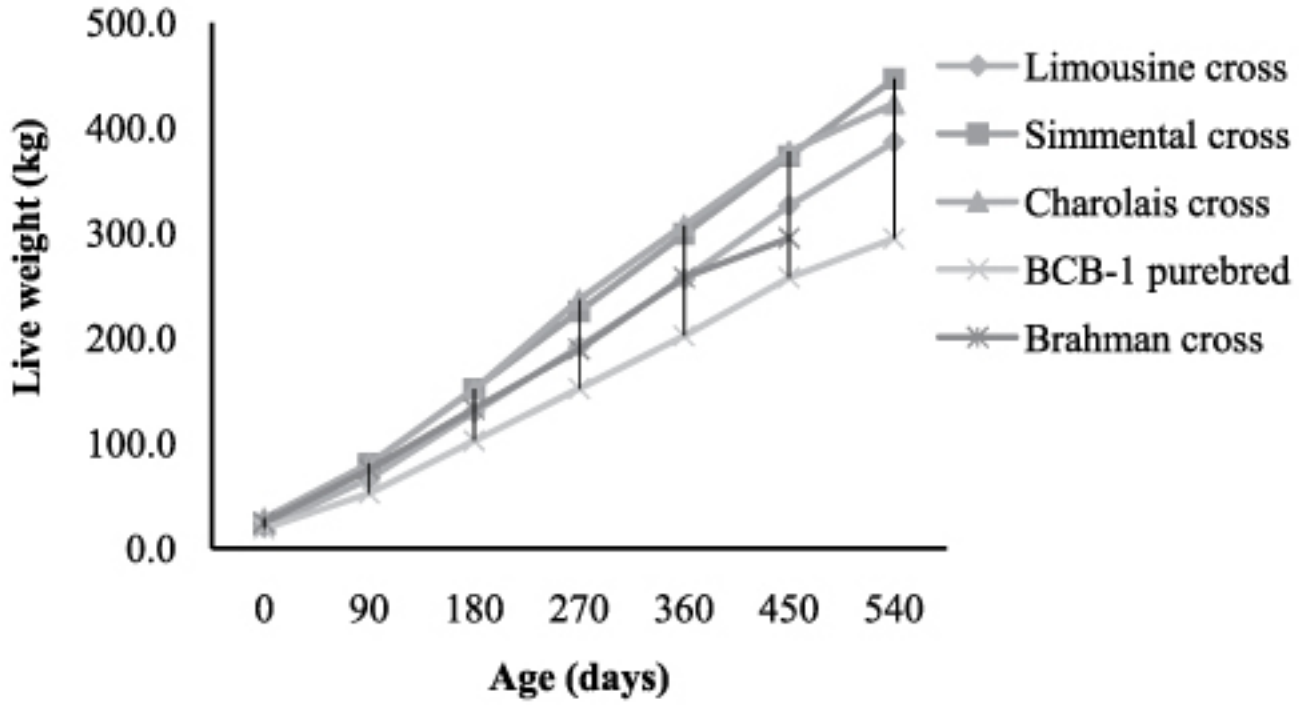

Figure 2. Growth curve of F_1 female progeny

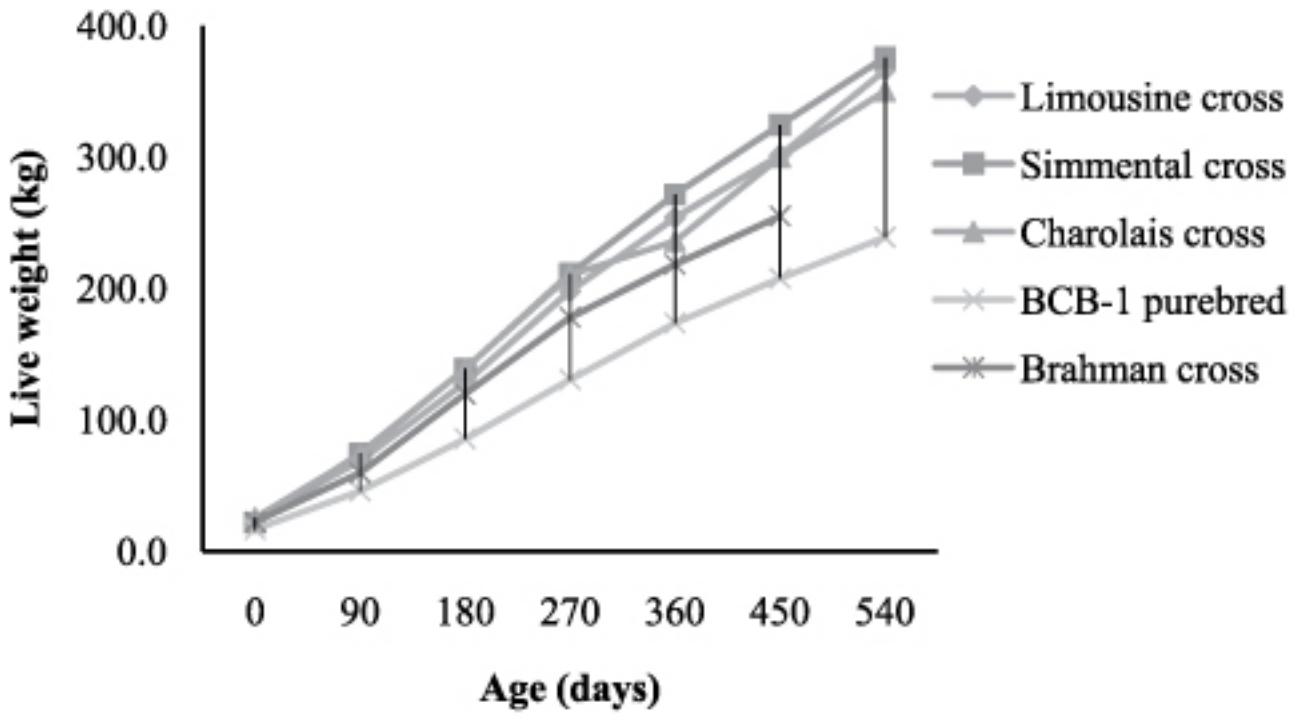

Table 6. Calf mortality of crossbred and BCB-1 progeny

Genotypes	Total calf born	Calf mortality		
		No. of calf died	Causes of death	% calf mortality
BCB-1×BCB-1	10	0	-	0
Limousine×BCB-1	11	1	Coccidiosis/ Bloody Diarrhea	9.09
Simmental×BCB-1	9	0	-	0
Charolais×BCB-1	9	1	Premature Birth	11.11
Brahman×BCB-1	12	0	-	0
Total	**51**	**2**	**Total 3.92**	

The main objective of the present study based on the dataset conducted in BLRI research farm, Savar, Dhaka was to study the comparative production performance of BCB-1 and its crosses of different beef sires. At all, stages, crossbred cattle as a whole were superior in live weight compared to BCB-1. The superiority of the crossbred cattle over the BCB-1 is a result of both additive and non additive gene effects. The additive

component of superiority represents a half of the additive genetic superiority of the *Bos taurus* sire breeds, while the non-additive genetic component represents the full amount of F_1 heterosis.

The traditional American Brahman was in general inferior to the continental breeds (Charolais, Simmental and Limousine). Waritthitham *et. al* (2010) reported the better growth performance and carcass quality of Charolais × Thai native compared to Brahman × Thai native cattle which partially agreed with the present study. Due to higher birth weight and faster growth rate Charolais was used to produce crossbred of Jeju native cattle (Cundiff *et al*., 1993). Heavier weight of Charolais compared with Brahman, other tropical and subtropical cattle are also well established. Peacock *et al*. (1981) reported 20% higher weaning weight for Charolais than for Brahman calves. In growth curve, charolais males were faster than other males. On the other, Simmental females were faster than other females. It may be due to number of calves belonged to both sexes in each genotype.

Calf scour and alopecia occurred in all genotypes. A total of 51 calves were born and out of that 2 calves were died due to coccidiosis and premature delivery. Under this breeding program, calf mortality was found as 3.92%.

Conclusion and Implications

Overall, significant breed differences were observed in growth performance. In conclusion, Charolais×BCB-1 is performing as the best among the five genotypes in terms of growth up to 18 months of age. More F_1 progeny is yet to be produced to evaluate their performance for the production of market beef cattle. Therefore, this breeding program should be continued for the coming years to achieve its goal.

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Determination of genetic variations among BLRI Cattle Breed-1 using microsatellite markers

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Abstract

Bangladesh Livestock Research Institute (BLRI) is the unique national livestock research organization in Bangladesh. The BLRI Cattle Breed-1 (BCB-1) was developed through selective breeding of indigenous Pabna cattle from Bangladesh. The study aimed at identifying the genetic variation of different generations of BCB-1 and its relationship with indigenous Pabna. A total of 20 ISAG-FAO recommended microsatellite DNA markers were used for this study. Blood samples were collected randomly from fifteen unrelated animals of 1st, 2nd, 3rd and 4th generation of BCB-1 and Pabna cattle. The allele number per locus ranged from 5 to 14 in the studied populations. The mean numbers of alleles ranged from 4.95 (BCB1-G3) to 7.55 (Pabna). The mean numbers of alleles per locus decreased in 3rd and 4th generation compared to 1st and 2nd generation of BCB-1. Pabna cattle showed 7.55 mean numbers of alleles. Both observed and expected heterozygosity decreased in subsequent generations among BCB-1. The observed heterozygosity of BCB1-G2 was 0.7257, which reduced to 0.6232 in BCB1-G4. The average observed heterozygosity values ranged from 0.6232 to 0.7393 with the lowest in BCB1-G4 (0.6232±0.0291) and the highest in Pabna (0.7393±0.0254). The genetic distance gradually increased from older to newer generations of BCB-1. First and second generations of BCB-1 have more genetic distance from 3rd and 4th generations. Pabna cattle showed more distance from all generations of BCB-1. Dendrogram based on DA genetic distance using unweighted pair group method of arithmetic means (UPGMA) indicated segregation of the four generations of BCB-1 into three main clusters. Cluster 1 consisted of 1st and 2nd generations and cluster 2 consisted of 3rd and 4th generation of BCB-1. All generation of BCB-1 of cluster 1 and 2 grouped together and separated from the cluster 3 of Pabna population. Mean number of alleles and heterozygosity decreased in subsequent generation and genetic distance between 3rd and 4th generation of BCB-1 also decreased. The findings suggest that BCB-1 is a unique cattle population and/or breed of Bangladesh can be conserved in pure form.

Key words: BCB-1, heterozygosity, microsatellite DNA marker

Introduction

Bangladesh Livestock Research Institute (BLRI) has developed BLRI Cattle Breed-1 (BCB-1) through selective breeding within indigenous cattle of greater Pabna district, which was evolved through admixture of Hariana, Tharparker and Sahiwal genetic materials with local cattle (Bhuiyan; 2008). BLRI collected these cattle and have been conserving them Ex *situ* since 1992. The selective breeding program was being conducted to improve milk and meat production performance of the genotype over the last 20 years. Selection pressure was put on lactation yield, live weight and red coat color of animals. These selection pressures resulted changes in the physical appearance and production performance of the cattle toward a distinct feature than that of their ancestors. The live weight of mature BCB-1 bulls ranged from 450 to 550 kg and cows ranged from 250 to 350 kg with their age varies from 5 to 6 years. The cows produce 900-1000 liters' milk in a lactation period of 240-260 days (Deb *et al*, 2008 and 2009). The coat color is red with a gradual shift to fawn or light brick at the ventral part of the body. The progress in milk production potential was very low (Deb *et al*, 2008 and 2009). However, the attractive coat color and body size of the bulls has a great market value. Over the last couple of years, BLRI systematically studied their morphological, production, reproduction, nutritional management, disease prevalence and beef characteristics. At present situation, the BCB-1 herd of BLRI research farm is progressed to 4th generation and few calves of 5th generation have already borne. However, due to selection pressure on BCB-1 the genetic parameters such as allele frequency, heterozygosity, and genetic distance among different generations might have changed; on the other hand, the genetic relationship of BCB-1 with indigenous Pabna

still unknown. Considering these facts, this study was aimed at identifying the genetic variations among different generations of BCB-1 and its relationship with indigenous Pabna cattle using microsatellite DNA marker.

Material and Methods

Sample collection, DNA extraction, and quantification

The BCB-1 herd of BLRI research farm has progressed to 4th generation and few calves of 5th generation have already borne. Blood samples were collected from 1st to 4th generation of BCB-1. Fifteen blood samples including males and females were collected from unrelated animals of each generation of BCB-1 and indigenous Pabna. Blood samples were collected in EDTA containing 4 ml vacutainer using 21G needle. The vials were gently tilted for mixing the blood with anticoagulant. The vial was marked with the date of collection, sex and genotype. The blood samples were carried into lab in ice box and stored at -20°C until extraction of genomic DNA (gDNA). The DNA was isolated from blood using a commercial kit (QIAGEN DNA Mini Kit) following manufacturer instruction. The DNA was quantified using 1% agarose gel electrophoresis and Nanodrop 2000 spectrophotometer.

Microsatellite polymorphism detection

The PCR amplification was done at Molecular Genetics Laboratory of Animal Production Research Division, BLRI and microsatellite genotyping work (Fragment Length Analysis) was done in a commercial laboratory named as 1st Base, Malaysia. All DNA samples were amplified in PCR machine using 20 pairs of microsatellite primers selected from ISAG-FAO recommendation (Table 1). All the microsatellite markers were forwardly labeled with a capillary based dye: FAM (blue), HEX (Green), TAMARA (yellow) and ROX (Red) for the purpose of genotyping. PCR was carried out in 15µl reaction mixture containing 4ng/µl of template DNA, 0.3µl each 10µM primer, 1µl of 20 mM dNTPs, 0.05 unit/µl of *Taq* DNA polymerase, 1.2 to 1.5mM/µl of MgCl2 and 1X PCR buffer. All PCR products were quantified by 2% agarose gel electrophoresis for checking the success of PCR amplification. The amplified DNA was genotyped by automated capillary DNA sequencer (Genetic Analyzer 3730 of Applied Bio-system, USA). The internal standard was prepared by adding 7.55µl of LIZ size standard to 0.45 µl of HiDi formamide. A total of 2µl of diluted PCR product of each co-loading was transferred to individual wells with 8µl of standard/formamide mix and denatured for genotyping. The genotyping results were processed by the GeneMapper v4.0 program which determines the allele sizes in each animal. A total of 71 samples including 13 from BCB-1 1st generation, 15 from BCB-1 2nd generation, 14 from BCB-1 3rd generation, 14 from BCB-1 4th generation and 15 from indigenous Pabna were amplified and genotyped.

Table 1: Selected ISAG-FAO recommended microsatellite markers.

SL. no.	Name	Primer sequence (5'- 3') Forward Reverse	Gene bank accession number	Allele range (bp)
1	INRA063	ATTTGCACAAGCTAAATCTAACC AAACCACAGAAATGCTTGGAAG	X71507	167-189
2	CSSM66	ACACAAATCCTTTCTGCCAGCTGA AATTTAATGCACTGAGGAGCTTGG	...	171-209
3	INRA037	GATCCTGCTTATATTTAACCAC AAAATTCCATGGAGAGAGAAAC	X71551	112-148
4	INRA005	CAATCTGCATGAAGTATAAATAT CTTCAGGCATACCCTACACC	X63793	135-149
5	HAUT27	AACTGCTGAAATCTCCATCTTA TTTTATGTTCATTTTTTGACTGG	X89252	120-158
6	TGLA227	CGAATTCCAAATCTGTTAATTTGCT ACAGACAGAAACTCAATGAAAGCA	...	75-105
7	BM1824	GAGCAAGGTGTTTTTCCAATC CATTCTCCAACTGCTTCCTTG	G18394	176-197
8	ETH152	TACTCGTAGGGCAGGCTGCCTG GAGACCTCAGGGTTGGTGATCAG	G18414 Z14040	181-211
9	HEL9	CCCATTCAGTCTTCAGAGGT	X65214	141-173

		CACATCCATGTTCTCACCAC		
10	BM1818	AGCTGGGAATATAACCAAAGG AGTGCTTTCAAGGTCCATGC	G18391	248-278
11	ILSTS006	TGTCTGTATTTCTGCTGTGG ACACGGAAGCGATCTAAACG	L23482	277-309
12	ILSTS005	GGAAGCAATGAAATCTATAGCC TGTTCTGTGAGTTTGTAAGC	L23481	176-194
13	INRA035	TTGTGCTTTATGACACTATCCG ATCCTTTGCAGCCTCCACATTG	X68049	100-124
14	MM12	CAAGACAGGTGTTTCAATCT ATCGACTCTGGGGATGATGT	Z30343	101-145
15	INRA032	AAACTGTATTCTCTAATAGCTAC GCAAGACATATCTCCATTCCTTT	X67823	160-204
16	HAUT24	CTCTCTGCCTTTGTCCCTGT AATACACTTTAGGAGAAAAATA	X89250	104-158
17	BM2113	GCTGCCTTCTACCAAATACCC CTTCCTGAGAGAAGCAACACC	M97162	122-156
18	ETH225	GATCACCTTGCCACTATTTCCT ACATGACAGCCAGCTGCTACT	Z14043	131-159
19	TGLA122	CCCTCCTCCAGGTAAATCAGC AATCACATGGCAAATAAGTACATAC	...	136-184
20	ETH10	GTTCAGGACTGGCCCTGCTAACA CCTCCAGCCCACTTTCTCTTCTC	Z22739	207-231

Data analysis

The genotyped data were analyzed using Microsatellite Toolkit program (http://animalgenomics.ucd.ie/sdepark/ms-toolkit/) (Minch, 1995) to calculate allele frequencies at each locus for each population, mean number of alleles per population and heterozygosity values (expected and observed). Dispan program (http://www.personal.psu.edu/nxm2/software.htm) (Ota, 1993) was used to calculate DA genetic distances between the populations and an unweighted pair group-method with arithmetic mean (UPGMA) phylogenetic tree was constructed using the PHYLIP 3.57c package (http://evolution.genetics.washington.edu/phylip.html) (Felsenstein, 1995).

Results and Discussion:

The four generations of BCB-1 and Pabna cattle showed polymorphism for microsatellite markers in the study. The allele number per locus ranged from 5 to 14 in the studied populations. The mean numbers of alleles ranged from 4.95 (BCB1-G3) to 7.55 (Pabna). The mean numbers of alleles per locus decreased in 3rd and 4th generation compared to 1st and 2nd generation of BCB-1. Pabna cattle showed7.55 mean numbers of alleles. Both observed and expected heterozygosity decreased in subsequent generations among BCB-1. The observed heterozygosity of BCB1-G2 was 0.7257, which reduced to 0.6232 in BCB1-G4. It indicated that heterozygosity decreased over the time among the generations of BCB-1 cattle. The average observed heterozygosity values ranged from 0.6232±0.0291 to 0.7393±0.0254 with the lowest in BCB1-G4 (0.6232±0.0291) and the highest in Pabna (0.7393±0.0254). The mean numbers of alleles along with observed and expected heterozygosity values per cattle population are presented in Table 2.

Table 2: Population statistics of different BCB-1 generation and Pabna cattle

Cattle populations	**Sample size**	**Loci typed**	**Mean number of alleles (MNA)**	**Observed heterozygosity**	**Expected heterozygosity**
BCB1-G1	13	20	5.45±2.04	0.6731±0.0291	0.6918±0.0359
BCB1-G2	15	20	5.75±1.55	0.7257±0.0258	0.7049±0.0291
BCB1-G3	14	20	4.95±1.23	0.6750±0.0280	0.6508±0.0378
BCB1-G4	14	20	5.30±1.95	0.6232±0.0291	0.6462±0.0484
Pabna	15	20	7.55±1.93	0.7393±0.0254	0.7828±0.0209

The genetic distance (Table 3) gradually increased from older to newer generation of BCB-1. 1st and 2nd generations of BCB-1 have more genetic distance from 3rd and 4th generations. Genetic distance between 3rd and 4th generation of BCB-1 decreased. The Pabna cattle showed more distance from all generations of BCB-1.

Table 3: DA genetic distance

	BCB1-G1	**BCB1-G2**	**BCB1-G3**	**BCB1-G4**
BCB1-G2	0.0867	0		
BCB1-G3	0.0949	0.0877	0	
BCB1-G4	0.0966	0.1010	0.0810	0
Pabna	0.1788	0.1574	0.1727	0.1711

Dendrogram based on DA genetic distance using unweighted pair group method of arithmetic means (UPGMA) indicated the segregation of four generations of BCB-1 into two main clusters in where Pabna cattle separated from these clusters (Figure 1).

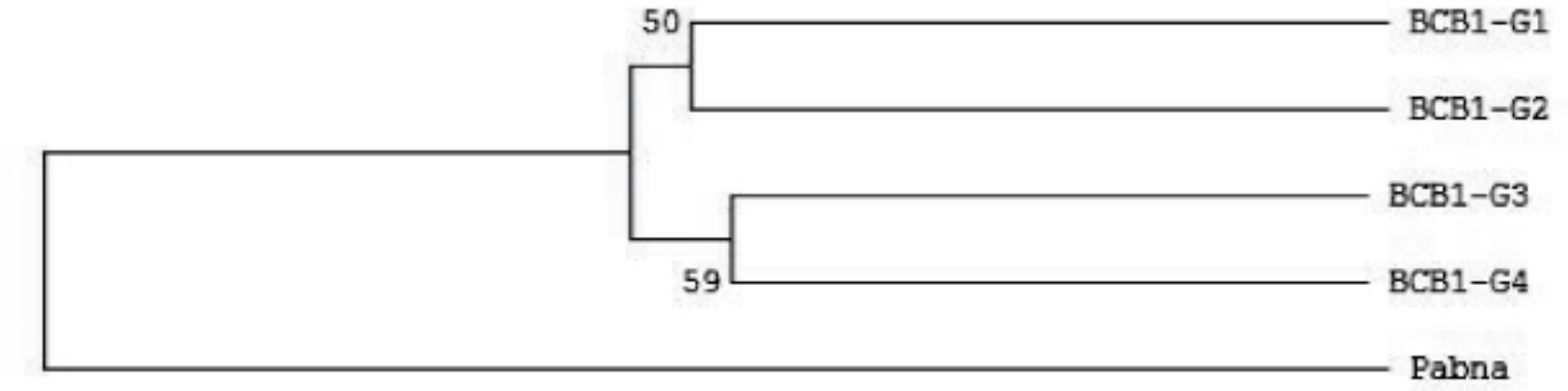

Figure 1: Dendrogram showing genetic relationship among four generations of BCB-1 and Pabna cattle.

Cluster 1 consisted of 1st and 2nd generations and cluster 2 consisted of 3rd and 4th generation of BCB-1. All generations of BCB-1 of Cluster 1 and 2 grouped together and separated from the Pabna population.

Because of applying selection pressure on BCB-1 for increasing meat and milk production and to refine red coat color, some unwanted alleles must have lost from subsequent generations. Mean number of alleles and heterozygosity decreased in subsequent generations. The genetic distance between 3rd and 4th generation of BCB-1 also decreased. As a result, it can be said that mean number of alleles and heterozygosity values reached to its fixed level in the last two generations of BCB-1. On the other hand, BCB-1 showed more genetic distance from its predecessor (Pabna) and also differed in mean number of alleles and heterozygosity value. The Pabna cattle, which was used in this study, were not from the foundation stock of BLRI was collected recently from greater Pabna district. The number of Pabna cattle has become rare in its habitant. Because of haphazard breeding with exotic semen in the greater Pabna district, the gene pool of Pabna cattle has possibly been diluted and which might have caused increased mean number of alleles and heterozygosity value in the present study but the values were much higher than the 1st generation of BCB-1. At present situation if the Pabna cattle were in its pure form then it would show close relationship with the 1st generation of BCB-1. All these findings suggest that BCB-1 can be considered as a unique population and/or cattle breed.

Conclusion and Implications

According to breed definition, cattle breed shows similar or unique phenotype and genotype through generation after generation. In 3rd and 4th generation, BCB-1 showed almost similar phenotype and genotype. Due to selection pressure the genetic characteristics of latest generations (3rd and 4th) of BCB-1 is distinct not only from its predecessor (Pabna) but also from its prior generations (1st and 2nd). Thus one would claim that BCB-1 is a unique cattle population or breed of Bangladesh. Further attempts are necessary to conserve it in a pure form and can be used for crossbreeding program for specific purpose(s) to get maximum benefit of hybrid vigor.

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Timed artificial insemination (TAI) in Bangladeshi buffaloes during the off breeding season

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Abstract

The study was designed to evaluate the efficacy of two different oestrus synchronization protocols in buffaloes for timed artificial insemination (TAI) during the off breeding season (March to September). A total of 60 lactating buffalo cows at ≥60 days postpartum were selected and were divided into two groups: CIDR-synch and Ovsynch protocols were conducted in Group 1 (n=20) and Group 2 (n=40), respectively. All buffalo cows were with a body condition score (BCS) ≥2.5. Buffaloes in group1 were synchronized by injecting GnRH on D0, inserting progestagen implant (CIDR) two days after GnRH injection, removing CIDR during injection of PGF2α on D7, injecting a second GnRH on D9 and TAI was performed 16 -20 h later (CIDR-synch protocol). Buffaloes in group 2 were treated by injecting GnRH on D0, PGF2α on D7, second GnRH on D9 and TAI was performed 16 -20 h later (Ovsynch protocol).TAI was performed with frozen-thawed semen of Italian Mediterranean buffaloes in all synchronized Bangladeshi buffaloes. Different epidemiological factors related to farm and animal information were considered and carefully recorded prior to the conduction of the experiment. Vaginal Electrical Resistance (VER) was recorded and ultrasound scanning of both ovaries was performed on D0, 7 and 10 to confirm estrus and to monitor ovarian follicular and luteal status. Pregnancy was diagnosed with transrectal ultrasonography after 35 days of AI. Higher pregnancy rate (45%) was observed in buffaloes of group-2 compared to that of group 1 (35 %/) that was non-significantly different ($p > 0.05$). The findings of the study suggest that, choice of protocol for oestrus synchronization has prominent roles on oestrus induction and improvement of pregnancy rate of water buffaloes when inseminated with frozen-thawed semen during off breeding season.

Key words: Buffalo, synchronization, timed artificial insemination, post-partum.

Introduction

Buffalo has never been addressed in Bangladesh as an important animal and it is a neglected species. In Asia, the domestic water buffalo is generally classified into two principal sub-species (Yue*et al*., 2013); the river type and the swamp type (Perera, 2008) and in Bangladesh also two types of buffaloes are found these are River type and Swamp type. Many buffaloes rear in saline coastal areas as herded animals, totally free as Bathan farming and mostly used for meat production.Bangladesh having 1.86 million buffaloes stands 9thin Asia in term of buffalo population (FAO, 1986). World's buffalo population is increasing day by day which is estimated to be 202 million represents approximately 11% of all bovine species in world (FAO, 2007); out of these, Asia alone represents 174.21 Million (FAOSTAT, 2008).Buffaloes play a prominent rule in livestock production in many Asian countries including Bangladesh. The river buffalo is triple purpose species contributing milk, draft work and meat.Due to their contribution and importance, buffaloes are being concentrated in particular agro-ecological zones of Bangladesh. Despite their important role in Bangladesh national economy, buffaloes are always neglected animals in Bangladesh. The production system are characterized by small number of animal with no or minimal input, low output and periodic destruction of animals by disease and mostly maintained under scavenging system with little or no inputs for housing, feeding or health care. The native buffalo are well adapted to the local environmental, low quality feed resources, housing facilities and scavenging system and the most important aspect is that their performance is

also good in terms of feed efficiency. The feed resource base for these animals is scavenging and consists of crop residues, household wastage, tree fodder, roots and tuber, grain by-product and anything edible found in the immediate environment. However, buffalo farmers usually face challenges in reproductive efficiency in female buffaloes by inherent late maturity, poor oestrus expression in summer, distinct seasonal reproductive patterns and prolong inter-calving intervals (Madan, 1988; Madan and Raina, 1984).As a result, fertility and pregnancy rates decrease dramatically. To overcome this type of problem and improve reproductive efficiency, different estrus and ovulation synchronization protocols like Ovsynch and CIDR Synch protocols have tobepracticed in Bangladesh. But in CIDR Synch drug expense, more labor cost, high level of management and good handling facilities are required. Ovsynch plays an important role in improving conception rates in farm animals. Among two synchronization protocol Ovsynch is easier and reliable for the farmer which is very helpful for the improving livestock production.The experiment was carried out to study the efficacy of two different oestrus synchronization protocols in buffaloes for timed artificial insemination (TAI) with frozen thawed semen during the off breeding season (March to September) in Bangladesh.

Objectives of the study:

i. To develop procedures incorrect oestrus/heat detection by studying vaginal electrical impedence using heat detector (first time in Bangladesh) and by studying follicular dynamics with transrectal ultrasonography.
ii. To evaluate synchronization protocol suitable for native water buffaloes in off breeding season.
iii. To study fertility of female buffaloes following timed AI with frozen-thawed semen.

Materials and methods

The present study was conducted at a commercial Breeding Farm 'LalTeer Livestock Ltd., Mymensingh, Bangladesh' from March 2017 to June 2017.

Experimental Animals and Management

A total of 60 indigenous lactating water buffalo cows at ≥60 days postpartum from the mentioned Breeding Farm were divided into two groups: Group 1(CIDR synch, n=20) and Group 2 (Ovsynch, n=40).The buffalo cows which were in cyclic and characterized as in good health and without a history of reproductive illness were selected for experimental protocol. Body condition scoring (BCS) of all the cows were in between 2.5 and 3.5 out of 5.0. The animals were fed with paddy straw, concentrate mixture, cut-and-carry grass and milling by-product according to their body weight and milk production.Vitamin premixes (Powder Renavit-DB®, Renata Animal Health, Mirpur, Dhaka, Bangladesh) were also supplied to the buffaloes with concentrate. All the selected buffaloes were dewormed by using Nitroxyline (Injection Niloxin®SKF,Bangladesh Ltd) 14 days before beginning of experiment.

Synchronization protocol and Timed Artificial Insemination (TAI)

CIDR Synch and TAI

Buffalo cows in Group 1 (n=20) were synchronized by intramascular injection of GnRH analogue (Buserelin 10μg; Receptal, Intervet, The Netharlands) on Day 0 and inserting progestagen implant containing natural progesterone 1.38gm (CIDR) two days after GnRH injection, removing of CIDR during the injection of PGF2α (cloprostenol 500μg; Ovuprost ®, Renata Animal Health, Mirpur, Dhaka, Bangladesh) on Day 7. On Day 9 the second GnRH (Buserelin 10μg; Receptal, Intervet, The Netharlands) was injected. TAI was performed 16-20h after the second GnRH injection using frozen semen from Mediterranean buffalo bull. Vaginal Electrical Resistance (VER) values were recorded at the time of FTAI, and ultrasound scanning of both ovaries was performed on D0, 7 and 10 to confirm estrus and to monitor ovarian follicular and luteal status.

Ovsynch and TAI

Buffalo cows in Group 2 (n=40) were synchronized by intramuscular injection of a GnRH (Buserelin 10μg; Receptal, Intervet, The Netharlands) on Day 0 followed by injection of PGF2α (cloprostanol 500μg,

Ovuprost, Renata Animal Health Ltd., Bangladesh) on Day 7. On Day 9 the second GnRH (Buserelin 10µg; Receptal, Intervet, The Netharlands) was injected. TAI was performed 16-20h after the second GnRH injection using frozen semen from Mediterranean buffalo bull. Vaginal Electrical Resistance (VER) values were recorded and ultrasound scanning of both ovaries was performed on D0, 7 and 10 to confirm estrus and to monitor ovarian follicular and luteal status.

Recording of Vaginal Electrical Resistance (VER)

The VER values of the buffalo cows were recorded on the protocol's Day 0, 7 and 10 using Electronic Oestrus Detector (Draminski Estrus Detector, Poland). The functional activity of the oestrus detector was tested and all hygienic precautions were taken before VER recording. The reading was taken three times and the mean value was calculated and noted.

Ultrasonographic examination for the estimation of follicular size and pregnancy diagnosis

Follicular development was monitored with B mode digital ultrasound equipment (Ultrasonic diagnostic instruments-vet (Digicare Biomedical Technology, Boynton Beach, Florida, USA) using 7.5 MHz high frequency linear array probe on Day 0 and 10, and pregnancy diagnosis at 35 days after TAI and data were recorded. All fecal materials from the rectum were removed before inserting the transducer. For orientation, the transducer was moved along the dorsal surface of the reproductive tract and then moved laterally to examine the ovaries. The transducer face was lubricated with a suitable coupling medium (Eco Gel™-250 mg, Eco-Med Pharmaceutical Inc., Ontario, Canada) and was usually covered by a lubricated plastic sheath before insertion. The transducer was then progressed cranially along the rectal floor to overlie the reproductive tract. The ultrasound monitor and operator's eye were at comparable level for accurate reading of ultrasound images. The enlarged Grafian follicle in ovaries with increased tonacity of uterus was regarded as buffalo in oestrus. Each ovary was scanned several times. The image was retained on the screen, and the size of the follicle was determined and recorded. Diagrams of the relative positions of the follicle as well as related ovarian structures were drawn.

Blood sampling and progesterone (P4) assay

Blood samples were collected properly at the protocol's Day 0, 10 and 21 from jugular vein of each animal using non-heparinized blood collecting tube for serum collection. The harvested serum samples were stored at -20ºC for further use to assay the progesterone profile.

Statistical analysis

Data from the research work were entered into Microsoft Excel-2007 work sheet and analyzed by Statistical Package for Social Sciences (SPSS) software version 20 using descriptive statistics.Continuous data from pregnancy rate were analyzed by chi-square test and VER values, follicular size were analyzed by paired T-test.

Results

After CIDR Synch and Ovsynch treatments buffalo cows showed different estrus signs like bellowing, frequent urination, swelling of the vulva and had mucus. The buffaloes had a variable degree of uterine tone and their cervices were open enough for easy passage of AI gun during insemination.

Pregnancy rate after implementation of synchronization protocol

Pregnancy rate of the water buffaloes after implementation of synchronization protocol is presented in the Table 1.

Table 1: Pregnancy rate after implementation of synchronization protocol

Experimental Group	Experimental cows	Pregnant cows	Non-pregnant cows	P value
Group-1 (CIDR Synch)	20	7 (35%)	13 (65%)	0.459
Group-2 (Ovsynch)	40	18 (45%)	22 (55%)	

*** indicates significant, where P<0.05.**
There is no significant variation of pregnancy rate in synchronized Group1-CIDR Synch (35%) and Group2 Ovsynch(45%) (P>0.05).

VER values of pregnant and non-pregnant cows during the synchronization protocol

VER values of pregnant and non-pregnant cows during the synchronization protocol is presented in the Table 2.

Table 2: VER values of pregnant and non-pregnant cows during the synchronization protocol

Experimental Group	VER (Ω)								
	Day 0			Day 7			Day 10		
	Pregnant cows	Non-pregnant cows	P-value	Pregnant cows	Non-pregnant cows	P-value	Pregnant cows	Non-pregnant cows	P-value
Group-1 (CIDR Synch)	417.14 ±32.93	473.08 ±26.78	0.218	235.71 ±11.30	265.38 ±17.50	0.262	204.29 ±6.85	230.00 ±2.26	0.000**
Group-2 (Ovsynch)	398.89 ±15.92	426.82 ±23.75	0.358	260.00 ±12.02	277.73 ±18.81	0.455	210.56 ±4.16	226.36 ±5.57	0.035*

**** indicates highly significant, where P<0.01. * indicates significant, where P<0.05.**
Result showed that at Day 0 and Day 7 the VER values were non-significantly lower in the cows which are getting pregnant after TAI than the cows of non-pregnant in both the experimental Groups. But on Day 10 the VER values were significantly (P<0.05) lower in the cows which were getting pregnant after TAI than the cows of non-pregnant in Group 1 and Group 2 respectively.

Follicular size of pregnant and non-pregnant cows during the synchronization protocol

Follicular size of pregnant and non-pregnant cows during the synchronization protocol is presented in the Table 3.

Table 3: Follicular size of pregnant and non-pregnant cows during the synchronization protocol

Experimental Group	Follicular size					
	Day 0			Day 10		
	Pregnant cows	Non-pregnant cows	P-value	Pregnant cows	Non-pregnant cows	P-value
Group-1 (CIDR Synch)	4.07±0.22	4.08±0.25	0.989	11.36±0.45	8.87±0.51	0.005**
Group-2 (Ovsynch)	3.87±0.14	3.86±0.17	0.971	10.65±0.37	8.60±0.29	0.000**

**** indicates highly significant, where P<0.01.**
Result showed that at Day 0 the follicular size was more or less similar in all the cows of both experimental Groups. But on Day 10 the follicular sizes were significantly (P<0.01) higher in the cows which are getting pregnant after TAI than the cows of non-pregnant in both the experimental Groups.

Discussion

Silent oestrus is a common problem in buffaloes even under good management and non-stressful periods of the year (Kamboj and Prakash, 1993; Abdallah, 2003) and the farmers cannot easily identify the cyclic buffaloes due to the nature of showing silent heat. CIDR Synch and Ovsynch with TAI were practiced in the present study in indigenous water buffaloes, where estrus detection is the main restrictions for satisfactory reproductive performance.

Result in the present study revealed that 45% buffaloes became pregnant when oestrus was induced by Ovsynch protocol and 35% buffaloes become pregnant when oestrus was induced by CIDR Synch protocol. The pregnancy rate obtained in Ovsynch protocol was more or less similar with the results of other experiments which ranged from 27.2 to 42.4% (Barusell*iet al*., 1997; Irikura*et al*., 2003; Negli*aet al*., 2003; Paul and Prakash, 2005). Negli*aet al*. (2003) observed that a conception rate of 36% after using the Ovsynch protocol in postpartum lactating dairy buffalo cows. Similarly, Paul and Prakash (2005) obtained a conception rate of 33.3% and Warriach*et al*. (2008) obtained 36.3% conception rate in buffaloes when oestrus was induced at breeding season. However, the obtained pregnancy rate of the present study is consistent with the earlier study conducted by Berber *et al*. (2002) who reported 56.5% pregnancy rate in buffaloes. The pregnancy rate obtained in CIDR Synch protocol is considered more or less similar with the findings of Xu and Burton (2000). In the CIDR Synch and Ovsynch protocol the first GnRH injection can successfully synchronize a new follicular wave 1-3 days after treatment (Negli*aet al*., 2003; Ali and Fahmy, 2007) and this wave results in the development of a new dominant follicle, which may be attributed to the subnormal P4 concentrations. Sub luteal circulating P4 levels have been reported to increase the frequency of LH pulses, and a prolonged growth phase of the dominant follicle (Bridge and Fortune, 2003). The subsequent injection of PGF2α increases the percentage of synchronized animals by lysis of both the cyclic CL and the CL resulting from ovulation of the dominant follicle (Pursley*et al*., 1995). After the PGF2α injection, if the dominant follicle did not ovulate, a new wave of small follicles needs several days to grow and become able to produce estradiol-17β, leading to an induction of the preovulatory LH-surge (Bridge and Fortune, 2003). It has been previously suggested that high P4 levels at the time of PGF2α application may be an important factor to improve conception rates for subsequent inseminations (Hussein, 2003; De Rensis*et al*., 2005). In order to increase synchrony of ovulation, a second dose of GnRH is injected to ovulate the preovulatory follicle at a precise time (Wiltbank, 1998). The second GnRH injection on Day 9 of the protocol causes an induced LH surge responsible for ovulation of the dominant follicle and formation of a new CL (Senger, 2003). Moreover, in the CIDR Synch the removal of CIDR device was intended to improve the precision of oestrus (Xu and Burton, 2000). However, the cause of difference in pregnancy rates among studies may be due to differences in breed of buffaloes, follicular number and size, oestrus induction protocols and agro-climatic conditions of study areas in different studies which emphasizes the requirement of selecting a suitable oestrus induction protocol for buffaloes that may result in satisfactory pregnancy rate without paying much time for oestrus detection.

Result revealed that VER values was significantly lower on the protocol's Day 10 (at the time of AI) in the buffaloes which were getting pregnant after TAI than the buffaloes of non-pregnant, which is the similar findings of Gupta and Purohit (2001),although the values of VER in the present study was higher than the findings of Gupta and Purohit (2001). Gupta and Purohit (2001) stated that VER can be used successfully to predict the stage of oestrous cycle, ovarian status and ovulation; and insemination at a low VER distinctly improves the conception rates in buffaloes.

Results showed a significant ($p<0.01$) effect of follicular size in the pregnant buffaloes after the application of CIDR Synch and Ovsynch protocol, which is more or less similar with the findings of other experiments (Berber *et. al*., 2002; Baruselli and Carvalho, 2005; Bartolomeu*et. al*., 2007). The time to ovulation after GnRH injection depends mainly on the diameter of the largest follicle at the time of injection (Hussein *et al*., 2002; Hussein, 2003) and it is a determining factor for the successful synchronization of ovulation and high conception rates (De Rensis*et al*., 2005). In a recent study, Campanile *et al*. (2008) have stated similar size of follicles in ovulated and non-ovulated buffaloes. However, the stage of follicular development (growth or regression phase) greatly affects its response to GnRH treatment (Dharani*et al*., 2010). Therefore,

understanding and considering follicular waves as a functional unit, rather than the estrous cycle, could help in the proper synchronization protocol for the enhancement of reproductive efficiency in river buffaloes. A good knowledge about follicular wave dynamics could facilitate the development of a methodology for influencing ovarian function and oestrus in both cyclic and non-cyclic animals.

Conclusion

On the basis of the results of this study, following conclusions may be made:

1. Results of the study confirm that the Ovsynch protocol has more effect on the efficiency of oestrussynchronization for successful fertility.
2. Follicular size at the beginning of the synchronization of oestrus insignificantly affects in pregnancy rate.
3. Ultrasound monitoring of the reproductive organs in buffaloes can be a suitable diagnostic tool for monitoring cyclicity and early pregnancy in Bangladesh.

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Feed & Nutrition

Economics and stakeholders participation in fodder production and marketing system under different climatic zones of Bangladesh

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Abstract
An investigation was carried out with the objectives to determine the economics and stakeholders participation of fodder marketing system under different climatic zones, namely saline prone area (Sathkhira), flood plain/river basin areas (Pabna), and semi A arid/drought prone areas (Chapainobabgonj). To achieve the objectives, Focus Group Discussions (FGDs), Key Informant Interview (KII), physical observations of market places were studied. In flood prone areas, developed formal fodder marketing system was prevailing but there was no market place. Farmers purchased cultivated grass from other land by spending BDT5000-6000/cut. Most of the farmer cultivated HYV green grass Napier grass,e.g. (*Pennisetum purpureum L*), Jumboo (*Sorghum bicolour; Sorghum sudanefe*) and leguminous grass e.g. *Khasari* (*Lathyrus sativus*) for their own farm. HYV green grasses cultivation was found more profitable (BDT1194/decimal/year) compared to rice production (BDT273/decimal/year) in flood prone areas. In Semi- arid area/drought prone areas, farmers usually leased the land by spending 12000-13000 BDT/decimal for collecting natural grasses that were grown under the shed of orchards/ fruit trees (Mango, Malta, jujube, Guava etc.). In saline areas, the shrimp & crab farming were dominating crops and which occupied most of the agricultural lands. Local grass like *Nona Shapna* (*Panicum sp.)* was produced in the dike of all Ghare areas round the year. Some cattle farmers' in saline area has taken leased the dike of Ghare for collection and production of native grasses which consists of 230-250 decimal of water body . Farmer usually leased the dike by spending BDT1000-1500 for 3-5 months. Regarding the stakeholders participation, it was observed that there were seven types of market actor were found in fodder marketing system. They were i. Sole HYV grass producer; ii.HYV grass Producer who had dairy cattle; iii. Middle man who purchased grass from producers and sold to local dairy farmers in a market place; iv. Vans/Rickshaws pooler who sold grass by mobile van to different places; v. Consumer, dairy farmers who purchased grass from other farms during lean period; vi.HYV grass seed shop; vii. Dairy farmer leased the land/dike of bill/ shed of the fruit garden for nursing & collecting native grass. The study further revealed that fodder production was profitable business as one unit investment of BDT could return 5-6 units of BDT i.e. BCR 1: 5.73.

Key words: Fodder production, Fodder business, Fodder value chain, Climatic zones

Introduction

The major effects of climate change for livestock sector are reduction in grazing area, fodder crisis due to low growth & yield of green grass that decrease the production of milk, egg and meat. All these will lead to loss in rural household income, dietary nutrition and unemployment (Rahman et al.2017). Fodder plays important role in economizing the cost of production of livestock product (Begum et al. 2014). Feeds and fodder cost constitute about 60-70 per cent of cost of milk production (Grover et al. 2008). Climate change is causing unusual behavior in temperature; rainfall, flooding pattern and saline water intrusion in coastal region are affecting either natural growth or damaging pasture grasses. Therefore, key policies issues consider on creation of pasture land; training of farmers on fodder cultivation and fodder. Preservation and quality control of animal and poultry feed (Bangladesh delta plan, 2015). The government and non-government organizations have been involved in the extension of improved grass varieties through distributing improved grass seeds/cutting and training to minimize the fodder crisis in farmer's level. Research organization has been given emphasis to development of production technology as well as adaptation trail of HYV grass and fodder

crops. In 1970 program for introducing high yielding fodder like Napier, Para, guinea, maize, cow pea etc were taken up in Central Cattle Breeding and Dairy Farm (CCBDF), Savar under the German assistance. Extension of green fodder at farmer's level and around the farm was carried out up to 1974-75 simultaneously. During 1977-79 the first pilot program for planting of fodder in one mile embankment by the Water and Power Development Authority (WAPDA) and Milk Vita under the cooperation of Bangladesh cattle development project in Bera upazilla under Pabna district. In early nineties similar program was taken in Meghna Dhanagoda project of Water Development Board in Chandpur district (Fattah, 2011).

In Bangladesh, there is a requirement of 70 million metric tons of green grass for cattle feed in a year but produced only 24 million metric tons. Thus, there is a deficit of animal feeds for about 60 percent, which are hampering the livestock development to a great extent (ProthomAlo, 2008). The total feed and fodder available for ruminant in the country was estimated to be 3234.14x103 ton in 2012 (BBS, 2012; Huque and Sarker, 2014). Other researchers also reported that the major feed for livestock in Bangladesh is straw. About 2 kg of straw is available per head per day and supplementation is limited to about 1 kg of green fodder plus marginal quantities of cereal and oilseed by-products (Zannat et al., 2012). The farmers' rearing animals under climate vulnerable areas utilizing natural grasses are more prone to higher methane production compared to animals rearing better feed resources though their availability was varied with the seasons and locations. The calculated total DMI (Kg/h/day) was the highest (14.14±1.06) in flood prone followed by drought (13.80±1.30) and saline areas (4.43±0.20), respectively (Rahman et al., 2017). Similarly, the milk production was also higher (12.06±1.19 litre/h/day) in flood prone area followed by drought (4.47±0.60. litre/h/day) and saline (1.83±0.11 litre/h/day.) areas, respectively (Rahman et al., 2017). The calculated total methane emission (g/h/d) was significantly higher in flood prone (478.31±36.36) and the lowest in saline (153.35±7.14) prone areas . Whereas, methane production per unit of milk yield, was the lowest in flood prone (46.55± 6.78) and the highest (110.48±21.69) in drought prone area and the difference was statistically significant ($p<0.05$) (Rahman et al.,2017).

The main constraint to forage production for feeding ruminants is the scarcity of land. Usually farmers do not want to spare cultivable land for fodder production instead of crop production. Recently, fodder production gets momentum as an income generation and employment opportunity in certain areas of Bangladesh, especially milk pocket areas, namely Madaripur, Munshigonj, Rangpur, Sirajgonj and Tangail,where small scale dairying, fattening and milk marketing facilities are prevailed (Sayeed et al. 2008). Though several districts were facing acute shortage of green fodder which an obstacle for sustainable fodder marketing system lead more vulnerable situation for ruminant production. Considering those situation, an investigation was undertaken to know below objectives.

i) To find out existing marketing system and determine the benefit of fodder business.
ii) To draw market map of fodder marketing system.
iii) To identify the stakeholders participation of fodder marketing system.

Materials and Methods

To achieve the above objectives several studies were done. Focus Group Discussions (FGDs), Key Informant Interview (KII), physical observations of market places were studied. The study was conducted in different agro-climatic zones of Bangladesh(Figure1), namely Flood Plain/River basin areas (Pabna), Semi-arid/Drought Prone areas (Chapainobabgonj), Saline Prone area (Sathkhira). Farmers having livestock were selected for the FGDs that who were utilizing natural grasses to livestock by cut & carry from natural habitats. The data collected from FGD was cross checked by a interviewing from the farmers.

Table 1. Locations of FGDs and number of participants.

Number of FGD	Name of the location	Number of participants	Nature of FGD
Flood prone area (Pabna)			
1	Bhabanee pur,Bhangura	12	Male group
2	Bowalmare, Bhangura	12	Male group
3	Par Bhangura	12	Mix group
Drought area (Chapainobabgonj)			
1	West Mirzapur,Fatahpur, Nachol	12	Male group
2	Srirampur,Nachol	11	Male group
3	Bandra ,Nachol	10	Mix group
Saline area (Satkhira)			
1	Zaowakhalee, Shamnagar	11	Mix group
2	Norarchok ,Debhata	12	Male group
3	Purbapara , Debhata	12	Female group

A total of 9 (nine) FGDs were conducted in different locations during the project periods to achieve the above stated objectives (Table 1). A total of 3 FGDs were carried out for each agro-climatic zone to find out fodder marketing system, benefit of fodder production and stakeholders participation in the fodder chain. Twelve participants were attended in each FGD, having 2 age groups i.e. older people (more than <50) and young people (not less than>40 year age). Two aged group of people were selected due to know real transformation of fodder marketing system. Participants were asked according to prescribed check list. Therefore, through this process all the participants had the opportunity to share real situation in their locality and the present practices related to fodder and livestock production systems.

Result and Discussion

After investigation, fodder marketing system, benefit of fodder production and stakeholders' participation in the fodder chain in different climatic vulnerable areas of Bangladesh are discussed below.

Fodder marketing system:

Cattle production depend on the agricultural bi-products like straw; green grass and cereal milling by-products that is close related on existing cropping pattern. Milking cows were supplemented with concentrate feed in addition to roughage. Farmers cultivate leguminous fodder crops e.g. Khesari (*Lathyrus sativus*), Maskalai , and HYV grass e.g. Napier grass (*Pennisetum purpureum L*) and Jamboo (*Sorghum bicolour Sorghum sudanefe*) grasses. In study areas, different cropping pattern were seen which were not fully supportive with livestock production system, except in flood prone areas. In flood prone areas, it was two crops (Aman-Boro) and now common practice is Boro-HYV green grass (HYV). According to flood shocks, therefore existing cropping calendar was found as very supportive to livestock rearing. Cropping pattern has been divided into three stages. These stages were before flood, during flood and after flood. Farmer faced shortage of green grass and concentrate feed during flood. During this time man and cattle lived together in same place which leads destocking of animal resources (Rahman, 2012). Farmers purchased cultivated grass from other land by spending BDT5000-6000/cut. Most of the farmer cultivated HYV green grass Napier grass, e.g. (*Pennisetum purpureum L*), Jumboo (*Sorghum bicolour; Sorghum sudanefe*) and leguminous grass e.g. Khasari (*Lathyrus sativus*) for their own farm. HYV green grasses cultivation was found more profitable (BDT 1194/decimal/year) compared to rice production (BDT 273/decimal/year) in flood prone areas (Figure 2).

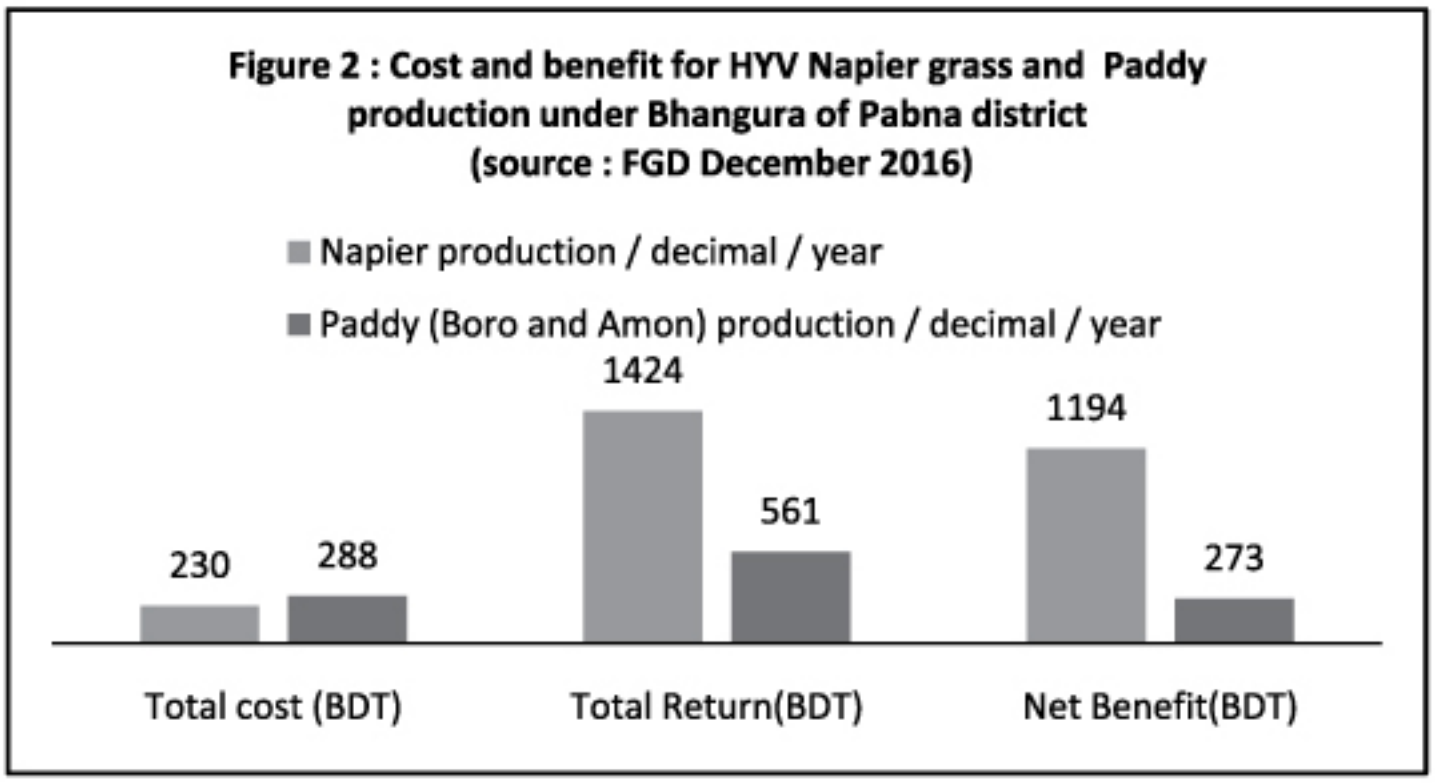


Figure 2 : Cost and benefit for HYV Napier grass and Paddy production under Bhangura of Pabna district (source : FGD December 2016)

In Semi arid area/drought prone, existing cropping calendar was not supportive to livestock rearing. Cropping patterns were consisting Boro-T Aman-Pulse/wheat. The feed sources of ruminant livestock mainly depend on crop residues and natural grasses. In areas, farmers usually leased the land by spending 12000-13000 BDT/decimal for collecting natural grasses that were grown under the shed of orchards/ fruit trees (Mango, Malta, jujube, Guava etc.). In saline areas, different vegetable cultivated in household yard for August to February. Salinity increased in the drought season, especially month from March-May. Saline water was found from 250-300ft of deep water. In this time, soil moisture has been decreased. Moreover low intensity of monsoon one of the reason for increasing soil salinity. In saline areas, the shrimp & crab farming were dominating crops and which occupied most of the agricultural lands. Local grasses, like Nona Shapna (*Panicum sp.*) was cultivated in the dike of all Ghare areas round the year. Some cattle farmers' in saline area has taken leased the dike of Ghare for collection and production of native grasses which consists of 230-250 decimal of water body . Farmer usually leased the dike by spending BDT1000-1500 for 3-5 months.

Different input cost and return for HYV green grass production demonstrated in Table 2. The percentage of input cost for higher for irrigation followed to seed/cutting and labour cost. Grass production cost was BDT8000-9000 for 33 decimal of lands. Selling cost per cutting was BDT9000-10000 and 5-6 cuttings were done in each year. Therefore, the benefit was found about BDT40,000-45, 000 in each year from 33 decimal lands. Table 2, showed that fodder production was profitable business as one unit of BDT could return 5-6 unit of BDT (BCR 1: 5.73). This findings support to the observation of Huque et al.,(2006) who calculated benefit cost ration as 1:3.40 for Nepier and it was much higher than other crops. Begum et al., (2014) also found the highest BCR (1:2.18) for producer cum seller group in Jessore district.

Table 2 : Input cost and return for HYV green grass production under Sitol bhaha of Potia

Description	BDT	% of total cost
Cost (BDT) of HYV grass for 33 decimal for one year		
Seed/ Cutting	2200	25.23
Fertilizer (Urea, TSP))	1100	12.61
Irrigation, 5 times/year	2500	28.67
Labour for weeding, cutting, harvesting and other	1920	22.02
Fencing	1000	11.47
Total cost	8720	
Benefit (BDT) for 33 decimal for one year		
Production (5 cuttings /year; BDT 10,000/Cutting)	50000	
Total return (BDT/Year)	41280	
Cost Benefit Ratio (BCR)	1:5.73	

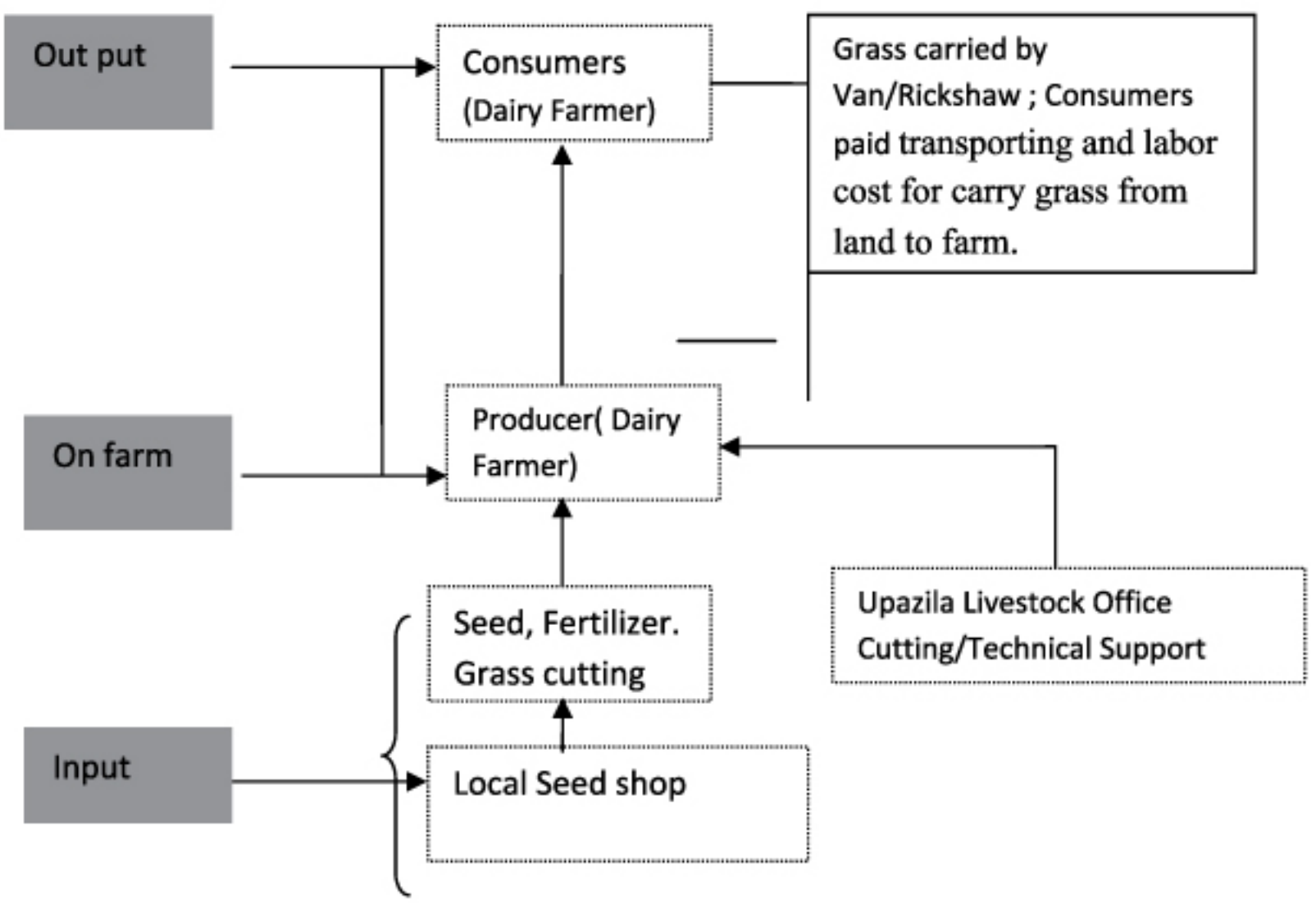
Source: KII, 2017

Fodder market map

Several types of fodder market map could be functioned in different areas. Fodder shop for selling & procuring was not established yet in Potia of Chittagong district. Most of farmer didn't bring fodder to market place due to avoid caring cost & taxation cost as all sellers must be paid certain amount of money to market committee. There were developed formal fodder marketing system but there were no market place in Bhangura of Pabna district. Dairy farmers had been purchased fodder from the land of other farmers who produce fodder for sale.

In our study areas we did not found market place for grass trading. It was not found formal marketing system in the areas of Upazilla level. But it was found in the in the district level. Farmers sold grass to other cattle farmers from land to land in river basin/flood prone areas e.g. Pabna & Chittagong. In Debhata of Satkhira and Nachal of Chapainobabgonj, it was practiced to sell native grass from the dike of Ghare and from orchards. Begum et al.,(2014) observed that there were no organized and regular marketing systems in Bangladesh. They noted that main channel of fodder marketing system were i) Fodder farmer ii) Fodder middle man and iii) Dairy owner. A market map on HYV green grass has been drowns in Diagram 1.

Diagram 1 Fodder market map under Potia upazilla of Chittagong district.



Stakeholders participation

Base on secondary review and field observation in different district, FGD, KII, seven types of stakeholders were observed in fodder marketing system in Bangladesh. Stakeholder participation of existing Fodder Marketing System in different areas of Bangladesh has been illustrated in Table 3. Roy et al., (2012) found three stakeholders found under fodder value chain in Maharpur district. These are fodder producers, middlemen and users of fodder. In our study highest numbers of stakeholders in fodder marketing system were found in Dinajpur district followed to Rangpur and Chittagong. Overall individual stakeholder was Dairy farmer who leased the land for cultivation grass followed to HYV grass producer who have dairy cattle, consumer, dairy farmers who purchased grass during lean period from other farm and HYV grass seed shop (Table 3).

Table 3: Stakeholder participation of existing Fodder Marketing System in different areas of Bangladesh (Source: FGD, KII, Field Observation and secondary review, October 2016- March 2017)

Area	Sole HYV grass producer	HYV grass Producer who have dairy cattle	Middle man who purchase grass from producers and sell to local dairy farmers in a market place	Vans/Rickshaws Pooler who sell grass by mobile van to different place of certain areas	Consumer, dairy farmers who purchases grass during lean period from other farm	HYV grass seed shop	Dairy farmer lease the land/dike of bill/ shed of the fruit garden for nursing & collecting native grass.	Score of total participation (Presence=2, absence=0)
Potia , Chittagong	√	√	×	×	√	√	√	10
Nachol, Chapainobabgonj	×	√	×	×	√	√	√	8
Bhangura, Pabna	×	√	×	×	√	√	√	8
Shamnagar,Satkhira	×	×	×	×	×	×	√	2
Debhata,Satkhira	×	×	×	×	×	×	√	2
Sadar ,Dinajpur	√	√	√	√	√	√	√	14
Kawnia,,Rangpur,	√	√	√	×	√	√	√	12
Lalpur,Nator	√	√	√	×	×	×	×	6
Overall participation of individual stakeholder	8	12	6	2	10	10	14	

Conclusion

Feeds and fodder are the most important inputs for livestock production especially dairying in Bangladesh. The farmers' of Bangladesh have adapted over generations to the risk of floods, droughts and salinity. They are rearing their dairy animals during natural calamities based on availability of conventional & non-conventional feeds and it was revealed that sometimes it's not sufficient to meet the maintenance and production. The study indicates that cultivation of HYV grasses is profitable where dairying is more dominating in animal agriculture though the present study revealed that formal marking system of fodder was observed but formal market place has not yet been established in study areas. However, grass hay in small bales & rolls, silage with plastic drum/bags in different weight, total mix ration (TMR) block may be improved the situation and in addition, effective participation of different stakeholders needs to be developed for sustainable fodder marketing system.

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Development of New Napier Cultivars through Gamma-ray Irradiation

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Abstract

Napier cultivars are potential fodder germplasms have been widely accepted among the dairy and cattle farmers in all over the country except few areas of coastal and saline zones. Therefore, an experiment was conducted to develop new Napier cultivars through use of gamma irradiation. Hence, two Napier cultivars (BN-3 and BN-4) were selected to assess the effectiveness of different dosages Gamma rays on survivability, growth behavior, yield traits, and nutritional value. For this purpose, 20 (Twenty) double node cuttings were treated at a time in gamma chamber for 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 Gy doses, respectively. The study revealed that effect of various dosages of gamma ray on the number of shoots initiation of BN-3 and BN-4 were linear decreasing with increasing gamma-rays dosages. Survival rates of BN-3 and BN-4 cuttings linearly decreasing with the increasing gamma-rays was highly negatively correlated (r^2=0.955 for BN-3 and r^2=0.963 for BN-4) and cuttings irradiated at 80 and above G*y* were all not survived. LD_{50} dosages for BN-3 and BN-4 were 28.18 and 27.54 G*y*, respectively. Biomass yield per hill was linear increasing with increasing gamma-rays doses in BN-3 mutants but in BN-4 mutant's biomass yield decreasing with the increasing gamma-ray dosages. In the mutants of BN-4, tiller height linear decreasing with increasing gamma-rays doses. Crude protein (CP) contents were significantly higher in 40 and 70 Gy treatment group and the lowest in 60 Gy treatment group of mutants from BN-4. Mutants identified from the different treatment groups from 10-70 Gy showed significant difference in tiller height. Crude protein contents were significantly higher in 60, 30(1), 10(3) Gy and lowest in 20(5), 20(7) 40(2) Gy treatment lines of mutants of BN-3. Mutants from 60(5), 50(2) and 40(17) Gy contained the highest proportion of leaf. Mutants from BN-4, CP content were significantly higher in 60, 30(1), & 10(3) Gy and lowest in 20(5), 20(7) & 40(2) Gy. Ten primers showed good quality reproducible bands and yielded a total of 97 amplified loci of which, 74 were polymorphic and accounting 76.29% of the total Loci. Among the ten primers and twelve mutant's line, overall average gene diversity was 0.21±0.17. The dendrogram showed that 70(18), 60(14), 50(2), 10(13) and 40(2) were highly distant from the other mutants line and control. This study confirmed that there were some degree of morphological, nutritional and genetic variation in mutant's line of BN-3 and BN-4, supporting RAPDs a powerful tool for the generation of potential fingerprinting diagnostics markers for Napier cultivars and assessment of genetic variations among them. Based on the performance, the best mutants will be identified and studied in further generations to develop new Napier cultivars adaptable under climatic stress conditions.

Key words: *Napier cultivars, Gamma-ray, Mutants, RAPD, genetic variation*

Introduction

The performance of ruminants depends on the year round availability of quality fodder in adequate amount. The situation of fodder production and availability throughout the year in Bangladesh is not satisfactory (Akbar and Khaleduzzaman 2009). Fodder production at farm levels is very limited. To increase the fodder availability to the growing animal's population and decreasing the land resources, research is required. The demand of fodder production is increasing because of limited livestock feed resources in the country. Improvement or development of new fodder will be helped to increase production and productivity of farm animals in the country. Napier grass is the most popular perennial fodder and recommended for smallholder crop-livestock farming systems. High yielding fodder improvement requires a long-term and multidisciplinary approach, viz. plant breeding, morphology, genetics, agronomy, pathology, physiology, molecular biology & biotechnology and animal nutrition etc (Gunckel and Sparrow 1961). Mutation breeding has become increasingly popular in recent times as an effective tool for crop fodder improvement and an efficient means supplementing existing germplasm for cultivar improvement in breeding programmes. Mutagens may cause genetic changes in an organism, break the linkages and produce many new promising traits for the

improvement of crop plants (Shah *et al*., 2008, Ahloowalia and Maluszynski 2001). Irradiation- induced mutations have succeeded in forage grasses and turfgrasses. Non-arrowing and broad-leaved mutants of rhodes grass with increased yield and crude protein, nitrogen and calcium accumulation have been isolated following gamma irradiation (Krishna *et al*. 1984). Bermuda grass cultivars of 'TifEagle', 'Tift 94' and 'Tifway II' were selected from gamma irradiated mutant lines (Burton 1985; Hanna *et al*. 1997; Hanna and Elsner 1999). Gamma ray irradiation has also been used in the breeding of centipede grass and Augustine grass (Busey 1980; Dickens *et al*. 1981). By use of gamma irradiation, a disease-resistant cultivar of *S. guianensis* has been released. In Bangladesh there is no single study was carried out to know the effect of Gamma irradiation on Napier cultivars. Therefore, the present study was under taken to assess the effectiveness of different doses of gamma radiation on growth behavior, yield traits, nutritional value and stress tolerances on Napier cultivars.

Material and Methods

Experimental Material

The experimental plant material used in the present study consisted of an important BLRI Napier-3 (BN-3) and BLRI Napier-4 (BN-4) having desirable attributes like early vegetative and high biomass yield.

Irradiation doses and experimental design

Twenty double node cuttings were treated at a time in gamma chamber for different time intervals *viz*. 8 sec, 17 sec, 26 sec, 34 sec, 42 sec, 51 sec, 59 sec, 1 min 8 sec, 1 min 17 sec and 1min 22 sec for 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 Gy doses, respectively in the department of Electronics, Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh. The central dosages rate at the time of irradiation was 4.22 KGy/hr. Each treatment consisted of 20 cuttings. The control cuttings were not irradiated. All the material were in planted in Complete Randomized Design with 20 replications having plot size of 10×8ft with intra row spacing of 27 inches comprising 5 cuttings per row and inters cutting distance of 27 inches. The planting was done in the month of February 2017 and normal agronomic practices were followed throughout the growth period.

D*ata collection*

The observations were recorded for average days need for shoot initiation, number of shoot initiation, survival rate, number of tillers per hill, tiller height and leaf & stem ratio.

Chemical analysis

The proximate components (DM, CP) of plants were done at nutrition lab, Animal Production Research Division, BLRI following the methods described by AOAC (1995).

Statistical analyses

Survival of the cuttings was scored 30 days after irradiation treatment. Survival rate was calculated as a percentage of cuttings with re-growth divided by the total number. The data-points obtained in this way were fitted to the probit function. $Probit\ (p) = a+b\sqrt{2}erf^{-1}(2p-1)$; where a is the y-intercept, b is slope and p is the normalized survival probability. The fit was performed by using the Excel and SPSS packages. The probit function can be easily used to determine any lethality dose (LD). An analysis of variance was done to determine the varietals differences. Collected data were analyzed statistically by using Compare Means (CM) procedure of One-Way Analysis of variance (ANOVA): Post Hoc Multiple Comparisons of SPSS 17.5 for Windows (SPSS Inc. 2010) following the method of Randomized Complete Block Design (RCBD).

DNA extraction

Each sample was reduced to a fine powder through mortar and pestles, with liquid N_2 addition, and transferred to a 1.5ml eppendrop tube. The total cell DNA was extracted using a commercial kit (Plant Genomic DNA, Promega, USA®). After the DNA extraction, DNA quantification was performed on NanoDrop Spectrophotometer. The image was revealed using the resources of the MiniBis Pro photo-documentation system.

DNA amplification

Amplification reactions were conducted with a total volume of 25 µL, containing Master Mix 12.5 µL, primer (10 pmol) 2 µl, Template DNA 1µl (50 ng), and water 9.5 µl. The polymerase chain reactions (PCR) were conducted as follows: 2 min at 95°C, continuing for 42 cycles (95°C for 30 sec, 30-40°C for 30 sec and 72°C for 1 min), and a final extension at 72°C for 5 min Amplification reactions were performed in the GeneAmp 9600 thermalcycler (Applied Biosystems). A total of 10 random primers were selected from Operon Technologies: OPA-01, OPA-09, OPA-13, OPA-16, OPC-02, OPC-06, OPAK-04, OPS-12, OPAW-09, and OPAW-19.

DNA electrophoresis

DNA was run on 1.2% agarose gel using 1x TBE buffer at 100 voltages for 32-36 min and staining with 1µl Green DNA stain. DNA fragments were visualized under ultra-violet light and gel image was captured by the Eagle-Eye II photo documentation system (Stratagene Inc.), and the images stored as JPEG archives.

Data analysis

RAPD bands were scored visually on the basis of 'presence (1)' or 'absence (0)' of bands of same molecular weight, respectively. For accuracy, three individual persons scored the bands separately. Bands which were not identified by the three persons were considered as non scorable. Scores in respect of all primers were pooled for constructing a single data matrix. This data matrix was used to estimate polymorphic loci, overall gene frequencies, gene diversity (NEI 1973), genetic distance (NEI 1972) and constructing an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram using POPGENE, version 1.31 (YEH *et al.,* 1999) and 'Tools for Population Genetic Analyses (TFPGA, MILLER, 1997) software packages. The size (in base pair) of each amplified RAPD band was determined by AlphaEaseFCTM Version 4.0 (Alpha Innotech Corporation).

Results and discussion

Days to initiation of shooting and days to completion of shooting (average shooting time) were delayed by higher doses of gamma irradiation (Fig.1). Delay in germination may be due to the inhibitory effects of gamma rays on cutting dormancy. The result shows that linear increasing of average days need for shoot initiation with increasing gamma-rays doses. Averages days need to shoot initiation of BN-3 and BN-4 cuttings irradiated at 0,10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 G*y* were 10, 13, 14, 14, 15, 15, 16, 16, 17, 18 and 18 days, respectively.

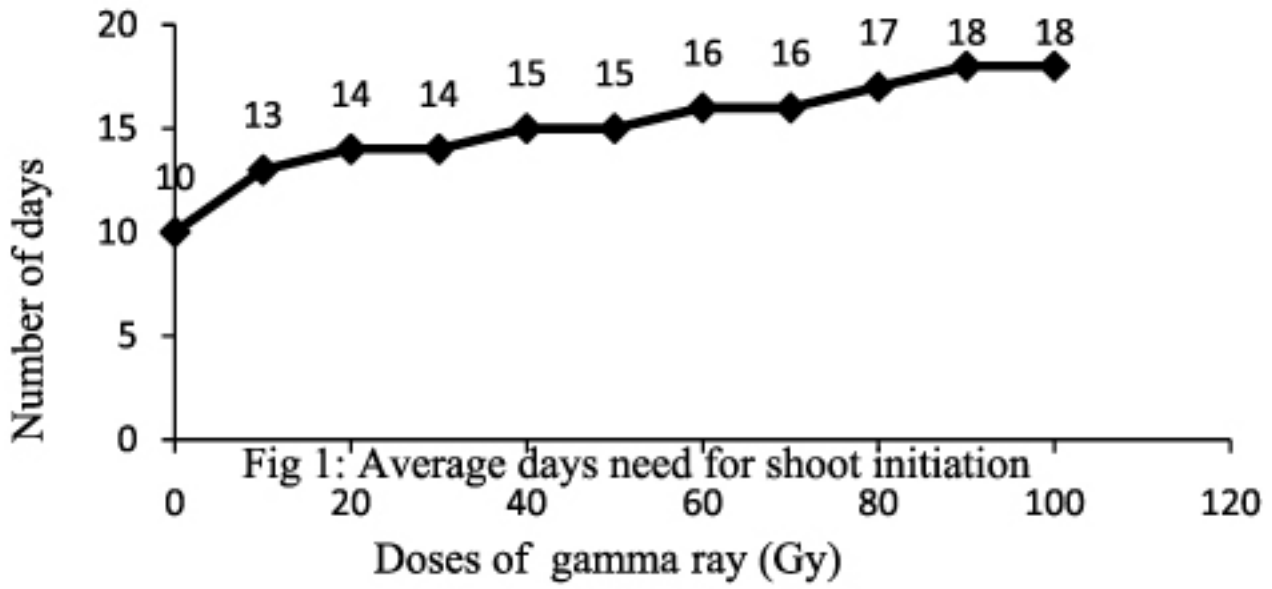

Fig 1: Average days need for shoot initiation

Fig. 1: Average days need for shoot initiation

The effect of various dosages of gamma ray on the number of shoots initiation of BN-3 and BN-4 are shown in the Fig 2 & 3. The result shows that linear decreasing number of the shoot initiation with increasing gamma-rays doses. Number of shoot initiation of BN-3 and BN-4 cuttings irradiated at 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 G*y* were 20, 20, 18, 15, 12, 10, 9, 10, 7, 5, 3 and 20, 20, 18, 16, 14, 11, 8, 8, 5, 3, 2, respectively.

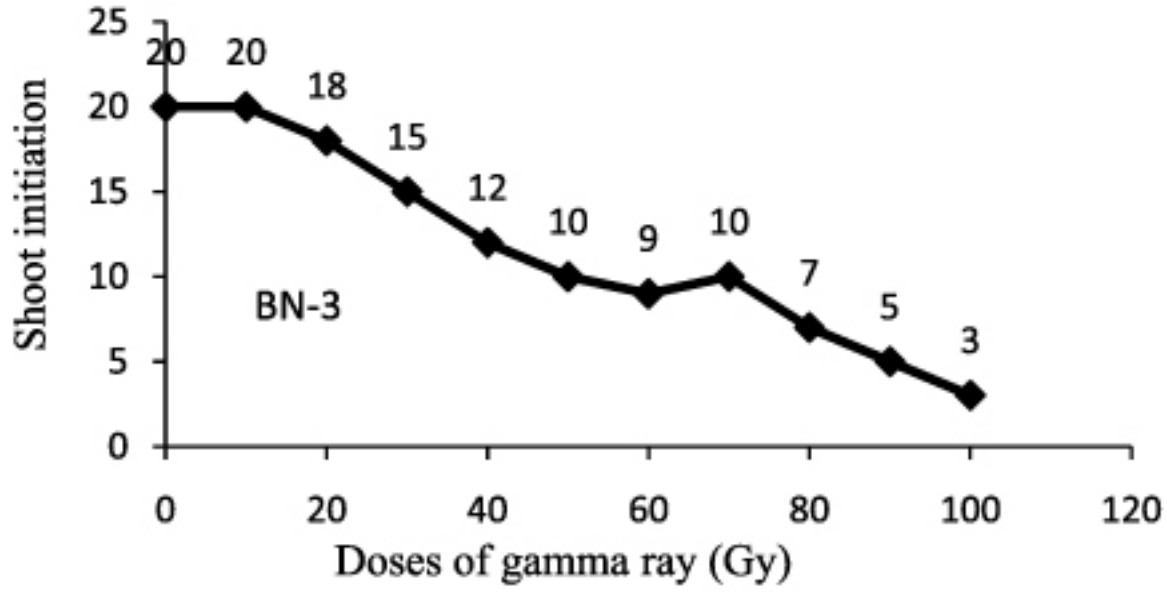

Fig. 2: Effects of Gamma ray dosage on number of shoot initiation of BN-3

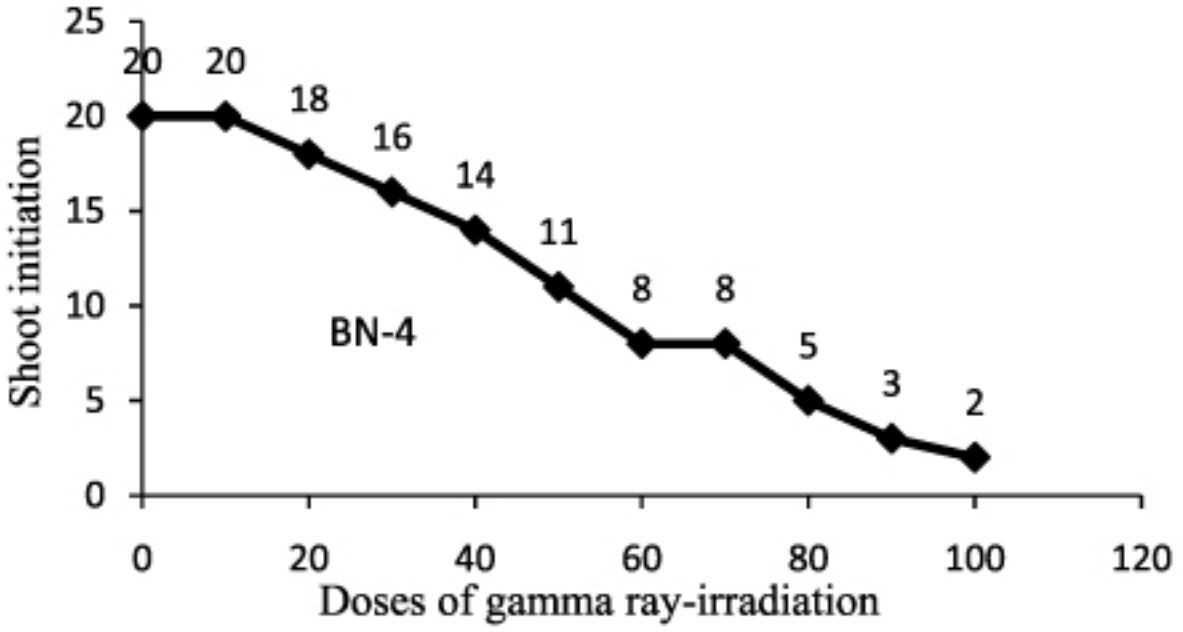

Fig. 3: Effects of Gamma ray dosage on number of shoot initiation of BN-4

Survival rates of BN-3 cuttings irradiated at 10, 20, 30, 40, 50, 60 and 70 G*y* were 95%, 80%, 60%, 45%, 30%, 25% and 15%, respectively. BN-4 cuttings irradiated at 80 and above G*y* were all not survived (Fig. 4). On the other hand, survival rates of BN-4 cuttings irradiated at 10, 20, 30, 40, 50, 60 and 70 G*y* were 95%, 75%, 60%, 50%, 40%, 20% and 15%, respectively. BN-3 cuttings irradiated at 80 and above G*y* were all not survived (Fig. 5).

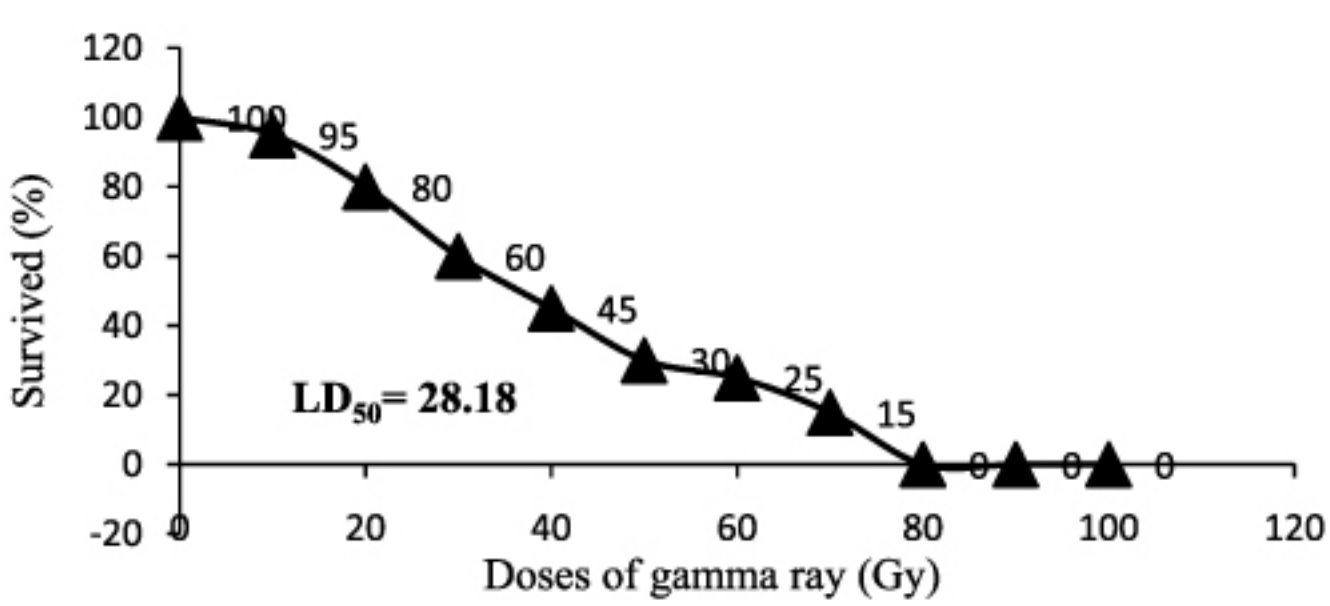

Fig. 4: Effects of Gamma ray dosage on survival of Napier grass (BN-3)

The result shows that linear reduction of survival rate with increasing gamma-rays was highly negatively correlated (r^2=0.955 for BN-3 and r^2=0.963 for BN-4). Probit analysis indicated that LD_{50} dosages for BN-3 and BN-4 were 28.18 and 27.54 G*y*, respectively (Fig. 4 and Fig. 5). The results however, reveal that BN-3 and BN-4 have a same tolerance to gamma irradiation.

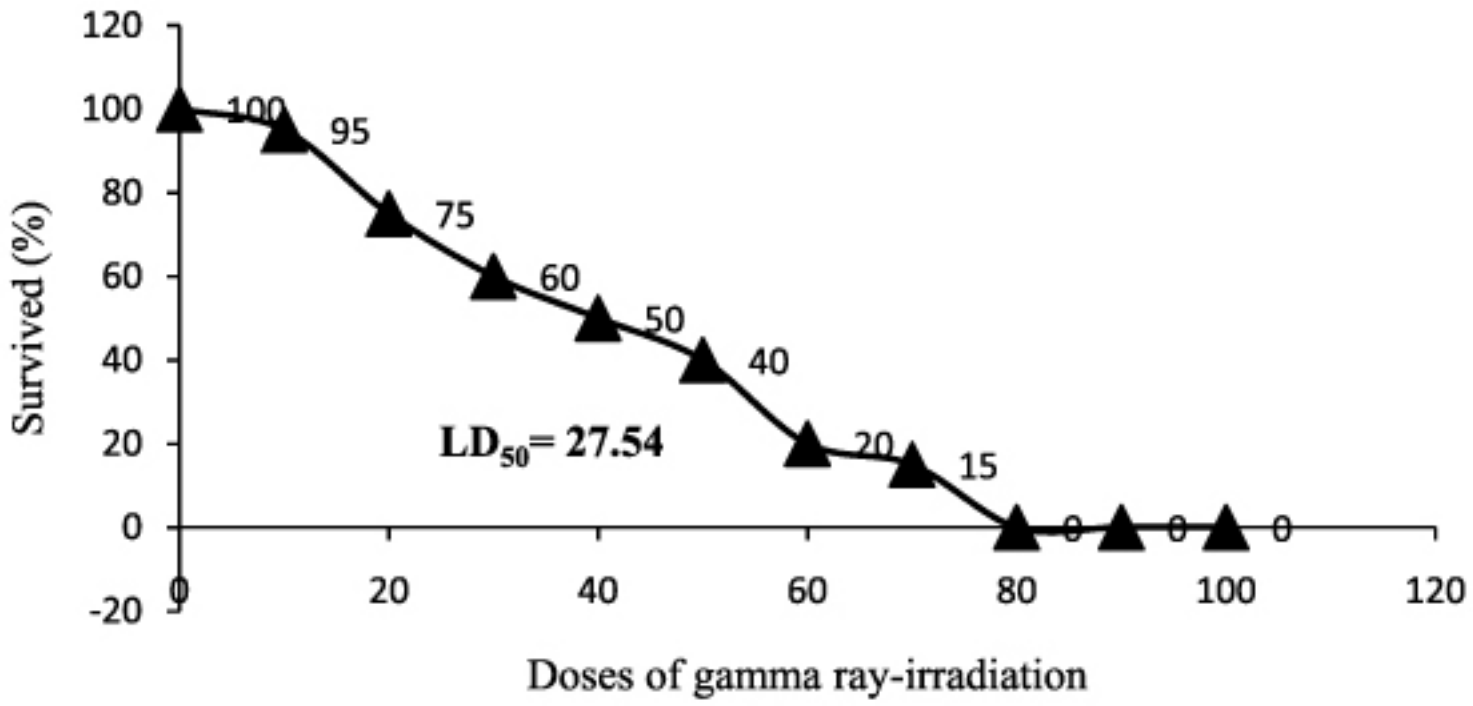

Fig. 5: Effects of Gamma ray dosage on survival of Napier grass (BN-4)

The effects of gamma ray dosages on tiller number and biomass yield (kg) per hill of mutants of BN-3 & BN-4 are shown in Table 1. Table 1 revealed that number of tiller per hill significantly differed ($p<0.05$) among the treatment groups. The number of tiller per hill of mutants of BN-3 was significantly higher in 30 Gy and the lowest in 60 Gy. On the other hand, number of tiller per hill of mutants of BN-4 was significantly lower in 60 Gy and the higher in control (0 Gy). Biomass yield was linear increasing with increasing gamma-rays doses in BN-3 mutants but in BN-4 mutant's biomass yield decreasing with the increasing gamma-ray dosages. From mutants of BN-3, biomass yield per hill were found higher in 70, 60, 50, 40 and 30 Gy. On the other hand, biomass yield per hill were found higher in control, 10, 20, and 30 Gy from BN-4 mutants.

Table 1: Effects of gamma ray dosages on tiller number and biomass yield per hill of mutants of BN-3 & BN-4

Sl. No.	Gamma ray doses	Mutants from BN-3		Mutants from BN-4	
		Tiller number/hill	Biomass yield/hill	Tiller number	Biomass yield/hill
1.	Gy-0	$13.18^{b}\pm2.42$	1.17	$11.65^{a}\pm1.37$	2.13
2.	Gy-10	$18.46^{ab}\pm1.84$	1.38	$10.35^{ab}\pm1.14$	2.50
3.	Gy-20	$18.23^{ab}\pm3.04$	1.52	$9.63^{ab}\pm1.03$	2.03
4.	Gy-30	$26.20^{a}\pm3.02$	2.08	$8.00^{ab}\pm0.74$	1.17
5.	Gy-40	$18.66^{ab}\pm0.88$	2.10	$8.41^{ab}\pm1.45$	0.56
6.	Gy-50	$17.50^{ab}\pm2.32$	2.12	$6.60^{ab}\pm1.28$	0.39
7.	Gy-60	$11.00^{b}\pm1.52$	2.23	$4.66^{b}\pm0.88$	0.35
8.	Gy-70	$12.33^{b}\pm1.45$	2.36	$6.00^{ab}\pm1.15$	0.50
	Pooled	16.96±1.11	1.87	9.28±0.49	1.20
	Sig	*	-	*	-

^{ab}Values in the same row with different superscripts differ significantly ($p<0.05$)

Tiller height did not significantly differ in BN-3 mutants (Table 2). The control, 70, and 10 Gy mutants of BN-3 contained the highest tiller height of 4.47, 4.40 and 4.39 ft, respectively. But in the mutants of BN-4, tiller height significantly differs and tiller height linear decreasing with increasing gamma-rays doses. Mutants of BN-3 from 50 and 40 Gy contained the highest proportion of leaf. On the other hand, mutants of BN-4 from control and 50 Gy contained the highest proportion of leaf.

Table 2: Effects of gamma ray dosages on tiller height and leaf: stem of mutants of BN-3 & BN-4

Sl. No.	Gamma ray doses	Mutants from BN-3		Mutants from BN-4	
		Tiller height (ft)	Leaf: stem	Tiller height	Leaf: stem
1.	Gy-0	4.47±0.46	542:458	5.64^{a}±0.34	612:388
2.	Gy-10	4.39±0.29	525:475	5.61^{a}±0.27	382:618
3.	Gy-20	3.76±0.25	533:467	4.81^{ab}±0.74	283:717
4.	Gy-30	3.14±0.37	545:455	3.92^{bc}±0.71	318:682
5.	Gy-40	3.30±0.35	570:430	3.46^{bc}±0.27	377:623
6.	Gy-50	3.69±0.20	580:420	2.91^{c}±0.32	655:345
7.	Gy-60	3.29±0.26	410:590	2.92^{c}±0.32	470:530
8.	Gy-70	4.40±0.29	340:660	2.90^{c}±0.66	350:650
	Sig	**NS**	**-**	***	-

NS= Non significant; ^{abc}Values in the same row with different superscripts differ significantly ($p<0.01$)

The results presented in Table 3 indicated that the DM content was significantly higher ($p<0.01$) in 70 Gy group (13.91) and the lowest in control (9.89) of mutants of BN-3. There was significant difference in control, 60 and 70 Gy group. On the other hand, the DM content was significantly higher ($p<0.01$) in 20 Gy group (14.03) and the lowest in control (10.19) of mutants of BN-4. There was no significant difference in other treatment groups. CP content significantly differs in 20 and 40 Gy treatment group of BN-3 mutants. There was significant difference in other treatment groups. CP content was significantly higher in 40 and 70 Gy treatment group and lowest in 60 Gy treatment groups of mutants from BN-4.

Table 3: Effects of gamma ray dosages on Dry matter (DM) and Crude protein (CP) of mutants of BN-3 & BN-4

Sl. No.	Gamma ray doses	Nutritional composition (%) of			
		Cloned of BN-3		Cloned of BN-4	
		DM	CP	DM	CP
1.	Gy-0	9.89^{d}±0.48	15.99^{a}±0.19	10.19^{c}±0.75	15.07^{cd}±0.25
2.	Gy-10	10.18^{cd}±0.14	16.20^{a}±0.38	11.54^{bc}±0.40	15.83^{bc}±0.25
3.	Gy-20	11.79^{bc}±0.91	14.66^{b}±0.18	14.03^{a}±0.74	14.41^{de}±0.23
4.	Gy-30	10.91^{cd}±0.26	15.93^{a}±0.50	13.07^{ab}±0.37	15.29^{cd}±0.31
5.	Gy-40	10.87^{cd}±0.39	16.38^{a}±0.19	12.10^{abc}±0.47	17.42^{a}±0.26
6.	Gy-50	12.78^{ab}±0.27	16.19^{a}±0.37	11.82^{bc}±0.06	16.70^{ab}±0.35
7.	Gy-60	11.48^{bcd}±0.53	16.33^{a}±0.09	13.53^{ab}±0.50	13.67^{e}±0.36
8.	Gy-70	13.91^{a}±0.31	15.95^{a}±0.06	12.71^{ab}±0.24	16.91^{a}±0.35
	Pooled	**11.12±0.24**	**15.89±0.14**	12.25±0.26	15.65±0.20
	Sig	***	**	***	***

^{abc}Values in the same row with different superscripts differ significantly ($p<0.01$)

Some mutants were selected on the basis of eye observation within treatment group and compared to the control (Table 4). Mutants identified from the different treatment groups from 10-70 Gy showed significant difference in tiller height where highest tiller height were obtained from 70, 10, 50 Gy and lowest tiller height were observed in 20 and 40 Gy. Mutants of BN-3 from 20, 10, 30 and 40 Gy contained the highest proportion of leaf. On the other hand, mutants of BN-4 from 70 and 10 Gy contained the lowest proportion of leaf compared to the control. The DM content was significantly higher ($p<0.01$) in 70, 20(5), 50 and 60 Gy group (13.91) and the lowest in 20(7) and 10(19) Gy compared to control mutants. There was significant difference of CP content selected mutant's lines. Crude protein contents were significantly higher in 60, 30(1), 10(3) Gy and lowest in 20(5), 20(7) 40(2) Gy treatment lines of mutants.

Table 4: Effects of gamma ray dosages on morphological and nutritional composition of selected mutants of BN-3

Sl. No.	Gamma ray doses	Mutants from BN-3				
		Number of Tiller specific hill	Tiller height (ft)	Leaf: stem	DM	CP
1.	Gy-0(1)	20	4.12^{bcd}±0.52	545:455	10.90^{cde}±0.23	15.62^{cd}±0.14
2.	Gy-0(2)	37	5.30^{a}±0.66	530:470	8.87^{f}±0.32	16.35^{bc}±0.17
3.	Gy-10(3)	14	3.40^{bcde}±0.15	600:400	10.50^{de}±0.17	17.65^{a}±0.04
4.	Gy-10(13)	23	3.46^{bcde}±0.14	690:310	10.06^{de}±0.28	15.70^{cd}±0.11
5.	Gy-10(19)	26	4.41^{abc}±0.40	408:592	9.99^{e}±0.21	15.26^{de}±0.39
6.	Gy-20(5)	10	2.53^{e}±0.33	717:283	13.78^{ab}±0.41	14.23^{f}±0.47
7.	Gy-20(7)	13	3.37^{bcde}±0.29	590:410	9.80^{ef}±0.16	14.43^{f}±0.24
8.	Gy-30(1)	35	3.26^{cde}±0.36	625:375	11.14^{cd}±0.40	17.03^{ab}±0.15
9.	Gy-40(2)	16	2.94^{de}±0.36	620:380	10.68^{cde}±0.36	14.82^{ef}±0.11
10.	Gy-50(2)	24	3.74^{bcde}±0.26	585:415	12.88^{b}±0.35	16.26^{bc}±0.44
11.	Gy-60(14)	27	3.36^{bcde}±0.30	555:445	11.61^{c}±0.61	17.66^{a}±0.18
12.	Gy-70(18)	15	4.52^{ab}±0.33	345:655	14.0^{a}±0.32	16.00^{cd}±0.06
	Sig	-	***	-	***	***

Mutants identified from the different treatment groups from 20-70 Gy showed (Table 5) significant differences in tiller height where highest tiller height were obtained from 10(10), 20(11) Gy and lowest tiller height were observed in 30(10), 50(2), 60(5) and 70(10) Gy compared to two controls. Mutants from 60(5), 50(2) and 40(17) Gy contained the highest proportion of leaf. On the other hand, lowest proportion of leaf contained at 10 and 20 Gy treatment groups. The DM content was significantly higher ($p<0.01$) in 70, 50(2), 40, 30(10) & 10(17) Gy lines and the lowest in 60(5) & 10(19) Gy compared to control mutants. There was significant difference of CP content selected mutant's lines. Crude protein contents were significantly higher in 60, 30(1), & 10(3) Gy and lowest in 20(5), 20(7) & 40(2) Gy treatment lines of mutants.
Please clarify the meaning of values presented in brackets above and below in the Table 5 2nd Column.

Table 5: Effects of gamma ray dosages on morphological and nutritional composition of selected mutants of BN-4

Sl. No.	Gamma ray doses	Mutants from BN-4				
		Number of Tiller specific hill	Tiller height (ft)	Leaf: stem	DM	CP
1.	Gy-0(1)	12	5.75^{ab}±0.73	580:420	8.81^{f}±0.29	14.67^{fg}±0.33
2.	Gy-0(2)	19	5.56^{ab}±0.32	545:455	11.57^{cde}±0.90	15.48^{def}±0.21
3.	Gy-10(10)	4	6.17^{a}±0.39	290:710	12.66^{bcd}±0.25	15.23^{ef}±0.26
4.	Gy-10(17)	9	4.63^{abcd}±0.61	405:595	11.66^{cde}±0.69	16.46^{bcd}±0.55
5.	Gy-10(19)	20	4.58^{abcd}±0.68	309:691	10.30^{e}±0.08	15.79^{cde}±0.17
6.	Gy-20(11)	16	5.66^{ab}±0.28	245:755	12.54^{bcd}±0.57	14.44^{fg}±0.24
7.	Gy-20(15)	14	4.92^{abc}±0.37	255:745	15.52^{a}±0.48	14.37^{fg}±0.47
8.	Gy-30(10)	5	2.60e±0.32	524:476	13.64^{b}±0.37	15.99^{bcde}±0.06
9.	Gy-30(19)	14	4.30^{bcde}±0.42	376:624	12.51^{bcd}±0.49	14.59^{fg}±0.13
10.	Gy-40(9)	17	3.74^{cde}±0.53	394:606	12.98^{bc}±0.44	17.08^{ab}±0.20
11.	Gy-40(17)	21	3.40^{cde}±0.40	410:590	11.22^{de}±0.38	17.76^{a}±0.43
12.	Gy-50(2)	9	2.92^{de}±0.28	415:585	11.95^{cd}±0.13	16.75^{abc}±0.38
13.	Gy-60(5)	10	2.92^{de}±0.32	662:338	13.59^{b}±0.52	13.78^{g}±0.45
14.	Gy-70(10)	8	2.90^{de}±0.66	320:680	12.85^{bc}±0.29	17.01^{ab}±0.41
	Sig		***		***	***

^{abcdefg}Values in the same row with different superscripts differ significantly ($p<0.01$)

A total of 10 RAPD primers were used in this study (Table 6). The average GC content of these primers was 60-70% and the band was obtained between 280bp-3000bp for these primers. All primers showed the private loci and produced total 18 PL (Elaborate as it appeared first). Ten primers showing good quality reproducible bands and efficiency in detecting polymorphism yielded a total of 97 amplified loci of which, 74 were polymorphic and accounting 76.29% of the total Loci and an average 7.4 polymorphic loci per primer. Among the ten primers and twelve mutants line, the average observed number of alleles was 1.76±0.43 and effective number of alleles was 1.33±0.32. The overall average gene diversity and information index were 0.21±0.17 and 0.33±0.23, respectively (Table 7). The primer OPA-09 and OPC-06 disclosed higher level of gene diversity (average 0.33 and 0.30) whereas gene diversity value determined by the primer OPA-01 was found to be lowers (average 0.12).

Pair-wise average gene diversity values in mutant's line of BN-3 were studied. The highest gene diversity was found between 70 Gy line rests of other treated lines including control whereas the lowest gene diversity value was found between Control 1, 2, 10(3), 10(19), 20(5) and 20(7) Gy mutants line. Shannon's information index was found highest between control 1, 2, 10(3), 10(19), 20(5), 10(3), 30(1) 20(7) and 50(2) mutants line whereas the lowest value was found between 30(1), 40(2), 50(2) and 70(18) Gy mutants line.

Table 6. RAPD primers with corresponding total loci with their size range, number and proportion of polymorphic and number of private loci observed in two control and ten mutants line of BN-3

Primer code	Primer Sequence (5'-3')	GC content (%)	TNL*	Size ranges (bp)	PL*	PPL* (%)	NPL*
OPA-01	CAGGCCCTTC	70	9	550-2100	7	77.78	3
OPA-09	GGGTAACGCC	70	9	300-2000	9	100	1
OPC-02	GTGAGGCGTC	70	7	680-1400	7	100	2
OPAK-04	AGGGTCGGTC	70	12	280-1900	11	91.67	3
OPS-12	CTGGGTGAGT	60	12	280-2500	8	66.67	3
OPAW-09	ACTGGGTCGG	70	8	460-3000	3	37.50	1
OPA-13	CAGCACCCAC	70	9	400-1650	5	55.55	2
OPA-16	AGCCAGCGAA	60	8	400-2000	7	87.50	1
OPAW-19	GGACACAGAG	60	14	460-3000	10	71.43	1
OPC-06	GAACGGACTC	60	9	500-2700	7	77.78	1
	Overall		**97**	**280-3000**	**74**	**76.29**	**18**

TNL*, total number of loci; PL*, polymorphic loci; PPL*, proportion of polymorphic loci; NPL*, number of private loci

Table 7: Summary of statistics of genetic variation for all loci (Nei, 1987) in control and mutants line of BN-3

Sl. No.	Primer's	na*	ne*	h*	I*
1.	OPA-01	1.78	1.14	0.12	0.22
2.	OPA-09	2.0	1.55	0.33	0.51
3.	OPC-02	2.0	1.36	0.24	0.40
4.	OPAK-04	1.92	1.20	0.16	0.29
5.	OPS-12	1.67	1.19	0.14	0.23
6.	OPAW-09	1.38	1.26	0.14	0.21
7.	OPA-13	1.56	1.10	0.08	0.18
8.	OPA-16	1.86	1.48	0.28	0.43
9.	OPAW-19	1.71	1.47	0.27	0.40
10.	OPC-06	1.78	1.54	0.30	0.44
	Mean	**1.76**	**1.33**	**0.21**	**0.33**
	St. Dev	**0.43**	**0.32**	**0.17**	**0.23**

*na = Observed number of alleles; *ne = Effective number of alleles; *h = Nei's (1987) gene diversity; *I = Shannon's Information index

A UPGMA dendrogram was obtained using the pair-wise genetic distance values computed for different mutants line to visualize genetic relationship among them (Fig. 6). The dendrogram showed two main clusters (a and b); the first (a) include 70 Gy line and the second cluster (b) includes others eleven mutants line including control. The second cluster is divided in two sub clusters (b1 and b2). In one of them (b2) are grouped the three mutants line viz., 10(13), 50(2) and 60(14) Gy. The other sub-cluster (b2) contains other eight mutant's line. The 70(18), 60(14), 50(2), 10(13) and 40(2) were highly distant from the other mutants line and control.

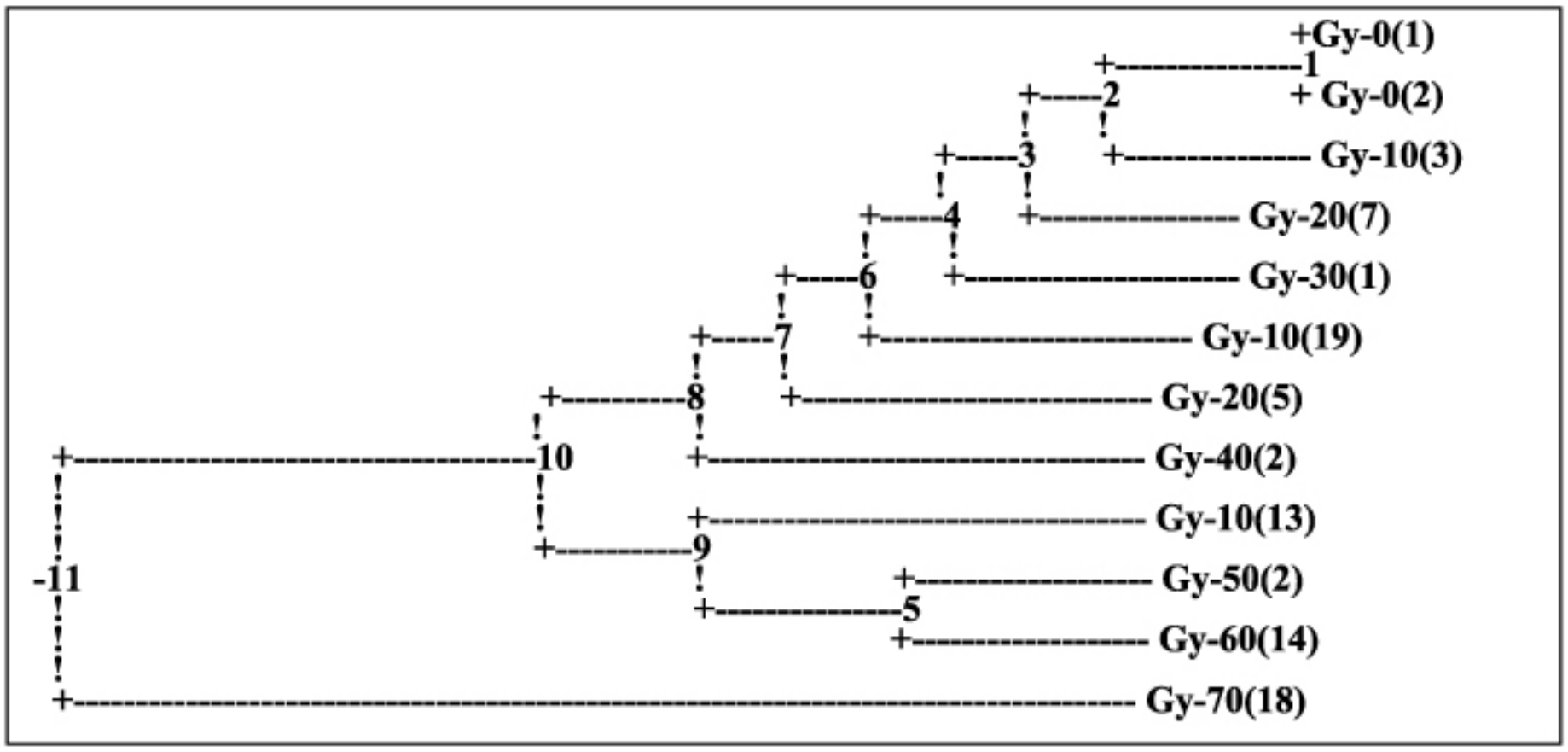

Fig.6. UPGMA dendogram based on Nei's genetic distance, summarizing data on genetic variation between mutants line of BN-3 according Random amplified polymorphic DNA (RAPD) analysis.

Conclusion

This study confirmed that there were some degree of morphological, nutritional and genetic variation studied in mutant's line of BN-3 and BN-4. Supporting RAPDs a marker used in BN-3 mutant lines from different gamma-ray irradiation, dendrogram showed distinct difference of mutant line of Gy-70(18) from other mutant lines.

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Buffaloes for dairying in South Asia: potential, challenges and way forward

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Abstract

Buffalo is considered the dairy animal for 21st century due to its higher adaptability and productivity in the changing climatic conditions. There is a large diversity in the buffalo genetic resources and South Asia is home of high yielding source promising buffalo breed of Murrah and Nail Ravi. South Asia is inhabitant of 151.49 million buffalo populations out of 194.29 million of global populations. Besides, about 79.74 % of Asia and 77.9 % of world buffalo populations are inhabitant in South Asian countries. During the last decade, the world buffalo population has been increased by approximately 1.49% annually. South Asia is currently producing 100.74 million metric tons of buffalo milk which is about 96.05 % of Asia and 93.19 % of world buffalo milk production. The share of buffalo milk is around 51.07% of the total milk production of the South Asia. The contribution of buffalo milk in India, Nepal and Pakistan are 51.2%, 66.6% and 59.5% respectively in total milk production. Among the South Asian countries, maximum milk is produced by India followed by Pakistan, Nepal, Bangladesh, Sri Lanka and Bhutan. The productivity of buffalo has been recorded 410 kg/buffalo/year, 1880 kg/buffalo/year, 1934 kg/buffalo/year and 867 kg/buffalo/year, 508 kg/buffalo/year in Bangladesh, India, Pakistan, Nepal and Sri Lanka respectively. Although most of the buffaloes are non-descript indigenous types and their production potential is not satisfactory. There are different production systems are prevailing such as zero input-low output, low input-medium output and high input-high output. Moreover, selective breeding for buffalo with the same breed under low input-medium output production system and grading up of non-descript buffaloes using improver breed/s under zero input-low output production system has been commonly practiced. The demand of milk has been increasing due to economic solvency and rapid pace of urbanization but most of the countries are deficit in production even it is challenging to meet the projected demand to achieve the SDG by 2030. To increase the productivity through genetic improvement of buffaloes could be important thrust areas to obtain projected demand of milk. Productive and reproductive efficiency can only be improved by adopting suitable breeding policies and good management practices.

Keywords: Dairying, SDG, South Asia, buffalo, milk.

Introduction

Buffalo is called the black gold of South Asia. Buffalo produces high quality, rich, creamier milk as well as lean meat with very low fat and cholesterol content. Its milk is suitable for specially dairy products owning to higher fat, lactose and solid not fat (SNF) contents. Buffaloes are resistant to several tropical diseases prevalent in South Asia, while enduring diverse harsh climates making it a preferred climate resilient livestock species. It has ensured survival for landless and small farmers in many parts of South Asia, while ushering fortunes for the elite buffalo breeders and entrepreneurs. Buffalo holds great hope for food security and poverty alleviation in South Asia, because of the largest population comprising diverse and the best buffalo germplasm of the world. Of the 194.29 million world buffalo population, 97% is concentrated in Asia and 57% in India alone (FAO, 2014). The increasing importance and popularity of buffalo species in the world is evident from its increasing population trends over the last decades with about 1.13% in Asia, 0.92% in Africa, 1.74% in Americas and overwhelming 5.22% in Europe (FAO, 2014). About 150 million households around the world are directly engaged in milk production involving primarily small and marginal households whereas almost the same number is indirectly involved in associated activities related to milk and meat which is the primary source of animal protein for human consumption. This region has a great biodiversity of buffalo germplasm, including the world famous buffaloes Murrah and Nili-Ravi - renowned for high milk production potential. The India and Pakistan share 69.32% and 23.19%, of world buffalo milk production respectively (FAO, 2014). About 71.4% of world buffalo meat is produced in South Asian countries (FAO, 2014). Although buffalo is an essential part of livestock in South Asian countries, it has

never been addressed in Bangladesh and always neglected despite their important role in the national economy.

Buffalo Population Dynamics in South Asia

According to FAO (2014) estimate, about 79.74 % of Asia and 77.9 % of world buffalo population are inhabitant in South Asia. The total buffalo population is around 34.80% of the total cattle and buffalo population in the region. In the region, buffalo population is highest in India followed by Pakistan, Nepal, Bangladesh, Sri Lanka and Bhutan. Buffalo population in Maldives and Afghanistan is not documented yet. Asian buffaloes dominate the world population, representing 92.52% of the worldwide population of 194.29 million (FAO, 2014).

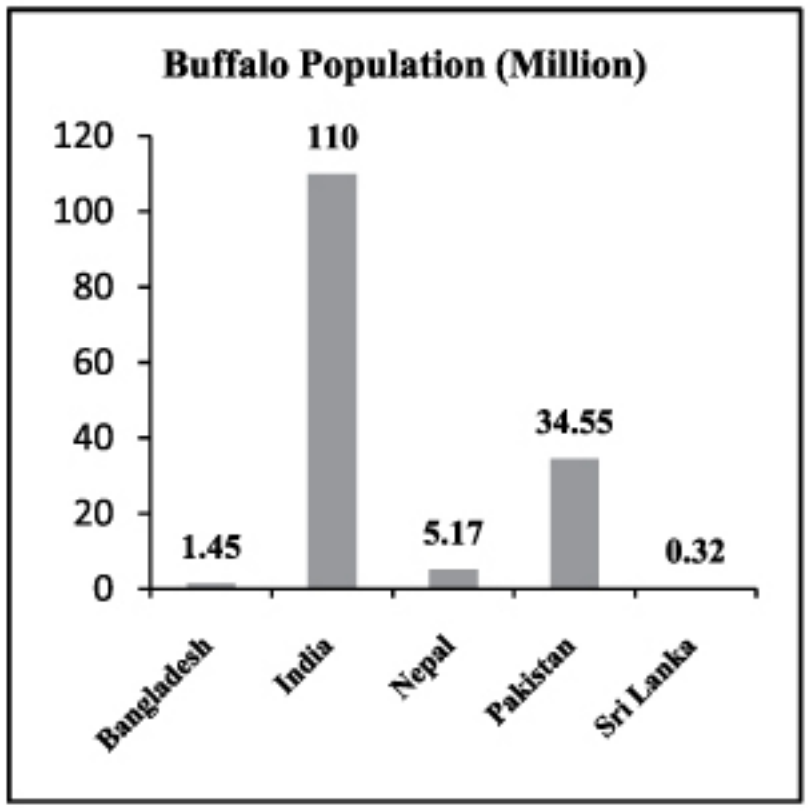

Figure 1. Buffalo population in South Asia

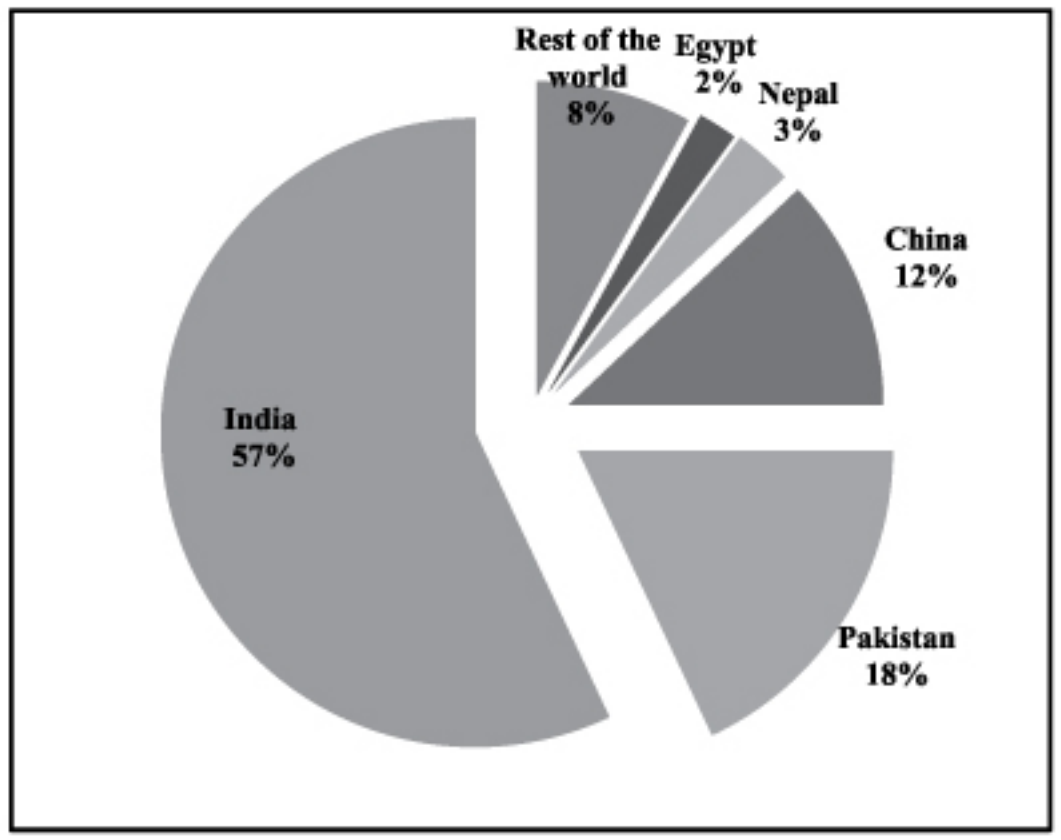

Figure 2. Buffalo population diversity in the world

Table 1. Buffalo populations in the world, in Asia and in South Asia

Region/ Country	Total population (million)	Share in world population (%)
World	194.29	100%
Asia	179.75	92.52%
India	110.00	56.61%
Pakistan	34.55	17.78%
Nepal	5.17	2.66%
Bangladesh	1.45	0.746%
Sri Lanka	0.32	0.16%
Bhutan	0.001	-

Source: FAO, 2014; Chakravarty, 2013

Within the Asian region, about 79.74% of buffaloes are in South Asia and the rest 20.26% in other countries (Chakravarty, 2013). The buffalo populations in the world, in Asia and in South Asia are presented in table 1. During last decade, world buffalo population has increased by 20.0 million head and 89.41% of that increases occurred in Asia, in fact, that population growth has been largely contributed by India and Pakistan (FAO, 2014). According to latest livestock census, the buffalo populations are 1.45, 0.001, 110, 5.17, 34.55 and 0.32 millions in Bangladesh, Bhutan, India, Nepal, Pakistan and Sri Lanka respectively (figure 1). The growth rate of buffalo population in the region was always in positive trend. In the region, total buffalo population was 121.18 million in 2000 and after the passage of time it became 151.49 million in 2014 (table 2).

Table 2. Growth rate of buffalo population in million in South Asia (2000-2014)

Year	Bangladesh	India	Nepal	Pakistan	Sri Lanka	Total
2000	0.89	93.83	3.5	22.66	0.30	121.18
2002	0.97	96.53	3.7	24.03	0.28	125.51
2004	1.06	99.72	3.9	25.50	0.30	130.48
2006	1.16	103.42	4.2	27.33	0.31	136.42
2008	1.26	106.01	4.4	29.00	0.31	140.98
2010	1.34	107.37	4.8	29.41	0.42	143.34
2012	1.44	108.70	5.13	32.68	0.41	148.36
2014	1.45	110.00	5.17	34.55	0.32	151.49

Buffalo Genetic Resources in South Asia: Regional buffaloes are mostly riverine type (2n=50), however, some swamp type (2n=48) are also available in some agro climatic zones. Buffaloes are considered the "dairy animal of 21st century" due to its high adaptation and sustainability in changing climatic conditions. This regional buffaloes comprises of indigenous non descript, crossbred and pure breed. The genotypic and phenotypic analysis of buffalo genomics has not done properly except India. National Bureau of Animal Genetic Resources (NBAGR) of India has successfully completed phylogenetic analysis of 13 buffalo breeds. The status of buffalo genetic resources in the region are presented in table 3.

Table 3. Documented buffalo genetic resources in South Asia

Country	Documented buffalo genetic resources
Bangladesh	Indigenous local called Deshi, Nili Ravi, Surti, Murrah, crossbred, swamp buffalo
Bhutan	Non Descript, Hyakule, Kagye, Murrah, crossbred and wild
India (13 Breeds)	Non descript, Murrah, Nili-Ravi, Jaffarabadi, Banni, Mehsana Marathwada Nagpuri, Pandharpuri, Bhadawari, Surti, Toda, Chilika, Kalahandi, cross breed, swamp and wild
Nepal	Non descript, cross breed, Gaddi, Parkote and Lime
Pakistan	Non descript, Nili-Ravi, Nili, Ravi, Kundhi and Azakheli
Sri Lanka	Non descript, cross breed, Lankan buffalo, wild buffalo, Murrah, Crossbred

Buffalo Milk Production in South Asia

South Asia is currently producing 100.74 million metric tons of buffalo milk which is about 96.05 % of Asia and 93.19 % of world buffalo milk production (FAO, 2014). The share of buffalo milk is around 51.07% of the total milk production of the South Asia. The contribution of buffalo milk in India, Nepal and Pakistan are 51.2%, 66.6% and 59.5% respectively in total milk production (FAO, 2014). Among the South Asian countries, maximum milk is produced by India followed by Pakistan, Nepal, Bangladesh, Sri Lanka, and Bhutan. The total milk production scenarios in South Asia are presented in table 4.

Productivity of buffalo in South Asia

The productivity of buffalo has been recorded 410 kg/buffalo/year, 1880 kg/buffalo/year, 1934 kg/buffalo/year and 867 kg/buffalo/year, 508 kg/buffalo/year in Bangladesh, India, Pakistan, Nepal and Sri Lanka respectively (FAO, 2014). Highest productivity recorded in Pakistan and lowest in Bangladesh. The production potentiality of buffaloes in different countries is presented in table 4. According to production potentiality, it is observed that Pakistan and India has the highest milk producing buffaloes

than others. Pakistani buffalo Nili-Ravi is the better performing buffalo in the world for milk production. The highest production recorded in elite herd is 2500 litre per 305 days lactation while average production at commercial herd is 1800-2500 litre per 322 days lactation (Pak Dairy Info, 2017). Average milk production of Pakistani buffaloes is 5-7 litre/day. India is home to great biodiversity of buffalo germplasm, including the world famous Murrah buffaloes-renowned for high milk production potential. The average milk production of Indian buffaloes is 5-6 litre/day. The productivity of buffaloes in different countries are given in table 5.

Table 4. Total milk production in South Asia

Country	Total Milk Production (MMT)	Buffalo Milk Production (MMT)	Percentage of buffalo milk (MMT)
Bangladesh	7.2	0.1	1.4
India	146	74.7	51.2
Nepal	1.8	1.2	66.6
Pakistan	42	25	59.5
Sri Lanka	0.25	0.045	18.0
Total	197.25	100.74	

Source: FAO, 2014 & Hamid et al., 2016

Table 5. Average buffalo milk production in different countries

Country	Average milk yield (kg/animal/year)
Pakistan	1934
India	1880
Vietnam	1000
Nepal	867
Sri Lanka	508
China	563
Bangladesh	410
Italy	815.9
Asia	1389

Buffalo Genetic Improvement

Genetic improvement of large number of buffaloes under different stakeholders in South Asia demands on quality buffalo male germplasm. The easiest way of multiplication of superior germplasm and genetic improvement of buffalo could be done through artificial insemination (AI). The production of quality male germplasm should be through progeny testing programme. In South Asia the animal breeding technology like AI are not even widely adopted because of non-availability of quality male buffalo germplasm at the door step of farmers, lack of breeding infrastructure, non-availability of quality fodders, lack of awareness of rural households and many other problems directly and indirectly associated with the genetic improvement of buffaloes. As a result, the productivity of buffalo is low in South Asia. The adoption of AI reduces the indiscriminate use of breeding bulls, inbreeding among animals and also the chance of reproductive problems of buffaloes due to various sexually transmitted diseases. The breeding policy would be

A. For organized dairy farm
1. Selective breeding of high producing buffalo using most adapted breeds in the institute/ commercial herds under high input-high output production system of the respective country.
2. Grading up of non-descript buffaloes using same improver breeds/s in organized dairy farm under low input- medium output production system in each country.

B. Un-organized dairy farm
1. Selective breeding for buffalo with the same breed under low input- medium output production system of the respective country.
2. Inter se mating of non-descript buffaloes using improver breed/s under zero input- low output production system in each country.

Nutrition and Health Care

To enhance productivity, farmers have to be given adequate awareness on balanced feeding of animals. The farmers will be supported with the supply of good quality seeds and seedlings to develop improved pasture where feasible. Farmers will be encouraged to utilize agricultural by- products to increase efficiency and reduce production cost. Awareness to farmers on fodder conservation crop residues enrichments technologies, contract fodder production to make fodder available for feeding the animals round the year will be pursued rigorously. Proactive animal health action plans to prevent infection and parasitic diseases as well as zoonoosis and effective laboratory diagnostic services, timely monitoring shall be instituted within the existing regional and national laboratories.

Support product processing and marketing facilities

More dairy groups will be formed and encouraged to take up buffalo farming and they will be supported with required product processing, diversification and marketing facilities. The dairy groups/cooperatives will be supported for establishment of vital infrastructures such as milk collection centers for collection and marketing of fresh milk, milk processing units for collection and processing of local dairy products and dairy sales counter in every town for marketing of products. Through periodic market information and research, preferred dairy products from buffalo milk will be explored. There is an opportunity for the production of mozzarella cheese from buffalo milk in Bhutan. Where feasible small to medium scale processing plants will be encouraged to diversify such products.

Capacity building

The farmers and extension workers lack skills and knowledge in improved buffalo breeding and husbandry practices. Need assessment will be done and training will be imparted to national and field including farmers on buffalo breeding and improved buffalo husbandry practices. Routine disease screening and preventive health care will be emphasized through timely up-dation of skill and knowledge of field staff. National Buffalo Information and Recording System (NBIS) shall be instituted which will ensure traceability for performance recording, disease out- breaks and will help in instituting progeny testing program in the long run. Where possible, awareness and sensitization will be given and farmers will be encouraged to form breeders groups and association to produce quality animals which will form the basis for sustainability of buffalo farming in the country.

Knowledge and skill of national professional will be enhanced to take up research and development works and a provision within national buffalo breeding program shall be created for communication of research findings to the farmers to enhance production. To strengthen networking mechanism and institutional linkages among the buffalo research and production centers within the region, with Asian and world buffalo federations for exchange of gemplasm, scientist visits and information exchange on latest research and development works to improve and sustain buffalo farming in the region.

Long term intervention areas for Buffalo breed improvement program

The combination of AI with Multiple Ovulation and Embryo Transfer (MOET) as advanced reproductive bio-technology approach shall be initiated in a long run which can immensely enhance the rate of genetic gain and productivity in buffalo population. The application of other bio-technologies such as embryo manipulation (splitting, sexing and cloning) for faster multiplication of superior germplasm from highly selected elite donors and facilitate to achieve the target of producing large number of superior bull calves/bulls and their adequate number of quality semen doses are other promising breeding options available for application. Molecular genetic studies for identification of genes of interest (milk quality and quantity traits and Marker Assisted Selection (MAS) are other breeding tools available for identification of superior animals and commercial exploitation of genetic potential of indigenous breeds. Besides, MOET, there may be a limited application of other advanced breeding tools for buffalo breeding in the region.

Advantages of buffalo husbandry over cattle

Work/Animal traction

The water buffalo is an important beast of burden in Asian farming. It is widely used to plough, level land, plant crops, puddle rice fields, cultivate field crops, pump water, haul carts, sleds and shallow-draft boats. It is also used to carry people, thresh grain, press sugar cane, haul logs, and more. Buffalo have an advantage over other draught animals in wet or muddy areas, with their large hooves. Their legs can withstand wet conditions better than cattle. However they are not as fast as cattle, horses or mules. This puts them at a disadvantage in dryer areas.

Therefore, the additional income every year through the sale of surplus milk is vital to their well-being and economic security.

Utilization of feeds: conversion of meat and milk

The most important and desirable quality of the water buffalo is its extraordinary capacity of utilization less digestible feeds (straw, sugarcane wastes etc.) than cattle. So it requires less concentrate feeds than cattle. This mean that it can produce excellent quality food meat and milk using only crop residues, pasture, and mineral salts, without the addition of supplement concentrates (Hamid et al., 2016).

Not so much diseases

Buffalo is high adaptable and healthily animal that can resist infectious and contagious diseases. However, they must receive the same vaccines, hygienic care and careful, dedicated attention, as do cattle. The females, because they produce nutritive milk, transfer to their calves more resistance to dangerous diseases. There is no report of mad cow disease in buffalo any part of the world.

Enrich soil fertility

Buffalo enrich soil structure and fertility while tracking paddy field. Each year, an adult buffalo produce 4-6 tones of wet manure plus additional urine as bio-fertilizer. This reduces the requirement of chemical fertilizers as well provide soil humus that chemical cannot provide.

More resistance to climatic hazard

Buffalo can survive against tidal wave better than any other livestock species. This is evidence from the cycles that occurs frequently in the coastal area of Bangladesh.

No housing requirement

Buffalo do not require expensive house as like cattle. It can even live in open air throughout the year. For 99% of buffaloes in Bangladesh, there is no house at all. This is true in many parts of world.

Inherent qualities as meat producer

Buffaloes have a unique ability to utilise coarse feeds, straws and crop residues converting them into protein rich lean meat. Hence buffaloes fit well in poor countries having poor feed resources. Buffalo properly managed and fed as a meat producing animal and slaughtered at 16 to 20 months of age yields a highly satisfactory top quality meat at a much lower cost than the cattle (Ranjan and Pathak, 1979). Since buffaloes have been used as draught animals for centuries, they have evolved with exceptional muscular development. Until recently, little thought was given to use them exclusively for meat production. Buffaloes are lean animals. The sub-cutaneous fat layer of the carcass is usually thinner than that on comparably fed cattle. Fat is low even under feed lot conditions.

More lean and less fat compared to cattle, has created a demand for it among health conscious consumers (Kondaiah, 2002). Buffaloes have higher degree of resistance and tolerance than cattle against many

diseases. They are reasonably productive up to 15 years of age. At 18 years and even more may produce calves. Buffaloes have excellent body weight gain compared to other species. The nutrient requirements of buffalo steer include 0.24 kg DCP, 1.8 kg TDN, 6.6 MCal ME, 14 g Ca, and 11 g P. On adlibitum and high concentrate (75:25) based rations the growth rate is 610 g/day with feed efficiency of 7:1. On all roughage rations (Green berseem/ berseem hay) the growth rate is 370 g/day with feed efficiency of 10:1 (Ranjan and Pathak, 1979).

Constraints of buffalo development in South Asia

- Lack of knowledge about the quality of buffalo milk and meat
- Lack of knowledge about feed utilization capacity, adaptability and disease resistance capacity of buffalo
- Lack of public awareness about buffalo husbandry
- Lack of high yielding buffalo breed. The indigenous buffaloes are low producers
- Scarcity of quality feeds, fodder and pasture land
- Lack of technical skills about buffalo production of farm holders
- Lack of long-term breed development policy and research programme
- No or limited facilities to breed improvement technologies
- Lack of breeding infrastructure
- Non-availability of records
- Non-availability of proven bulls
- Lack of coordination within research and government organizations, universities, NGOs and beneficiaries

Recommendations of buffalo development in South Asia

- Develop/strengthen breeding infrastructure in the region
- In intensive production system, continuous up gradation of native buffaloes in the plain land with imported semen of Murrah, Nili-Ravi or Mediterranean breed
- In semi-intensive production system, crossbreeding of native buffaloes with Murrah or Nili Ravi and fixed 50% exotic bloods followed by inter se mating
- In low input production system, crossbreeding of native buffaloes with Murrah or Nili Ravi and fixed 50% exotic bloods followed by inter se mating
- Breeding, feeding and disease preventive measures should be addressed properly
- Reproductive biotechnology should be utilized
- Public awareness for buffalo production by different media; Radio like TV, newspaper etc. should be created
- Quality fodder seed production farms should be established. Increase the land for fodder production should be ensured
- Development of manpower by technical training should be ascertained
- National research and international collaboration should be strengthened
- Buffalo milk and meat market and infrastructure should be developed
- Private investment is to be explored and encouraged to invest in buffalo development infrastructure including marketing of milk and meat products in the country

Conclusion

The buffalo dairying in the region is characterized by small-scale, scattered and unorganized dairy animal holders; low productivity; inadequate and inappropriate animal feeding and health care; lack of assured year-round remunerative producer price for milk; inadequate basic infrastructure for provision of production inputs and services; inadequate basic infrastructure for procurement, transportation, processing and marketing of milk; and lack of professional management. Low productivity of dairy buffaloes is a

serious constraint to dairy development in the region. The productivity of dairy buffaloes could be increased by crossbreeding the low yielding non-descript buffaloes with high yielding selected indigenous purebreds or suitable exotic breeds in a phased manner. The breeding policy should not only focus on milk yield but should also provide for the production of good quality breeding bulls. Upgrading of nondescript indigenous buffalo through selective breeding with high yielding purebreds, such as Murrah, Nili Ravi, should be given high priority in all areas where buffalo are well adapted to the agro-climatic conditions.

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Feeds and Fodder Production in Bangladesh: Present trend, availability, future requirements and challenges for dairying in 2030

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Abstract

Livestock production and agriculture are complimentary to each other and both are crucial for overall food and nutrition security. Livestock is directly or indirectly linked to all 17 SDGs and faces many challenges to achieve sustainability while responding to an important increase in demand for animal products.Feeds and Fodder are an agricultural term for animal feed and fodder crops are those plants that raised to livestock.Although, the fodder production gets momentumbut at present, the country faces a net deficit more than 40% dry matter, 65% crude protein and 60% metabolizable energy. The recent growth rate of livestock is not matching with the requirement of feeds andfodder in the country. The net availability of green grass including cut and carry systems and grazing on natural grasses not more than 2.5 kg fresh /h/day.A total of 4.464 million tons of commercial feed is produces in Bangladesh for dairy, beef, broiler, layer and fish. The share of broiler, layer and fish are 46.1 %, 27.65 % and 20.16%, respectively in the country. The share of dairy and beef in the country is 3.81% and 2.24 %, respectively and in terms of million tons which are 0.17 and 0.1 respectively for dairy and beef production.To meet the increasing demand of milk and meat in the country, 130.0 and 27.0 thousand tons commercial feed for dairy and beef may be required annually. In addition to livestock feeding, the global human population set to rise from 7.0 billion to 8.00 billion by 2030 and 9.00 billion by 2050, opportunities for food producers in Bangladesh to represent to their grower's market place are considerable. FAO has analyzed global dairy trends as far as 2050. Their analysis predicts that as income rise, people are generally preferring to spend a higher share of their food budget on animal protein (AP), so meat and dairy consumption tends to grow faster than that of food crops. This paper will highlight the present trend of feeds and fodder production, their availability, predicts the future requirements and challenges to achieve those requirements for supporting the dairying in 2030.

Key Words: *Feeds and fodder, Trend of fodder production, availability, requirements and challenges*

Introduction

Livestock is one of the important sub-sectors of Agriculture. It contributes in the GDP is about 1.60% and growth rate of GDP is about 3.32% (Table 1) and about 20% population in the country are directly and 50% peoples are indirectly depending on livestock for their livelihoods.Cattle share the highest relative importance of milk (98.0%), meat (60.0%) and hides (60.0%) production in the country. The compound growth rate of milk production is about 27.80% considering the base year production in 2007-08, and per capita availability is about 158ml/head /day in place of 250 ml per capita requirement of milk. As per DLS (2016) the daily per capita availability of meat including chicken is about 122g against the recommended requirement of 120g (Table 2). Even though food production is considered as the priority consideration for livestock development, achievement of sustainable development goals (SDGs) by 2030, in addition, is considered as an important tool for poverty reduction, income & employment generation and sustaining environment. The present milkand meatproduction and future projection by the 2030 is shown in Table 3.

Table 1. Livestock contribution in GDP

	2009-10	2010-11	2011-12	2012-13	2013-14	2014-15	2015-16	2016-17
GDP (Base : 2005-06)	2.06	1.98	1.9	1.84	1.78	1.73	1.66	1.6
Growth rate of GDP	2.51	2.59	2.68	2.74	2.83	3.1	3.21	3.32

Source: DLS (2017).

Table 2. Projected demand and supply of milk by 2030

Commodity	Demand*	Production	Availability	Demand	**Projected demand 2030**	**Projected availability 2030**
Milk (MMT)	14.86 (250 ml/h/d)	9.28	157.97ml/h/d	5.58	17.69 (250 ml/h/d)	15.6 MMT (225 ml/h/d)
Meat (MMT)	7.13 (120g/h/d)	7.15	121.74g/h/d	Surplus		

Estimated population at present:162.90 million and Projected demand is estimated as per BBS (2010) based on growth rate of population @ 1.37%, the total population by 2030 assuming to be 193.96 million

Table 3. Trend of milk and meat production for the last 10 years

	Year										
Products	2007-08	2008-09	2009-10	2010-11	2011-12	2012-13	2013-14	2014-15	2015-16	2016-17	Growth rate (%)
Milk (MMT)	2.65	2.29	2.37	2.9	3.46	5.07	6.09	6.97	7.27	9.28	27.80
Meat (MMT)	1.04	1.08	1.26	1.99	2.3	3.62	4.52	5.86	6.15	7.15	65.28

Growth rate is determined by using the formula= [{(Current year-base year)/Base year}x100] /Total year

Livestock dynamics

The annual growth rate and population of livestock are presented in Table 4. It shows that the growth rate of cattle and buffalo, are 0.45, 2.50%, respectively. The growth rate is determined by using the data on 2005-06 as base year. It is estimated that considering the present growth rate of cattle and buffalo, theprojected population by 2030 will be 25.36 and 2.09 million, respectively.

Table 4. Present status and projected cattle and buffalo population (million) by 2030

Livestock Species	**2005-06**	**2006-07**	**2007-08**	**2008-09**	**2009-10**	**2010-11**	**2011-12**	**2012-13**	**2013-14**	**2014-15**	**2015-16**	**2016-17**	**Growth rate**	**2019-20**	**2024-25**	**2029-30**
Cattle	22.8	22.87	22.9	22.97	23.05	23.12	23.19	23.34	23.48	23.64	23.78	23.93	0.45	24.25	24.80	25.36
Buffalo	1.16	1.21	1.26	1.30	1.35	1.39	1.44	1.45	1.46	1.47	1.47	1.48	2.50	1.59	1.80	2.09

Projection for the year of 2030 (considering base year 2017) = [{(Growth rate /100) +1} Defense between current and projected year(13)] × Base year population

Dynamics of feed & fodder production

Feeds and forage production at farm levels is one of the priority tasks along with breeding policy implementation, strengthening veterinary services, enforcement of hygienic regulations and legal frame

works, introduction of good manufacturing practices of traditional dairy products and development of human resources for expediting dairy development in the country.A diet of ruminant animals consists of roughage and concentrate. Crop residues (rice and wheat straw, maize stover, sugar cane by-products, pulse crop shreds, banana pseudostem), green grasses (winter and monsoon forage and fodder, natural green biomass, water and crop weeds) and tree foliage and leaves (jackfruit, leucaena, mulberry, other edible tree leaves and bushes) are major sources of roughage for the ruminant of Bangladesh (Table 3). Losses due to the absence of preservation and processing facility or knowledge gaps of users, natural calamity, movement problems of roughages; and their otherwise uses affect availability to animals. Concentrate feeds, on the other hand, mostly of grain by-products (Table 3) considered to have required nutritional quality but, its quantitative supply is not sufficient to support the demand. This has made feed problem for livestock raising more acute, and made dairy and fattening farming most competitive and challenging to sustain under open market trading policy. Feeding to satisfy appetite, one of the pre-conditions for supplying nutrition to ruminants, requires availability of bulk that comes from roughages; and roughage quality is one of the basic requirements for optimizing intake and digestion kinetics in the rumen to make production efficient and economic. Farmer's problem of both quantity and quality of roughage that fluctuates on cropping, seasons and regions hinders dairy and fattening cattle production in the country.

Present trend of fodder production

The trend of area coverage in acres of black gram and Khashari as fodder crops in different years are shown in Table 5 & Fig. 1. It shows from the Fig. 1 that the area coverage of both the crops are negatively correlated with the year.

Table 5. Trend of area coverage (acres) for fodder production from 2006-07 to 2015-2016

Years Name of fodders	**2006-07**	**2007-08**	**2008-09**	**2009-10**	**2010-11**	**2011-12**	**2012-13**	**2013-14**	**2014-15**	**2015-16**
Maskalai/ Black gram (acre)	57505	58918	61303	79287	28475	77298	79046	27616	29545	29227
Kheshari (acres)	245630	201426	212210	212313	229147	230787	261993	69893	72707	70609
Total	**303135**	**260344**	**273513**	**291600**	**257622**	**308085**	**341039**	**97509**	**102252**	**99836**

Source: BBS (2016)

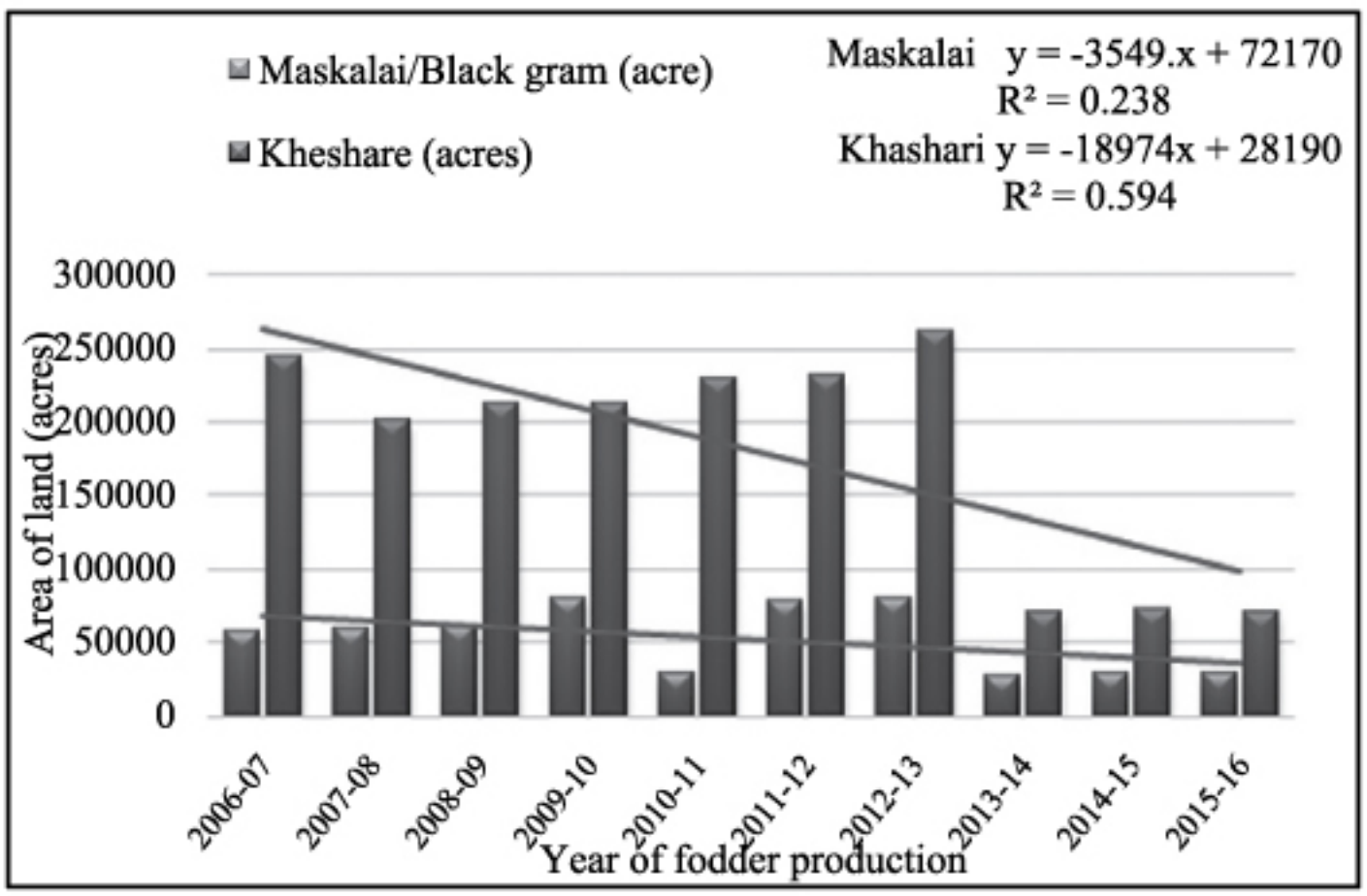

Fig 1. **Trend of area coverage (acres) for fodder production from 2006-07 to 2015-2016**

The trend of area coverage (acres) of Bhadoi and Rabi fodders for the last 10 years in the country are shown in Table 6. The Fig. 2 shows that there is a liner relationship in area coverage of Bhadoi and Rabi fodder with the increment of year. Further, it is indicated that with the increase of year, the unit area is increased by 295 and 530 for Bhadoi and Rabi fodder, respectively.

Table 6. Area coverage (acres) of Bhadoi and Rabi fodder from 2006-07 to 2015-16

Years Name of the fodder	**2006-07**	**2007-08**	**2008-09**	**2009-10**	**2010-11**	**2011-12**	**2012-13**	**2013-14**	**2014-15**	**2015-16**
Bhadoi fodder (acres)			1999	3101	3205	2564	2729	2755	4186	4935
Rabi fodder (acres)	32515	30174	36701	36872	37285	37227	36958	36808	37307	36613
Total	**32515**	**30174**	**38700**	**39973**	**40490**	**39791**	**39687**	**39563**	**41493**	**41548**

Source: BBS (2016)

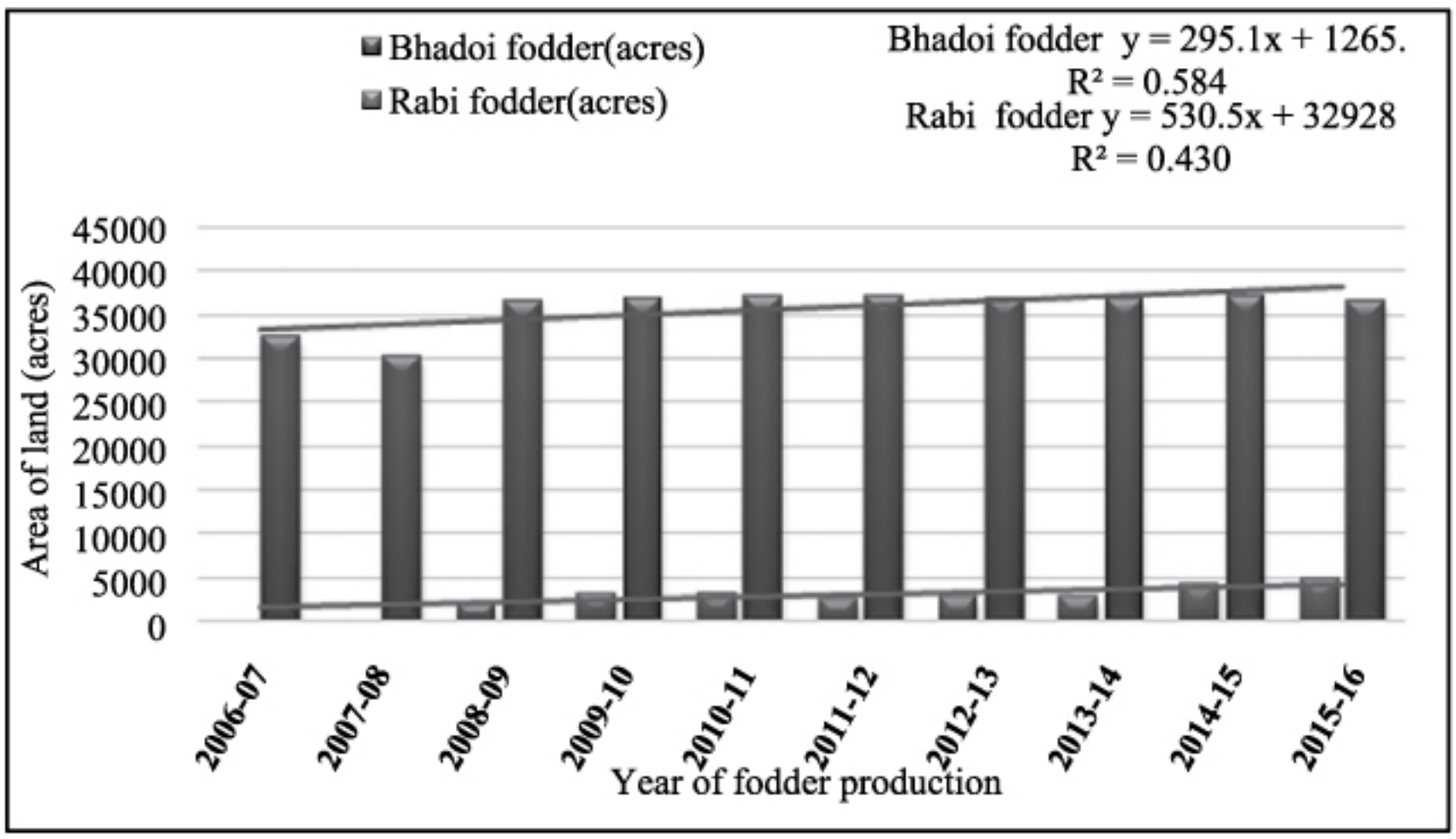

Fig. 2. Trend analysis of area coverage (acres) of Bhadoi and Rabi from 2006-07 to 2015-16

Feeds, nutrients availability and future projection (2030)

Based on the annual production of different crops and the extraction rates of feed the total production of feeds is estimated and presented in Table 7. The total annual production dry Matter (DM) production 69576 x1000 tons including green grasses and non-conventional crop residues. Considering the harvest losses and other uses, the net availability of DM is 44082x1000 tons and crude protein and ME availability are 3324.3 x1000 tons and 244368 x1000 MJ, respectively. However, based on the present cattle and buffaloes population, the requirements of nutrients are estimated which is shown in Table 8. Its indicated that the present total demand in DM, crude protein (CP) and metabolizable energy (ME) is 60.40 million metric tons, 6.00 million metric tons and 545633 million MJ, respectively. This indicates a negative feed balance in Bangladesh for dried biomass, crude protein and metabolizable energy. The projected nutrients requirement by 2030 is 65.44 million metric tons DM, crude protein 6.49 million metric tons and 590518 million MJ for 25.36 million cattle and 2.08 buffaloes, respectively.

Table 7. Total feed production and availability (DM,CP and ME) to livestock in Bangladesh

Feed resources	Production, fresh (1000 tons)	Production ,DM (1000 tons)	DM availability to livestock (1000 tons)	CP availability to livestock (1000 tons)	ME availability to livestock (in 1000 MJ)
1. Crop residue					
Rice straw	45123	40628	18316	549	64098
Wheat straw	1483	1456	728	22	1566
Maize straw	3000	2730	1638	66	6388
Potato leaves	17037	4259	3833	153	12267
2. Oil seed cakes/meals	847	771	694	215	6397
3.Pulses	270	243	219	35	1531
4.Grains					
Rice	34710	31527	696	56	4521
Wheat	1348	1213	142	20	926
Maize	1000	890	89	8	579
Other cereals	2.82	2.4	0.24	0.1	156
5. Grain by products					
Rice police	3220	2898	232	32	1159
Rice bran	2777	1522	213	213	6542
Wheat bran	108	97	5.5	1	23
Broken rice	3471	3123	2388	239	1719
Pulses bran	657	858	46.8	7	281
6. Other by product					
Sugarcane tops	4491	4087	3065	41	32449
Sugarcane bagases	4491	4087	3065	41	32449
Molasses	281	2	1.6	0.2	17
Banana leaves	706	141	113	5.6	1121
7. Grasses					
Green grass	34650	8316	8316	1.58	68856
Sweet potatoes	782	391	156	28	782
Mango kernel	181	127	127	13	695
Total	122765	69576	44082	3324.3	244368

Source: Year book of Agricultural Statistics (2016) and by-products of all crop residues are determined based on extraction factors and harvesting loss.

Feed nutrients supply and demand balance

The nutrients supply and demand balance is presented in Table 9. Though the estimated availability of DM is about 73% and its deficiency is 27% but CP & ME deficiencies are 59% and 55% in the present year. The extrapolated projected demand of DM by 2030 shows that if the current of growth rate of livestock and crop production are maintained then the supply and demand of nutrients will still be negative. If the body weight and higher producing animals are maintained,then the projected DM will not be sufficient.

Table 8. Present requirements and future projection of nutrients for dairying by 2030

Species	Year	No of Animals (M)	No of Livestock unit (M)	Ave. LW (kg)/h	DM (MMT) /Year	CP (MMT) /Year	ME (MMJ) /Year
Cattle	2016-17	23.93	20.6	300	56.33	5.63	510790
Buffalo	2016-17	1.48	1.3	350	4.06	0.37	34842
Total	**2016-17**				**60.40**	**6.00**	**545633**
Cattle	2030 (Projected) Growth rate 0.45 (estimated from 2005-06 to 2016-17)	25.36	21.8	300	59.70	5.97	541314
Buffalo	2030 (Projected) Growth rate 2.50 (estimated from 2005-06 to 2016-17)	2.09	1.8	350	5.74	0.52	49203
Total	**2030 (Projected)**				**65.44**	**6.49**	**590518**

Calculation is based on total population converted into standard livestock unit (LU) considering the species and age with as per Ramachandra et al, (2007); DM, CP and MJ are calculated based on 2.5%, 750 CP/h/d and 70 ME MJ/h/d.

Table 9. Feed balance, feed availability and feed requirement of cattle in Bangladesh

	Dry Matter (MMT)	Crude Protein(MMT)	Metabolizable Energy (MMJ)
Availability	44.08	3.33	244368
Requirement	60.40	8.24	545633
Balance (1000 tons)	-16.32	-4.91	-301265
Balance (%)	-27.02%	-59.58%	-55.21%

Author's own calculation

Availability of commercial feed

A total of 4.464 million tons of commercial feed is produces in Bangladesh for dairy, beef, broiler, layer and fish. The share of broiler, layer and fish are 46.1 %, 27.65 % and 20.16%, respectively in the country. The share of dairy and beef in the country is 3.81% and 2.24 %, respectively and in terms of million tons which are 0.17 and 0.1 respectively for dairy and beef production (Alltech, 2017). To meet the growing demand of milk and meat in the country, 130.0 and 27.0 thousand tons commercial feed for dairy and beef may be required annually.

Challenges of Fodder Production

1. Change in crop and animal agriculture

Land scarcity, seed unavailability of economic fodder crop, absence of production technology to match with the ecosystem (land, climate, cropping system) and economy of dairy farming are the major factors that affect introduction of a forage crop in an existing farming system. Subsistence farms with close interlinking of crops, livestock and household were the predominant feature in Bangladesh agriculture before the intensification of high yielding variety (HYV) of rice cultivation to meet the increasing cereal demand due to population pressure. About 67.5% of the total land area is cultivable and covered with single, double and triple crops resulting in a crop intensity of 180%; and 7.4% is cultivable waste. Rice, Jute, Sugarcane and Wheat are the major crops and which covers about 72.8, 4.50, 1.10 and 5.30%,

respectively of the gross cropped area. Moreover, population growth along with urbanization has been limiting the total operated and gross cropped area of the country (BBS, 2016), and sparing no additional land for fodder cultivation and squeezing common land, being used mainly for grazing by local animals.

During the last four decades rice cultivation has been intensified affecting close interlinking of crop, livestock and households. Traditional legume and non-legume crop rotations or crop and livestock integration are replaced by single crop for grain production and affected soil health of many regions of the country. Legume cropping supported livestock keeping and helped soil fertility through recycling nitrogen and facilitating animal grazing during a part of crop cycle, especially, in the winter season. The long changes in crop production systems have impacts on production performances of farm animals, especially, of large ruminants, and ultimately it has brought a change in production system of animal agriculture in the country. There are other challenges for fodder production in country are given below.

2. **Increased availability of protein rich feeds and fodder**

As stated above quantitative deficiency of crude protein is a major treat for dairy production in the country. Options for increased production and supply of high quality protein feeds and fodder should be explored. Expansion of pulse and oil seed crops will definitely help be increased production of food and feed. Moreover, potential plantation crops may be identified and brought into production system for commercial production of high quality feed following the existing social plantation systems.

3. **Gradual squeezing of grazing & pasture areas**

Due to continuous changes of cropping pattern & HYV rice production, the scope for production of fodder is getting scarce day by day. There is high pressure on land for production of food for human being which is also not enough for ever growing human population. Moreover, more and more land is being utilized for industry, housing and urbanization.

4. **Natural non-synchronization of feed production & availability**

Change of cropping pattern: Replacement of pulses and oilseed cultivation by the boro-paddy causes serious reduction of nutritious feed for ruminants e.g. pulse straw, pulse bran and oilcakes.

5. Loss/otherwise use of crop by- products

The straw and other crop residue used as fuel and other purposes: It is estimated that 30 to 40 percent of the total straw and crop residue are used as fuel and other purposes.

6. Technology transfer problems

Improper use of non-conventional feedstuffs: Technology has not yet been developed to utilize the non-conventional feed stuff like slaughter house waste, crop residue, food processing for the use of waste

7. Limitations in enriching of HYV fodder germplasm bank

Foddergermplasm bank is unavailable in districts and upazilla levels.

8.Competition human with livestock

Livestock farming leads to direct competition for food with human and livestock in the country. The situation is unlikely to be reversed in near future unless country achieve self-sufficiency in food grain production and fodder production proves more profitable to farmers.

9. Fodder seed bank unavailability at regional level

The most difficult factor for the farmer to continue Napier cultivation is the availability of cuttings. The inundation of Napier fodder land by floodwater requires repeated plantation of the grass every year. This urges supply of Napier cuttings from other sources, that are met at present by the BLRI. The institute in

cooperation with the Milk Vita demonstrated farmers to keep cuttings at their homesteads, roadsides and highlands available to other farmers. Farmers use to conserve grass cuttings following the systems. In addition, some farmers of high land area which is not affected by floodwater are trained to raise Napier grass after several cuttings to use as sources of seed for the next year, and in addition to sell seed cuttings to other farmers. This has developed another sources of income from Napier grass for the farmers who grow Napier up to maturity after having several harvests of green fodder. It is reported that a farmer may earn about Tk.247,500.0 from a hectare of land if seed cuttings are sold to other farmers.

10.Climate change and salinity

Climate changeand salinity are the major facing by the country now-a-days. More than 30% of the cultivable land in Bangladesh is in the coastal area. Out of 2.86 M ha of coastal and offshore lands and about 1.056 M ha of lands are affected by different degrees of salinity (Hussain et. al., 2012).

Conclusions and Recommendations

Livestock is suffering from huge shortage of green fodder. Very little efforts are made for awareness building among the farmers for HYV fodder cultivation. Land scarcity, inadequate extension work and absence of fodder seed bank are main reasons for green fodder shortage in the country.

Recommendations

1. Establish infrastructure & manpower capacity in potential AEZs for testing and adopting suitable fodder crops such as saline, shed loving and draught tolerant. To address the feed problems in those areas, salt tolerant native/natural fodder germplasm would be identified and also collect from national and international organizations.
2. Establish capacity for fodder breeding and agronomical trials.
3. Adoption and establish facilities for post-harvest fodder and feed technology.
4. Strengthening the germplasm conservation facility and establish linkage with national and international organizations & research institutes for exchanging high yielding fodder germplasm.
5. Establish capacity for fodder seed production and marketing.
6. Fodder production and conservation for chars, bathan and other fodder producing areas.
7. Establishment of fodder seed bank/ germplasm centre in major districts of the country
8. Introduction of fodder into agro-forestry system through efficient utilization of land, tree fodder & shrubs.
9. Identification of noble plant like Moringaas commercial fodder production
10. Integration of various fodder germplasm matching with the existing cropping systems.
11. Establish a research center in the name "**Fodder & Grassland Research and Development Centre**" at the campus of BLRI may support the requirements of the farmers. This centre will bring benefit for the development of dairy and fattening farms through gradual increase in the number of quality human resources. In addition, local feed problems would be addressed through research and will strengthen the research, training and extension linkage.

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Commercialization of fodder production among smallholder dairy producers in the saline prone coastal areas of southwest Bangladesh: A Research-NGO linkage

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Abstract

To know the market driven commercial fodder production among the beneficiaries under SaFaL working areas in the districts of Khulna, Bagerhat, Satkhira, Jessore and Narail of southwest region of Bangladesh the study was conducted. For this purpose, a total of 102 demonstration plots of BLRI developed saline tolerant variety of HYV (Napier BLRI-3), 58 sweet Jumbo and 21 Alfalfa having 6.37, 2.97 and 0.84 acres land were used, respectively. From demonstrations in 102 farmers' field of 6.37 acre land, a total of 4038 farmers are producing Napier BLRI-3 fodder in 90.20 acre of lands in addition some farmers' were used road side and pond side lands. Out of total farmers, about 46 % farmers were cultivated HYV Napier fodder and more than 50 entrepreneurs were produced fodder commercially and sold on daily or weekly basis in 6 fodder markets in addition to feed their own dairying. The average biomass yield was 152.5kg/decimal in demo plots and 141kg/decimal in farmers' replicated plots. The average milk production was increased initially by 0.5 -2.5 litre/cow/day. During the project period the trend of increased was 11% in 1st year, 18% in 2nd year, 32% in 3rd year, 36% in 4th year and 38% in 5th year (6 months). The results also indicated that the feed cost was decreased about 30-35% and increased the households income about 71% in dairying due to cultivation of high yielding fodders. The commercial fodder producers were sold about 804 kg of fodder in 6 markets everyday (134 kg per day per market) with an average income of about Tk. 2412/day (Tk. 402 per market @ of Tk. 3/kg); In addition, a total of 50 lead farmers and the entrepreneurs were also engaged in selling of fodder cuttings. Therefore, it may be concluded that a comprehensive livestock development programme in the salinity prone area with the participation of GOs and NGOs needs to be developed for sustainable smallholder dairy farming in climatic vulnerable southwest areas of Bangladesh.

Key Words: Commercialization, fodder production, smallholder dairy, saline & coastal and linkage.

Introduction

The coastal belt of Bangladesh is likely to be most vulnerable area in the country in particular southwestern region in the event of climate change effects. The impact of climate change perceived enormously in terms of increasing natural disasters. Almost every year, it experiences disasters of one kind to another such as-tropical cyclones, storm surges, floods, draughts which causes heavy loss of life, crop and property and also jeopardizing development activities. Most climate change impacts in Bangladesh likely to come from the south due to situation of Bay of Bengal and the adjoining North Indian Ocean. These waters are the sources of tropical cyclones and storm surges, coastal erosion, monsoon wind, evaporation for monsoon rainfall, floods and draughts. It is now a common phenomenon of this region on the other hand salinity is an acute problem for crop production and livelihoods activities. Livestock production is also a big challenge. The arsenic contamination is also coming in to the face.

As the entire agriculture sector is climate sensitive similarly, livestock and fisheries sub-sectors are also affected extremely to the climate change effects. There are limited human, institutional and financial capacities to anticipate and respond to the direct and indirect effects of climate change. The adaptive capacity of the affected poor communities to climate change clearly needs to be increased. As we are now locked into inevitable changes to climate pattern and suffering from disasters, strategy for risk reduction and adaptation to the threat needs to be capitalized and it is no longer a secondary option.

The government and a good number of NGOs are working for the development of south-western region in different way and component-wise activities are in place but the research and development work is very limited in this region as a result visible outcome and positive impact is not yet observed in livestock sub-sector as anticipated. However, present need awaked the community for adaptation to the environment and strategic planning are undertaking by the government as well as development partners. Meanwhile, some of the donors and NGOs are working together with the technical support of the respective public institutions for the development of livestock sub-sector in the context of climate change effects in the southwestern region of the country. Feed and nutrition is one of the most important segments of which deficiencies leads to the vulnerability for livestock development resulting from the climate change effects. The potentials of fodder production in this region is neglected due to salinity, prawn culture and land scarcity. It is a snap shot of private sector experiences in fodder production that can be the valuable feed source of livestock production in the southwest region to promote income generation creating job opportunities.

Livestock and feed situation

The cattle farmers are heavily dependent on dry rice straw cultivated throughout the country. In the regional context the dry straw is deficient in southwestern part compared to northwestern area because farmers in this region involved in vegetables cultivation and prawn culture. On the other hand rice is the main cash crop for the farmers of northwestern part. Average 35% household keep cattle in southern delta area with a number of 2.9 cattle per household (Sarker et al., 2017). Crop residues and their by-products are the common feed for livestock and poultry depends on yearly crop production situation and those are reduced due to the natural calamities resulting food crisis and price hike. It was estimated that the total roughage production in the country was 51.06 million ton in 2012 where 5.78 million ton is available in the form of cut-and-carry and road side grazing (Huque and Sarker, 2014). The annual availability of green grass varies with the regional context, seasonal variation and dairy production system. Around 53% (27.32 million ton) of the annual total production of fibrous biomass is available to the animals as feed and bulk of the rest is spoiled and used for other purposes (Huque and Sarker, 2014). Three major feed ingredients namely grains, cereal milling by-products and oilcakes are commonly used as animal feed in the form of mixed concentrate feed in the country. Among the concentrate feed ingredients corn has the lion's share of 41% and used about 2.04 million ton per year mainly for commercial poultry (Sarker et al., 2009). Among the cereal milling by-products 58% (2.91 million ton) is used for ruminants and poultry. The rest of the ingredients covered from oilcakes of different oilseeds for both ruminants and poultry (Asia Dairy Network-2014). The country also produces 72,000 ton of molasses every year of which most are exported and used for ethanol production locally (BBS, 2016).

Roughages and concentrate

The annual dry matter requirement of total ruminant in the country is more than 73.80 million ton which needs to be cover from both roughage and concentrate at a ratio of 2:1 that accounts the yearly demand of about 49.20 million ton of roughage (Straw and green grass) and 24.61 million ton of concentrate mixture (grains, milling by-products, oil cakes etc.). The total dry matter roughage production is about 51.06 million per year that surpluses by 3.8% of its annual demand but surprisingly the losses due to natural calamities and other uses results 44.6% deficit of its total annual demand (Sarker et al., 2017). This yearly deficit needs to be addressed and strategically planned to overcome for retaining the productivity of animals. The domestic production of concentrate feed of about 5.02 million ton met only 20% of the total annual requirement. Increasing demand of compound food was observed from the field survey due to structural change of dairy and beef sector in Bangladesh (Uddin et al., 2013) and that supports field observation conducted by Change Maker in 2016. The requirement of livestock feed need to be fulfilled by increasing production, best uses of existing resources, reduce spoilage, discourage to use other purposes and finally import the ingredients for concentrate feed preparation.

Green grass

Most livestock farmers are dependent mainly on the natural grasses produced locally but limiting due to the crop intensity. Among the HYV fodder available in the country Napier is considered as a prime green grass usually cultivated commercially and non-commercially due to its unique nature and profitability. In 2016 the

Change Maker projected the Napier Value Chain and stated that a total of about 34.65 million ton of Napier is being fed annually to the cattle of the country and 50% farmers grow Napier for consumption of their own cattle and remaining are used for their own cattle as well as sold from field or at fodder markets. In the sale volume one third is traded by the large wholesalers while another two third goes to the farmers through retailers. Price at retailers are 20% higher than those of wholesalers. The market actors of Napier grass are-cutting provider, traders, wholesalers and retail market actors. The channel starts from Napier grass cutting producers (DLS, BLRI, university, research centre, NGOs, farmers and other organizations). The farmers sell cutting grass to the Farias (65%) and wholesalers (35%). Among the buyers 60% is bought by large farmers (More than 10 cattle) and 40% by medium (6-10 cattle) and small (1-5 cattle) farmers. There is also integrated Napier grass producers who are mostly commercial and semi-commercial cattle farmer and produced about 50% of Napier grass, medium and small cattle farmers produced about 40% and rest are by traditional cattle farmers.

Materials and Methods

The NGO activities on HYV fodder production and strategic planning for the availability of roughages to reduce the vulnerability of livestock feeding in the SaFaL working areas sporadic development activities on livestock development are being implemented for sustainable livelihoods by few NGOs in southwestern region. The practices are mostly at subsistence level. The SaFaL (Sustainable Agriculture Food Security and Linkages) dairy programme started to work during the period of (2012-13) with a mandate for developing and strengthening milk supply chain dealing with backward and forward markets. Dairy production is one of the components of SaFaL programme in addition to horticulture and aquaculture. Considering the present situation of southwest region, status of dairy production and markets, climate change effects, social and economical status of the community SaFaL came forward to transform the marginal, smallholder and medium farmers from subsistence farming to enterprise farming. With this view in mind, the following market driven activities have been undertaken in SaFaL working areas in the districts of Khulna, Bagerhat, Satkhira, Jessore and Narail of southwest region. Tools were used for data collection from the different strata are as follows:

1. Area selection- FGD, PRA and household survey;
2. Group formation- Milk producer group consist of 50-60 farm households per group;
3. Lead Farmer selection- To mentor two groups by each Lead Farmer;
4. Clustering- Each cluster consists of 8-12 groups;
5. Governance- Formation of 7 members executive committee (3 would be women);
6. Problem identification- Identify local problems for dairy production, prioritization & time bound plan to overcome problems so far identified;
7. Social Mapping- Listing of existing resources and infrastructures;
8. Business plan development- Develop entrepreneurship and calculate return on investment for profitable and sustainable dairy business;
9. Training and capacity building- Through technical sessions, group cohesion, gender, DRR, leadership etc.;
10. Skill development- Refresher, internship, practical orientation, demonstration, demo result sharing through FFD, learning visit and coordination meeting;
11. Improved management practice- Hands on training;
12. Input provisions- Through agro-input shop establishment and women feed seller development;
13. Services- Develop CLSP for livestock health service, AI service provider and One-stop service centre;
14. Marketing- MCC establishment, formal milk processors and local milk processors;
15. Research- Hydroponics fodder production with Interdisciplinary Centre for Food Security (ICF), BAU and BLRI; and
16. Linkages- Both public sector (DLS, BLRI and BAU) and Private sector (BRAC, ACI, ACI Godrej, Winning Agro, SK+F etc.).

Problem identification and prioritization through participatory process

The "Dairy Welfare and Development Wheel" based activities has been undertaken and arranged based on the farmers' requirement locally. The "Dairy Welfare and Development Wheel" has been developed jointly in consultation with the experts from the Netherlands and Dairy Team of SaFaL programme and adapted. It is very easy to understand and memorize the dairy production tips whatever the academic level of the participants. There are 10 fundamental requirements have been identified and arranged in a wheel on priority basis through voting system in participatory way to avoid any nepotism and to justify the reality. Considering the cattle feed deficiency and requirements in the local area fodder production was ranked 4 among 10 parameters of Dairy Wheel.

Results and discussion

Common livestock feeds were usually inadequate during dry and monsoon season in the country and also the same scenario in the southwestern region. It was primarily due to land scarcity and shortage of feeds. Limited or non-availability of green grass was also evident during the same period. Productive and reproductive performances of dairy cows are affected due to inadequate and imbalanced feed supplementation. Most farmers provide concentrate supplements to the cows partially i.e. below the requirement without considering the body condition, physiological status and milk yield. Only poor quality roughage (straw) was provided *ad libitum* and green grass based on the availability. If the feed supplement was provided from green source the partial replacement of concentrate feed is possible that reduces the pressure on costly concentrate feed as well as reduces the production cost.

On the contrary farmers' are coping with the scarcity of cattle with varieties of feed sources. The farmers of saline prone coastal belt acted differently and supplemented very poorly. As per recommendation and trained the farmers to cultivate HYV fodder (Saline tolerant variety) in the existing landscape considering the environmental factors and the potentials of dairy business. They were also suggested for preparing dry straw processing (UMS), silage and hay preparation as well as home-made fodder production to address the food crisis during lean period as well as to provide nutritional support. Pre-intervention conditions was observed in the field such as-

The traditional practice

1. More than 95% farmers were not acquainted with HYV fodder and cultivation technique;
2. Importance of green grass feeding and the beneficial effects on cattle were not known;
3. They used to feed their cattle dry straw, water hyacinth, local grass, road or pond side mixed grass and non-descriptive green grass;
4. Most cattle (90%) were suffering from worm infestation causing diarrhoea that creates nuisance and un-hygienic condition to keep the cattle shed dry and clean;
5. Cows were ill health, repeat breeding and reproductive problems were common and most cattle were malnourished;
6. Low productivity due to genotypic characteristics, traditional management practices and climate change effects;
7. High incidence of common diseases, nutritional deficiency and skin diseases.
8. Very limited access to services, inputs and green fodder;
9. High rate of infertility (Repeat breeding, conception rate was very low, delayed heat show, high calving interval);
10. Land scarcity is acute due to prawn and fish culture and vegetables production;
11. Salinity, arsenic contamination, water logging, flood, rain fall and draughts are common climate change effects;
12. High rate of mortality and morbidity; and
13. Farmers' were losing 35-50% income every day.

Mitigation process undertaken to overcome the situation

1. In the context of southwestern region and local environment identified the problems on cow rearing and prioritized them. Understanding the problems mitigation measures were planned on priority basis through participatory planning process and implemented accordingly;
2. Developed Linkages with BLRI for the cuttings of saline tolerant variety of HYV (Napier BLRI-3) and technical support and with BRAC for Sweet Jumbo fodder seed;
3. Capacity building of dairy farmers on different HYV fodder cultivation technique and inter-cultural management practices;
4. Fodder demonstration plots were established with 102 farmers for Napier BLRI-3 in 6.37 acre land, 58 Sweet Jumbo in 2.97 acre land and 21 Alfalfa in 84 decimal lands. Recently German and Pack Chong (Imported from Thailand) cuttings distributed to 132 and 38 farmers for trial in 25.71 acre land and 1.88 acre land, respectively.
5. Organized Farmers' Field Day (FFD) and group meeting for demo result sharing among the Milk Producer Group Members;
6. Motivated the farmers to multiply the production, extension among others and commercialization;
7. Introduced home-made hydroponics fodder production system in homesteads of 26 farmers and 7 entrepreneurs as an innovative technology; and
8. Capacity building of farmers on fodder storage system specially silage and hay preparation for disaster and lean period.

Production results of Napier fodder

1. From demonstrations in 102 farmers' field of 6.37 acre land presently, a total of 4038 farmers are producing Napier BLRI-3 fodder in 90.20 acre of lands in addition some are using road side and pond side lands even small pieces in the homesteads not counted.
2. About 46 % farmers are cultivating HYV Napier fodder and increasing over time;
3. More than 50 fodder entrepreneurs are producing fodder commercially and sold on daily or weekly basis in 6 fodder markets in addition to their own consumption;
4. The average yield was estimated 152.5kg/decimal in demo plots and 141kg/decimal in replicated farmers' field.
5. The average milk production increased initially by 0.5 -2.5 litre/cow/day. During the project period the increasing rate was in 1st year 11%, 2nd Year 18%, 3rd Year 32%, 4th year 36% and 5th year 38% (6 months) although other improved management practices were in place;
6. Improved body and health condition (Weight, performance and disease incidence);
7. Reduced repeat breeding and increased fertility;
8. Green fodder are available in the community and created enthusiasm for fodder cultivation;
9. Decrease feed cost about 30-35% and increased HH income about 71% in dairy;
10. Presently average everyday sale is about 804 kg fodder in 6 markets (134 kg per day per market) with an average income of about Tk. 2412/day (Tk. 402 per market @ of Tk. 3/kg); and
11. 50 Lead Farmers and the entrepreneurs are engaged in selling fodder cutting and some time they offer to the farmers free of cost for promoting fodder production.

Production results of other fodder

In addition to the Napier other varieties of fodder were selected for trials considering the environmental factors and natural calamities such as Jumbo, Alfalfa, Pack Chong, German, and Hydroponics. The production results are as follows-

1. Jumbo-88.5 Kg/decimal/cutting
2. Alfalfa-38 Kg/decimal
3. Pak Chong (Thailand)-Not yet harvested
4. German (BLRI)- Not yet harvested
5. Hydroponics- 6.85 Kg/Kg wheat seed

Hydroponics fodder production

It is a unique system of fodder production and practiced in Europe and Asia. Considering the land scarcity, concentration of salinity, seasonal variation and climate change effects, feed scarcity during dry and monsoon season hydroponics fodder (HF) production system has been introduced first time in Bangladesh through SaFaL programme. We jointly worked with Interdisciplinary Centre for Food security (ICF) of BAU, Mymensingh for feasibility study and found its potentiality in dairy production. The initial trial was conducted with wheat, maize and barley seeds. The wheat was found suitable on its availability, cost and volume of production.

The uniqueness was due to- home made production system, women can easily cope with the production technique, less labour intensive, free from contamination, availability within one's reach, require very limited land (72 sq.ft. is enough to produce fodder for rearing 2 cows) and can be produced vertically saving land, less costly and nutritious.

Beneficial effects are- can be produced within 7-9 days, requires no soil except water, well accepted by cattle, increases milk production, improves milk quality and whiteness of milk colour, improved reproductive health, improved fertility reducing repeat breeding, nutritional value specially CP content is about 4 times higher than the Napier grass and reduced feed cost replacing concentrate feed without affecting production.

Challenges are- initial investment is high, heat and humidity sensitive, non-availability of quality seeds round the year, black point and blast diseases are threat to wheat seed, fungal infection in hot weather, sometimes seeds are costly and require intensive care.

The progress of hydroponics fodder (HF) production so far achieved

1. On-station trial and nutritional value analysis of HF including cow performance in collaboration with ICF of BAU;
2. Established 26 field trials and demonstrations on HF production and performance of dairy cow;
3. Established 7 HF entrepreneurs for commercial HF production to promote business;
4. High nutritional value obtained compared to other fodders;
5. Crude Protein content of HF plant is 4 times (29-32%) higher than the Napier (7-8%);
6. On-station trial produced 6-7 kg HF from 1 kg wheat seed & in field trials it was 7-8 kg;
7. Milk production increased to 11% during on-station trial & field trials showed 31%- 45% increment;
8. Milk fat content was more than 4% that adding value to the milk price;
9. Reduced feed cost to about 41.03% resulting low production cost that contributes to income;
10. Farmers' additional income increased to Tk. 201 per day (In case of a dairy cow of 5 lit/day milk production);
11. An entrepreneur produced 32 kg HF everyday and used 16 kg for his own cows and sold initially 16 kg to two farmers and earned Tk. 60 everyday (@Tk. 3.75/kg as a promotional sale);
12. Body condition and health status was improved reducing disease incidence;
13. Reduced repeat breeding and improved fertility showing 88% conception rate; and
14. Further in-depth studies are on-going with ICF.

Conclusions and Recommendations

1. A comprehensive livestock development programme in the salinity prone area with the participation of all stakeholders to be developed to avoid any duplication and fashionable activities usually not sustained;
2. Market focused and business oriented activities need to be identified and facilitated for inclusive business environment;
3. Emphasize the research considering the climate change effects in the coastal belt and develop suitable technologies for SW region;
4. Develop National Feed Inventory;
5. Develop fodder germ-plasm bank for saline resistant variety;

6. Facilitate the private sector fodder nursery development through entrepreneurship and sustainable business model;
7. Involve private sector for business promotion and investments;
8. Create M4P on the livestock commodities;
9. Develop service and supply facilities and create accessibility; and
10. Establish livestock research centre or sub-centre for SW region especially in Satkhira.

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Feeding effect of straw based Total Mixed Ration (TMR) on milk yield, milk composition and rumen parameters in Red Chittagong cows

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Abstract
An experiment was conducted to determine the effect of feeding total mixed ration (TMR), on milk yield, milk composition and rumen parameters in Red Chittagong Cows (RCC) for a period of 70 days. A total of 15 milking cows were selected and divided into 3 treatment groups having 5 cows in each considering their milk yield. Existing feeding practice at the farm was considered as control group (T_0). In another two groups, animals were fed TMR (60% roughages: 40% concentrate, contained 16% CP), where one fed as block (T_1) and another as mash form (T_2). In control feeding (T_0) 82% Napier -3 silage, 5% wheat bran 3% wheat broken 2.5% Kheshari bran, 1.5 % soybean meal,3% maize crushed, 0.5% salt, 2.5% DCP were used. In treatment groups, 60% rice straw,1% wheat bran, 1% kheshari bran, 25% soybean meal, 10% molasses, 0.5% salt and 2.5% DCP were used in T_1 and T_2 , respectively. TMR blocks were prepared manually by hand pressing contained 5 kg materials with the dimension of 9cm×9cm×9cm. To determine the rumen parameters, rumen liquor was collected at post feeding intervals of 0, 3, 6, 12, 24 and 36h. Fresh feed intakes were significantly ($p<0.001$) higher in T_0 group than those of animals in T_1 and T_2 groups. DM intake, CP intake and %DM intake on live weight was significantly higher in T_1 group. Milk production was significantly ($p<0.001$) differed among treatment groups. The Highest milk yield, lactose, milk protein content were observed in $T_1$and T_2 ($p>0.05$) groups and lowest in T_0 group except milk fat. Body weight gain of animals did not differ significantly ($p>0.05$) among the treatment groups. Post feeding period and treatment had no significant effect on concentration of rumen parameters, except total volatile fatty acids (TVFAs) and ammonia-nitrogen (NH_3-N). TVFA and NH_3-N were significantly higher in T_1 and T_2 groups than T_0 group. Finally, it may be concluded that rice straw TMR, either in block or mash form gives better results in terms of milk yield, milk composition, and concentration of TVFAs and NH_3-N in the rumen which helps in the microbial protein synthesis and enhance higher milk production.

Keywords: Densified TMR, crop residues, nutrient digestibility, rumen fermentation, milk yield, milk composition

Introduction

Livestock plays a vital role in the natural resource-based livelihood of the vast majority of the population living in developing countries. However, most of these regions face the problem of acute shortage of feed resources. The pastures are degraded and poorly managed and the area under green forage crops is shrinking due to increase in human population and urbanization. As a result, the basal feeds available for ruminants in these regions are the crop residues which have low nutritional value and are bulky and fibrous. Due to lack of effective management of these resources, unfortunately they are being burnt in some countries, causing environmental pollution. The digestibility of crop residues and other low quality forages can be increased through the action of rumen microbes by strategically mixing nitrogen and minerals that are deficient in these feed resources. The increase in digestibility of crop residues and low quality forages, in turn also increases their intake. Both these phenomena enhance the efficiency of nutrient utilization from these feed resources in animal food chains. In the recent years, the concept of feeding complete rations comprising of fibrous crop residues to dairy animals became popular among the farmers. The objective of complete rations is to provide a blend of all the feed ingredients including roughages without giving any choice to the animal for selection of specific ingredient (Khan et al., 2010).

Animals of the country mainly depend on rice straw for their nutrition and its contribution to livestock feeds is more than 90% of feed energy available in ruminant's diet (Tareque and Saadullah, 1988). An estimate shows that there is a shortage of 39.8 % dry matter, 38% total digestible nutrients and 43% digestible crude protein. Another estimate shows that there is a shortage of 66.5% green fodder and 89.9% concentrates in the country. The availability of straw is more than that of our requirements.

Straw based densified TMR is a new concept of a complete balanced ration for feeding dairy animals as well as a novel system of delivering nutrients to bovines. Each TMR block is a total ration composed of both roughage and concentrates for a cow or a buffalo for 24 hours and supplies all the major nutrients, including micro nutrients required by the animals. The merits of complete rations are related to a stable environment for rumen fermentation, minimal fermentation losses and fluctuation in release of ammonia and enhancement in utilization of low grade roughages (Venkanna *et al*., 1997). Besides these complete rations facilitates control ratio of roughage to concentrate, provides uniform feed intake, reduces feed wastage, enhances nitrogen balance and milk production and reduces the cost of feeding (Hundal et al., 2004; Lailer et al., 2010). The roles of complete feed blocks are to increase productive and reproductive efficiency, enhance immuno-protective ability, reduce helminthic load and decrease ruminal methanogenesis of animals. This can also be effectively used as a disaster management strategy, by setting up fodder banks, to save the animals from death during natural calamities like floods and droughts. Hence, the study was undertaken to know the feeding effect of densified complete feed on the performance of dairy cows.

Materials and Methods

Location and duration of experiment

The research was conducted at Pachutia Research farm of Bangladesh Livestock Research Institute (BLRI), Savar Dhaka for a period of 70 days from 31 May to 8 August, 2016.

Composition and preparation of feed

Rice straw as a crop residue was chosen for the roughage component of TMR collected from Savar upazilla and chopped by a chopper machine before preparation of TMR. Rice straw along with other concentrates were analyzed to determine the DM (88.88, 20.39, 90.0, 91.69, 90.55, 80.0, 89.11, 90.01 and 88.55%, respectively for rice straw, Napier silage, wheat bran, Khesari bran, soybean meal, molasses, wheat broken, crushed maize and til oil cake) and CP (4.61, 10.25, 14.96, 12.48, 43.76, 4.50, 12.16, 8.50 and 30.03%, respectively for the same ingredients).After nutritional evaluation, all the concentrate ingredients (40%) were well mixed manually and then those mixture were properly mixed with roughage(60%) contained 16% CP and made in a block form by pressing manually in a hand machine that formed a dimension of 9×9×9c.cmand a gross mass weight of about 5 kg.

Dietary treatments and layout of the experiment

A total of 15 RCC milking cows were selected and divided into 3 treatment groups having 5 milking cows with almost same average initial milk yield in each group Animals were in early lactation and their body weight in 180-200 kg. One group was considered as control (T_0) and fed regular feed supplied to the animal at the farm. This 100kg conventional diet composed with 82 kg Napier -3 silage, 5 kg wheat bran , 3kg wheat broken 2.5kg kheshari bran, 1.5 kg soybean meal,3 kg maize crushed, 0.5 kg salt,2.5 kg DCP. Another two groups were fed TMR (60% roughages: 40% concentrate), in which one was fed TMR block (T_1) and another TMR mash (T_2). In each 100 kg TMR mixture the amount of rice straw was 60kg and rest 40 kg concentrate composed with 25 kg soybean meal, 10 kg molasses, 1 kg wheat bran, 1 kg khesari bran, 2.5 kg di-calcium phosphate and 0.5 kg table salt.

Feeding and management of animals

All the experimental animals were stall fed with zero grazing. Every day required amount of molasses and water solution was properly mixed with previously made TMR mash and provided twice daily (once in the morning at 8.00 am and rest in the afternoon at 4.00 pm) *ad libitum.* Always fresh, clean and safe drinking water was supplied to the animals.

Digestibility trial

In order to determine digestibility of supplied feed, a conventional digestibility trial was carried out at the middle of the feeding trial having 7 days collection periods. During that period, daily feed intake, feces and urine voided were recorded and collected individually. At the end of the collection period the feces were composites together and then proximate components of each of the samples were analyzed in Animal Nutrition Laboratory at BLRI.

Milk analysis

Milk samples were collected from each cow at 15 days interval and were analyzed for fat, protein, lactose, SNF and total ash contents by milk analyzer (Lactostar, Funk Gurbar).

Rumen liquor collection

At the end of the trial, rumen liquor was collected at the intervals of 0, 3, 6, 9, 12, 24 and 36 hours after feeding by using stomach tube having 0.15mm internal diameter and 150cm long plastic tube.The stomach tube was moistened and animal's mouth was opened by placing thumb in the region without teeth. The tube was then passed over the back of tongue and enters into the esophagus. A vaccum pump was used to apply suction to draw ruminal fluid. The fluid was obtained by lowering the animals head until fluid runs from the tube. Approximately, 50 ml of rumen liquor was collected from two animals in each group. The sucked fluid was filtered by a cheese cloth. The p^H was measured immediately after collection, using the digital P^H meter. Then, samples were stored at -18 °C for subsequent laboratory analysis. Total volatile fatty acids (TVFAs), total nitrogen (TN), ammonia nitrogen (NH_3-N), total solids (TS), non-protein nitrogen (NPN), and protein nitrogen (PN) were also measured from the samples collected from those hours.

Chemical analysis

The feed samples, leftover and faeces were analyzed by the method of AOAC (2005) for determination of DM, CP, OM and Ash, while, ADF and NDF by Van Soest et al., (1991).All the samples were analyzed in duplicate and mean values were recorded.

Statistical analysis

The data were analyzed using the "SPSS 17.0" statistical program. Duncan's Multiple Range Test (DMRT) which is a post-hoc test to measure the specific differences between pairs of means was also done to compare between two different treatment means for various parameters.

Results and Discussion

Feed intake

The fresh feed intakes of animals of different groups are shown in Table 1. Total DM intake, CP intake, and %DM intake on live weight was significantly higher in T_1 group (fed TMR block) than other two groups. The result was in agreement with Lailer et al. (2010) and Sehgal and Jha (2008) who described that densified TMR block significantly improves DM intake. The results of the present study are also in corroboration with the findings of Verma et al. (1996), Nagpal et al. (2005), Gupta et al. (2006), Lade et al. (2007) and Sharma et al. (2010).

Bargo et al. (2002) studied on the performance of Holstein Friesian cows feeding with three different diets viz. pasture plus concentrate, pasture plus partial TMR and TMR (non-pasture) and found significantly higher DM intake for cows fed TMR which is coincided with this study. They found highest milk yield, fat and protein content in cows fed only TMR which is in agreement with this study. CP intake was highly significant in block at the present study which strictly supported with the findings of Chander (2011).The CP intake differed significantly ($p<0.001$) among animals of different treatment groups. The highest CP intake was observed in T_2 and lowest in T_1. Though, animals of all treatment groups were supplied diet containing same % of CP but variation of CP intake could be due to variation of DM intake from diet.

Table 1. Feed intake of animal for different treatment groups

Parameters	Treatment group			
	T_0 (Control)	T_1 (TMR block)	T_2 (TMR mash)	Significance level
Fresh feed intake (kg/day)	16.82^{c}± 0.18	14.66^{b}±0 .10	14.12^{a}±0 .20	***
DM intake (kg/day)	3.30^{a}± 0.03	4.54^{c}±0 .03	4.37^{b}±0 .06	***
CP intake (kg/day)	0.40^{a}±0 .004	0.54^{c}±0 .002	0.52^{b}±0 .007	***
%DM intake on LW	1.66^{a}±0.12	2.06^{b}±0.09	1.80^{ab}±0.07	**

DM-Dry matter; LW-Live weight of animal; ***-p<0.001means with uncommon superscript within the same row differed significantly (p<0.01)

Milk yield

Milk production of cows for different treatment groups are shown in Table 2 which varied significantly (p<0.001) among groups. Highest average daily milk yield (both whole and fat corrected) was observed in T_1group and lowest in T_0 group, although there was no significant difference betweenT_1 and T_2 groups.

Table 2. Milk yield of different treatment groups

Parameter	T_0 (Control)	T_1 (TMR block)	T_2 (TMR mash)	Significance level
Initial average daily milk yield (ltr)	2.74±0.31	2.74 ±0.21	2.75±0.21	NS
Finalaverage daily milk yield(ltr)	2.75^{a}±0.02	3.02^{b}±0.02	2.93^{b}±0.03	***
Fat corrected daily average milk yield (ltr) (4.5% fat)	2.95^{a}±0.10	3.24^{b}±0.05	3.14^{b}±0.04	*

NS-non significant (p>0.05)***-p<0.001 means with uncommon superscript within the same row differed significantly (p<0.01) [FCM=.4 *Milk yield + (15*milk fat in kg]

The results obtained in this study is coincided with Lailer et al. (2010) who reported that densified complete block significantly improves milk production performances of animal. The result also agreed with the recent study found by Walli (2015) who conducted an experiment with straw based densified TMR block and got a positive effect on milk production. Bargo et al. (2002) studied performance of milk production of Holstein Friesian cows feeding with three different diets viz. pasture plus concentrate, pasture plus partial TMR and TMR (non-pasture) and found significant difference of milk yield which agreed well by this study. They found highest milk yield in cows fed only TMR which is in agreement with this study. Hundal et al. (2004) conducted an experiment with densified TMR block and found no significant changes for milk yield which is contradicted with this study. This contradiction may be due to difference of breed, formulation of feed, feed ingredients, stage of lactation, environment etc.

Milk composition

Changes of milk compositions of cows for different treatment groups are given in Table 3. The table shows that milk fat, protein and lactose differed significantly (p<0.05) among treatment groups, while not differed for SNF. Apparently, highest milk fat was observed in T_2 group and lowest in T_0 group. However, there were no differences between T_0 and T_1 and between T_1 and T_2.Although, milk protein was higher in T_1 than T_0 and T_2, but did not differ with T_2.

Table 3. Milk composition of different treatment groups

Parameter	T_0 (Control)	T_1 (TMR block)	T_2 (TMR mash)	Significance level
Fat(%)	4.52^{b}±0.18	4.91^{ab}± 0.12	5.03^{a} ±0.15	*
Protein(%)	3.82^{b}±0.1	4.17^{a}±0.03	4.07^{a}±0.03	***
Lactose(%)	5.78^{b} ±0.1	6.01^{a}±0.05	5.87^{ab}±0.04	*
SNF(%)	10.92±0.08	11.09±0.1	10.9±0.05	NS

NS-non significant (p>0.05) ***-p<0.001 means with uncommon superscript within the same row differed significantly (p<0.01)

Bargo et al. (2002) in their studyfound highest milk fat and protein percentages in cows fed TMR compared to cows fed partial TMR and pasture plus concentrate which is in agreement with this study. It is evident that there are lots of genetic and non-genetic factors responsible for variability of milk composition like breed, heredity, dietary regime, time and frequency of milking, season etc.

Body weight changes

Total body weight gain at the end of the experiment is given in Table 4. Although, there were changes of body weight, but differences were not significant ($p>0.05$) among cows of different groups. The result is in agreement with Mahal et al. (1997) who reported no differences in live weight changes for roughage and concentrates ratio of 60:40.

Table 4. Changes of animal's body weight for different treatment groups

Parameters	Treatment group			
	T_0 (Control)	T_1 (TMR block)	T_2 (TMR mash)	Significance level
Total body weight gain (kg)	12.28± 3.55	16.34± 1.32	33.72± 1.89	NS
Daily weight gain (kg)	0.175±0.05	0.233±0.01	0.481±0.02	NS

LW=Live weight of animal; NS-non significant ($p>0.05$); means with uncommon superscript within the same row differed significantly ($p<0.01$).

Though, animals were more or less mature and all were at milking stage, emphasize was given for increasing milk yield, thus body weight gain is not expected in such condition. However, all dietary treatments were subjected to constant the level of roughage and concentrate ratio (60:40), and in such situation difference of weight gain is unexpected among treatment groups. This is agreed with Sporndly (1986) who concluded that changing the ratio of roughage to concentrate from 62:38 to 50:50 significantly increased live weight gain . But the result is contradicted with Bargo et al. (2002) who found significantly higher body weight gain for cows fed TMR.

Nutrient digestibility

Digestibility of nutrients in feed supplied to animals of different groups is illustrated in Table 5, which shows to have significant ($p<.001$) differences of DM, OM, CP, ash and NDF digestibility among treatment groups. In all cases, digestibility of the said components in supplied feed was significantly higher in T_1 than those of other two groups. On the other hand, ADF digestibility did not differ significantly among groups.

Table 5. Digestibility (%) of DM, CP, OM, Ash, ADF and NDF for different rations

Nutrient digestibility (%)	Treatment			SE
	T_0(Control)	T_1(TMR block)	T_2(TMR mash)	
DM*	51.38^{c}	63.84^{a}	61.21^{b}	1.49
CP*	52.49^{b}	63.53^{a}	60.50^{a}	1.57
OM*	54.01^{b}	66.58^{a}	65.11^{a}	1.56
Ash*	33.24^{b}	50.85^{a}	50.05^{a}	2.32
ADF	53.72	58.85	56.33	1.20
NDF*	52.74^{b}	57.68^{a}	57.13^{ab}	1.01

Values are means of fivefold samples; SE: standard error; means withinthe same rowwith different superscripts (a-c) differ significantly ($p<0.01$).

The result is comparable with the findings of Konka *et al.*(2015) who conducted an experiment with three different crop residues based complete rations (crop residue and concentrate with ratio of 60:40 in buffalo at India) and found significant difference of *in vitro* DM digestibility. They reported DM digestibility from 55.45% to 58.59% which closely agrees with this study. This result was also similar with the findings of Lailer et al. (2010) and Jha (2008) who described that densified TMR block significantly improves DM digestibility. The higher digestibility of DM obtained in TMR block might be due to more soluble carbohydrates in the form of starch which agrees well by Sardar et al. (1996). Wang et al. (2004) and Shojaeian and Thakur (2007) reported that the increase DM digestibility appeared to be associated with higher NDF digestibility which confirms by this study. The values obtained for CP digestibility of TMR in the present study were in agreement with the values of 62.22%, 61.40% and 59.65%, respectively ($p>0.05$) for maize stover, red gram straw and black gram straw based complete rations (Konka *et al.*,2015). The values of NDF obtained in this study were also similar to the values (53.23 to 56.33%) found by Konka et al. (2015). But the values of ADF in this study were higher than the values (42.10 to 44.73%) obtained by Konka et al. (2015).It was also reported that digestibility of complete feeds can be enhanced by the additions of relatively small quantities of specific nutrients such as protein or soluble carbohydrates (Khan et al., 2010).

Rumen fermentation

Table 6, shows the rumen fermented products in different incubation period after feeding for different treatment groups. The result shows that rumen p^H had no significant differences ($p>0.05$) in different hour of incubation in different treatment groups. The result of the present findings is similar with the observation of Williams and Christian (1959). They reported no corresponding changes in rumen p^H value in different treatment groups. Smith and Rode (1972) reported that the variations between 6.0 and 6.8 in ruminal p^H provide maximum activity of cellulytic organisms. This finding was contradicted with the observation of Gray et al. (1954) who shows that after the fermentation peak level reached two to four hours after feeding, thereafter p^H increases.

There were significant differences observed in TVFA in different periods post feeding (0, 3, 6, 12, 24 and 36h) and treatment groups. The concentration of TVFA was increasing after 3h post feeding, reached a maximum level at 12h and then reduced at 24h and increasing thereafter as shown in Figure1. This result is highly corrugated with the findings of Reid et al. (1957), however, contradicted with Balch and Campling (1962) who observed no consistent variation in the proportions of the TVFA withtime after feeding. The concentration level of TVFA depends on ruminal microbial activity, hydrolysis of protein and NPN (Tomar and Sengar, 1999; Samanta et al., 2006; Venkanna et al., 1997). TVFA production is an indicator of carbohydrate digestion especially the crude fiber (Girdhar and Balaraman, 2005).

The changes of TN concentrations in different treatment groups ($p>0.05$) were found to be increased slowly up to 6h post feeding and then decreased gradually ($p>0.05$). The TN at 0-3h and at 36h post feeding for any of the treatment groups was almost similar. The highest level of PN concentration was obtained at 24h post feeding, although the changes for period of fermentation and treatment groups were not significant. The highest level of NPN concentration was obtained at 12h post feeding, although the changes for period of fermentation and treatment groups were not significant.

Table 6. Rumen fermentation parameters in different treatment groups according to different hours

Parameters	Period (hour)	Dietary treatments				Level of Sig.
		T_0	T_1	T_2	SEM	
P^H	3	6.54	6.33	6.61	2.64	NS
	6	4.51	4.40	4.54	2.64	NS
	12	6.4	6.48	6.63	2.65	NS
	24	6.67	6.46	6.54	2.67	NS
	36	6.51	6.67	6.59	2.68	NS
VFA(mM/100	3	10.5	11.5	11.52	4.56	**
ml)	6	15.6	11.31	9.19	5.98	**

	12	18.5	17.5	17.0	6.80	**
	24	11.0	12.9	11.5	4.69	**
	36	15.5	18.5	16.5	6.87	**
TN(g/100ml)	3	1.9	1.7	1.4	0.68	NS
	6	2.3	2.0	2.15	0.87	NS
	12	1.90	2.50	2.10	0.88	NS
	24	1.55	1.85	2.60	0.83	NS
	36	1.8	1.9	1.8	0.74	NS
NH_3-N(g/100ml)	3	13.9	14.0	12.8	5.53	*
	6	10.6	11.6	11.15	4.53	*
	12	9.36	9.63	9.30	3.84	*
	24	17.61	23.64	23.90	8.86	*
	36	9.05	9.15	8.73	3.66	*
PN(g/100ml)	3	1.0	1.1	1.1	0.45	NS
	6	1.2	1.1	1.0	0.36	NS
	12	0.9	0.9	1.0	0.69	NS
	24	1.4	1.9	1.8	0.69	NS
	36	1.0	1.45	1.30	0.56	NS
NPN(g/100ml)	3	0.91	0.63	0.30	0.428	NS
	6	1.1	0.90	1.15	0.51	NS
	12	1.15	1.0	1.6	0.13	NS
	24	1.2	0.15	0.05	0.13	NS
	36	0.8	0.45	0.5	0.23	NS

**-$p<0.01$,*-$p<0.05$NS-non significant ($p>0.05$); means with uncommon superscript within the same row differed significantly ($p<0.01$)

NH_3-N concentrations differed significantly for periods of fermentation and treatment groups. The changes of NH_3-N concentrations in different treatment groups were found to be decreased gradually towards 12h post feeding, but again increasing and reached a maximum level at 24h and decreasing thereafter (Figure2).According to Carvalho et al. (1997), the reduction in ruminal NH_3-N concentration can be explained by the increase in energy availability in the rumen, allowing higher use of ammonia for microbial growth, with consequent reduction in ammonia loss due to synchronization in the carbohydrates and protein degradation.

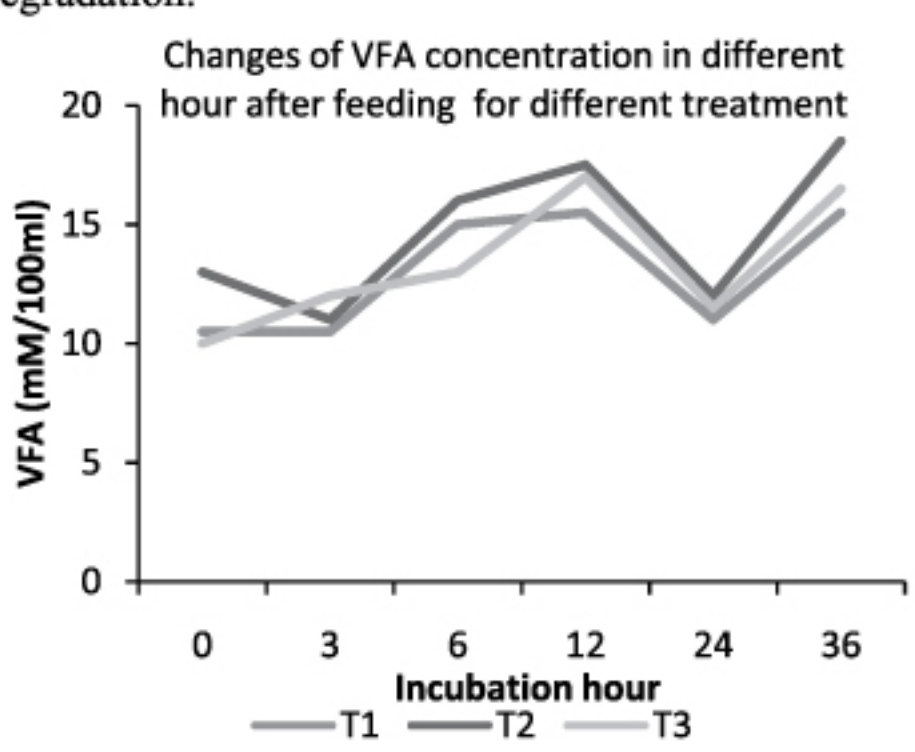

Figure.1. Changes of TVFA concentration in different fermentation period

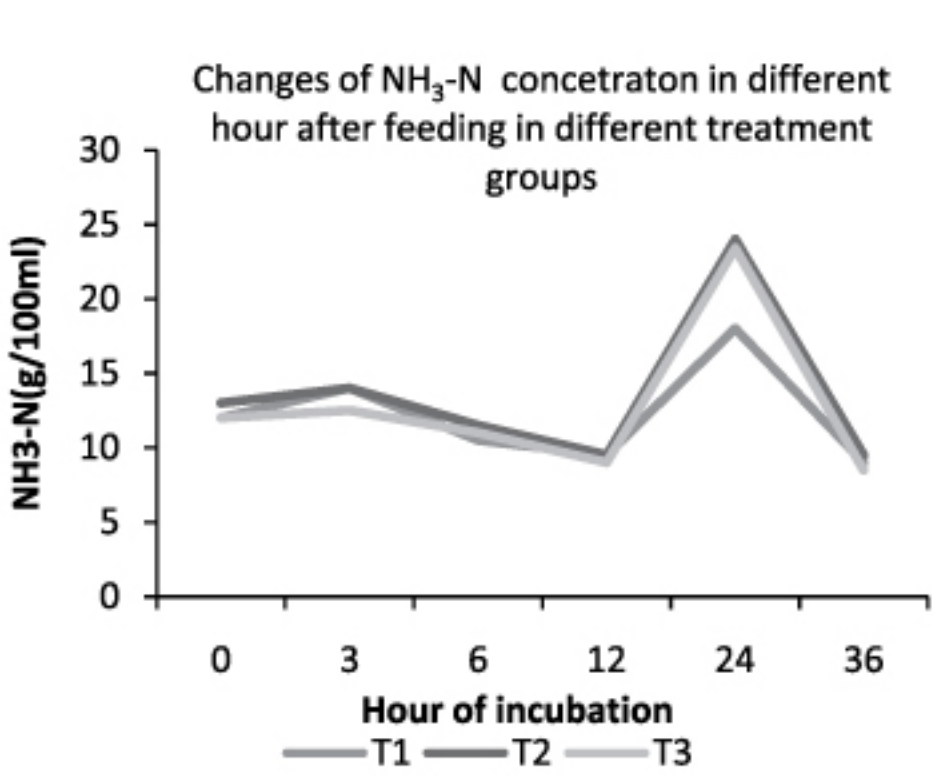

Figure.2. Changes of NH_3-N concentration in different fermentation period

Conclusion

The results of the present experiment revealed that the ration T_1 and T_2 are superior compared to T_0 in terms of milk yield, milk composition, the digestibility (DM, CP, OM, Ash, and NDF), TVFA and NH_3-N production. However, there were no significant differences of performance between TMR block and TMR mash.TMR block is more suitable because it is easy to handle and required low space for storage.

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Study of system modeling of Moringa (*Moringa oleifera)* fodder agronomy and its nutritional response to ruminants

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Abstract

The experiment was carried out to evaluate the system modeling of Moringa fodder agronomy and their feeding response to ruminants. Three different germplasm of Moringa such as Local White Seed Moringa (WSM), Black Seed Moringa (BSM-L) and Black Seed Moringa from FAO regional office, Bangkok (BSM-T) are available in Bangladesh. The annual biomass yield of BSM-L tops (114.5 t/ha fresh; 22.7 t/ha DM) was significantly higher than that of WSM (29.0 t/ha fresh; 5.80 t/ha DM) or BSM-T (83.5 t/ha fresh; 16.0 t/ha DM). No significant difference in chemical composition (224.9, 222.4 & 223.8 $g.kg^{-1}$ DM of crude protein (CP), respectively, and 450.9, 455.3 & 435.4 $g.kg^{-1}$ DM of neutral detergent fiber, respectively) or nutritional value (47.4, 46.7 & 45.3% of potential, and 62.8, 64.2 and 63.6% of effective degradability of dry matter) was found for the cultivars. BSM-L had a significantly higher survivability (97.2%), prune number per plant (3.50) and RGR (15.6 mg DM/day) than WSM (25.0%, 2.30 & 4.20 mg DM/day) or BSM-T (55.6%, 3.10 & 10.8 mg DM/day) respectively. In terms of biomass production and its nutritional values, the variety of local Black Seed Moringa (BSM-L) may be considered for commercial purposes to ruminant animals. It's also revealed that, Moringafeed could be replaced the whole locally mixed concentrate mixture including the 23% of soybean meal as well as reduce the cholesterol level in the blood. All these data shows Moringa could be a good quality fodder for ruminants.

Key words: Moringa cultivars, Biomass and nutritional value, Milk product

Introduction

Gradual transformation of traditional subsistence livestock farming into input supported dairy or beef production system has been demanding more quality feeds & fodders. Moringa (*Moringa oleifera),* a plant fodder being researched and found responsive to increasing production and productivity of small (Sultana *et al*. 2014) and large ruminants (Foidl *et al*. 1999; Sanchez *et al*., 2006 ;), was identified as one of the best options for Bangladesh (Huque and Sarker, 2014) for supporting growing quality feed demands. Options for Moringa fodder production were tested (Foidl *et al*., 2001), but locally, it is known as a source of drumsticks and, to a lesser extent, as leafy vegetable. Asexual propagation at homesteads without any input intervention or competition for land is followed for vegetable production irrespective of agro-ecological conditions of the country. Sexual propagations were established (Huque *et al*, 2014), but varietal responses on biomass production, contribute to increased availability of protein rich quality feeds and fodders in the country. Impacts of season on the biomass & its response to ruminant animals; and cost effectiveness introduction into existing cropping system competing with available cash crops are burning questions remain unaddressed. Without developing systems for cost effective biomass production, value additions or animal production, the unconventional plant fodder may not suitable variety of Moringa out of domestic or regional plant biodiversities, investigate pathologies of seeds and saplings, develop agronomical practices for maximizing biomass production and productivity, determine the quality of biomass in lab and in animals, and to determine cost effective biomass production on farm. Keeping the above factors in consideration the present research work was undertaken to (i) identify suitable cultivar(s) of Moringa for year round feed production (ii)

determine feeding impacts of Moringafeed (M_f) on dairy cattle, (iii) test on farm production performance of selected Moringa cultivar.

Materials and Methods

The agronomical trial was conducted at the Cattle Research Station of the Bangladesh Livestock Research Institute (BLRI) from August 2014 to December 2015. Three different cultivars of Moringa were used in the present programme. The seeds of the two cultivars, White Seed Moringa (WSM)) and Black Seed Moringa (BSM-L) were collected from selected local sources. The third cultivar having black seeds was collected from Thailand (BSM-T). The three cultivars of Moringa (taxonomical identification not completed yet) are entitled according to their seed color, considered as a major phenotypic difference. A uniformly plain land area of 97.2 m^2 was divided into four blocks, each of 24.3 m^2 separated by 1.0-meter wide walking alleys. Each block was again divided into three experimental plots, each of 8.1 m^2 for the planting of 90 saplings at a space of 0.3 m x 0.3 m per sapling. The blocks and plots were arranged in a Randomized Block Design to determine production responses of the three cultivars of Moringa. The saplings were transplanted in predesigned experimental plots. Before transplantation, the soil of the plots was ploughed and fertilized with a basal dose of cattle dung at the rate of 3.0 t/ha and a mixture of TSP (Triple Super Phosphate) and MP (Murate of Potash) of a ratio of 30:15 kg per hectare. The urea N at the rate of 90kg/ha was top dressed when the plants were initially established in the research field and it was repeated at each harvest. All other agronomical practices e.g. weeding, irrigation etc were common for all cultivars. After a post-transplantation growth period of 90 days, branch tops with leaves were harvested at a 60 days interval keeping an average stem height from the ground of 40 cm. The plants were allowed to grow after each cut and fertilized accordingly. A total of six cuts were given. The biomass yield of each experiment in six (6) different cuts of a year (Dec-Jan, Feb-Mar, Apr-May, Jun-July, Aug-Sep and Oct-Nov) was added to determine the annual yield of biomass production. Survival rate (% of saplings grew after transplantation), the number of prunes per plant, defoliation rate (% of total leaf biomass defoliated), and the growth rate of biomass were determined at different harvesting times. Fresh tops were harvested avoiding any surface water on plants and weighed on a top loading balance and the fresh yield per plot was recorded. Fresh yield (kg or ton) was converted to DM yield $plot^{-1}$ ha^{-1} according to the equation of DM yield $plot^{-1}$ = Weight of fresh material × (%) DM.

The tops were manually separated into stems and leaves to determine stem to leaf ratio and weighed accordingly. Representative samples of tops, stem or leaves were taken to determine fresh dry matter, total ash, crude protein (CP) and ether extract (EE) according to AOAC (1990); and neutral detergent Fiber (NDF) or acid detergent Fiber (ADF) and acid detergent lignin (ADL) according to Van Soest (1990). All the analyses were done in the animal nutrition laboratory of the BLRI. The tops and stems were chopped manually at a range of 0.03 m to 0.05 m, dried in the sun, and milled for chemical analyses of the biomass of different harvests.

The data on rainfall, temperature and humidity were collected during the period of 14 September 2014 to 23 September 2015. The relative growth rate (RGR) of different cultivars was calculated using the equation of $RGR = \frac{lnW_2 - lnW_1}{t_2 - t_1}$ described by Hoffmann and Poorter (2002); *w*here, ln = natural logarithm, t_1 = time one (in days), t_2 = time two (in days), W_1= Dry weight of plant at time one (in grams), W_2= Dry weight of plant at time two (in grams). Heat Index, a measure of how hot it really feels when relative humidity is factored in with the actual air temperature, was calculated from the HI chart of National Weather Service of the US Department of Commerce.

Three local growing bulls of an average live weight of 225 kg fitted with rumen cannulae (14 cm diameter & 9 cm length) were used to determine rumen degradability *in-sacco* of dry matter of Moringa tops. The animals were fed Napier and German grass mix *ad libitum* and the roughages were supplemented with locally mixed concentrate mix of wheat bran, sesame oil cake, Kheshari (*Lathyrus sativus*) bran, di-alcium phosphate (DCP) and common salt at the rate of 1.0% of the live weight. Having the animals adjusted to the diet for at least three weeks Dacron bags (7x16 cm, pore size 45 µm) containing the samples (2 g each) of three Moringa tops (oven dried, milled and passed through a sieve of 1.0 mm size) were incubated in the rumen following the

method described by Ørskov *et al.* (1980). Considering animals and three periods as replication the samples of Moringa tops of three cultivars were incubated at 0, 8, 16, 24, 48 and 72 hrs in the rumen. Each hour of incubation of a sample of each cultivar was repeated in three animals in a period, and the incubations were repeated in three different periods. The degradation kinetics of DM were determined by fitting the disappearance values to the equation $P= a+ b (1-e^{-ct})$ Ørskov and McDonald (1979), where P represents the disappearance after time t. Least-squares analyses were used for the estimation of rapidly degradable fraction (a), slowly degradable fraction (b) and the rate of degradation (c). The effective degradability (ED) of Moringa tops were estimated using the equation of McDonald (1981), where ED= a+bc/(c+k), and 0.05 rate constant (k) was considered.

Considering the three Moringa cultivars as treatment, their responses to biomass production performances (yield, growth rate, pruning efficiency and ratio of botanical fractions), and nutrient yield and contents (DM, CP, ADF, NDF or ADL) were analyzed in an ANOVA of a Randomized Block Design (RBD) using general linear model of SPSS-17.0 statistical software program in a computer. Any significant differences in the rate and extent of the DM degradability *in-sacco* of Moringa tops of different cultivars were analyzed using an ANOVA of 3x3 Latin Square Design.

For testing the Moringa feed (processed Moringa tops containing CP:ADF ratio of 1:2) on milk production, fifteen BCB-1 cows of third or fourth parity after 3 to 4 weeks of calving were selected and divided into three dietary groups having five animals in each considering their live weight and ex-post factor of milk yield . A group of cows fed a control diet consisting of 50:50 ratio (DM basis) of Napier silage and a concentrate mixture of rice bran-38%, wheat bran-23%, Soybean meal-23% Crushed wheat 12%, Mineral mixture-3% and Salt-1%. The concentrate of the control diet was replaced by Moringafeed at 50% maintaining a ratio of 50:25:25 respectively; in diet T_1 and at 100% maintaining a ratio of 50:0:50 respectively in diet T_2, and the diets were fed for 60 days. All the three concentrate mixture was iso-nitrogenous, and the daily energy and CP requirement of the cows were calculated according to BSTI (2008). Feeding response of different diets on different parameters was analyzed in an ANOVA of a Completely Randomized Design (CRD).

i). Varietal Performances
Results and Discussion

The effect of different cultivar of Moringa on survival rate (%), the number of prunes per plant, growth rate (kg/ha/day) and defoliation rate (%) are shown in Table 1. The survivability of BSM-L was the highest (97.2%) followed that of BSM-T (55.6%) and WSM (25.0%); and the difference between the cultivars were significant ($P<0.001$). A similar trend was found in the RGR of the cultivars (15.6, 10.7 & 4.2 mg/day, respectively, $P<0.001$). Both the black seed cultivars having a significantly higher average number of prunes (3.5 vs. 3.1 prunes/plant) were bushier than WSM. The average defoliation rate of all the cultivars varied from 2.4 to 4.0% and it did not differ significantly ($P<0.46$). A higher survivability of *M. oleifera*and its growth have also been reported by Ezekiel Edward *et al.* (2014); Benevides (1994); Richter *et al.* (2003); Sanchez *et al.* (2006) and Mendieta-Araica*et al.* (2011).

Table 1: Performances of survivability and growth of different cultivars of *Moringa oleifera* (means±SE; n=4)

Parameters	Moringa cultivars			Significance		
	BSM-T	WSM	BSM-L	Overall mean	Overall SE	Level
Survival rate (%)	$55.6^{b} \pm 10.1$	$25.0^{c} \pm 5.3$	$97.2^{a} \pm 2.7$	59.3	9.6	P<0.00
No of prunes/plant	$3.1^{a} \pm 0.2$	$2.3^{b} \pm 0.2$	$3.5^{a} \pm 0.1$	2.9	0.2	P<0.01
Annual RGR (mg/day)	$10.8^{a} \pm 1.8$	$4.2^{b} \pm 3.0$	$15.6^{a} \pm 1.2$	7.4	2.76	P<0.00
Defoliation rate (%)	2.6 ± 0.14	3.7 ± 0.9	3.1 ± 0.6	3.1	0.4	P<0.46

BSM-T= Black Seed Moringa of Thailand; WSM= White Seed Moringa; BSM-L= Black Seed Moringa of local origi

Biomass yield

The effect of three different cultivars on fresh or dry matter (DM) yield of tops, leaf and stem fractions of Moringa and their leaf to stem ratios are shown in Table 2. The annual fresh (114.51 t/ha) and dry matter (22.73 t/ha) yield of BSM-L tops were significantly (P<0.001) higher than that of BSM-T (83.52 t/ha and 16.03 t/ha) or WSM (29.01 t/ha and 5.79 t/ha). Similarly, the annual fresh or dry matter yield of stem (75.82 t/ha and13.19 t/ha) was the highest for BSM-L, followed by BSM-T (51.61 t/ha and 9.07 t/ha) and WSM (18.24 t/ha and 3.75 t/ha) and the difference was significant (P<0.001). The annual fresh or dry matter yield of leaves was 38.7 t/ha and 8.50 t/ha for BSM-L and 31.9 t/ha and 7.62 t/ha for BSM-T and differed significantly (P<0.05) between the two cultivars. Both BSM-T and BSM-L had a significantly (P<0,001) higher fresh or DM yield of leaves than that of WSM (10.6 t/ha and 2.3 t/ha). The average leaf to stem ratio of BSM-L was 0.45 and it reflects that almost a half of the whole tops dry matter was shared by leaves. The ratio varied from 0.56 to 0.58 for WSM and BSM-T. Nevertheless, the variation in the leaf to stem ratio among the cultivars was not significant (P<0.42) (Table 2).

Table 2: Biomass production and composition of botanical fractions of different *Moringa oleifera* cultivars

Parameters	Moringa cultivars			Significance		
	BSM-T	WSM	BSM-L	Overall mean	Overall SE	Level
	Fresh yield(t $ha^{-1}year^{-1}$)					
Tops	83.5^{b}±12.0	29.0^{c}±6.7	114.5^{a}±4.7	75.7	11.5	P<0.00
Stem	51.6^{b}±9.7	18.2^{c}±4.9	75.8^{a}±4.5	48.5	7.9	P<0.00
Leaf	31.9^{b}±2.5	10.6^{c}±1.7	38.7^{a}±1.3	27.1	3.7	P<0.00
	Dry matter yield(t $ha^{-1}year^{-1}$)					
Tops	16.0^{b}±2.3	5.8^{c}±1.3	23.6^{a}±1.1	14.9	2.3	P<0.00
Stem	9.1^{b}±1.7	3.8^{c}±1.0	13.2^{a}±0.8	8.7	1.3	P<0.01
Leaf	7.6^{a}±0.6	2.3^{b}±0.4	8.5^{a}±0.3	6.1	0.8	P<0.00
Leaf: Stem	0.58±0.10	0.56±0.06	0.45±0.02	0.53	0.04	P<0.42

BSM-T= Black Seed Moringa from Thailand; WSM= White Seed Moringa of local origin; BSM-L= Black Seed Moringa of local origin

Table 3 shows the chemical composition of different biological fractions of the three Moringa cultivars. BSM-L had a significantly (P<0.05) higher fresh dry matter of tops (206.3 g kg^{-1}) than BSM-T (191.9 g kg^{-1}) and lower (P<0.05) fresh leaf dry matter (222.5 g kg^{-1}) than the latter (235.0 g kg^{-1}) or WSM (233.5.0 g kg^{-1}). The ash content of BSM-L (66.8 g kg^{-1}) was significantly (P<0.01) lower than BSM-T (83.4 & 77.4 g kg^{-1}, respectively). All other chemical components (CP, ADF, NDF, EE and ADL) in the tops, stem or leaves of three cultivars did not differ significantly (P>0.05). Their average contents for the three cultivars were 223.7, 419.8, 442.8, 277.3 & 208.2 g kg^{-1}, respectively for tops; 124.8, 632.5, 711.5, 87.4 and 248.2 g kg^{-1}, respectively, in stem; and 299.4, 215.2, 343.3, 106.2 and 326.8 g kg^{-1}, respectively in leaves (Table 3).

Table 3: Chemical composition of different Moringa cultivars and their botanical fractions

Parameters	Moringa cultivars			Significance		
	BSM-T	WSM	BSM-L	Overall mean	Overall SE	Level
	DM(g kg^{-1})					
Tops	191.9^{b}± 2.3	198.6^{ab} ± 5.51	206.3^{a} ± 1.1	198.9	2.54	P<0.05
Stem	161.5± 3.5	162.9± 5.3	165.7 ± 4.3	163.4	2.36	P<0.80
Leaf	235.02^{a} ± 2.7	233.50^{a} ± 2.4	222.4 ± 2.20^{b}	230.3	2.13	P<0.01
	Ash(g kg^{-1})					
Tops	83.4^{a}± 0.6	77.4^{b} ± 1.4	66.8^{c}± 1.7	75.8	2.1	P<0.01
Stem	70.0± 3.8	67.8± 2.2	63.3 ± 3.3	67.1	1.86	P<0.35

Leaf	86.6 ± 2.8	79.6 ± 1.1	80.7 ± 2.5	82.3	1.51	P<0.11
			CP(g kg^{-1} DM)			
Tops	223.8±1.7	222.4±1.9	224.9±2.3	223.7	1.1	P<0.68
Stem	126.1±4.5	122.2±2.6	126.2±5.2	124.8	2.31	P<0.75
Leaf	305.1±3.4	296.8±4.0	296.3±2.8	299.4	2.17	P<0.32
			ADF(g kg^{-1} DM)			
Tops	422.6±51.1	422.1±7.8	414.7±0.4	419.8	13.43	P<0.97
Stem	619.6±5.4	656.1±17.7	622.0±10.2	632.5	9.23	P<0.20
Leaf	212.65±2.5	217.75±7.3	215.08±4.9	215.2	2.56	P<0.31
			NDF(g kg^{-1} DM)			
Tops	435.4^{b}±0.75	455.3^{a}±1.12	450.9^{a}±3.40	447.2	6.58	P<0.934
Stem	724.7±6.2	707.2±9.1	702.7±7.4	711.5	5.44	P<0.24
Leaf	351.3±3.8	339.9±4.2	338.6±1.4	343.3	2.97	P<0.13
			EE(g kg^{-1} DM)			
Tops	277.1±1.9	277.8±0.3	277.1±1.9	277.3	0.72	P<0.92
Stem	87.7±0.4	86.5±1.5	87.9±0.7	87.4	0.51	P<0.37
Leaf	106.3±0.8	105.8±0.5	106.3±0.8	106.2	0.35	P<0.83
			ADL(g kg^{-1} DM)			
Tops	205.5±1.4	209.1±0.9	209.8±0.7	208.2	0.96	P<0.11
Stem	232.8±1.30	231.8±1.3	231.6±0.1	248.2	0.53	P<0.48
Leaf	332.6±3.4	328.7±0.5	319.1±8.8	326.8	3.52	P<0.43

BSM-T= Black Seed Moringa of Thailand; WSM= White Seed Moringa; BSM-L= Black Seed Moringa of local origin; DM, Dry Matter; CP, Crude Protein; ADF, Acid Detergent Fiber; NDF, Neutral Detergent Fiber; EE, Ether Extract; ADL, Acid Detergent Lignin;

Similar CP levels between 290 and 320 g.kg^{-1} was reported by Al-Masri (2003) and Soliva *et al.* (2005). The CP of stem did not vary significantly among the cultivars and the average content was 124.8 g kg^{-1}. The CP content of even Moringa stem of different cultivars was higher than that of Napier or Guinea grass (109.0 g kg^{-1} DM and 91.7 g kg^{-1} DM, respectively; Ansah *et al.* 2010 or Fadiyium *et al.* 2010). The CP contents of Moringa tops reported in this study are within the range of 193.0 to 264.0 g kg^{-1} DM, reported earlier by Makkar and Becker (1996 &1997); Foidle *et al.* (1999); Aregheore (2002); Al-Masri (2003); Manh *et al.* (2003); Sultana *et al.* (2014) and Minson (1990). The average ADF (419.8 g kg^{-1}), NDF (442.8 g kg^{-1}) or ADL (208.2 g kg^{-1}) content of the tops of three different cultivars were similar to those reported by Makkar and Becker (1996); Foidle *et al.*(1999); Aregheore (2002) and Al-Masri (2003). However, different stem to leaf ratio of a harvest affected the level of different cell wall components in different cultivars of Moringa.

Degradation kinetics

Table 4 shows that the calculated soluble fraction (a) was significantly higher for BSM-L tops (23.0%) than BSM-T (20.9%) and WSM (21.53%); while the rate constant (c=0.08) of BSM-L was significantly (P<0.001) lower than that of the later two cultivars (0.12 and 0.13, respectively). The rate of rumen dry matter degradation of WSM tops was the highest (0.13, P<0.01) followed by 0.12 of BSM-T and 0.08 of BSM-L. The potential (b) or effective degradability of tops of the three cultivars ranged from 45.3 to 47.4 %, and 62.8 to 64.2 % at a rate constant of 0.05 passage rate and their differences among the cultivars was not significant (P>0.05).

Table 4: Rumen degradation kinetics of different Moringa cultivars

Parameters	Moringa cultivars			Significance		
	BSM-T	WSM	BSM-L	Overall mean	Overall SE	Level
a	20.9^{b}±0.7	21.5^{ab}±0.2	23.0^{a}±0.2	21.81	0.37	P<0.03
b	45.3±1.7	46.7±1.3	47.4±0.6	46.1	0.73	P<0.50
c	0.12^{a}±0.01	0.13^{a}±0.05	0.08^{b}±0.07	0.11	0.08	P<0.01
Effective degradability (%)	63.6±0.4	64.2±1.1	62.8±0.52	63.5	0.51	P<0.14
RSD	2.9±0.9	2.4±0.7	4.4±0.5	3.27	0.49	P<0.23

BSM-T= Black Seed Moringa of Thailand; WSM= White Seed Moringa of local origin; BSM-L= Black Seed Moringa of local origin;

Seasonal effect on Moringa production

The average HI was 20, 23, 31, 30, 29 and 29°F, and monthly total rainfall was reported as 12, 86.5, 332, 364, 130 and 28 mm during December-January, February-March, April-May, June-July, August-September, and October-November harvesting periods, respectively (Fig1), and the fresh tops yield (t/ha/cut) varied according to the variations in HI and rainfall in a year. The yield was the lowest (average 0.76 t/ha/harvest) during the dry (monthly total rainfall 12.0 mm) and cool (HI 20^{0}F) months (December to January) of a year. The yield peaked during the dry and hot period, from April to May (average 23.4 t/ha/harvest) with the rise of HI (31^{0}F) and rainfall (332 mm). A further increase in rainfall affected peak productions (17.2 to 22.4 t/ha/cut) during the hot and humid months of the year. Nouman *et al.* (2013) reported a suitable ambient temperature range of 27^{0}C to 35^{0}C in Nicargua. Moreover, Moringa can grow on a wide range of soils (Martin 2007) and may not compete with floodplain arable fertile land used mostly for staple food crop production.

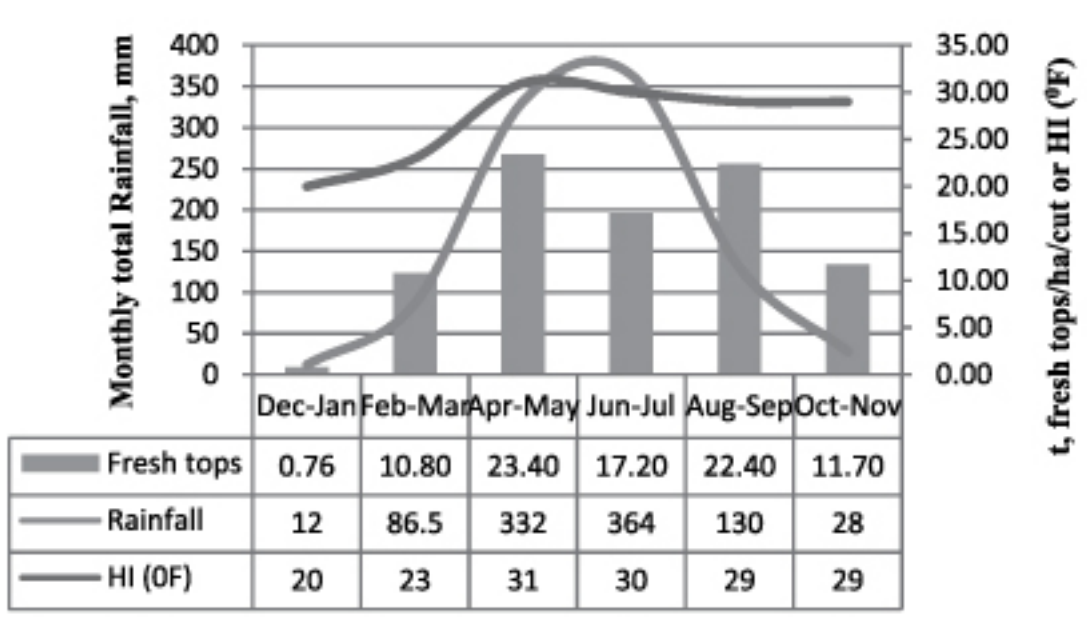

Fig1: Seasonal impacts on annual Moringa production

The daily RGR of all three cultivars was affected by seasons, and it varied from 0.61 mg to 2.88 mg in dry & cool months and rose to daily 8.86 mg to 13.26 mg in dry and hot months (Fig2). With the rise of HI and rainfall, the RGR of BSM-L was the highest (0.61 to 10.47) followed by BSM-T (1.03 to 8.86) and WSM (2.88 to 13.26).

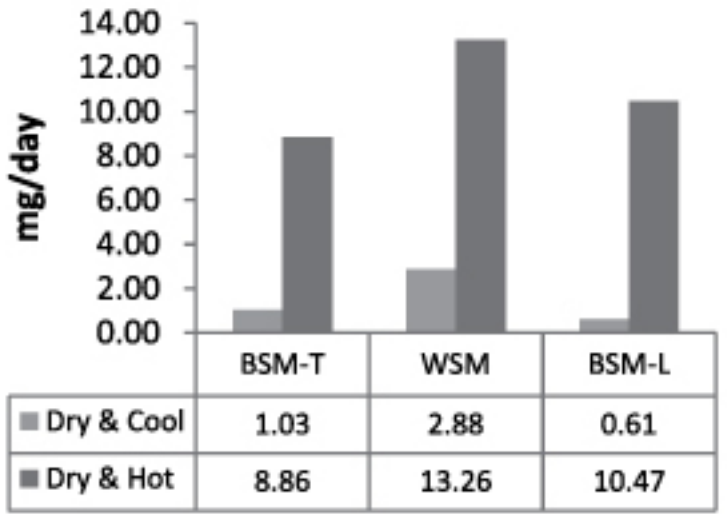

Fig2: Seasonal impact on Relative Growth Rate of Moringa

Thus, considering biomass production and its quality in terms of chemical composition and nutritional values to animals Black Seed Moringa may be cultivated as a plant fodder crop for the production of feed for ruminant animals.

ii). Feeding impacts of Moringafeed(M_f) on dairy cows

The replacement of concentrate mixture by Moringafeed had no effect on daily DM, CP or ME intake or daily milk yield of the cows (Table 2). But, the daily live weight gain of cows and their calves increased significantly ($P<0.05$) when Moringafeed was added to the control diet. It decreased blood cholesterol from 204.5 mg/dl in the control to 111.5 mg/dl in the cows fed T_2 diet, without showing any significant ($p>0.05$)

Table 2: Effect of supplementation of moringa feed on the nutrient intake and production of BCB-1 cows

Parameters	Experimental rations			Significance	
	T_0	T_1	T_2	Overall SE	Level
Total dry matter intake(kg/day)	5.8±0.3	5.9±0.4	5.6±0.4	0.2	NS
Total CP intake(g/day)	796.5±28.5	793.9±34.8	778.2±43.2	19.3	NS
Total ME intake(MJ/day)	52.1±2.1	52.9±4.6	49.9±3.7	1.9	NS
Ave.daily milk production(kg)	3.30±0.4	3.31±0.6	3.40±0.8	0.34	NS
Daily weight gain	206.8^{c}±27.6	246.0^{b}±30.8	300.6^{a}±4.2	20.7	*
Daily wt gain (g) of calves	321.4^{b}±84.4	370.8^{a}±96.4	369.9^{a}±25.0	40.7	*

change in fat, SNF, Lactose or CP content of milk. The relationship between ADF or NDF digestibility with % of Moringafeed (M_f) in the local mixed concentrate mixture is shown in Fig 1. It was revealed that the increase of Moringafeed to the treatment diet higher the digestibility of ADF or NDF. The ADF digestibility was linearly ($R^2=1$) increased with the increase of Moringafeed in the diet. The Moringafeed also effects the NDF digestibility ($R^2=1$). So, 100% Moringafeed is totally replaced the local mixed concentrate and also increased the feed digestibility.

iii). **On-farm production performances**

For evaluating cost effective on farm production of Moringa fodder, a 100 decimal cultivable land in Gaibandha district was leased in and a farmer was allowed to cultivate BSM-L following the agronomy being practiced on-station. The on-farm biomass production of BSM-L shows that the yield of the first two harvests was even better than that was found on station (26.67 ton vs. 19 ton). The land is being maintained for the production of Moringafeed and its cost effectiveness will be compared with the existing cash crops of the area. Its production on farm and manufacturing could be turned into commercial initiatives increasing the availability of climate-strss-reducing-feeds (CSRF) to farmer. Fostering the innovation may further the opportunity of sustainable industrialization of cattle feed.

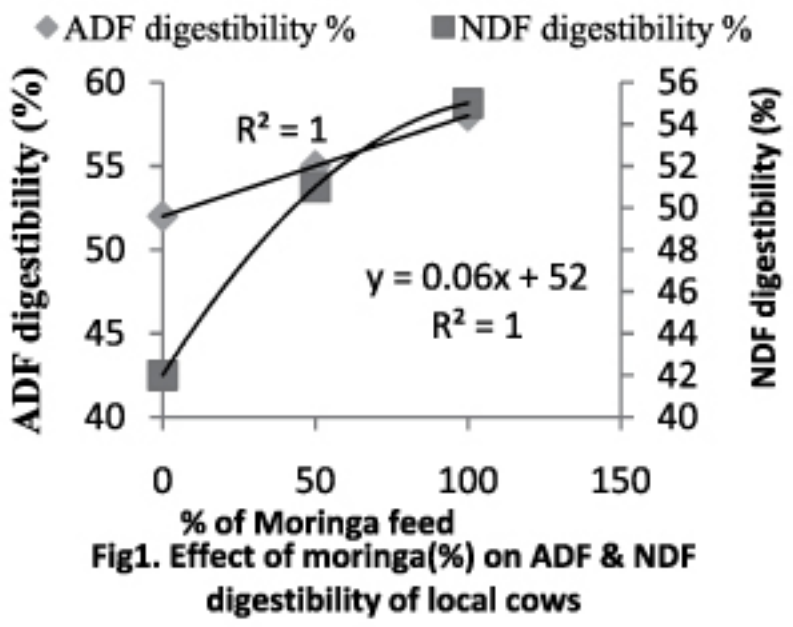

Fig1. Effect of moringa(%) on ADF & NDF digestibility of local cows

Conclusion

The result so far generated shows, Local Black Seed Moringa (BSM-L) is the suitable cultivar on the basis of quantity and quality and it may increase daily live weight gain of cows and calves with a significant reduction in milk cholesterol. However, the effect of Moringafeed on milk yield and quality needs to be determined through further research. The seasonal impacts on Moringa production on station and on farm needs to be determined through continuation of its cultivation in subsequent years.

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Dairy buffalo production system under semi-intensive condition at the Coastal area of Bangladesh

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Abstract
The study was conducted to investigate the management system, reproduction and production parameters using pre-tested questionnaire during the period from January to July 2017 at Bhola sadar upazila of Bhola district in Bangladesh. Thirty households of dairy buffalo farmers under semi-intensive system were selected and interviewed. The buffaloes were provided with very minimum housing facilities. Both the grazing and confinement time were found similar (around 6 hrs in a day) and they spent almost 3 hrs in a day for wallowing. Local green grass such as Helencha Enhydra fluctuens), Water hyacinth (Eichhornia Crassipes),Durba(Cynodon dactylon) and Dol (Saccrolepsis indica); dry roughage such as paddy straw (Oryza sativa) and kheshari (Lathyrus sativus) hay; and concentrate such as rice husk, wheat bran, pulse and oil cake were more usable feed resources. A total of 89, 100 and 57 percent farmers were provided dry roughage (straw), green roughage (local grass) and concentrate feed respectively to lactating buffaloes Among those, the average dry roughage, green roughage and concentrate feed intake were 15.59±7.46, 6.23± 4.01 and 1.62±0.75Kg DM/day/head, respectively. The average dry period (day), weaning age (day), service per conception (number), age at first pregnancy (month), age at first caving (month), calving interval (month), gestation period (month), post partum heat period (month) were 42.80±26.44, 201.88±77.63, 1.11±0.32, 35.37±9.44, 46.56±6.80, 14.21±2.09, 10.04±0.23 and 3.94±1.51, respectively. A total of 91.7%, buffalo cow received natural breeding whereas artificial insemination (AI) was followed only 4.2%. The highest number of the buffalo cows showed heat in early morning (54.2%), followed to early night (22.2%) and late night (9.3%). The average lactation length (days), average milk yield (litre/day) and lactation yield (litre/lactation) were 197.44±29.06, 2.39±0.83 and 469.52±163.71, respectively. Farmers sold 92% milk to Ghose and 8% used for home consumption. A total of 57.89% farmers received advance money (BDT 3000-10000) from Ghosh for monthly basis. Average technology adaptation capacity were 16.34,11.53,12.01,13.46,23.07,17.30 and 6.25% for practice on concentrate feed, urea-molasses straw/blocks, fodder crop production, Artificial Insemination, de-worming, vaccination and vitamin-mineral premix, respectively. But only 17.4 , 4.3 , 8.7 and 8.7% farmers fully adopted technologies for use of concentrate feed and Artificial Insemination, de-worming and vitamin-mineral premix. It therefore be concluded that technological transformation could be boost up to national milk production by developing of management practise of dairy buffalo under semi-intensive system.

Keywords: Dairy buffalo, Semi-intensive, Management practice, Coastal area, Technology

Introduction

The dynamics of buffalo production systems in South Asia Region transforming day by day due to increasing the population more rapidly specially in Asia for its emerging role in economic development (Dhanda, 2013). Buffalo production system varies widely in accordance with climate, soil and socio economic opportunities in Bangladesh (Saadullah, 2012). Buffalos are always grazed through long way of inland nearby in the case of extensive feeding practice in coastal areas. In these areas, approximately 22.23% of farmers' supplies only straw and 95.55 % straw with roughage and 8.87% of farmers' supplies only concentrate to their buffaloes. A total of 11, 5 and 84% farmers reared buffalo for milk purpose, meat purpose and milk and meat purpose, respectively (Nahar, et al., 2014). According to Saadullah (2011) in semi-intensive production system, buffaloes are kept mainly for specific purposes, i.e. either for milk or for meat production. It is an important livestock resource in several countries of South Asia and the Mediterranean regions. Buffalo is playing a leading role in the national economy by producing milk, meat and draught power in India and Pakistan. Out of the total milk produced in India and Pakistan, contributes about 57 and 68%, respectively (FAO, 2010). Due to high fat content of buffalo milk, it is the most preferred species and called Black Gold of Pakistan

(Bilal, et al., 2006). However, being the closest neighbour, Bangladesh has never included buffalo in policy level despite of their important role in the national economy. Climatic condition of Bangladesh is nearly similar to India and has lots of river and marshy lands that is favourable for raising buffaloes. Recently Bangladesh government, private sector and research organization has been given more emphasis on Buffalo production. The availability of milk in Bangladesh is only about 125.59 mL/h/d of about 49.76% (DLS, 2017). Whereas, the availability of milk in India, Pakistan, Nepal and Srilanka is about 227, 520, 140 and 142 mL/h/d, respectively (Khan, 2014). This figure indicates that Bangladesh need to give more emphasis on the milk production to fulfil the national demand. The indigenous dairy cows are low producers and the crossbred cow has the limitation regarding disease resistance, repeat breeding etc. Use of other mammalian livestock species for milk production could help to improve the scenario (Siddiki, 2017) and the success of India and Pakistan dairy industry based on buffalo milk might be a good indication for Bangladesh. However, in Bangladesh, buffaloes are low producers because of poor genetic potentialities, poor nutrition, delayed age at puberty, seasonality of breeding, longer calving interval, poor semen quality in males, high calf mortality and poor management practices (Shamsuddin et al., 2001; Qureshi, 2009). There are few reports available regarding the socio economic status of buffalo farmers, productive and reproductive performance of buffaloes, and management practices of buffalo in different localities of Bangladesh (Faruque et al., 1990; 1995; Sarker et al., 2013; Amin et al., 2015; Nahar, 2015). Nevertheless, still there is an ample space to contribute in strengthening the baseline data on buffalo production in Bangladesh. Therefore the present study was designed to investigate the reproduction and production characteristics along with existing management of dairy buffalo under semi-intensive system in Bhola district of Bangladesh.

Materials and Methods

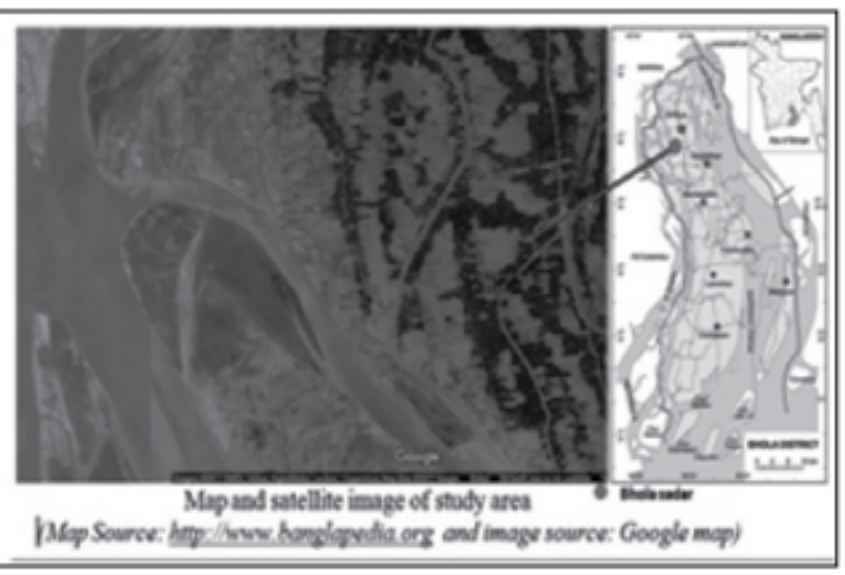
Farm management research involves collection of data from individual farmers. Farm survey data can be gathered by three methods these are: i) Direct observation ii) Interviewing respondent and iii) Records kept by respondents (Dillon and Hardaker, 1993). Among the methods, the interviewing and direct observation methods are completely well fitted to achieve the objectives of the study as the farmers of Bangladesh do not usually maintain records and accounts of their farm operation. The current study therefore follows the mixed methodology mode of Greene et al. (1989) focusing mainly on triangulation which can investigate the same issue with the same unit of analysis (Mitchell, 1986).

Location of study areas

The study area was Vhalu Miar Char, Sub-district: Bhola sadar, District: Bhola, Bangladesh. This is a coastal area of Bangladesh having high buffalo concentration (10.1-32.3 buffaloes/1000 people (Huque and Khan, 2017).Under the Köppen Climate Classification, Bhola is under Tropical Monsoon Climate (Wikipedia, 2017).

Table 1. Geographical information and satellite image of study area.

Name of the Sub-District	Location in Bangladesh	Human population density	Type of farm
Vhalu Miar Char, Bhola sadar	22.6903° N& 90.6525°E	480/km^2	Semi-intensive



Map and satellite image of study area
(Map Source: http://www.banglapedia.org and image source: Google map)

Farmer selection

Buffalo farmers were randomly selected from three villages, which have had at least one milking buffalo with calf and reared buffalo under semi-intensive management system. Farm visit, farm observation, secondary review, community discussion were held in the period of six moths which was from January to July 2017.

Preparation of questioner

Structured questionnaire was developed according to variable of the objectives. Variables like productive and reproductive performances, feeding and management practices with related problem for buffalo farming were considered. The questionnaire was pre-tested in the selected area. The preliminary schedule was edited on the basis of experience gained in pre-testing and then the questionnaire was finalized. The schedule contains both open and closed-ended question.

Data collection

Qualitative and quantitative information were collected from semi-intensive farm. Data were collected directly from 30 farms by the experience enumerators. Researcher monitored the enumerators and support as required by direct visit and mobile communication. Moreover, more than 40 milking buffalo farmers were directly contacted by the researcher for validation of the information. Discussion was also conducted with the cowboys (hired labours) who are responsible for the care and management of the buffalo and the farm as a whole, to confirm and recheck the information that were collected from the household buffalo farms which fulfil the triangulation methods.

Period of Study

Farm visit, farm observation, secondary review, community discussion were held in the period of six moths which was from January to July 2017.

Data Analysis

After collecting data from field, these were edited and coded. The data was then transfer to MS Excel 2007 for processing and summarizing. The tabular technique mainly used to analyze the data and derived meaningful finding by using simple statistical measures like mean, percentage and ratio by using SPSS16 version.

Results and discussion

Household composition

The average member was 5.91±2 per household of buffalo farmers. Figure2 demonstrate the household composition of semi-intensive buffalo farmers. In the household, the earning member, without earning member, school-college-university going children(students), children of school aged who are not attending school and children not of school aged were 27, 22, 23,9 and 19%, respectively. It is illustrated that earning members were more than non earning members but those non earning members involved in several household work as well as in buffalo husbandry, those members were mostly women, some were aged people. A total of 9% of school aged children not attending school, found to be a working force in household buffalo rearing under semi-intensive system.

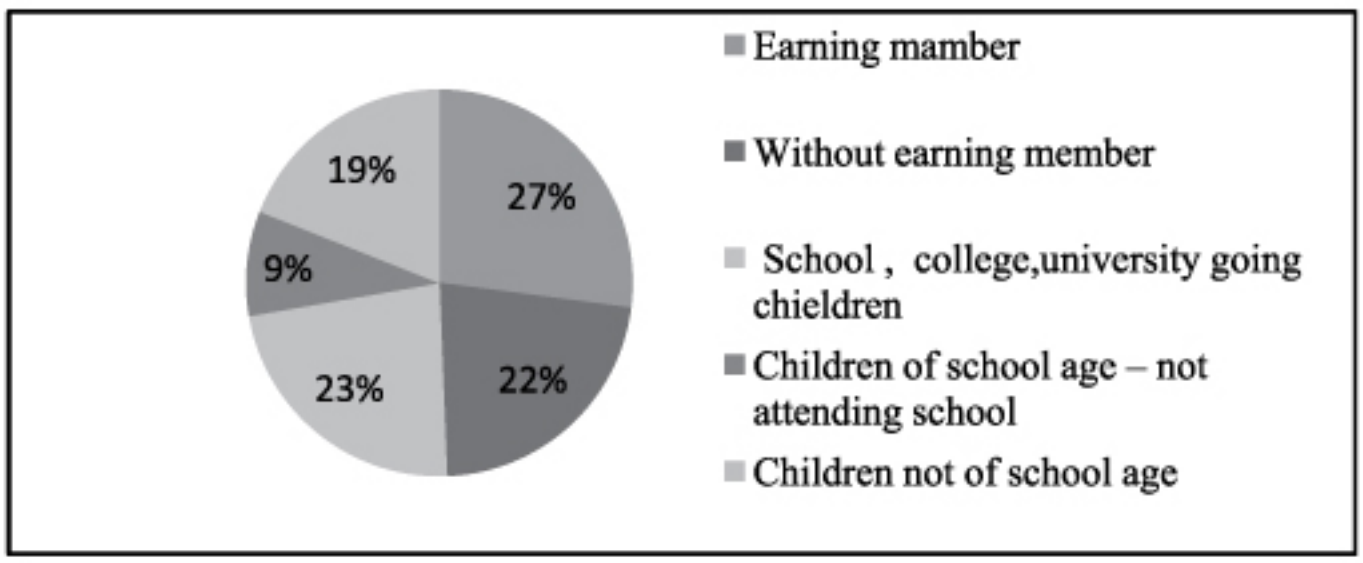


Figure 1. Household composition of buffalo farmer

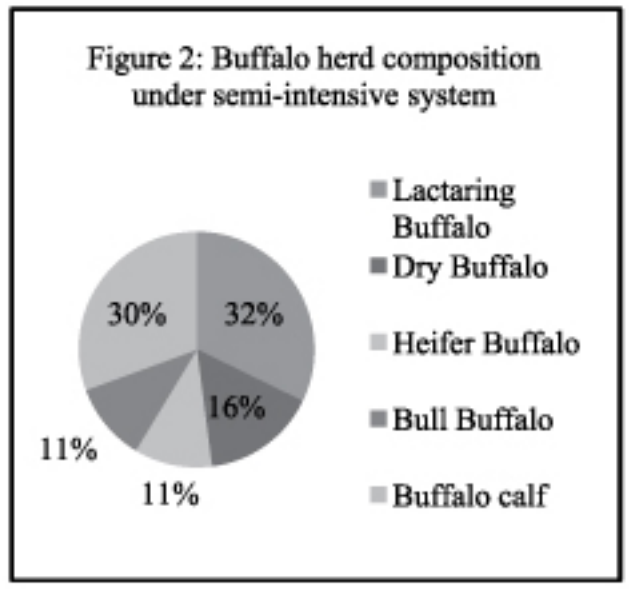
Buffalo herd composition

Farmers kept different type of buffalo in the herd. The average herd size was found 1.80±0.12. The average herd size in household subsistence farming (HF) and semi-intensive farming (SIF) buffaloes were about 1-3 and 4-15 animals, respectively (Huque and Borghese, 2013).The herd was composed mainly of lactating buffalo, dry buffalo, buffalo heifer, buffalo bull and buffalo calf. The average number of lactating buffalo, dry buffalo, buffalo heifer, buffalo bull and buffalo calf were 1.01±1.01, 0.78±1.72, 0.89±1.85, 1.06±1.62 and 1.02±1.94, respectively under semi-intensive system (Table 2). This result support to the findings of Uddin et al.(2016) who reported that 82% of the farmers having 1 to 3 buffalo in coastal areas of Bangladesh. Figure 2 demonstrated herd composition of buffalo farmers which illustrated lactating buffalo, Dry buffalo, Heifer buffalo, Buffalo bull and Buffalo calf were 32, 16,11,11,30%, respectively. Faruque (2000) also found that the percentage of milking buffaloes, dry buffaloes, heifer buffaloes, bulls and calves were 35,15,16, 2 and 32, respectively which is very similar of our finding though number of bulls were relatively few. But it was sufficient number bull for breeding purpose in coastal areas (Akbar et.al.2009).

Table 2. Average number of buffalo holding under semi-intensive system in Bhola district.

Type of buffalo	Mean±SD (N)	Min	Max
Lactating Buffalo (No/HH)	1.85±1.01 (39)	1	4
Dry Buffalo(No/HH)	1.72±0.78 (19)	1	3
Heifer Buffalo(No/HH)	1.8±0.89 (13)	1	3
Buffalo Bull (No/HH)	1.62±1.06 (13)	1	4
Buffalo calf (No/HH)	1.94±1.02 (37)	1	4
Overall	1.80±0.12		

N, Number of observation; SD, Standard Deviation



Housing system of buffalo

Different types of housing were seen in the study area. Farmers' constructed floor by brick, some shed has roof using tin or Golpata (Nipa fruticans). Most of the house had no boundary wall and roof. Buffalo were kept on the yard in open raised place at day-night. Uddin et al. (2016) observed that dairy buffaloes were kept under semi-intensive system in the wet season and only 15% farmers provided shed having only roof but no concrete floor. Akbar et al. (2009) mentioned that most of the dairy buffalo were not housed in extensive system of bathan areas but buffaloes which were in the plain land sometime had an enclosure, only roof made by straw or tin without wall and floor was always muddy. The average grazing time of buffalo was 6.04±1.52 hour in a day and rest of the time (5.91±1.70 hour/day) it was kept in confinement shed situated in household yard (Table 3). The average wallowing time of buffalo was found 2.78±1.34 hrs./day (Table 3). Saadullah (2012) described that about wallowing of buffalo in rural areas of Bangladesh and mentioned that usually the beast has its own wallow, but sometimes the whole village herd will lie down together in mud wallows remaining asleep or ruminating till evening. By about afternoon they emerge out of their mud wallows covered with grey slime and graze on road side and other aquatic herbage till late in the evening. After this, they move to dry grounds where they remain till the day-dark. Siddiki (2017) noted that majority (85%) of the buffalo farmers followed average grazing period of 6-8 h in a day and lowest grazing period (<6h) was found in Trishal and Lalpur Upozila (10%).

Table 3. Average scavenge, confinement and wallowing time (hrs/day).

Variable	Mean±SD	Minimum	Maximum
Grazingduration(hrs/day)	6.04±1.52	2	8
Confinement (hrs/day)	5.91±1.70	3	10
Wallowing (hrs/day)	2.78±1.34	1	6

Feeding system

The available feed base were straw e.g. Paddy straw (Oryza sativa; local green grass e.g. Dol (Saccrolepsis indica), Dubla (Cynodon dactylon), Halancha (Enhydra fluctuens), Sesbania, Water hyacinth (Eichhornia Crassipes) and kheshari (Lathyrus sativus); Concentrate feed e.g.oil cakes, wheat bran, pulses, rice polish, soybean and broken maize were found in the study area. Among those feed resources, local green grass such as Helencha (Enhydra fluctuens), Water hyacinth (Eichhornia Crassipes), Durba (Cynodon dactylon) and Dol (Saccrolepsis indica); Straw such as Paddy straw and kheshari hay ; and concentrate such as rice polish, wheat bran, pulses and cack were more usable by the farmers. Islam et al. (2002) studied on identification, screening and nutritive value of forages available throughout the Bangladesh and identified more than fifty different type of local green grasses from different Agro Ecological Zones (AEZs) in Bangladesh among which most of those were same found in this study. They noticed that baksha, lota, poa, khesari, beju, matikalai, kolmi, gamma, badam, durba, chailla, helencha, shama were mostly common and more potential native grasses. Name of available feeds and fodders fed to dairy buffaloes are presented in Table 4.

Table 4. Available feeds and fodders fed to dairy buffaloes.

Location	Feeds and fodders	Availability
Bhola sadar	Durba (Cynodon dactylon), Dol (Saccrolepsis indica) grass	Year round available but production is more in summer than winter
	Helencha(Enhydra fluctuens)	Available but production is more in rainy season
	Dhaincha (Sesbania spp.)	Available but production is more in summer than winter
	Water hyacinth (Eichhornia Crassipes)	Available but production is more in rainy season
	kheshari (Lathyrus sativus) hay	Seasonal but production is more in winter than summer
	Paddy straw (Oryza sativa)	Year round available
	Rice husk	Year round available
	Wheat bran	Late winter
	Broken maize	Year round available
	Pals	Late winter
	Mustard oil cake	Late winter
	Soybean cake	Late winter

A total of 89, 100 and 57 % farmers were provided dry roughage (straw), green roughage (local grass) and concentrate feed respectively to lactating buffaloes under this system. Among those, the average dry roughage, green roughage and concentrate feed intake were 15.59±7.46, 6.23± 4.01 and 1.62±0.75Kg DM/day/head, respectively. Nahar et al. (2015) found that 22.23% farmers supplied only straw, 95.55% farmers supplied straw with roughages, 8.89% farmers supplied only concentrate under extensive system in Bhola district. In the Ganges-Brahmaputra flood plain, dairy buffaloes were reared in a semi-intensive system in which farmer raised buffaloes with minimum inputs. They were allowed to graze on natural pasture or rod side in day time and were corralled at night (Akbar et al., 2009). The HF buffaloes were reared under stall feeding with 6-7 hours grazing and around backyard or public land with very little feed supply. The SIF buffaloes were raised in combination of seasonal based household during rice cultivation and free range system during common land free which was mostly upper part of coastal areas (Huque and Borghese, 2013). According to investigation of Uddin et al. (2016), in the household farming, after morning milking, buffaloes were allowed to graze in fallow or road side land up to evening that covers approximately 8-9 hours per day.

From the evening to next morning, animals were tied up in homestead and they were offered mainly rice straw with little concentrate mixtures (wheat bran, rice bran, rice polish etc.).

Table 5. Average feed intake (DMkg/day/head) of lactating buffalo under Semi-intensive system in Bhola district.

Items	Mean±SD	Minimum	Maximum
Concentrate feed	1.62±0.75	0	3
Straw	15.59±7.46	4	36
Green grass (cut and carry)	6.23±4.01	2	13

Productive and reproductive performance

The average lactation length of indigenous buffalo cows were 197 days under semi-intensive system (Table 6). But Karim et al. (2013) found 286 days of lactation period in similar kind of buffaloes under extensive production system in Mathbaria and Pirozpur, which was 290 days in Pathorghata and Barguna. The findings of this study were also more or less similar to the findings of other authors (Faruque et al., 1990; Faruque and Amin, 1995 and EI-Kirabi, 1995). The average milk yield (L/d) and lactation yield (L/lactation) were found as 2.39±0.83 and 469.52±163.71, respectively (Table 6). The average dry period (day),weaning age (day), service per conception (Number), age at first pregnancy (month) , age at first caving (month), calving interval (month) ,gestation period (month), post partum heat period (month) were 42.80±26.44, 201.88±77.63, 1.11±0.32, 35.37±9.44, 46.56±6.80, 14.21±2.09, 10.04±0.23 and 3.94±1.51, respectively (Table 6). Similar findings were also reported by other authors (Karim et al., 2013, Faruque et al., 1990 and Shabede et al., 2003).

Table 6: Productive and reproductive trait of lactating buffalo under semi-intensive system.

Parameters	Mean±SD	Minimum	Maximum	Highest frequency percentage
Lactation Length (d)	197.44±29.06	150	270	180 (37%)
Milk yield (L/d)	2.39±0.83	1	5	2.3(18.2%)
Lactation yield (L/lactation)	469.52±163.71	196	916	392(15%)
Dry Period (d)	42.80±26.44	30	90	30 (60%)
Weaning age(d)	201.88±77.63	7	365	180 (24%)
Service Per Conception (Number)	1.11±0.32	1	2	1 (88%)
Age at first Pregnancy (month)	35.37±9.44	3	48	34 (34%)
Age at first caving(month)	46.56±6.80	34	58	46(28%)
Calving interval (month)	14.21±2.09	11	18	16 (15.4%)
Gestation period (month)	10.04±0.23	10	11	10(80%)
Post Partum heat period (month)	3.94±1.51	2	6	3(29.2%)

L, liter; d, day.

Marketing

All of the farmers sold milk to Ghosh. About 57.89 percent farmers received advance money (BDT3000-10000) on monthly basis from the Ghosh and 42.11 percent farmer didn't receive the same. The higher milk selling price was BDT 55.71±5.34 for the farmer who didn't take advance from Ghosh but selling price was relatively low (BDT 52.72±4.10) who had taken advance (Table 7). It also noted that milk market was more volatile in case of the farmers who didn't take advance from Ghosh as CV% was higher (9.59%) than other (7.77%). The present finding is in agreement with the earlier findings of (Raha, 2010) who reported that price of milk was set below by BDT 5/liter in the case of advance payment.

Table 7 : Milk marketing scenario in Bhola district.

Group	% of farmers	Amount of advance money (BDT/Month) Min-Max	Average milk price price(BDT/L)	SD value	CV%
Farmers who taken advance	57.89	3000-10000	52.72	4.10	7.77
Farmers who didn't taken advance	42.11	-	55.71	5.34	9.59
Overall	100				

Technology adaptation

Adaptation levels of technologies for dairy buffalo farmers were very poor due to lack of public awareness and inadequate knowledge about buffalo husbandry (habitat, feeding, breeding & health care). Only 17.4 % farmer adopted for providing concentrate feed to their animal. Adaptation level of technologies e.g. use of urea-molasses straw/blocks, cultivation of fodder crops, use of Artificial Insemination(AI), practice for de-worming, practice for vaccination and provide vitamin-mineral premix from farmers were 0 ,0, 4.3,8.7,0 and 8.7%, respectively. Average adaptation capacity of technologies were 16.34, 11.53, 12.01, 13.46 23.07, 17.30 and 6.25% for use of urea-molasses straw/blocks, cultivation of fodder crops, use for AI, practice for de-worming, practice for vaccination and provide vitamin-mineral premix from farmers, respectively. Among those technologies, the capacity of technology was higher for practise of de-worming and vaccination though those were not followed regularly (Table 8). Creation of public awareness by different media; i.e. Radio, TV, Internet, Newspaper about the impotence of buffalo milk and meat as well as the good practice of dairy buffalo management might be improved the adaptation level of technologies.

Table 8. Technology adaptation of dairy buffalo farmers.

Technical intervention	Level of adaptation(Frequency)			Average capacity of individual technology (Per cent)
	Never	Tried but not adopt	Fully adopted	
Provide concentrate feed	69.6	13	17.4	16.34
Use of urea-molasses straw/blocks	95.7	4.3	-	11.53
Cultivation of fodder crops	91.3	8.7	-	12.01
Use for Artificial Insemination	82.6	13	4.3	13.46
Practice for de-worming	-	91.3	8.7	23.07
Practice for vaccination	34.8	65.2	-	17.30
Provide vitamin-mineral premix	52.2	39.1	8.7	6.25

Breeding

Recently government buffalo project has been started AI in Bhola district, but AI facilities were not available, even yet not popularized because of insufficient AI worker as well as unavailable of buffalo semen and low conception rate. Farmers didn't get AI worker in right time when buffaloes showed heat. Traditionally farmers kept buffalo bull to bred buffalo cows in these areas. It means that there was two breeding system followed in Bhola district. A total of 91.7% buffalo cow received natural breeding whereas, AI was covers only 4.2% of buffalo cows. Akbar et al. (2009) reported that the sufficient number of breeding bull was kept by the farmer in coastal areas and there were no fertility problem. However, inbreeding and abortion due to use same bull was common. He also noted that Low oestrous detection (i.e. 30%) is a major challenge that limits efficiency of AI use. According to farmer's observation, buffalo cows showed heat in different time. A few number of buffalo cows (4.2-5.2%) showed heat in day time but higher number of the buffalo cows showed heat in early morning (54.2%), followed to early night (22.2%) and late night (9.3%)

(Table 9). Saadullah (2012) stated that the buffaloes show their maximum activity during the night and breeding take place mainly during night. Therefore present study has been supported to the information of Saadullah (2012) though a few number of buffalo cows showed heat in day time (4.2-5.2%).

Table 9. Type of breeding and time of heat showed by buffalo cows.

Variable	Value
Type of breeding	
Natural breeding (% of buffalo)	95.9
Artificial insemination (AI) (% of buffalo)	4.1
Time of showing heat	
Morning (% of buffalo)	54.2
Noon (% of buffalo)	4.8
Afternoon (% of buffalo)	5.2
Evening (% of buffalo)	4.2
Early night (% of buffalo)	22.2
Late night (% of buffalo)	9.3

Calf management

Snuff-short of buffalo calf management shown in below Table 10. Most of the farmers kept buffalo calves beside of mother shed near to farmer home. Calves passed time in open place in a day. Few farmers kept newborn calf with lactating mother. Farmers raised the pith of the shed and using straw as bedding materials for calves. Mainly colostrums and milk were used for feeding of new born calf. After one month later, farmers stared to provide soft new growing green grass, wheat bran, rice bran and some farmers additionally supplied molasses to the calves. All farmer fed colostrums to buffalo calf and 17% farmer used separate manger. Buffalo calf fed colostrums 1.60±0.82 litre/day maintainingwith2.30±1.32 hours' interval. A total of 13.04 % farmers provide concentrate feed to calves. Amount of average concentrate mixture provided to buffalo calves were150-200 gm/day and percentage of calf mortality were18.60±20.52 (Table 10). The buffalo calves in those farms suffered from a number of diseases finally succumbing to death. The most common and frequent occurring disease in calves was diarrhoea. Besides these diseases calves were also suffering with other disease like skin diseases, eye infection, parasite infestation (endo & ecto), naval ill and pneumonia.

Table 10. Management of buffalo calves.

Parameter	Value
Frequency of colostrums feeding	100%
Providing water by separate manger (% of farmer)	17.4
Use of vaccine (% of farmer)	56.5
Average amount of colostrums feeding (Litre/day)	1.60± 0.82
Average of interval of colostrums feeding(hrs)	2.30±1.32
Use milk replacer	None
Providing concentrate mixture (% of farmer)	13.04
Amount of concentrate mixture(gm/day)	150-200
Calf mortality (%)	18.60±20.52

Hygienic measure followed for milking

All of the farmers tried to follow some hygienic measure for milking. Hand milking is usually followed in the study areas. Farmers washed their hands, milking pots and also cleaned milking parlour/platform before milking. All of the farmers sucked udder of buffalo by the calf for stimulating hormonal affects before starting milking. Moreover, the udder health situation of milking buffaloes was aggravating as mastitis was rising as of the notification of farmers. Islam et al., (2016) isolated pathogens (i.e. Coagulase Negative

Staphylococci (CNN), *Staptococcus spp*. and Bacillus spp. and *Staphylococcus aureus*) from milk sample in Bangladesh including coastal areas that were responsible for mastitis in lactating buffaloes.

Waste management

Manure was kept to the pit in the household yard (HHY). The house was cleaned once a day and waste kept besides the house. Manure used as organic fertilizer after decompose. An adult buffalo produced 4-6 tons/year of wet manure plus additional urine as bio-fertilizer which reduced the requirement of chemical fertilizers as well as provided soil humus that chemicals could not provide (Hamid et al., 2016).Manure pit was situated 2-20 (min-max) feet distance from the shed. Some people (22%) did not followed good practice for waste management. They kept manure near to the shed.

Conclusion

The productive and reproductive performance of indigenous dairy buffalo was not similar to high producing buffalo milk producer's countries (i.e. India and Pakistan). Scientific management and improved breeding system could minimize this situation. Optimum use of feed resources and their treatment whenever needed becomes evident from the present study. Improved adaptation level of technologies would be ensured by creating public awareness and by providing door-step service to the buffalo farmers. It is therefore, can be concluded that technological transformation is important to boost up of the productivity of the dairy buffaloes under semi-intensive system.

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Zoonosis

Molecular characterization of *Bacillus anthracis* from soils in selected districts of Bangladesh

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Abstract

Bacillus anthracis is the causative agent of anthrax which is zoonotic disease. Molecular techniques were used to confirm the presence of *Bacillus anthracis* bacteria in soil. Soils (n=128)samples from Sirajganj, Pabna, Kushtia and Mymensingh districts were tested of which the bacterium was isolated from 21 soils and all the blood and meat samples by standard cultural, morphological, and molecular techniques (PCR) in the period of June 2016 to May 2017. Gene sequencing of one isolates was done commercially and phylogenetic trees were constructed with the MEGA5 (www.megasoftware.net) software. After genome sequencing it resembles with full length 16S rRNA gene of*Bacillus anthracis.* Phylogenetic analysis of 16S rRNA gene of*B. anthracis* Sirajganj-1revealed that the organism is almost similar to other established strains of *B. anthracis*available in Genbank.Genbank accession no. of *B. anthracis* Sirajganj-1 is MF353929.

Keywords: Anthrax; zoonosis; PCR; Genbank; Bangladesh.

Introduction

Gram positive *Bacillus anthracis* bacterium is the causative organism of anthrax which is acute infectious, soil borne, toxigenic, large rod shaped, non-motile, only obligate pathogen within the genus bacillus, spore forming and 1.0-1.5 × 3-10 µm in size (Gomes *et al.,* 2010). The endospores of this bacterium is highly resistant and if conditions are right through environmental contaminations and disseminations, maintain the bacterium in the environment outside of living hosts for decades between epidemics. Neutral to slightly alkaline soil, calcareous, vegetative or humic rich soil, drought followed by heavy rainfall, long day length, high mean ambient temperature, opened dead animals without burning, flooding pastures with contaminated water or dumping infected carcasses in streams, ponds or rivers, grazing animal on infected pastures, contaminated feed, feeding animals with uprooted and unwashed grass, feeding water hyacinth, decreasing of vulture population and slaughtering sick animals are commonly known risk and environmental factors of Anthrax outbreak (Van Ness and Stein 1956; Kasianenko*et al*., 1984; Kaufman, 1990; Titball*et al*., 1991; Gates *et al*., 1995; Moazeni-Jula*et al.,* 2004; Oaks *et al*., 2004; Biswas*et al*., 2011).

Every year sporadically outbreaks of animal anthrax occurred in some districts of Bangladesh which is followed by human anthrax later. Now it is believed to be enzootic disease of Bangladesh. Published reports about existence of anthrax in animals in Bangladesh date back to 1948. Anthrax was periodically reported both in animals and humans in Bangladesh between 1949 and 1986. Mustafa (1984) isolated anthrax bacillus from Elephant in Bangladesh. Samad and Hoque (1986) mentioned the occurrence of anthrax in 62 cattle and 27 humans in Pabna milk shed areas of Bangladesh during 1980-84. Reported and published findings shows that there were 450 animal cases and 725 human cases in Bangladesh during 1980 to October 2010 (Ahmed *et al*., 2010), 1320 cutaneous anthrax cases were found until July 2016 (Islam *et al*., 2017).

In Bangladesh, the disease is more prevalent in the Kushtia, Sirajganj, Pabna districts which contained the

country's largest cattle populations. *B. anthracis* forms spore in unfavorable conditions, which is resistant to heat and most chemical disinfectants, and this spore may persist in soil as viable for several decades (Dragon *et al*., 2001). Soil is a potential associate of spore survival and ecological conditions of repeated outbreak of anthrax (Minett, 1950; Meth *et al*., 2002). This is especially so in tropical and sub-tropical environments including Bangladesh. The actual environmental risk factors of anthrax outbreak are not well established in any part of the world so far and no previous work has done in Bangladesh on soil for repeated anthrax outbreak. Molecular techniques are the best method for rapid and confirmatory detection of *Bacillus anthracis* from soil (Nazir*et al*., 2015; Patra*et al*., 1998).

Evidence indicated that anthrax targets endothelial cells causing vascular leakage of fluid and cells and ultimately hypovolemic shock. The disease occurs worldwide. In some countries, specific areas are known to favor the survival of anthrax bacterium spores in the soil and are thus subject to occasional outbreaks. *Bacillus anthracis* spores can remain viable and inactive in the soil for many years. During this time, they are a potential source of infection for grazing livestock but generally do not represent a direct infection risk for humans. After entry into the host, the spores of *Bacillus anthracis* germinate and yield capsulated, toxin producing bacilli. Septicemia and toxiemia rapidly lead to death in infected mammals. The virulence of the Bacillus is partly due to the production of a tripartite virulence factor: protective antigen (PA), lethal factor (LF) and edema factor (EF). These factors together kill phagocytes, damage capillary walls and interfere with blood clotting leading to edema, shock and death. The bacilli are covered by an antiphagocyticpolyglutamic capsule. The capsule covers an S-layer sequentially composed of two distinct proteins. Recently, PA was also found to be associated with spores (Chabot *et al*., 2004).

As emerged zoonotic disease its immense need to detect the anthrax bacteria and molecular methods is a confirmatory method to detect the bacterium. It will be very helpful then to better predict about the presence of anthrax pathogen locally and increase the value of public health measures.

Materials and Methods

Sample collection

The research work was conducted during the period from June2016 to May 2017 at the Department of Microbiology and Hygiene, Faculty of Veterinary Science (FVS), Bangladesh Agricultural University (BAU), Mymensingh-2202, Bangladesh. A total of 128 soil samples and 3 clinical specimens were collected randomly from anthrax positive reported or anthrax suspected carcass disposal sites or burial sites, livestock habitats and livestock pasturing sites of the study regions(Sirajganj, Pabna, Kushtia). The collected environmental samples, which contained soil, animal feces, bones and remnant of carcass. Approximately 400 gm of surface soil to a maximal depth of 2 feet were collected using a spade and transferred to double layered plastic bags cautiously and tagged. One blood sample was collected from anthrax suspected dead cattle at Sujanagarin Pabna in a 5 ml plastic syringes aseptically and cautiously and transferred to the laboratory. Two meat samples were collected in nutrient broth from anthrax suspected dead cattle at Ullahpara in Sirajganj and brought to the laboratory at the Department of Microbiology and Hygiene, BAU.

Cultural identification

Primary growth was performed by inoculation into selective media (PLET) and incubated at 37^0C for overnight. In the laboratory 1 gm of each soil sample in sterile distilled or deionized water was processed through heating at 80±5°C for 30 minutes by using the hot water bath and 90-100 µl plated on to selective PLET medium (HIMEDIA,India). The colonies show typical cultural characteristics of *B. anthracis* on PLET medium. The positive samples were also sub- cultured in some others bacteriological media (nutrient agar, bovine blood agar, horse blood agar and nutrient broth to observe the colony characteristics. Staining characteristics were observed by Gram staining method.

Method of extraction of genomic DNA by boiling method

The genomic DNA of each *B.anthracis*isolate was extracted by boiling method. Single colony of each isolate was inoculated into 200 µl of distilled water followed by boiling for 10 min. After boiling the samples were

immediately kept on ice for few minutes for cold shock. Finally, centrifugation was done at 10000 rpm for 10 min. The supernatant was collected and used as DNA template for PCR.

Amplification of *Sap*, Protective antigen(PA) of *pX01*, Capsule(*pX02*), 16SrRNAgenes in *B. anthracis* by PCR:

To amplify Sap gene of *Bacillus*genus specific primers were used (Table 1). The total volume of PCR mixture was 25 µl consisting of 12.5 µl PCR master mixture, 2 µl of each primers, 4 µl of template DNA, nuclease free deionized water 4.5 µl. The thermal profile of PCR for Sap, Protective antigen (PA) of pX01, Capsule (pX02) were 94◦C, 2 min for initial denaturation, 94◦C, 30 sec for denaturation, 52◦C, 30 sec for annealing, 72◦C, 1 min for elongation and 72◦C, 5 min for final extension and the holding temperature was 4◦C. The thermal profile of PCR for 16SrRNA was 95◦C, 2 min for initial denaturation, 95◦C, 30 sec for denaturation, 50◦C, 40 sec for annealing, 72◦C, 1.5 min for elongation and 72◦C, 10 min for final extension and the holding temperature was 4◦C.

Table1. Primers used in this study with sequences

Primer Name	Gene Targeted	Primer Sequence (5´-3´)	Amplicon size (bp)	Reference
Sap-1	Sap in genomic DNA	CGCGTTTCTATGGCATCTCTTCT	639 bp	Cheun*et al.* (2003)
Sap-2		TTCTGAAGCTGGCGTTACAAAT		
Pag-5	Protective antigen(PA) of pX01	TCCTAACACTAACGAAGTCG	596 bp	Ramisse et *al.* (1996)
Pag-8		GAGGTAGAAGGATATACGGT		
Cap-1	Capsule(pX02)	CTGAGCCATTAATCGATATG	846 bp	Ramisse et *al.* (1996)
Cap-2		TCCCACTTACGTAATCTCTTCT		
8 F	16S rRNA gene	AGTTGATCCTGGCTCAG	1504 bp	Claudio *et al.* (2002)
1492 R		ACCTTGTTACGACTT		

Sequencing of PCR products

For purification of PCR product, PCR Purification Kit(Qiagen, USA) was used according to manufacturer's instructions. About 1 µl of the purified PCR product was electrophoresed on 1% agarose gel to examine the status of purified DNA. The purified PCR products of the isolates were sequenced directly from both directions using PCR primers from an overseas commercial sequencing laboratory (1st BASE, Malaysia). The raw sequence data were first checked for its quality and then edited and assembled with the MEGA5 (www.megasoftware.net) software. The sequences generated in the present study were aligned with other related sequences downloaded from the GenBank using MegAlign (DNASTAR Inc., USA) and MEGA5 (www.megasoftware.net) software. Multiple alignmentswere done with Clustal W algorithm and Maximum likelihood phylogenetic trees were constructed with MEGA5 program.

Results and Discussion

Initially, the *B. anthracis* were screened on the basis of characteristics colony morphologies on PLET agar. Out of 128, 21 samples were suspected as *B. anthracis* based on Growth of rough creamy-white 1-2 mm size colony in PLET agar medium colonies. Each sample was then sub-cultured onto blood agar. All 21 suspected *B. anthracis* isolates produced non-hemolytic grey colonies in horse blood agar. The pure cultures of suspected *B. anthracis* isolates were subjected for Gram staining. In Gram's staining method, the organism revealed gram-positive, violet color, large rod shaped, single, paired or long chained in appearance.

A total of 21 isolates were confirmed as *B. anthracis*by amplifying genus specific Sap primers. Out of 21

isolates of *B. anthracis* 11 samples were found positive for pX01 gene (Figure2; Table 3) and 7 samples were found positive for pX02 gene (Figure 3; Table 3).

Table-2: Prevalence of *B. anthracis* in examined soil and clinical samples

Sampling area	Sample type	Examined (N)	Positive (N)	Prevalence (‰)
Suzanagar, Pabna	Soil	20	8	40
Doulotpur, Kushtia	Soil	28	3	10.71
Mymensingh	Soil	50	0	0
Ullapara, Sirajganj	Soil	30	10	20
Total		**128**	**21**	**16.40**

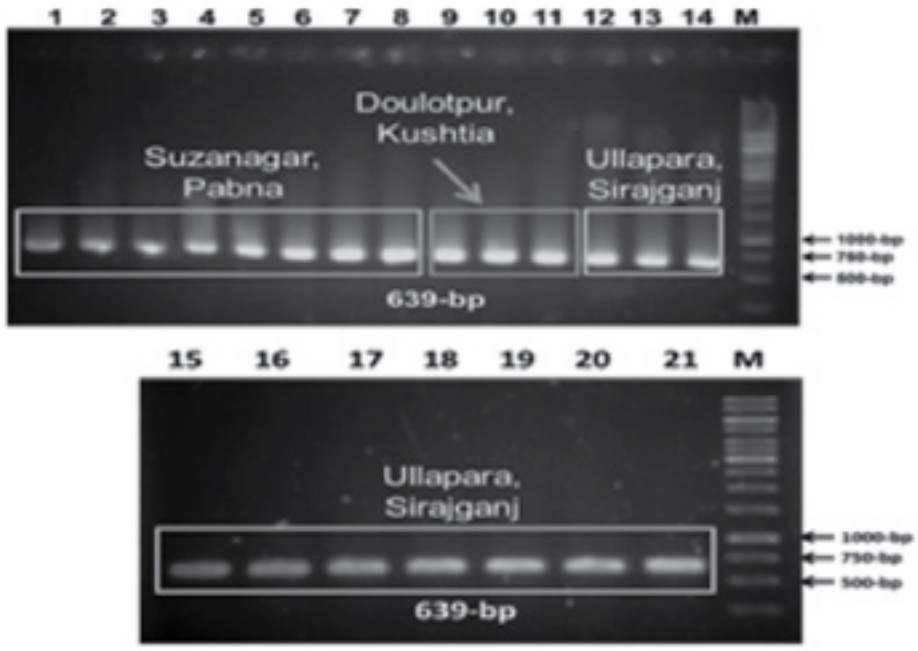

Figure 1. Amplification of Sap gene (639bp) specific genomic primer; Lane M: 1 kbp DNA ladder, Lane 1-21: positive for *Sap* gene.

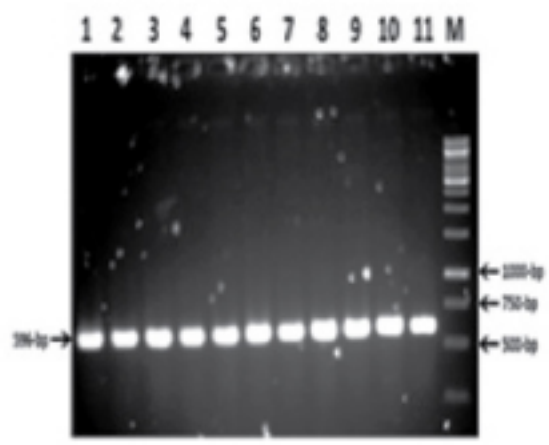

Figure 2: Amplification of pX01 gene (596 bp) specific primer; Lane M: 1 kbp DNA ladder, Lane 1-11: positive for pX01 gene.

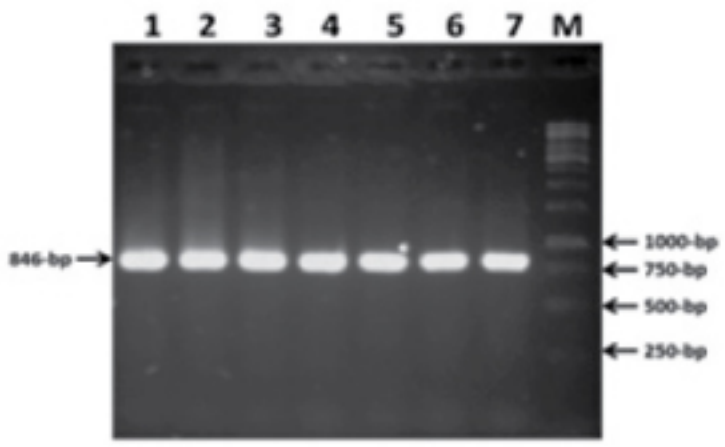

Figure 3: Amplification of pX02 gene (846 bp) specific primer; Lane M: 1 kbp DNA ladder, Lane 1-7: positive for pX02 gene.

Table-3: Summary of pX01 and pX02 plasmids in *B. anthracis* from different locat

Location	**Samples collected**	**Positive by PCR**	**pX01**	**pX02**	**pX01+pX02**
Suzanagar, Pabna	20	8	3	4	1
Doulotpur, Kushtia	28	3	2	1	-
Mymensingh	50	-	-	-	-
Ullapara, Sirajganj	30	10	6	2	2
Total	**128**	**21**	**11**	**7**	**3**

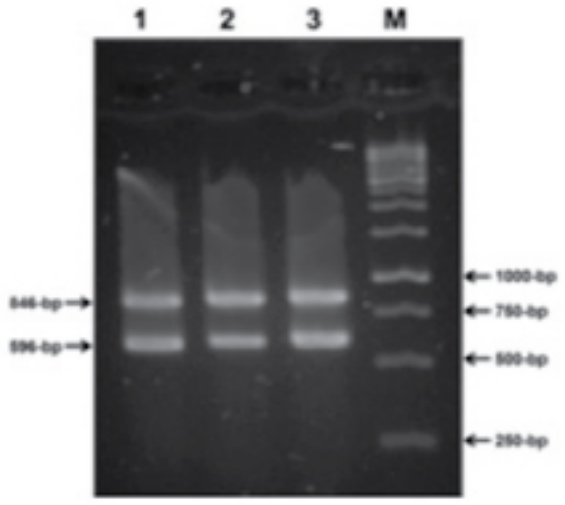

Figure 4: Amplification of pX01 andpX02 gene specific primer; Lane M: 1 kbp DNA ladder, Lane 1-3: positive for pX01 andpX02 gene.

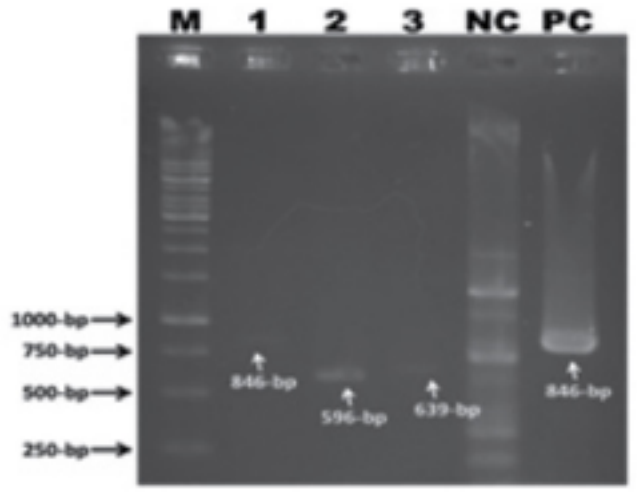

Figure 5: Amplification of *B. anthracis* specific gene (*Sap* gene), *pX01* gene (*Pag*gene),*pX02*gene (*Cap* gene) of *B. anthracis*.Lane M: 1 kbp DNA ladder; Lane 1:*pX01 plasmid* specific gene (*Pag*gene); Lane 2:*pX02 plasmid* specific gene (*Cap* gene); Lane 3: *B. anthracis* specific gene (*Sap* gene) with amplification size 639 bp; Lane NC: Negative control; Lane PC: Positive control.

In this research there were 21(16.40%) *B. anthracis* isolates were isolated by selective PLET agar medium from 128 soil samples. Among the positive samples, the highest percentage 40% was from Pabna district. The deposition of anthrax bacteria in soil has been examined in details in several investigations. After the deliberate contamination of the Gruinard Island during the World War II, sampling was regularly performed during 40 years the spores were, almost without exception, isolated from the top 6cm of soil (Manchee*et al.,* 1981). Similar studies were carried out in northern Canada and south Sudan and Isfahan in Iran (Dragon *et al.,* 2001; Ramachandran*et al*., 1988 and Moazeni-Jula*et al*., 2004). Moazeni-Jula*et al*. (2004) isolated 9 (15%) isolate from 60 soil specimens. Dragon *et al.* (2005) cited the theoretical sensitivity of isolation of *B. anthracis* in PLET medium was 10 spore/g of soil sample. All the works of this study conducted for the isolation and identification of *B. anthracis* from soil samples are matched with the mentioned results of "Manual for Laboratory Diagnosis of Anthrax" by World Health Organization (WHO, 2003) and "OIE Terrestrial Manual 2008" by World Organization for Animal Health (OIE) and "Basic Laboratory Protocols for the Presumptive Identification of *Bacillus anthracis*" by Centers for Disease Control and Prevention (CDC, 2001).

Among the 21 positives isolates, 1 sample (termed as Sirajganj-1) was sequenced from main source "1st BASE, Malaysia" by using 16S rRNA gene containing forward and reverse primers with an amplicon size 1504-bp. The sequence of the 16S rRNA gene has been widely used as a molecular clock to estimate relationships among bacteria (phylogeny), but more recently it has also become important as a means to identify an unknown bacterium to the genus or species level. The 16S rRNA gene sequences of *B. anthracis* have high levels of sequence similarity (>99%) that support their close relationships shown by DNA hybridization. Therefore, the phylogenetic analysis of 16S rRNA gene of*B. anthracis* Sirajganj-1revealed that the organism is almost similar to other established strains of *B. anthracis*.

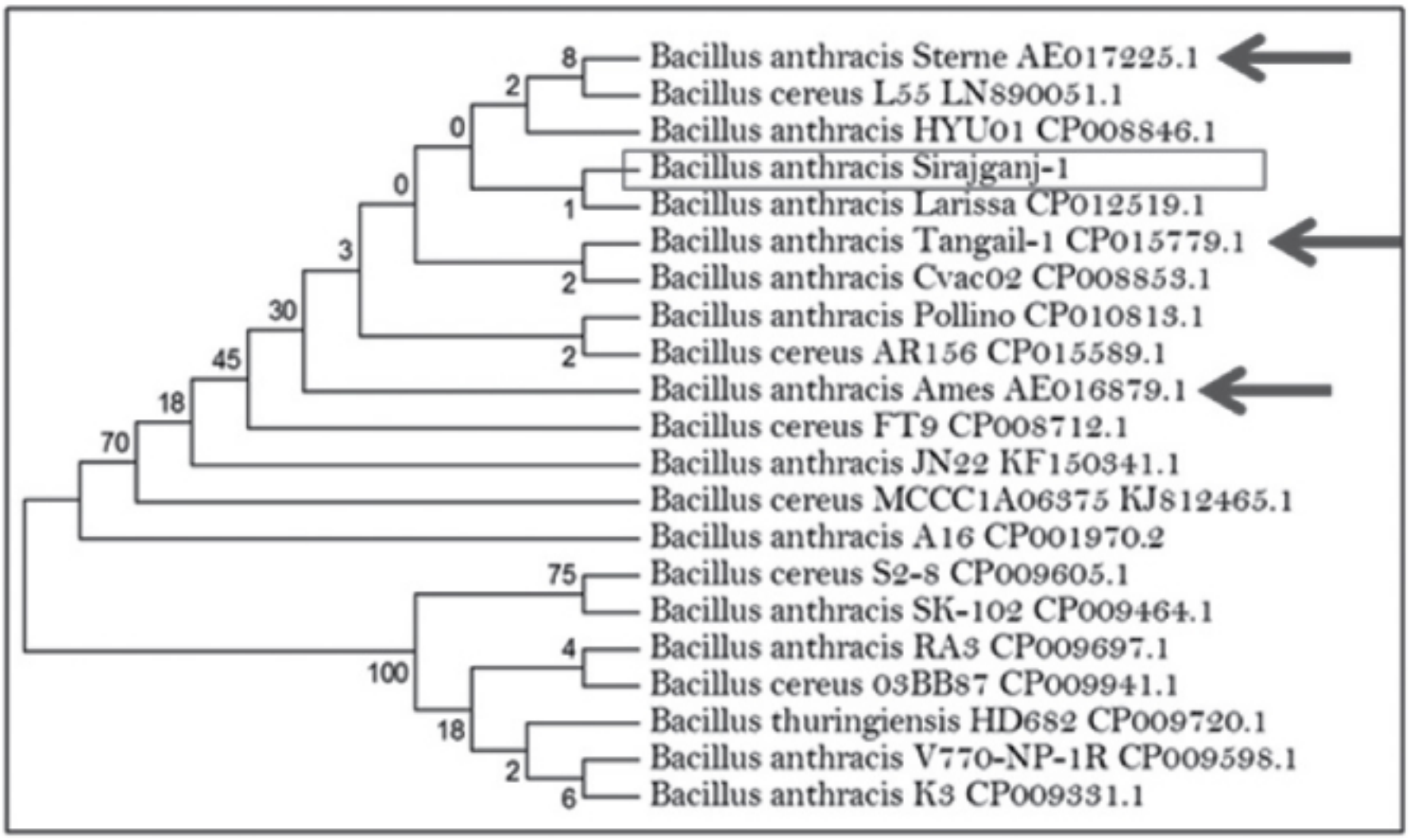

Figure 23: Phylogenetic analysis of 16S rRNA gene of *Bacillus anthracis.* Red rectangle indicates our isolate.**GenBank accession no. MF353929**

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Campylobacter species from Bangladesh Agricultural University dairy farm: isolation, molecular identification and antimicrobial susceptibility profiles

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Abstract

Campylobacters are generally regarded as the most common bacterial cause of human gastroenteritis worldwide and the species *C. jejuni* is responsible for 80% to 93.4% of the human campylobacteriosis cases depending on different geographic areas. Several studies revealed that ruminants may play an important role in the epidemiology of this zoonosis.The study was conducted for isolation, identification and characterization of *Campylobacter* species from Bangladesh Agricultural University dairy farm during the period of January to June, 2016. A total of 80 samples (faecal samples of calves, heifers and cows; milk samples of cows) collected from Bangladesh Agricultural University dairy farm were subjected to bacteriological isolation and identification by using cultural, biochemical and molecular techniques. Furthermore, the isolated bacteria were characterized by antimicrobial susceptibility test. *Campylobacter* like organisms were presumptively identified in 20 samples. Out of 20 positive *Campylobacter* isolates, 14(17.5%) were *Campylobacter jejuni* and the rest 6(7.5%) were *Campylobacter coli*. The *Campylobacter* species were isolated and identified by culturing on blood agar base no- 2 with 5% sheep blood, where *Campylobacter* spp. produced gray color spreading colonies. Isolates were biochemically positive to catalase and oxidase tests and in hippurate hydrolysis test some of the isolates (n=6) did not develop any purple color that indicated the isolates were *C. coli* and some of the test isolates (n=14) developed purple color that indicated the isolates were *C. jejuni*. All *Campylobacter* isolates were positive by 16s rRNA gene based PCR. Antimicrobial susceptibility test was performed to know the susceptibility and resistance patterns of the isolates to different antimicrobial agents. *Campylobacter jejuni* were resistant to amoxicillin, erythromycin, azithromycin and susceptible to gentamicin, ciprofloxacin, norfloxacin and streptomycin. Furthermore, *Campylobacter coli* were resistant to amoxicillin, erythromycin and susceptible to gentamycin, ciproflaxacin. Out of 20 *Campylobacter* isolates, 100% of the isolates were detected as multidrug resistant.

Key Words: dairy farm; Campylobacter species; molecular identification; antimicrobial susceptibility profiles

Introduction

Campylobacter spp. are Gram negative, microaerophilic, curved or spiral rods shaped under the family Campylobacteriaceae (Brenner and Krieg, 2006). At least a dozen of *Campylobacter* spp. have been associated with human disease and the most common are *Campylobacter jejuni* and *Campylobacter coli* (Ryan and Ray, 2004). Human infection may occur by direct contact with infected animals or by consumption of contaminated unpasteurized milk, dairy products, untreated water and raw meat and animal and domestic birds have been considered as one of the most important reservoirs of food- borne infection for humans (Mezher et al., 2016). The importance of milk as a source of human gastroenteritis caused by *Campylobacter* spp. was confirmed by the European Union summary report on food-borne disease outbreaks (European Food Safety Authority, 2015). It is assumed that contamination of raw milk derived most commonly by

Campylobacter spp. from secondary fecal contamination during the milking process (Oliver et al., 2005). The consumption of raw milk has been the most important source of *Campylobacter* infection (Heuvelink et al., 2009). Longer life span of dairy cattle than beef cattle can lead to long-term shedding of *Campylobacter* spp. and these cattle serve as a long-term reservoir (Hakkinen et al., 2007). Indirect exposure to cattle faeces through environmental contamination is considered a high risk to humans (Devane et al., 2005). The ideal environment for optimal growth of *Campylobacter* spp. requires an atmosphere containing approximately 5% O_2, 10% CO_2, and 85% N_2 at 37°C to 42°C (Corry et al., 2003). The selective blood-containing agar are recommended medium that are used for culture of *Campylobacter* spp. (Corry et al., 2003) although alternative media may be used. *C. jejuni* may remain viable for up to 9 days in feces, 3 days in milk (Silva et al., 2011). Contamination of raw milk with *Campylobacter* spp. mainly associated with fecal contamination (Park, 2002).

The concerns for human health are the inappropriate use of antibiotics in cattle production and the development of antimicrobial resistant strains of bacteria. The increasing rate of human infections caused by antimicrobial-resistant strains of *Campylobacter* spp. makes more difficult to clinical management of cases of campylobacteriosis (Levin, 2007). *Campylobacter* spp. with resistant to antimicrobial agent has been reported in many parts of the world (Chen et al., 2010; Kabir et al., 2011; Kabir et al., 2015).

In Bangladesh several studies such as occurrence, molecular detection and antibiotic sensitivity test of *Campylobacter* spp. in poultry have been performed (Kabir et al., 2014; Kabir et al., 2015). However, there are no documented reports exist yet on the occurrence and antimicrobial resistance of *Campylobacter* spp. in dairy farm where milk is widely consumed in Bangladesh. Therefore, the present study was performed to isolate and identify *Campylobacter* spp. inhabiting faeces and milk originating from Bangladesh Agricultural University dairy farm and to assess antibiogram profiles of the isolated *Campylobacter* spp.

Materials and Methods

Collection and transportation of samples

A total of 80 samples (60 faecal samples and 20 milk samples) were collected from BAU dairy farm during the period of January to May, 2016. Then it was immediately brought to Bacteriology Laboratory of the Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh through cool chain maintaining. After that, the samples were processed immediately for the isolation and identification of *Campylobacter* spp.

Isolation of *Campylobacter* spp.

Isolation of *Campylobacter* spp. were carried out by filtration method (0.45 µm filter; Biotech, Germany) as described by Shiramaru et al. (2012).

Identification of *Campylobacter* spp. by biochemical tests

Differentiation of isolated *Campylobacter* spp. with supporting growth characteristics were subjected to various biochemical tests such as catalase, oxidase and hippurate hydrolysis test according to the procedures followed by Foster et al. (2004).

Preparation of DNA templates

DNA templates were prepared with boiling method described by Hoshino et al. (1998).

16S rRNA gene based PCR for identification of the genus *Campylobacter*

The 16S rRNA gene was selected for the identification of the genus *Campylobacter*. Primers (Invitrogen, USA) used for the amplification of 16S rRNA gene are shown in Table 1. The reaction mixture (20 µl) was prepared by mixing 10 µl master mixtures (Promega, USA), 1 µl forward primer (10 pmol), 1 µl reverse primer (10 pmol), 3 µl DNA template and 5 µl deionised water. The PCR reactions were carried out using a thermocycler (Astec, Japan) with the following programme: initial denaturation with 1 cycle of 5 min at 94°C,

30 cycles each consisting of denaturation with 30 s at 94°C, annealing with 30 s at 47°C, extension with 1 min 30 s at 72 °C and a final extension step of 10 min at 72 °C. PCR products were analyzed by 1.5% agarose (Invitrogen, USA) gel electrophoresis and the bands were visualized with UV light after staining with ethidium bromide (0.5µg/ml) for 10 minutes in a dark place. Bands were visualized and images were captured on a UV transilluminator (Biometra, Germany).

Table 1. Primers used for the various PCR.

Primer	Sequence (5'-3')	Target	Amplicon size (bp)	Reference
16S9F 16S1540R	GAGTTTGATCCTGGCTC AAGGAGGTGATCCAGCC	16S rRNA gene	1530	Samosornsuk et al., 2007

Antimicrobial susceptibility test

All *Campylobacter* spp. were tested against eight (amoxycillin, azithromycin, ciprofloxacin, erythromycin, gentamicin, norfloxacin, strepmycin and tetracycline) commonly used antibiotics (HiMedia, India) by disk diffusion method as described by Luangtongkum et al. (2009). The zones of growth inhibition were compared with the zone size interpretative standards as described by Clinical and Laboratory Standard Institute (2015). *E. coli* ATCC 25922 was used as a quality control organism in this study. All susceptibility data were confirmed on at least two separate experiments.

Results

Isolation and identification of *Campylobacter* spp. by conventional methods

Results of percentages (%) of *Campylobacter* spp. available in faecal and milk samples are shown in Table 2. A total of 80 samples [faecal (60) and milk (20)] were subjected for isolation of *Campylobacter* strains by filtration method. *Campylobacter* spp. produced grey color spreading colonies on Blood agar base no. 2 media after 48 hrs of incubation at 37ºC under microaerophilic condition (5% O_2, 10% CO_2 and 85% N_2). In Gram's staining under microscope, the organism revealed Gram negative, pink color, small curved shape arranged as single or pair. Out of 60 faecal samples only 16 (26.66%) were positive for *Campylobacter* spp. and out of 20 milk samples, 4 (20%) were positive for *Campylobacter* spp. *Campylobacter* like organisms were then subjected for biochemical tests. All the isolates of *Campylobacter* spp. (n=20) produced bubbles were found positive in catalase test. All the isolates of *Campylobacter* spp. (n=20) produced deep blue color within 10 seconds were found positive in oxidase test. In hippurate hydrolysis test some of the isolates (n=6) did not develop any purple color that indicated the isolates were *C. coli* and some of the test isolates (n=14) developed purple color that indicated the isolates were *C. jejuni.*

Table 2. Percentages (%) of *Campylobacter* spp. available in faecal and milk samples.

Types of sample	Species	No. of sample	No. (%) of *Campylobacter* isolates		Overall no. (%) of *Campylobacter* spp.
			C. jejuni	*C. coli*	
Faecal	Calves	20	2(10)	0(0)	**20 (25)**
	Heifers	20	4(20)	2(10)	
	Cows	20	5(25)	3(15)	
Milk	Cows	20	3(15)	1(5)	
Total		**80**	**14(17)**	**6(7.5)**	

Molecular identification of *Campylobacter* spp.

Genus specific (16S rRNA gene) polymerase chain reaction (PCR) was performed. 1530 bp fragment of targeted gene was amplified successfully (Figure 1). All Campylobacter isolates gave specific amplification.

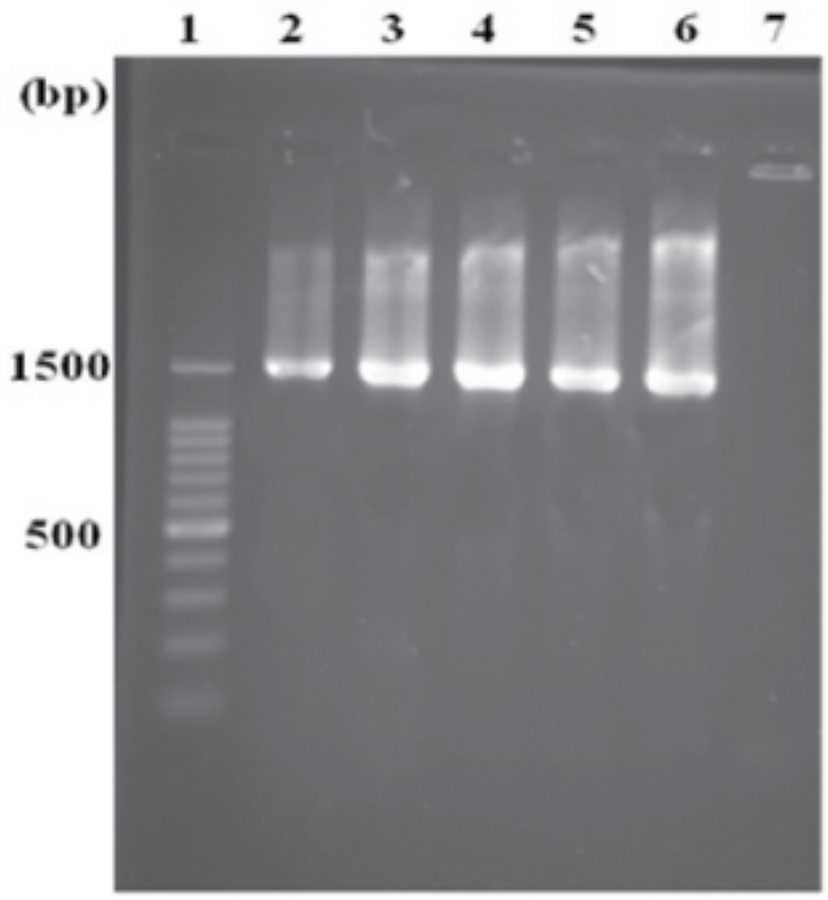

Figure 1. Detection of *Campylobacter* spp. by 16S rRNA gene based PCR. Lanes: 1, 100 bp DNA ladder (Promega, USA); 7, negative control; 2-6, tested positive samples.

Antibiogram profiles of isolated *Campylobacter* spp.

14 isolates of *C. jejuni* were subjected to antimicrobial susceptibility testing against 8 selected antibiotics (Table 3). Among all the isolates 10 (71.42%) were susceptible to gentamicin, 4 (28.57%) were susceptible to norfloxacin, 4 (28.57%) were susceptible to ciprofloxacin, 5 (35.71%) were susceptible to streptomycin and 1 (7.14%) was susceptible to azithromycin. Most of the isolates were resistant to amoxicillin 12 (85.71%), tetracycline 12 (85.71%), azithromycin 12 (85.71%), erythromycin 14 (100%). Furthermore, 8 (57.14%) were resistant to streptomycin, 7 (50%) were resistant to norfloxacin, 2 (14.28%) were resistant to gentamicin and 4 (28.57%) were resistant to ciprofloxacin. 6 (42.85%) isolates were intermediate resistant to ciprofloxacin, 3 (21.42%) were intermediate resistant to norfloxacin, 1 (7.14) was intermediate resistant to streptomycin, 2 (14.28) were intermediate resistant to amoxicillin. Six isolates of *C. coli* were subjected to antimicrobial susceptibility testing against 8 selected antibiotics (Table 4). Among all the isolates 4 (66.67%) were susceptible to gentamicin, 3 (50%) were susceptible to ciprofloxacin, 1 (16.67) was susceptible to norfloxacin, 1 (16.67) was susceptible to azithromycin, 2 (33.33%) were susceptible to streptomycin. Most of the isolates were resistant to amoxicillin 4 (66.67%), tetracycline 4 (66.67%), erythromycin 6 (100%), streptomycin 3 (50), norfloxacin 3 (50%), azithromycin 3(50), gentamicin 1(16.67) and ciprofloxacin 1(16.67). Moreover, 2(33.33%) were intermediate resistant to ciprofloxacin, 2 (33.33) were intermediate resistant to norfloxacin, 2 (33.33%) were intermediate resistant to azithromycin, 2 (33.33) were intermediate resistant to tetracycline and 1(16.67%) was intermediate resistant to gentamicin. The result of antimicrobial resistance patterns of *C. jejuni* and *C. coli* are summarized in Table 5. Out of 14 C. *jejuni* isolates, 3 (21.42%) was resistant to 1 agent (AMX), 2 (14.29) were resistant to 1 agent (E), 1 (7.14%) was resistant to 1 agent (AZM). Furthermore, 1(7.14%) was resistant to 2 agent (AMX- TE), 2(14.29) were resistant to 2 agent (AMX-S) respectively. Moreover, 1 (7.14%) was resistant to 3 agent (AMX-S-TE), 2 (14.29) were resistant to 3 agent (E-S-CIP), 1 (7.14%) was resistant to 4 agent (AMX-NOR-AZM-TE) and 1 (7.14%) was resistant to 5 agent (AMX-S-E-AZM-TE) respectively. Out of 6 *C. coli* isolates, 4 (66.67%) were resistant to 1 agent (AMX), 1(16.67) were resistant to 2 agent (AMX-E) and 1(16.67) was resistant to 3 agent (AMX-S-TE) respectively. In this study, multidrug resistant *Campylobacter* spp. were identified by considering resistant to 2 or more drugs as described in Table 5. A total of 14 *Campylobacter jejuni* were isolated and 8 (57.14) were identified as multidrug resistant. Out of 6 *Campylobacter coli* 2 (33.33) were identified as multidrug resistant.

Table 3. Antimicrobial susceptibility pattern of *C. jejuni* (n=14) identified by the disk diffusion method.

Antimicrobial agents	Number (%) of *C. jejuni*		
	S (%)	I (%)	R (%)
Amoxicillin	0(0)	2(14.28)	12(85.71)
Tetracycline	0(0)	2(14.28)	12(85.71)
Gentamicin	10(71.42)	2(14.28)	2(14.28)
Erythromycin	0(0)	0(0)	14(100)
Azithromycin	1(7.14)	1(7.14)	12(85.71)
Ciprofloxacin	4(28.57)	6(42.85)	4(28.57)
Norfloxacin	4(28.57)	3(21.42))	7(50)
Streptomycin	5(35.71)	1(7.14)	8(57.14)

Table 4. Antimicrobial susceptibility pattern of *C. coli* (n=6) identified by the disk diffusion method.

Antimicrobial agents	Number (%) of *C. coli* isolates		
	S (%)	I (%)	R (%)
Amoxicillin	0(0)	2(33.33)	4(66.67)
Tetracycline	0(0)	2(14.28)	4(66.67)
Gentamicin	4(66.67)	1(16.67)	1(16.67)
Erythromycin	0(0)	0(0)	6(100)
Azithromycin	1(16.67)	2(33.33)	3(50)
Ciprofloxacin	3(50)	2(33.33)	1(16.67)
Norfloxacin	1(16.67)	2(33.33)	3(50)
Streptomycin	2(33.33)	1(16.67)	3(50)

Table 5. Results of antimicrobial resistance pattern of *Campylobacter* spp.

Isolates	Resistance profiles	No. of resistant isolates (%)	No. of multidrug resistant isolates (%)
C. jejuni (n=14)	No resistance demonstrated	-	8 (57.14)
	Resistant to 1 agent AMX	3(21.42%)	
	Resistant to 1 agent (E)	2(14.29%)	
	Resistant to 1 agent (AZM)	1(7.14%)	
	Resistant to 2 agents (AMX- TE)	1(7.14%)	
	Resistant to 2 agents (AMX-S)	2(14.29%)	
	Resistant to 3 agents (AMX-S-TE)	1(7.14%)	
	Resistant to 3 agents (E-S-CIP)	2(14.29%)	
	Resistant to 4 agents (AMX-NOR-AZM-TE)	1(7.14%)	
	Resistant to 5 agents (AMX-S-E-AZM-TE)	1 (7.14%)	
	Total resistant isolates	14 (100%)	
C. coli (n=6)	Resistant to agent AMX	4(66.67%)	2 (33.33)
	Resistant to 2 agent (AMX-E)	1(16.67%)	
	Resistant to 3 agent (AMX-S-TE)	1(16.67%)	
	Total resistant isolates	6(100%)	

Discussion

To our knowledge, this is the first study investigating the prevalence *of Campylobacter* spp. in dairy farm of Bangladesh. Cultural examination, staining characteristics, biochemical tests and finally PCR were performed for the characterization of the *Campylobacter* spp. and the colony characteristics were exhibited grey color which was supported by several researchers (Doyle, 1990). The routine detection of *Campylobacter* spp. in

laboratories is based on a culture method using selective media and subsequent phenotypic identification by biochemical methods which was supported by several researchers (Jamshidi et al. 2008). The only biochemical test for discriminating between *C. jejuni* and *C. coli* is based on hippurate hydrolysis activity which were also used by a number of researchers (Shiramaru et al., 2012; Kabir et al., 2011; Kabir et al., 2014).The current study recorded 16 (26.66%) and 4 (20%) *Campylobacter* spp. from 60 faecal and 20 milk samples respectively during the study period. Out of 80 samples, 14 (17%) isolates were *C. jejuni* and the remaining 6 (7.5%) isolates were *C. coli*. Ramonaite et al. (2013) also recorded *C. jejuni* and *C. coli* from dairy farm in Lithuania. PCR primers targeting 16S rRNA gene of *Campylobacter* spp. were amplified 1530 bp fragments of DNA confirmed the identity of *Campylobacter* spp.(Fig. 1). All *Campylobacter* isolates were positive to 16S rRNA gene based PCR which is supported by Kabir et al., 2011; Kabir et al., 2015.

Despite of the fact that *Campylobacter* is common in cattle herds, our study revealed a moderate prevalence (20%) in BAU dairy farm, Mymensingh, Bangladesh as shown in Table 2. Most other comparable studies reported prevalence between 5% and 78.5% (Ramonaite et al., 2013; Sasaki et al., 2013). Since these studies vary in sampling design, cultural methods and conditions, a direct comparison of the results is difficult. However, our data contribute to previous discussions that cattle are significant reservoirs for *Campylobacter* spp. and could be a source of animal and human infections. The present study recorded that *Campylobacter* spp. can be found more frequently in faecal samples than milk samples.

In the antimicrobial susceptibility testing most of the isolates were sensitive to ciprofloxacin, gentamicin and all the isolates were resistant to amoxicillin, erythromycin. These findings are close to the findings of several researchers (Butzler, 2004). The current study also recorded some multidrug resistant *Campylobacter* spp. in collected samples of BAU dairy farm. Out of 20 isolates, 57.14% *C. jejuni* and 33.33% *C. coli* were detected as multidrug resistant. Resistant profiles of multidrug resistant *Campylobacter* spp. was close to the result of some researchers (Luangtongkum et al., 2009; Kabir et al., 2014). Findings of this study suggested that multidrug resistant *Campylobacter* spp. isolated from dairy farm might be an important concern for veterinary practitioners.

Conclusions

The findings of the present study revealed the presence of multidrug resistant *C. jejuni* and *C. coli* in faeces and milk samples that are not only harmful for cattle itself but also are harmful for consumers on milk consumption. Nevertheless, more studies are needed to clearly understand the genomic diversity in *C. jujuni* and *C. coli* as well as molecular mechanisms for the development of antimicrobial resistance.

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Bacteriological and Molecular detection of *Staphylococcus* spp. and antibiotic resistant mecA and *blaZ* genes in milk from selective dairy farms in Mymensingh

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Abstract

Staphylococcal mastitis is a major and common problem of dairy cattle all over the world including Bangladesh. The present study was undertaken for the isolation and molecular detection of *Staphylococcus* spp. in milk, determination of prevalence of both coagulase positive and negative staphylococci with their characterization from selective dairy farms in Mymensingh. For this purpose 63 bovine milk samples were collected aseptically from two different farms, BAU dairy farm and Nitish Bohumukhi farm, Fulbaria, Mymensingh and transferred to bacteriology laboratory, Dept. of Microbiology and Hygiene, BAU, Mymensingh by maintaining cool chain. The milk samples were subjected to various cultural, morphological, biochemical and molecular tests for the isolation and identification of *Staphylococcus* spp. The antimicrobial profiles were investigated by disc diffusion method and amplification of antibiotic resistant genes by PCR. Out of 63 samples, the prevalence of *S.aureus* and *S. epidermidis* were 25.39% and 11.11% respectively on the basis of ability to ferment in mannitol salt agar (MSA), β- hemolysis on 5% sheep blood agar, ability to coagulate rabbit plasma and by PCR. The overall prevalence of *Staphylococcus spp.* was 35 (56%). Off those 35 samples, 16 (47%) and 7 (20%) were confirmed as *S. aureus and S. epidermidis* by amplification of *nuc* and icaA genes respectively. The Staphylococcal isolates showed 100% sensitive to methicillin and 100% resistant to penicillin which were confirmed by PCR. Whereas the isolates were found 80% and 75% sensitive to ciprofloxacin and erythromycin respectively. β-lactamase producing *blaZ* gene was amplified from all isolates, but no methicillin resistant *mecA* gene was amplified from the isolates in this study. The presence of *Staphylococcus* spp in raw milk indicate that the cattle might be at risk of mastitis.

Key words: Staphylococcus spp, nuc, icaA, mecA, blaZ

Introduction

Staphylococci are the main etiological agents of mastitis and subclinical mastitis in dairy cows (Unal *et al.*, 2010), comprises 45 species and 21 subspecies (Bergeron *et al.*, 2011). Although *Staphylococcus aureus* has been identified as one of the most important pathogenic organism causing mastitis in cattle, coagulase-negative staphylococci (CNS) are increasingly becoming recognized as etiologic agents associated with intramammary infections (IMI) in most countries (Unal *et al.*, 2012 and Taponen *et al.*, 2009). However, their clinical/pathogenic relevance when cultured from milk remains a point of discussion. Some consider them as true mastitis pathogens with important virulence factors (Zhang *et al.*, 2000), a high level of antimicrobial resistance (Rajala *et al.*, 2009), and the ability to cause chronic infections (Gillespie *et al.,* 2009).

Today more than 15 CNS species have been identified that cause IMI in dairy cows. Because of the increasing significance of CNS in bovine mastitis, identification of CNS at the species level is needed to develop effective control strategies for CNS mastitis (Sawat *et al.*, 2009).

The primary cause of mastitis is a wide spectrum of bacterial strains. The organisms that cause mastitis in large animals are *Staphylococcus aureus, Streptococcus agalactiae,* other Streptococcus and Coliforms (Kader *et al.*, 2002; Sudhan *et al.*, 2005; Sharma and Maiti, 2010). Studies from Asian countries also report *S. aureus* as the chief etiologic agent of mastitis in cattle and buffalo (Kang-Hee *et al.*, 2001; Sharma *et al.*, 2007; Abdel-Rady and Sayed, 2009; Rahman *et al.*, 2010; Sharma and Maiti, 2010).

Antimicrobial resistance is an important health problem worldwide (Carmeli et al., 1999 and Cosgrove et al., 2006). The development of resistance in bacteria has been described to the extensive therapeutic use of antimicrobials or with their use as growth promoters in animal feed production (Rogues et al., 2007, Sapkota

et al.,2007 and Silbergeld *et al.,* 2008). Methicillin- resistant *S. aureus* (MRSA) was first described in 1961, shortly after the introduction of methicillin. *S. aureus* becomes methicillin resistant by acquisition of the *mec* gene which encodes a modified penicillin binding protein (PBP2a) that has a low affinity for β-lactams (Normanno *et al*., 2008, Lim *et al*., 2002 Fuda *et al*., 2004). The Identification of mastitis pathogens is important to select appropriate antimicrobial therapy. Bacterial resistance to β -lactam mechanisms include production of β -lactamases, encoded by the *blaZ* gene and production of a low affinity penicillin-binding protein (PBP2a). MR-CNS in large animal may act as important reservoirs for the transfer of antimicrobial resistance genes (Hanssen *et al.,* 2004). It has been suggested that *Staphylococcus epidermidis,* after horizontal transfer, facilitates the potential of *S. aureus* to resist antibiotic treatment, manifesting in methicillin-resistant *S. aureus* (MRSA).

The study was undertaken to determine the prevalence of *Staphylococcus aureus* and *Staphylococuus epidermidis* in raw milk from selected dairy farms in Mymensingh including antibiotic resistance profile of the isolates by conventional and molecular method.

Materials and Methods

Sample collection

A total of 63 raw milk samples (approximately 10 mL from each) from apparently healthy dairy cows were collected aseptically from Bangladesh Agricultural University (BAU) Dairy Farm and Nitish Bohumukhi Dairy Farm , Fulbaria, Mymensingh, Bangladesh in sterile test tube and immediately transferred to the laboratory of the Department of Microbiology and Hygiene, Bangladesh Agricultural University to accomplish laboratory works.

Isolation and identification of *Staphylococcus spp*

The collected milk samples (0.01 mL) were streaked onto 5% sheep blood agar (HiMedia®, India) incubated at 37°C for overnight. The presumptive colonies of *Staphylococcus spp.* were further sub-cultured onto mannitol salt agar (MSA) to get pure culture. The isolates were identified as *Staphylococcus spp.* on the basis of Gram staining, colony morphology on MSA (HiMedia®, India), hemolytic patterns on blood agar catalase and coagulase tests. Further the isolates were confirmed by amplification of *S. aureus* specific *nuc* gene and *S. epidermidis* specific *icaA* gene.

Extraction of bacterial Genomic DNA

To perform PCR the genomic DNA was extracted by boiling method. In brief for the extraction of genomic DNA, a single colony was dissolved in 100µl of DW and boiled for 10 minutes. After boiling the tubes were immediately placed on ice for cold shock followed by centrifugation at 10,000 rpm for 10 minutes at 4˚C. The supernatant was collected which was further used as template DNA.

Table 1: Oligonucleotide primers used in the study

Primer Name	Gene Targeted	Primer Sequence (5′-3′)	Amplicon size (bp)	References
nuc F	*nuc*	5′GCGATTGATGGTGATACGGTD 3′	279	Dewanand *et al.,* 2007
nuc R		5′AGCCAAGCCTTGACGAACTAAAGC 3′		
icaA F	*icaA*	5′GACCTCGAAGTCAATAGAGGT 3′	814	Wilma *et al.* 1999
icaA R		5′CCCAGTATAACGTTGGATACC 3′		
mecA F	*mecA*	5′AAAATCGATGGTAAAGGTTGG 3′	533	Dewanand *et al.* 2007
mecA R		5′AGTTCTGGCACTACCGGATTTTGC 3′		
blaZ F	*blaZ*	5’ TCAAACAGTTCACATGCC 3’	877	Rosato *et al.,* 2003
blaZ R		5’TTCATTACACTCTGGCG 3’		

PCR conditions for amplification of *nuc, icaA, mecA and blaZ* genes

Multiplex PCR reaction was performed to detect *Staphylococcus aureus* specific *nuc* gene and methicillin resistant mecA gene in isolated *S. aureus* in a gradient thermal cycler (Eppendrof, Germany). The thermal profile of PCR was set denaturation at 95℃ for 5 minutes followed by 30 cycles, initial denaturation, 95℃ for 1 minute, annealing 55℃ for 45 seconds, elongation at 72℃ for 1 minute and final extension 72℃ for 10 minutes and the holding temperature was 4℃. The thermal profile of PCR for *icaA* gene was set initial denaturation 95℃ for 30 seconds, annealing at 50℃ for 30 seconds, elongation at 72℃ for 1.5 minute and final extension 72℃ for 10 minutes. The thermal profile of PCR for *blaZ* gene was initial denaturation at 90℃ for 30 seconds, annealing 46℃ for 30 seconds, elongation 72℃ for 45 seconds. Each reaction was performed at 30 cycles and final extension was 72℃ for 10 minutes at 4℃.

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility assay was performed according to the recommendation of Clinical and Laboratory Standards Institute (CLSI) (formerly National Committee for Clinical Laboratory Standards (NCCLS, 2013) by disc diffusion method. Susceptibility of *Staphylococcus spp* isolates to different antimicrobial agent was measured in vitro by employing the modified Kirby-Bauer (Bauer et al., 1966) method. β–lactamase producing *blaZ* gene was also detected by PCR.

Results

A total of 63 raw milk samples were collected and subjected for various bacteriological and molecular methods for isolation and identification of Staphylococcus spp with their antimicrobial resistance pattern. Out of 63 samples, 35 were suspected as *Staphylococcus spp* on the basis of hemolytic properties on 5% sheep blood agar and development of bright yellow or white pin point colonies. Off these 16 isolates produced β hemolysis (Figure 1) followed by fermentation of mannitol salt agar and rest of the isolates showed γ hemolysis or no hemolysis on blood agar (Figure 2) as well as no fermentation of MSA which are similar with the findings of Mariela *et al*., 2015 and Wilma *et al*., 1999 for *S. epidermidis*.

Figure 1: β hemolysis on 5% sheep blood agar by *Staphylococcus aureus*.

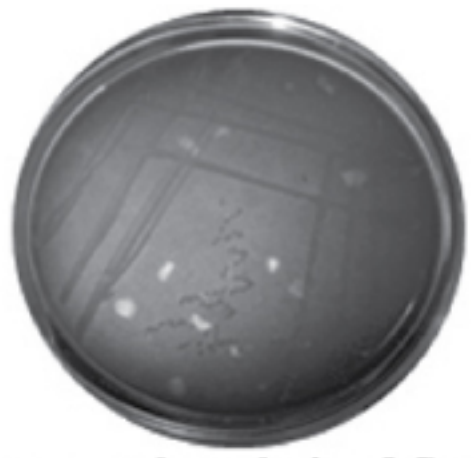

Figure2: No or γ hemolysis of *Staphylococcus epidermidis* on 5% sheep blood agar.

Figure 3: Fermentation of MSA by *S. aureus*.

Figure 4: No fermentation of MSA by *S. epidermidis*.

In Gram's staining, the organisms were found Gram-positive, cocci shaped arranged in grape like clusters which are the unique Characteristics of *Staphylococcus* species (Figure 5)

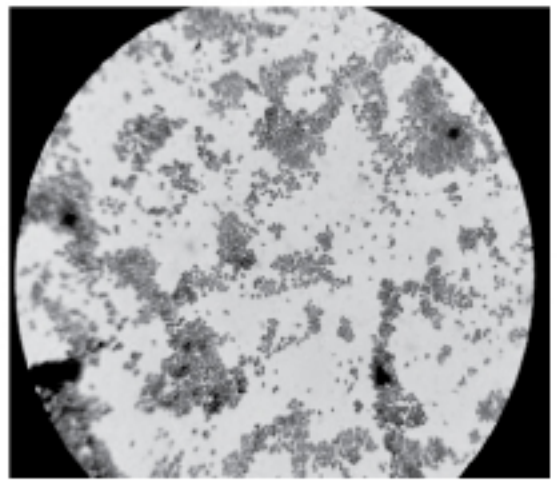

Figure 6: Gram's staining of *Staphylococcus* spp showed coccoid or spherical shaped organisms arranged in clusters under light microscope (100×).

Catalase test was performed to differentiate Staphylococci from Streptococci. A total of 35 samples were found catalase positive (Figure 6). Out of 35 isolates, 16 were found coagulase positive indicated that the isolates are *S. aureus*. (Figure 7) and other 19 gave negative result indicated that they are coagulase negative Staphylococci termed as CNS.

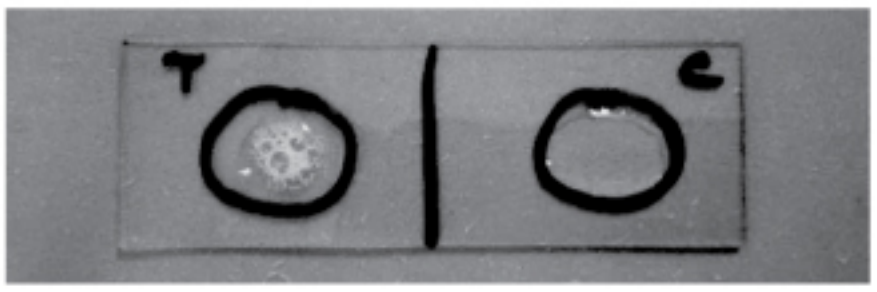

Figure 6: Result of Catalase test, T: Catalase positive, C: Negative control.

Figure 7: Result of Coagulase test, T: Coagulase positive, C: Negative control

In this study the prevalence of coagulase positive *Staphylococcus* was found 25.39% (16/63) and CoNS was found 30.15% (19/63). From all 16 isolates of S. *aureus* nuc gene of 279 bp were successfully amplified (Figure 8) which is the specific gene of *S. aureus*. Out of 19 isolates of *Staphylococcus* spp only 7 samples were found positive for *icaA* gene of 814 bp (Figure 9) which indicates these seven isolates are *Staphylococcus epidermidis*. Farm wise the prevalence of *Staphylococcus* spp was found 51.42% and 60.71% in Nitish farm and BAU dairy farm respectively. The overall prevalence was 55.55%.

The isolated *Staphylococcus* spp. were found 100% resistant to Penicillin and from all isolates of *S aureus* β-lactamase producing *blaZ* gene of 877 bp size were successfully amplified (figure11) which confer the resistance of isolates against penicillin. On the other hand, phenotypically and genotypically the isolates were found 100% sensitive to methicillin. From these isolates no methicillin resistant *mecA* gene of 533 bp size were amplified confers the isolates are methicillin resistant. The isolates were found 55%, 65% and 85% intermediately resistant to Tetracycline, Azithromycin and Gentamycin respectively. The isolates showed 80%, 75% and 70% sensitivity to Ciprofloxacin, Erythromycin and Chloramphenicol respectively. The antibiotic sensitivity patterns of the isolates are presented in figure 10.

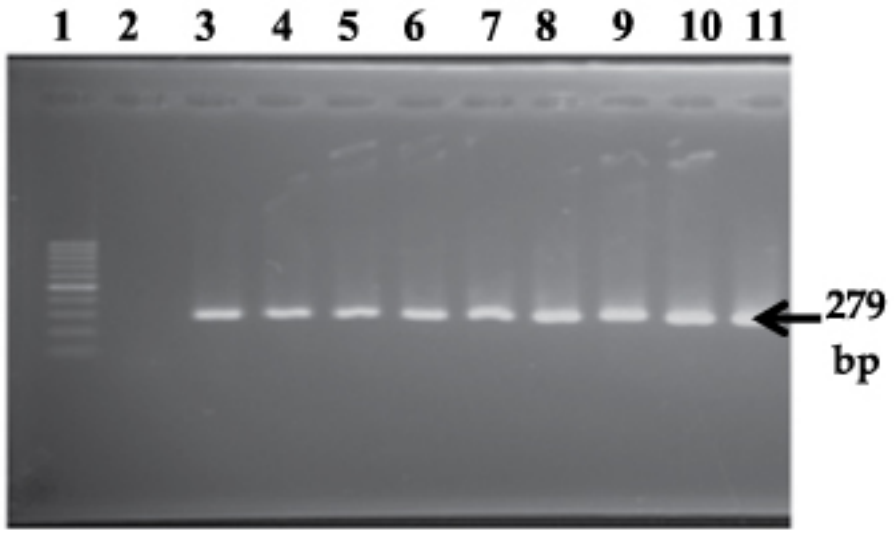

Figure 8: Agarose gel electrophoresis of *nuc* gene of *S. aureus*. Amplicon size: 279 bp, Lane (L) 1= 100bp ladder, L-3 to 10= amplified *nuc* gene of *S. aureus* from milk samples, L-2= negative control, L-11= positive control.

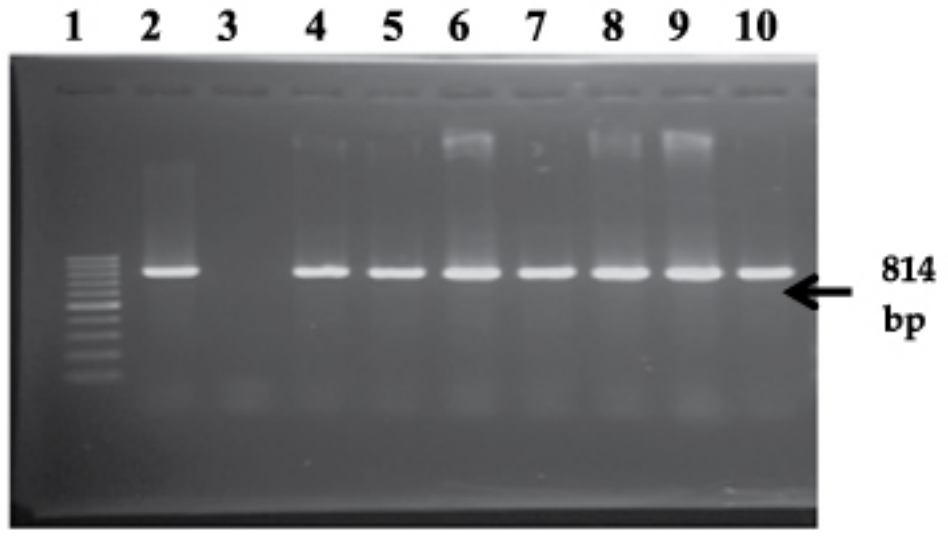

Figure 9: Agarose gel electrophoresis of *icaA* gene from *Staphylococcus epidermidis*. Amplicon size: 814 bp Lane (L) 1= 100 bp ladder, L-2= positive control, L-3= negative control, L- 4 to10= amplified *icaA* gene of *S. epidermidis* from milk sample).

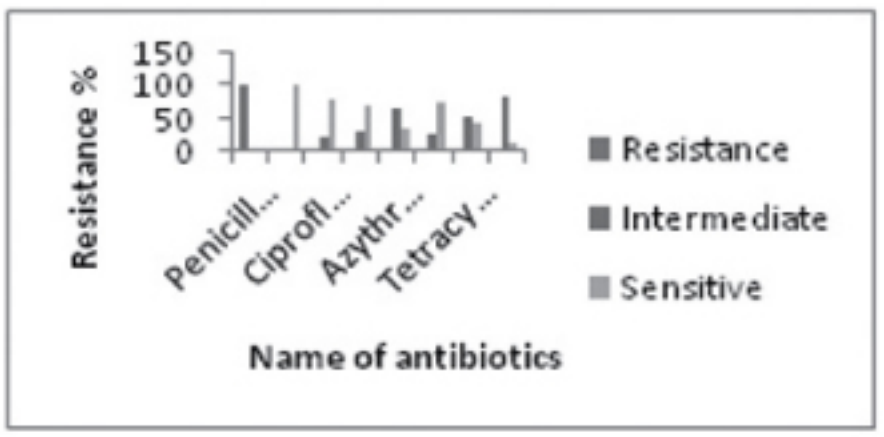

Figure 10: Susceptibility pattern of *Staphylococcus* spp to various antibiotics.

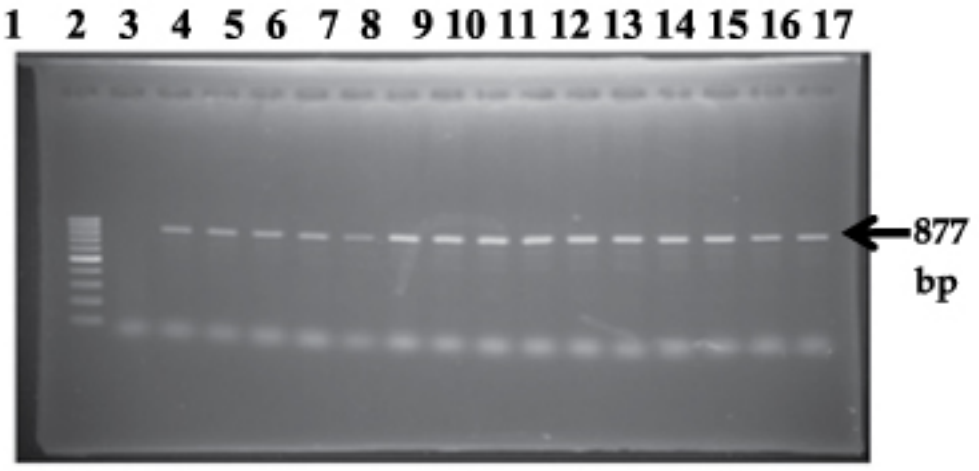

Figure 11: Agarose gel electrophoresis of beta-lactamase producing *blaZ* gene from *Staphylococcus* spp. Lane (L-1=100 bp ladder, L-2= negative control, L-3 to L-17= amplified *blaZ* gene from *Staphylococcus* spp. of test samples.

Discussion

The present study was designed for the molecular detection of *Staphylococcus* spp in bovine milk from selective dairy farms in Mymensing with determination of antibiotic profile by disc diffusion and molecular methods. The *Staphylococcus* spp was isolated and identified on the basis of cultural, Gram's staining, biochemical and detection of *Staphylococcus aureus* and *Staphylococcus epidermidis* specific nuc and icaA genes respectively by PCR. The prevalence of *S aureus was* detected 25.39% and CNS was 30.15%. The findings of our study is higher than the result of Hashemi *et al*.,(2011) and Atyabi *et al*., (2006). Hashemi *et al*., reported that the staphylococci as the most common isolated bacteria in milk and the prevalence of Coagulase positive and CNS Staphylococci were 19.56% and 12.53% respectively whereas Atyabi *et al*., 2006 found 30.27% for CNS and 2.89% for *S. aureus*. The finding of our study indicates that both CNS and pathogenic Staphylococci present in mammary gland of healthy milking cattle and may contribute to clinical and subclinical mastitis. The high incidence of *S. aureus* in milk is also indicative of poor hygiene measure during production, handling and distribution (Shusma *et al*., 2012). The proper heat treatment followed by the refrigeration can minimize the chance of infection with *S. aureus*. In our country it is commonly noticed that during boiling of milk, the temperature cannot rise up to the boiling point or even if it reaches but consumers do not boil it for long time. The presence of *S. aureus* in milk is of two importances one is its public health importance and another one there is chance of development of subclinical and clinical mastitis.

Bovine CNS has traditionally been considered as skin flora opportunists (Hogan *et al.,* 1987). Dairyman may act as potential source for colonization of these species in bovine udder tissue, as these species are commonly

found in the skin and the mucous membrane of human. This hypothesis is supported by identification of *S. epidermidis* as the third most common CNS (Nagase *et al.,* 2002) responsible for intramammary infection. PCR based molecular detection of *Staphylococcus* spp may contribute to identify the organisms more specifically than other method and many researchers identified *Staphylococcus* spp from milk by PCR (Jahan *et al.,* 2014, Mariela *et al.,* 2015).

The isolated *Staphylococcus* spp. were found 100% resistant to Penicillin which supports the study of Thaker *et al.,*(2013) who showed 100% resistance to Penicillin G. Resistance to penicillin by *Syaphylococcus* spp. due to production of β- Lactamase enzymes that destroy or modify the functional activity of antibiotic drug e.g Penicillin. Coagulase positive Staphylococci was highly sensitive to Ciprofloxacin, Erythromycin and Chloramphenicol which supports the findings with Shekhan *et al.,* (2011) who showed *S. aureus* is highly sensitive to these antibiotics. In case of CNS they showed intermediate resistance to Gentamycin, Azythromycin and Tetracycline which slightly differ from the study of Farid *et al.*, (2015) because they showed *S. epidermidis* are highly resistance to Gentamycin, Azythromycin and Tetracycline from human urinary tract. This result may quite differ because *S. epidermidis* is considered as common skin commensal in human skin. No MRSA was found in our study. Jahan *et al.,* (2014) could not detected any MRSA in raw milk because methicillin is not widely used in livestock for that reason all isolates were 100% sensitive to methicillin.

Conclusion and Implications

The present study was undertaken for the molecular detection of *Staphylococcus* spp. in bovine milk from selective dairy farms in Mymensingh. A total of 55.55% isolates were confirmed as Staphylococcus spp. on the basis of colony morphology, staining characteristics and molecular basis. Among the isolated organism highest prevalence of both *S.aureus* and *S.epidermidis* was found in BAU dairy farm 28.57% and 17.85% respectively. The PCR was subsequently used to screen the isolated organism by amplifying *S.aureus* specific *nuc* gene and *S.epidermidis* specific *icaA* gene. The antibiotic test indicated that isolated Staphylococcus spp were completely resistance to penicillin but showed sensitivity to ciprofloxacin, erythromycin, chloramphenicol and oxacillin. The presence of antibiotic resistant strain of *Staphylococcus* spp in milk indicates that the organisms might be come from cow's udder which may lead to clinical or subclinical mastitis of dairy cattle and may act as potential health hazard for those who consume raw or improperly boiled milk.

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Q fever in cattle with reproductive disorders: Prevalence and molecular diagnosis

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Abstract
*Coxiellaburnetii*is the causative agent of Q fever in human, animals and ticks are considered to be the natural primary reservoirs of *C. burnetii*and are responsible for the transmission of the infection in wild and domestic animals. Q-Fever is widely distributed throughout the world but a scant study is available in ruminants in Bangladesh. The objectives of this study were to determine the prevalence of *Coxiella (C.) burnetii*infection in cattle with the history of reproductive disordersand to detect the organism from ticks through molecular tools. A total of 102 ticks and 120 milk samples from cows with the history of abortion and reproductive disorders were collected conveniently from the different areas in Bangladesh. The milk samples were tested by IDEXX Q-Fever ELISA test kit and *Coxiellaburnetii*DNA was detected by multiplex quantitative PCR. The overall prevalence was 21.67%(26/120) in milk samples and the DNA of *C. burnetii* was found only in one tick (0.98%) out of 102. The prevalence of Q fever varied in different districts, age groups, type of breeding and reproductive disorders but the differences were not significant statistically. The study is still in progress with the collaboration of National Reference Laboratory for Q fever, Friedrich-Loeffler-Institut, Jena, Germany.

Keywords: Q fever, *Coxiellaburnetii*, cattle, ticks, Bangladesh

Introduction

*Coxiellaburnetii*is the causative agent of Q fever in human and animals and ticks are considered to be the natural primary reservoirs of *C. burnetii*responsible for the spread of the infection in wild animals and for transmission to domestic animals (Norlander, 2000; Pluta et al., 2010). Q fever and is transmitted by more than 40 tick species (Psaroulaki et al., 2006)Q-Fever (or Coxiellosis) is widely distributed throughout the world. *Coxiellaburnetii,* is present in virtually all animal kingdoms, including arthropods, but the disease affects mostly humans, cattle, sheep and goats (OIE, 2015).Cattle, sheep and goats are primary *C. burnetii* reservoir hosts (Arricau-Bouvery and Rodolakis, 2005). Although abortion is related to *C. burnetii* in sheep and goats, it is reported not to cause any clinical diseases in these animals (Lang, 1990). But when infected, shed the bacteria via vaginal excretion and the placenta during parturition as well as in milk, faeces and urine (Berri et al., 2000; Vaidya et al., 2010; Pritchard et al., 2011; Muskenset al., 2012). It is reported that *C. burnetii* microorganisms can persist for 150 days following birth or abortion and that in milk for up to 32 months (Rodolakiset al., 2007).

It has been reported that the pathogen can infect humans and animals by inhalation of contaminated aerosols of urine, faeces, placenta and by consumption of raw milk and dairy products as well (Norlander, 2000).

Diagnosis of Q- Fever in ruminants, including differentiating it from other abortive diseases, traditionally has been made based on microscopy on clinical samples, coupled with positive serological results (Lang, 1990). Direct detection and quantification by PCR and serological ELISA should be considered as methods of choice for clinical diagnosis (Sidi-Boumedineet al., 2010). Q fever has been reported in nearly all parts of the world, including Bangladesh, effective diagnosis is vital for effective eradication and control measures.

To control the spread of *C. burnetii* among animals to humans, the detection of shedders of *C. burnetii* and the knowledge of the prevalence of the infection are imperative. Only few reports on the prevalence of Q fever in animals are available in Bangladesh (Chakrabarttyet al., 2016abcd; Haideret al., 2015; Rahman et al., 2016a). Haideret al. (2015) reported seroprevalence of Q fever in cattle and goats that were brought to veterinary hospitals in Netrokona, Dinajpur, and Chittagong districts in Bangladesh. Rahman et al. (2016a) reported herd-level prevalence of Q fever in dairy cattle using bulk milk (Satkhira, Rajshahi, Chittagong), animal-level seroprevalence in cattle, goats and sheep originated from herds (Mymensingh, Sherpur, Dhaka) with previous history of abortion and detection of *Coxiellaburnetii* DNA from sheep placenta. Presence of *C. burnetii* in ticks has not been reported in Bangladesh. The objectives of this study were to determine the prevalence of *C.burnetii*infectioninruminants,and to detect *C. burnetii* DNA from ticks.

Materials and methods

Study area and Sampling

The study was conducted in Kurigramsadar, Shahjadpur, Pabnasadarand Mymensinghsadar duringDecember 2014 to September, 2017. 10 ml Milk samples were collectedinto sterile plastic tubes from each cattle and were stored at -20°C. Then sent from Bangladesh to Germany by world courierservice (in dry ice)following recommended sample transportation rules ofInstitute of Bacterial Infections and Zoonoses, Federal Research Institute for Animal Health, Jena, Germanyfor serology. Cattle with the history of reproductive disorders (retained fetal membrane, abortion, anestrous, repeat breeding)were selected purposively for sampling.

The ticks were handpicked from selected cattle, identified and sorted according to the area, species, sex and developmental stages. Later, ticks were gathered into groups of 1 to 7 ticks as to the area, species and gender for DNA extraction. The numbered tick groups were placed in aluminium foil and preserved at -20°C. The ticks were identified morphologically with the aid of a photographic microscope using the tick identification keys (Beati and Keirans, 2001; Walker et al., 2003).

Serology (ELISA)

Upon arrival, the samples were centrifuged, the fat fraction was removed and discarded, and the non-fat fraction was frozen to be tested for antibodies at a later time. The samples were tested for antibodies against C. burnetiiusing the commercial CHEKIT® Q fever antibody ELISA Test Kit (Idexx, Liebefeld-Bern, Switzerland). The ELISA plates were coated with C. burnetiiinactivated phase 1 and phase 2 antigens. The optical densities (OD) of the samples were corrected by subtracting the OD of the negative control. The remaining non-fat fraction of the milk samples was frozen and stored for possible later purposes like determination of C. burnetii. The results were expressed as S/P values and estimated as the ratio between OD of the sample (S) and the OD of positive control (P) included in the test kit. According to the instructions from IDEXX S/P≥40% was considered positive, S/P<30% was considered negative, and results in the interval 30%≤S/P<40% were considered to be intermediate

DNA extraction, multiplex PCR and real-time PCR:

Frozen ticks were triturated thoroughly in a mortar. The total DNA from ticks was extracted using NucleoSpin Tissue kit (Macherey-Nagel GmbH, Duren, Germany). Maximum 50 mg triturated tick sample was transferred to an eppendorf tube and processed as described by the manufacturer. The DNA extracts were stored at -20º C until amplification.

DNA quantification was performed with a Taq Man based real-time PCR assay targeting the transposase

element IS1111 as described by Klee et al. (2006). The real-time PCR assay was performed with the primers and the conditions (Table 1 & 2). Samples were considered positive with a cycle threshold (Ct) < 40 (Boarbiet al., 2014).

Real-time PCR was performed in a total volume of 25 µl containing: 2.5 µl of 10× PCR buffer, 2.0 µl of 50 mM MgCl2, 2.0 µl of 10 mM dNTP, 2.0 µl of 10 pmol forward and reverse primer, 0.75µlof10pmolFAM/TAMRAduallabelledprobe(FAM-TCATCAAGGCACCAATGGTGGCCA-TAMRA) (in deviation to thepublished minorgroove binder probe; Panning et al., 2008), 8.5 µl sterile water, 0.25 µl of 5 U/ µl thermostable polymerase DNA (Invitrogen, Germany), and 5 µl of extracted DNA. Cycling conditions in a 7500fast real-time PCR system (Applied Biosystems,USA) were as follows: 94℃ for 2 min, and 45 cycles of 94℃ for 15 s and 60℃ for 30 s. The reagents for real-time PCR were from Invitrogen, Germany; primers and probes from Genecam, Germany. As positive control C. burnetii DNA (Genecam, Germany) and negative control (water) were used. Sample values falling below cycle threshold (Ct) of 38 were considered positive.

Table 1. Primer for IS 1111 Real-time PCR

Oligo	Name	Sequence (5′ to 3′)
Primer	Cox- R	CCCCGAATCTCATTGATCAGC
	Cox- F	GTCTTAAGGTGGGCTGCGTG
Probe	Cox- TM	6FAM-AGCGAACCATTGGTATCGGACGTTXTATGG—PH

Table 2. PCR Conditions

Cycler: Stratagene MX 3000	Temperature	Time
Initial denaturation	50°C	2 min
	95°C	10 min
Denaturation, Cycles 50	95°C	15 secs
Anneal / Elong. Cycles 50	60°C	30 secs

Results and discussion

The overall prevalence was 21.67%in the cattle. The DNA of *C. burnetii* was found only in one tick (0.98%) out of 102(Table 3, Fig 1). The prevalence of Q fever was highest in Kurigram district (30%), crossbred cattle (24.2%), artificially inseminated cows (23.5%), and in cows with the history of anestrous (33.3%) than Pabna district, indigenous cattle, naturally served cow and repeat breeder cows respectively. However, these differences were not significant statistically (Table 4.).

Table 3. Prevalence of Q fever in cattle, goats, ticks and human

Species	Tested	ELISA		Prevalence	PCR
		Positive	Intermediate		
Milk	120	26	7	21.67%	-
Tick	102	-	-	-	1

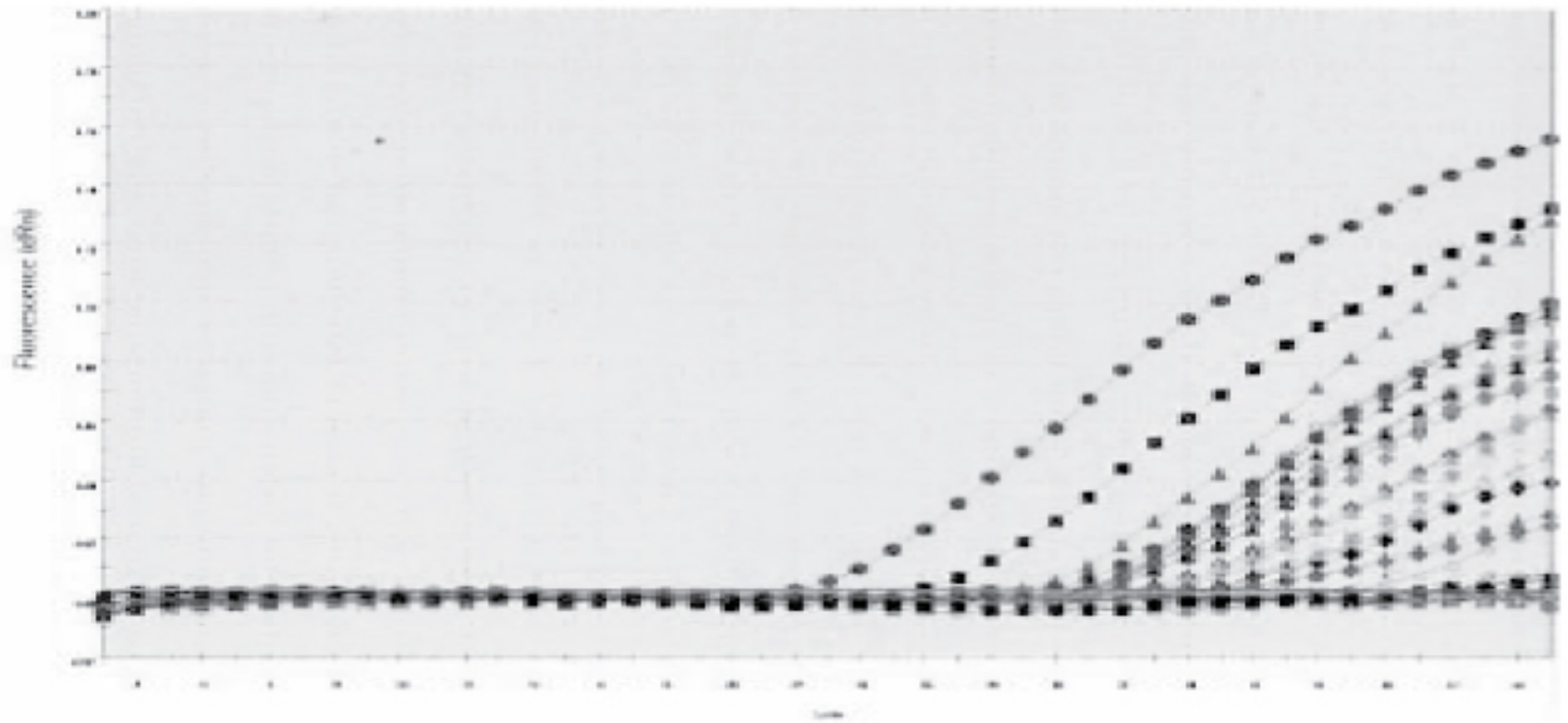

Fig 1. Real time PCR amplification plots from DNA extracted from Tick of Bangladesh. The ct-results for the positive tick sample are marked yellow

Table 4. Contingency tables and chi-square test conducted to evaluate the association between explanatory variables and Q feverstatus in cattle with reproductive disorders in Pabna, Kurigran, Mymensingh and Sirajganj districts in Bangladesh, 2014 – 2017.

Variable	Category	Tested	Prevalence (%), (95% CI)	P-value
District				0.08
	Pabna	18	0 (0–21.9)	
	Kurigram	20	30 (12.8–54.3)	
	Mymensingh	42	21.4 (10.8–37.2)	
	Sirajganj	39	28.2 (15.5–45.1)	
Age (years)				1.0
	Three to 5	72	22.2 (13.6–33.8)	
	More than 5	47	21.3 (11.2–36.1)	
Breed				0.33
	Indigenous	24	12.5 (3.3–33.4)	
	Cross	95	24.2 (16.3–34.3)	
Breeding				0.44
	Natural Service	17	11.8 (2.1–37.7)	
	Artificial Insemination	102	23.5 (15.9–33.2)	
History of reproductive diseases				0.59
	Repeat breeding	12	8.3 (0.43–40.2)	
	Abortion	60	21.7 (12.5–34.5)	
	Anestrous	6	33.3 (5.9–75.9)	
	Retention of placenta	41	24.4 (12.9–40.6)	

In this study we showed a prevalence 21.67% in dairy cattle with reproductive disorders. The prevalence of the infection in dairy cattle with reproductive disorders has been reported in several countries (Lang, 1990) The serological methods are useful for carrying out preliminary surveys of infection but they do not allow for the identification of *C. burnetii* shedding animals. When the positive serological results are found the PCR is the method of choice to trace shedders.

Serological methods are of limited validity because they often fail to detect *C. burnetii* shedding animals and show different sensitivities (Kittel bergeret al., 2009; Roussetet al., 2009). Moreover, serological diagnosis of Q fever in the early stage of infection can be unsuccessful due to the time-frame of seroconversion spanning 3–4 weeks post infection (Howe *et al*., 2009).

The epidemiology of Q-fever in Bangladesh is essentially unknown, but there are some studies that reported detection of *C. burnetii* in various clinical samples in Bangladesh.The overall prevalence was reported to be 6.1% and 7.6% in dairy cattle and goats respectively with the history of abortion and reproductive disorders (Chakrabartty et al., 2016abcd). Rahman et al. (2016) reported overall seroprevalence of Q fever as 6.38% in domestic ruminants originated from herds with the history of abortion.*Coxiellaburnetii* DNA was detected from only one sheep placenta in Bangladesh (Rahman et al.,2016). Whereas, Haideret al. (2015) detected immunoglobulin G for *C. burnetii* in 0.7% (8/1149) of ruminants where 0.65% (4/620) was in cattle and 0.76% (4/529) in goats by using enzyme-linked immunosorbent assay (ELISA) and Immunofluorescence assay (IFA). Serologic and PCR evidences indicateanimals in Bangladesh are exposed to *C. burnetii*but there is noinformation available regarding the presenceof this agent in specific vectors. This reportpresents the first detection *C. burnetii*inticks in Bangladesh. Detection of IS 1111gene in ticks may suggest that ticks can be an important vector for *C. burnetii*in Bangladesh. Ticks acquire *C. burnetii*during blood meal on infected animals and could transmit the bacterium to the other mammals during the next blood meal, or by aerogenic spread of dried tick faecal excretions, maintaining *C. burnetii*in animals.

Conclusion

This study and previous reports from Bangladesh indicate that Q fever is prevalent in livestock in Bangladesh. This study further added that it also present in ticks. Q fever is a zoonosis and as prevalent in livestock, the possibility of this disease in humans can't be ruled out in Bangladesh. Humans especially those occupationally exposed to animals should be screened for Q-fever regularly.

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Microbiological quality of raw and pasteurized milk obtained from local market

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Abstract

Milk is a nutrient rich food item. But sometimes it may be harmful to people due to contamination and adulteration. Occasionally people consume raw milk on their farm because of convenience and a preface for the taste of raw milk. In digestion point of view there is no harm to drink raw milk because raw milk digest easily and maximum benefits from bioactive components of milk can be obtained from raw milk. Milk borne diseases like Tuberculosis, Typhoid, Paratyphoid, Diphtheria, Septic sore throat etc. could be spread through raw milk to human beings. The study focuses on microbiological quality of raw and pasteurized milk obtained from local market. A total of 40 raw milk samples and 30 pasteurized milk samples were collected from different locations of Savar. Total viable bacterial count (TVBC) and total coliform count (TCC) was performed followed by molecular detection of *Escherichia coli* DNA for detection of pathogenic and non-pathogenic strains of this organism in milk. The ranges of TVBC and TCC in raw milk samples were from $1.2x10^8$ to $9.6x10^9$ CFU/ml and $1.0x10^4$ to $4.5x10^5$ CFU/ml, respectively. About 42.5% Sample (17/40) was found negative for total coliform count. On the contrary, the quality of pasteurized milk, in terms of microbial load, was better than raw milk. The range of TVBC in pasteurized milk samples was from $1.0x10^2$ to $2.2x10^3$ CFU/ml, slightly lower than that of recommendation of Bangladesh Standards and Testing Institution (BSTI). Interestingly, 33.3% (10/30) pasteurized samples were found negative for TVBC and TCC. The PCR parameters were optimized by using different combinations of 16srRNA gene based primers and could amplify a 584 bp product that was specific for *E.coli.* This data clearly indicate that microbiological quality of milk especially raw milk is very poor.

Key Words: Microbes, Raw milk, Pasteurized Milk, *E. coli*

Introduction

Milk is considered the most important dietary component for young mammals that has a major influence on human health, particularly in advanced countries where the people are well-nourished. It is a common source of proteins and microelements for human. Considering the most perishable food item, in recent years, scientists have been trying to find out the way to increase the shelf life & quantity of milk without hampering the quality. Major portion of milk is water in which other nutrients, i.e. fat, protein; lactose (carbohydrate of milk), minerals and vitamins are present either in solution, colloidal suspension or emulsion in water. Gross composition of cattle milk are- water 86.8%, fat 4.6%, lactose 4.7%, protein 3.2%, ash 0.7%, T.S. 13.2%and S.N.F. 8.6% (Prasad, 1997). Composition of milk changes during the lactation period and giving different amounts of components important for nutrient supply, growth and development. In Bangladesh total milk production was ~7.3 million ton in the financial year 2015-16 and 5.23 million ton up to February of 2016-17 (BER, 2017). All over the world people fulfill approximately 13% of their protein requirement from milk and milk products.Milk may become harmful for people due to contamination and adulteration. Sometimes people consume raw milk on their farm because of convenience and a preface for the taste of raw milk. From digestion point of view there is no harm to drink raw milk because of raw milk can be digest easily and maximum benefits from bioactive components of milk can be obtained from raw milk. The main problem of raw milk consumption is the presence of pathogenic bacteria in milk. It is very difficult to produce milk free from pathogenic bacteria. Milk borne diseases like Tuberculosis, Typhoid, Paratyphoid, Diphtheria, Septic sore throat etc. could be spread through raw milk to human beings (De, 2003). For this reason, milk should be pasteurized to kill the pathogenic bacteria that are present in milk. Consumption of adulterated milk can also cause additional health problems. For example, the milk adulterated with contaminated water is a serious health hazard because of potential water borne diseases. The adulteration of detergent in milk may cause food

poisoning. Raw milk is a well-known good medium that supports the growth and multiplication of many kinds of microorganisms due to its complex biochemical composition and high water activity (Oliver *et al.*, 2005). Many microorganisms can get easy access to raw milk such as *Escherichia coli (E. coli)*.It is an environmental pathogen which is found in the immediate surroundings of the cow such as the soil, grass, manure and the bedding of housed cows. It is therefore easy for the organism to be found in the udder of cow thereby gaining entrance to the milk. It's a true pathogen with public health significance. *E. coli* produces toxins that destroy cell membrane and can also directly damage milk producing tissues which can cause bovine mastitis. It inhabits the intestinal tract of animals and humans. Though most *E. coli* are harmless, some are known to be pathogenic, causing both severe intestinal and extra-intestinal diseases in man (Kaper *et al.,* 2004). The presence of food-borne pathogens in unpasteurized raw milk either directly or indirectly increases the risk of ingestion and transmission of food-borne pathogens and ingestion of potentially harmful toxins. Recovery and counting of *E. coli* is used as reliable indicator of fecal contamination and indicates a possible presence of entero-pathogenic or toxigenic microorganisms. Several studies have been reported

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on microbial quality of milk marketing in Bangladesh (Hasan et al., 2015; Uddin *et al.,* 2011).Present study was undertaken to enumerate microbiological quality of milk along with determination of *E. coli* fromboth unpasteurized raw milk and pasteurized milk samples collected from local market.

Materials and Methods

Collection and transportation of milk samples

A total of 40 raw and 30 pasteurized milk samples were collected from local market, Balibhodra and Sreepur bazaar, Ashulia, Savar, Dhaka (Table 1). Raw milk samples were purchased from farmers/sellers who sell milk just beside the street in an open environment. Raw milk samples were purchased within 9:00-10:00am over a period of month. Soon after purchase raw milk samples weretransferred from seller's container to our sterile containers to avoid further contamination. Then the containers were placed into ice box having ice pack and transported immediately to the laboratory of Animal Biotechnology Division, National Institute of Biotechnology. Raw milk samples were processed in the day of collection. Pasteurized milk of three different companies, name not mentioned, rather categorized as Group-1, 2 and 3 (Table 1) were purchased from local shop in one-time point. Pasteurized milk samples were stored in refrigerator in shop. Before purchase expired date mentioned in each pack were checked carefully. No milk samples beyond expired date were found during purchase.

Table 1: Summary of milk samples collected from local markets.

Type of sample	Processed technique	No. of sample
Raw milk sample	N/A	40
Processed milk (Group-1)	Pasteurization	10
Processed milk (Group-2)	Pasteurization	10
Processed milk (Group-3)	Pasteurization	10

Determination of total viable bacterial count (TVBC)

About 10ml raw milk was added with 90ml of 0.1% sterile peptone water (1:10 dilution), mixed thoroughly and incubated at 37ºC for 24 hours. Then serial 10-fold dilutions were made upto 10^{-8} dilution. About 0.5ml of 10-fold diluted milk sample (10^{-1} to 10^{-8}) was transferred and spreaded onto plate count agar (PCA) and incubated at 37°C for 24 hours. The number of colonies within the range of 30-300 in a particular dilution was counted and adjusted volume to calculate TVBC/ml. The plates were screened for the presence of discrete colonies after incubation period and the actual numbers of bacteria were estimated as colony forming unit in per ml (CFU/ml) sample.

Isolation and identification of *E. coli*

Isolation and identification of *E. coli* was performed according to the method described earlier(APHA 1992).Ten-fold diluted peptone enriched samples were streaked on selective media, EMB agar, for *E. coli* and incubated at 37°C for 24 hours. Total coliform was counted based on observation of metallic sheen and expressed in CFU/ml. Single colony was further sub-cultured to prepare pure culture. Identification of *E. coli* was performed on the basis of colony characteristics, biochemical tests viz. indole production, methyl red, Voges Proskauer test. Confirmatory diagnosis was done based on molecular analysis of 16s rRNA.

Extraction of DNA from *E. coli*

Individual colony of *E. coli* was grown in Luria-Bertani (LB) broth, spin for 5 minutes at 10,000 rpm to get bacteria. Obtained pellet was resuspended in 467µl TE buffer followed by repeated pipetting. Then about 30µl of 10% SDS, 3µl of 20mg/ml proteinase K was added, mixed thoroughly and incubate for 1 hour at 37˚C. Upon incubation equal volume of phenol-chloroform-isoamyl alcohol (P:C:I 25:24:1) was added and mixed thoroughly but very gently to avoid shearing the DNA, by inverting the tube until the phases are completely mixed. The mixture was then centrifuged at 12,000 rpm for 10 minutes. Aqueous, viscous supernatant ~450µl was taken into a fresh microcentrifuge tube and equal volume of PCI was added, extract thoroughly and centrifuged at 10,000 rpm for 5 minutes. The supernatant was transferred to a fresh tube and 1/10th volume of 3M sodium acetate, 0.6 volumes of isopropanol was added, mixed thoroughly and kept on ice for 10 minutes. The mixture was then centrifuged at 13,000 rpm for 15 minutes. Then the supernatant was discarded and 1ml of 95% ethanol was added and kept at room temperature for 5 minutes. Then centrifuged at 12000 rpm for 10 minutes. Liquid was decanted followed by drying of DNA pellet and resuspension of pellet in 50 µl of TE buffer containing 7.5 µl of RNase. Extracted DNA was stored at either 4˚C for short time or at -20˚C for long time.

Primers used for PCR

A genus specific PCR was performed to amplify 16S rRNA of *E. coli* using previously published primers (Table 2).

Table 2. Primers sequences with product size and reference.

Primer	Sequence (5´-3´)	Size (bp)	Reference
E.coli 16E1 (F)	GGAGTAAAGTTAATACCTTTGCTC		
E.coli 16E2 (R)	TTCCCGAAGGCACATTCT	584bp	Tsen *et al.*, 1998
E.coli 16E3 (R)	TTCCCGAAGGCACCAATC		

Polymerase chain reaction (PCR)

PCR was performed in 25µl scale. The PCR mixture was consisted of 12.5 µl of 2xPCR master mix, 1.0 µl forward primer, 1.0µl reverse primer, 5µl of DNA template (50-400ng) and nuclease free water to adjust volume 25µl. Thermal condition was initial denaturation at 95°C for 5 min
followed by 35 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. Final extension was performed at 72^{0}C for 5 min. Upon completion of PCR the reaction mixture was hold 4°C for 5 minutes. DNA band was visualized in 1.5% agarose gel stained with ethidium bromide using transilluminator.

Results and Discussion

This research work focused on microbiological quality of milk. For this purpose, two types of milk viz. raw and pasteurized milk samples were randomly collected from Balibhadra and Sreepur bazaar, Ashulia, Savar, Dhaka and analyzed. Results are presented and discussed.

Microbiological quality of raw milk samples

A total of 40 raw milk samples were investigated during the study period. In the laboratory, milk samples were processed and tested for TVBC and TCC. The result of TVBC and TCC is presented in Table 3. From the table it is evident that all the raw milk samples tested (100%) under this experiment contains huge number of viable bacteria- ranges from $1.24x10^8$ to $9.6x10^9$CFUper ml of milk. In case of TCC, about 57.5% (23/40) of samples contain *E. coli* while 42.5% (17/40) was negative for coliform count. The coliform concentration was fond to ranges from $1.0x10^4$ to $4.45x10^5$. The most frequent cause of high bacterial content is normally as a result of poor sanitation of milking system.

Table 3: Total viable bacterial count (TVBC) and total coliform count (TCC) of different milk samples collected from local market.

Milk	Total viable bacterial count		Total coliform count	
	No. positive/ No. tested (% positive)	No. negative/ No. tested (% negative)	No. positive/ No. tested (% positive)	No. negative/ No. tested (% negative)
Raw milk	40/40 (100)	0/40 (0)	23/40 (57.5)	17/40 (42.5)
Bacterial load CFU/ml	$1.2x10^8$ - $9.6x10^9$	-	$1.0x10^4$ - $4.5x10^5$	-
Pasteurized Milk	20/30 (66.66)	10/30 (33.33)	0/30 (0)	30/30 (100)
Bacterial load CFU/ml	$1.0x10^2$ - $2.2x10^3$	-	-	-

Microbiological quality of pasteurized milk samples

A total of 30 pasteurized milk samples of three different brands were randomly collected from different shops of Balibhadra, Ashulia, Savar, Dhaka during the study period. In the laboratory, milk samples were processed and tested for TVBC and TCC. The result of TVBC and TCC in pasteurized milk is presented Table 3.All three brands (100%) are found positive for total viable bacterial count while found negative for coliform count. Of the samples tested, 66.66% (20/30) samples were found positive for TVBC. Total viable bacteria were not found in 33.33 (10/30) of pasteurized milk samples. In one brand (Group-1) about 80% (8/10) samples were foundpositive for TVBC and bacterial load ranges from $1.0x10^2$ to $2.2x10^3$CFU/ml of milk. In case of brand 2 and 3 (Group 2 and 3) about 60.0% (6/10) samples in each brand contain viable bacteria. In these two brands concentration of bacteria was found to be varied from $1.1x10^2$ to $2.5x10^3$CFU/ml in (Group-2) versus and $1.5x10^2$ to $1.6x10^3$ CFU/ml in (Group-3).

Molecular detection of *E. coli*

PCR was performed with representative samples (n=15). We could consistently amplify 584bp fragment of DNA using *E. coli* specific primers. A representative image is shown in Figure 1. From the figure it is clear that there is no amplification of DNA in lane 1 and 2 (non E coli bacterial samples). But DNA band is prominent in samples placed in lanes 3-8 containing *E. coli* DNA.

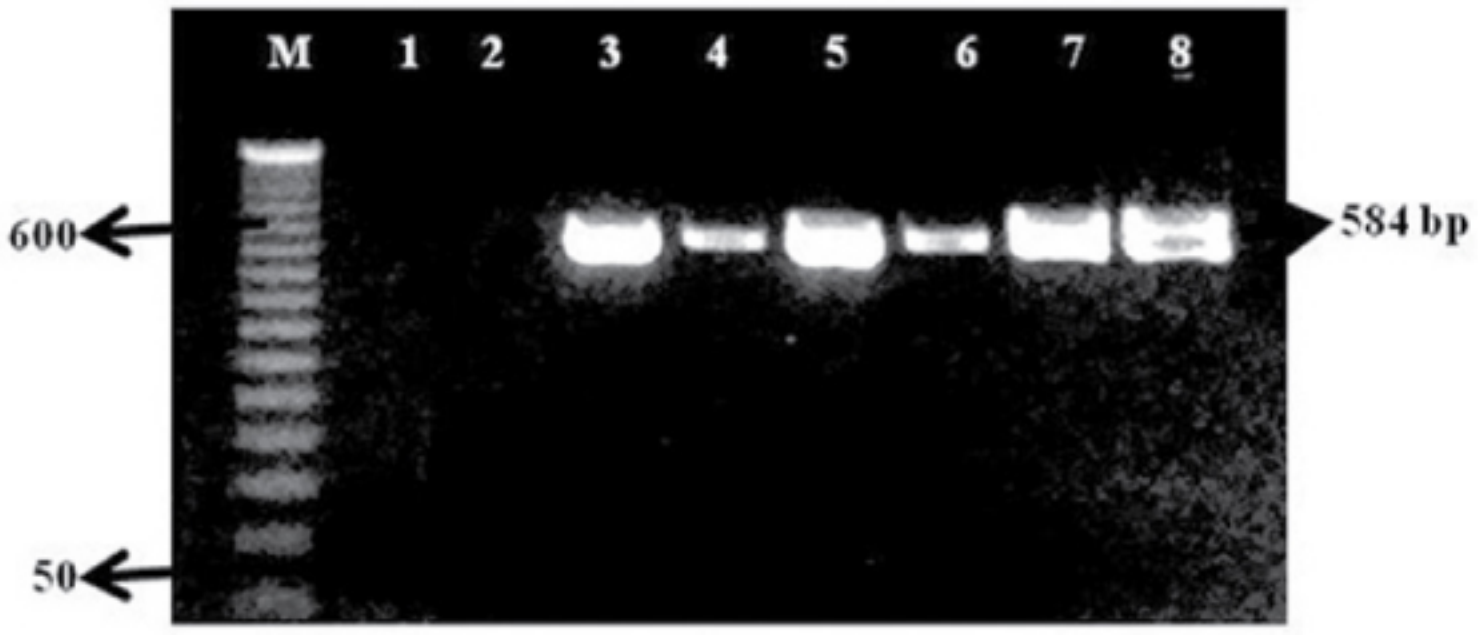

Figure 1: Amplified of 16SrRNA from *E. coli*, isolated from milk samples. M: Marker; Lane 1-2: Non *E. coli* bacterial sample; Lane3-8: *E. coli* sample. PCR product of 584bp is marked

Microbiological quality of raw and pasteurized milk

Milk is known to be the most complete food found in nature (Marjan *et al*., 2014, Hossain*et al*., 2010). But at the same time, it is highly vulnerable to bacterial contamination and hence is easily perishable (Kim *et al.,* 1983, OECD. 2005). Food borne diseases are of great concern around the world. However, this is an important issue in developing countries where poor sanitation is maintained during collection and processing of different types of food. Milk is considered as an excellent medium for growing of many microorganisms. Milk can be contaminated with several bacteria during milking process from the milking personnel, utensils used for milking. Besides, microorganisms may enter the udder through teat canal, and the bacteria may come out through milk (Smith *et al.,* 2007). *Staphylococcus aureus* and *Escherichia coli* are the two major contaminants of milk. The presence of the pathogen in milk largely depends on fecal contamination, and the presence of pathogen in feces mainly originates from feed contamination. The most frequent cause of high bacterial load in the milk is normally due to improper cleaning of the milking system. Bacterial count may be high due to milking dirty udders, maintaining an unclean milking and housing environment and failing to rapidly cool milk to less than 40°F (Saxena *et al.,* 2013). From the results of this study regarding microbial count in milk evident that all the raw milk samples tested under the experiment contains huge number of viable bacteria- ranges from $1.24x10^8$ to $9.6x10^9$in per ml of milk. In case of TCC, about 57.5% (23/40) samples contain *E. coli* while 42.5% (17/40) was negative. The coliform concentration was found to ranges from $1.0x10^4$ to $4.45x10^5$. Aaku *et al.,* (2004) have found 5.5×10^6CFU/ml and 10^6 to 10^7CFU/ml of the total number of microorganisms in raw milk, respectively, which were comparatively lower than this experiment. Hossain *et al.,*(2010) conducted an experiment in India and found that the bacterial count in raw milk ranged from 1.75×10^6 to 1.22×10^8CFU/ml. Banik *et al.,*(2014) analyzed microbiological quality of raw, pasteurized, UHT milk samples collected from different locations in Bangladesh and reported that raw milk samples were substandard in terms of TVBC and TCC. The range of TVBC and TCC in raw milk samples was 1.3×10^7 - 5.2×10^8CFU/ml and 1.0×10^4 - 4.2×10^4CFU/ml, respectively. On the contrary, the quality of pasteurized and UHT-treated milks was excellent. The TVBC range in pasteurized milk samples was reported from 1.1×10^2 - 1.8×10^3 to CFU/ml, slightly lower than that recommended by the Bangladesh Standards and Testing Institution (BSTI, 2002).

Generally, raw milk from a healthy cow contains a low microbial load (less than 1000/ml), but this count may increase up to 100 times or more when milk is left at room temperature (Richter *et al.,* 1992). Moreover, in cold seasons, low temperatures delay the bacterial growth rate, but milk preserved into bars in cold water well can reach a temperature of 15°C that can allow bacterial growth, up to 15 times in 12 hrs; unlike storage temperature of milk (4 to 7°C), where growth is minimal. The high contamination in raw milk samples indicated insufficient hygiene at milking (Aggad *et al*., 2010); during collection and transportation or insufficient industrial treatment. There are several reasons for the occurrence of bacterial contamination in the

pasteurized milk samples such as defect in pasteurization machinery, to survive even after pasteurization, and contamination in the post-pasteurized process due to poor processing and handling conditions and/or maintenance of substandard hygienic practices by working personnel. The TVBC and TCC in pasteurized milk samples in this study ranged from $1.1x10^2$-$2.5x10^3$CFU/ml (Group-2) and $1.5x10^2$-$1.6x10^3$ CFU/ml (Group-3) respectively. It is found that about 80% (8/10) of samples of Group -1 pasteurized milk contained viable bacteria that ranged from $1.0x10^2$ to $2.2x10^3$CFU/ml of milk. In case of Group -2 and Group -3, about 60.0% (6/10) samples of each brand contained viable bacteria. Banik *et al.,*(2014) examined the TVBC of the pasteurized milk samples in his study that ranged from 1.1×10^2 to 1.8×10^3 CFU/ml, which was slightly lower than that recommended by BSTI and USPHS (not exceeding 20,000 CFU/ml) (BSTI, 2002; Jay, 2003). Hossain *et al.*(2010) reported 7.5×10^7 to 1.24×10^8CFU/ml bacterial count in pasteurized milk samples. Banik *et al.,*(2014) found that the average coliform count in the raw milks ranged from 2.0×10^5 to 1.0×10^4CFU/ml which was higher than that obtained by Saitanu *et al.,*(1996) who found TCC of <1000 CFU/ml. The presence of *E. coli* in milk is a common indicator of fecal contamination.

E. coli was isolated from 23 out of 40 raw milk samples. A study by Uddin *et al.,*(2011), reported total coliform count of 1.0×10^4 to8×10^6CFU/ml. Several authors previously reported about the higher prevalence of *E. coli*. Banik *et al.,*(2014) found TCC in the raw milks that ranged from 2.0×10^5CFU/ml to 1.0×10^4 CFU/ml. Reasons for higher coliforms counts in raw milk may be as a result of poor hygiene, contaminated water, unsanitary milking practices, and improperly washed and maintained equipment (Marjan *et al.,* 2014). Coliform bacteria are supposed to be absent in pasteurized milk because they can't survive the pasteurization temperature. Due to defect in pasteurization process or post pasteurization contamination which includes contamination in packaging materials, defects in pipe lines, TCC may be detected in the pasteurized milk samples (Dey *et al.,* 2013). In this study as expected, the TCC was not found in the different pasteurized milk samples. According to USPHS not over 10 colonies is acceptable for 'Grade A' pasteurized milk (Jay *et al.,* 2003). There are many things which pose favorable environment for bacterial contamination. The unclean hands of worker, poor quality of milk, unhygienic conditions of manufacturing unit, inferior quality of material used and water supplied for washing the utensils could be the source of accelerating the bacterial contamination of milk products and the post manufacturing contamination (Marrier, 1973; Kumar and Sinha, 1989; Kulshrestha, 1990). Fecal coliforms usually indicate recent fecal contamination, because these bacteria cannot survive apart from the intestine for a long time and their number is generally proportional to pollution degree produced by feces (Aggad *et al*., 2010; Ravanis and Lewis, 1995). The presence of high numbers of coliforms in milk provides an index of hygienic standard used in the production of milk, as unclean udder and teats can contribute to the presence of coliforms from a variety of sources such as manure, soil, food, personnel and even water (Bille *et al*., 2009). The coliform standard for "Grade A" milk should not exceed 10 CFU/ml (Frazier and Westhoft 1958). When the fecal coliforms are numerous, they may lead to food poisonings

(Aggad *et al*., 2010). Serious pathogenic coliforms are *E. coli*, *Salmonella*, *Shigella* spp. And others (Bille *et al*., 2009). The presence of *E. coli* in raw and pasteurized milk samples was also be verified by PCR, along with cultural and biochemical tests. 16S rRNA gene of *E. coli* was identified by using methodology described earlier (Tsen et al., 1998). We used naturally contaminated raw milk samples collected from Balibhadra and Sreepur bazar, Ashulia, Savar, Dhaka, Bangladesh. The specific 584 bp DNA fragment was detected from representative (n=15) milk samples. Caro *et al.,*(2006) investigated the presence of *E. coli* O157:H7 in raw ewe s milk samples by a multiplex PCR assay for the presence of rfbO157 and fliCH7 genes. It seems that 16S rRNAbased PCR assay with using a combination of 16E1, 16E2 and 16E3 primers may be a valuable tool for detection of *E. coli* in milk and other dairy food products.

Conclusion

The present study has shown total bacterial count as well as coliform counts in raw and pasteurized milk samples collected from local market. The presence of large numbers of coliform in foods is highly undesirable. The presence of microorganisms in milk may be harmful to human health. Therefore, frequent inspection of the marketed milk should be carried out to check whether they meet the minimum legal standards. The overall hygienic conditions surrounding the production and handling of milk should also be

monitored. From the present study, it can be concluded that the microbiological quality of most of the raw milk samples tested were not satisfactory as indicated by their high bacterial loads and presence of coliform bacteria. However, pasteurized milk is found good as no coliform was detected.

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Detection and Quantification of Antibiotics Residue in Milk Collected from Non-commercial and Commercial Dairy Farms in Mymensingh District of Bangladesh

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Abstract:

The residue of Veterinary antibiotics (VAs) is becoming an obstinate health hazard and environmental problem in all over the world. However, residual levels of antibiotics and distribution are still obscure. This project systematically analyzed VAs residue in milk samples collected from Mymensingh district of Bangladesh. A questionnaire survey was conducted to understand farmer's regarding VAs in dairy product. All respondent farmers were multidrug users and only a quarter had residual knowledge (35.49%). However, most of them (86.08%) didn't respect the withdrawal period. Both identification (TLC) and quantification (HPLC) methods were applied to screen four antibiotics namely gentamycin (GEN), sulpha drugs (SUL), oxytetracycline (OTC) and amoxicillin (AMX) in 60 milk samples. Occurrence and concentration (mean±SD) of GEN, SUL, OTC and AMX are 10.83% and 28.81±20.85 mg/kg, 8.33% and 17.72±9.48 mg/kg, 23.33% and 16.50±11.48 mg/kg, 17.50% and 11.75±10.69 mg/kg respectively. Overall, this work explores the perspective of VAs residue in dairy farmers' level and builds a foundation for potential adverse effect of VAs residue in antibiotics resistance phenomenon through milk samples in human food chain.

Introduction

Veterinary antibiotics (VAs) are used worldwide in livestock and poultry farming to control disease, enhance growth and feed conversion efficiency and promote health (Zhang, Luo, Wu, Huang, & Christie, 2014). Continuous and frequent use of those VAs causes resistance population of bacteria which is an ordinary and inevitable phenomena. Prescriber or veterinarian compliance towards antibiotics prescription guidelines is a key to limit the spread of resistant bacteria.

Presence of antibiotics residues in food above the maximum acceptable level has been detected worldwide by different group of researchers (Chowdhury et al, 2015). Presence of VAs in milk could be due to treatment purpose like intra-mammary infusions of antibiotics for treating mastitis (92%), injections (6%), and other causes 2% (Booth et al, 1998). Presence of VAs residues in milk leads failure in validation of certain quality control tests. For human concern, antibiotic residues in food of animal origin produces potential threat to direct toxicity in human (cancers, allergic reactions, etc.), and low levels of antibiotic exposure results in alteration of microflora, and the possible development of resistance (Chowdhury et al, 2015).

Antibiotic residues in food samples like in milk is detected by screening methods and chromatographic techniques. However, microbiological assay techniques have been recommended as its offer simplicity but the bioassay methods lack specificity, and provide only semi-quantitative measurements of residues detected and sometimes produce false positives (Kuritto et al 2000). Chromatographic techniques, such as thin layer chromatography (TLC), and high pressure liquid chromatography (HPLC), and capillary electrophoresis (CE), have been used extensively for study of antibiotic residue in the food cycle.

No data regarding antibiotic residue in milk is available in Mymensingh division of Bangladesh. Therefore, the present work is taken to detect the level of antibiotic residues in milk from local and commercial farms in Mymensingh district of Bangladesh.

Materials and methods

Selection of Study Area

From April 2016 to March 2017, survey of farmers was carried out in 5 upazilla of Mymensingh district of Bangladesh both from commercial and non-commercial dairy farms; Sadar, Bhaluka, Muktagacha, Trisal and Gouripour.

Collection of samples

In total, 60 (sixty) milk sample were collected, both from commercial and non-commercial farm. Samples were collected in falcon tube and transferred to laboratory by ice bag container and stored at -18°C until extraction. Sample extraction was performed according to Zhao et al., 2010. Quantification of residues in samples was obtained and calculated from standard curves interpolated by the software (Chromeleon 6.8). Detection and chromatographic conditions were performed according to Zhao et al., 2010. Chromatographic parameters are tabulated in Table 1.

High Performance Liquid Chromatography (HPLC) analysis

The liquid chromatography system consists of Dionex UHPLC Ultimate 3000 (Thermo scientific, USA) equipped with a pump, an auto-sampler, a UV-Vis detector, C18 column (Acclaim® 120, 5µm, 120Å, 4.6 X 250 mm). For FQs and TCs; 278 nm and 356 nm wave length were used respectively. The detection parameters for antibiotic residues are summarized in Table 1. The concentration of antibiotic residue in the samples was calculated with reference to calibration curves that were prepared from working solutions of FQs and TCs using concentrations of 0.1, 0.5, 1, 2, 5, and 10 mg/L in methanol. Line curve fits were used for all analytes and correlation coefficients were > 0.99.

The response for each of the antibiotics detected with the HPLC methods was evaluated for linearity and the limits of detection and quantification for the instrument (LOD and LOQ) were determined using calibration curves containing six concentration levels (0.1, 0.5, 1, 2, 5 and 10 mg/L). LOD and LOQ were determined using the standard deviation of the response (σ); based on the calibration curve - the residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines was used as the standard deviation and the slope of the calibration curves (S) (ICH Steering Committee, 1996) as follows:

$LOD = 3.3\ \sigma/S$

$LOQ = 10\ \sigma/S$

Recovery studies for milk samples were carried out with antibiotic residue free milk sample at 1mg/kg of antibiotics. The average recoveries for all targeted analytes in the spiked sample ranged from 65.7% to 78.3%, with relative standard deviation (RSD) below 16%, confirming the validity of the methodology applied for the experiment.

Table 1: Detection parameters of analyzed antibiotics

Project	Parameter
HPLC	Dionex UHPLC Ultimate 3000
Column	Acclaim® C18 column (120, 5µm, 120Å, 4.6 X 250 mm)
Detector	UV-Vis
Wavelength	278 and 356 nm for FQs and TCs respectively
Column temperature	30°C
Flow rate	0.8 ml/min
Mobile phase	Mobile phase A: Acetonitrile Mobile phase B: 1% Acetic acid (pH 2.6)

Gradient elution	0 min: 10% A 18 min: 37% A 20 min: 80% A 22 min: 80 % A 25 min: 10 % A

Statistical Analysis

Surveyed data were stored in Microsoft Excel 2010 and further analysis was done by SPSS IBM 20 for descriptive statistics (IBM Corp. Released 2011).Responses to questionnaire were presented in simple frequency.

Results and Discussion

Farmers' perspective about usage and withdrawal period of antibiotics

A total 60 farmers were surveyed; 50% commercial and the rest were non-commercial farms. All farms (100%) uses more than one antibiotic. Out of 60 farmers, 35.49% farmers having the antibiotic residual knowledge and 64.51% have no knowledge about antibiotic residue in milk or food cycle. Only 32 % farmers have knowledge about withdrawal period. different types of Amoxycillin (AMOX), Oxytetracycline (OTC), Doxycycline (DOX), Sulfonamides (SUL), Gentamicin (GEN) and are mainly used. The prevalence of antibiotic usage in dairy farms in our study in Bangladesh was 60%. This finding is comparable with the reports of other studies; 52% (Turkson, 2008) and 100.00% (Wadoum *et al.*, 2016) who reported high usage of antibiotics in animal production.

Occurrence and detection of antibiotic residue in milk sample

All samples were screened by TLC for GEN, SUL, OTC and AMX. Samples found positive in TLC were further investigated with HPLC. Occurrence and concentration (mean±SD) of GEN, SUL, OTC and AMX are 20.83% and 28.81±20.85 mg/kg, 8.33% and 17.72±9.48 mg/kg, 23.33% and 16.50±11.48 mg/kg, 17.50% and 11.75±10.69 mg/kg respectively (Table 2)

Table 2: Occurrence and detected concentration of four antibiotics in milk samples

VAs	Occurrence (%)	Minimum (mg/kg)	Maximum (mg/kg)	Mean (mg/kg)	Standard deviation
GEN	10.83	1.66	60.42	28.81	20.85
SUL	8.33	2.15	37.41	17.72	9.48
OTC	23.33	2.83	39.48	16.50	11.48
AMX	17.51	1.75	35.41	11.75	11.05

In this study, survey of the dairy farmers shows that farmers have poor knowledge about antibiotics residue and withdrawal time of drugs. Through TLC, screening was confirmed and residue of four antibiotics were assessed through HPLC. Residue of all four antibiotics are above the maximum residual limit of the antibiotics concerned.

Use of tetracycline for treating various bacterial infection leads to residues accumulation in commercial milk. Our observations are coincided with Mehran et al., 2011 who found a similar result. Amoxicillin residues were found more frequent in the milk of commercial dairy cows. The present result is supported by (Khaskheli et al, 2018) as amoxicillin is a drug of choice for treating mastitis, but most of the time withdrawal period is not maintained. It may cause the presence of amoxicillin residues in milk.

Antibiotic residue over MRL is might be due to indiscriminate use and improper monitoring system for antibiotic withdrawal period of specific antibiotics in the dairy farms. Lack of knowledge about withdrawal period also should be credited for these higher residues. Presence of antibiotic residues above tolerance level induced allergic reaction which is a potential risk for public health safety. Indiscriminate and irrational use of antibiotics in livestock without following withdrawal period may result in unexpected residues in food supplies and could cause serious health hazards to consumers.

Conclusion

The present study is undertaken to visualize a figure of antibiotic used in dairy production in Bangladesh and this reports explores the perspective of VAs residue in dairy farmers and builds a foundation for potential adverse effect of VAs residue in antibiotics resistance phenomenon and in human food chain through milk sample.

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Antimicrobial resistance in food producing animal leads to human health hazard

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Abstract:
Antimicrobials have been used since the 1940s and have led to a dramatic reduction in illness and death from infectious diseases. Antimicrobials in animal food production systems have resulted in the emergence of antibiotic resistant bacteria that can be transmitted to other animals and humans—directly by contact and indirectly via the food chain, water, air, and manured and sludge-fertilized soils. The concern over resistance emergence and spread to people by therapeutic and nontherapeutic use of antimicrobials has led to conflicted practices and opinions. In recent decades, antibiotic resistant strains of bacteria are considered as an emerging global threat to both animal and human health as infection with those bacteria can result in increasing incidence of treatment failure and severity of disease. In this article, mechanism of development of antibiotic resistance in bacteria in food producing animals, possible ways of entering those antibiotic resistant strains of bacteria in human food chain and its global threat will be shortly discussed.

Key Words: Antimicrobials, Resistance, Bacteria, Human, Hazard

Introduction:

Antibiotics are low to medium molecular weight compounds exhibiting a variety of chemical and biological properties. This is mainly employed for chemotherapeutic and prophylactic purposes and also used as feed additives to promote growth and improve feed efficiency. In spite of these benefits, there is considerable concern from public health, food safety, and regulatory perspectives about the use of antimicrobials in food-producing animals. Over the last two decades, development of antimicrobial resistance resulting from agricultural use of antibiotics that could impact treatment of diseases affecting the human population that require antibiotic intervention has become a significant global public health concern. The safe and effective use of antibiotic in animal production has received considerable attention in most of the countries in the world. However, the antibiotic residues from milk,meat and egg may persist for longer period after treatment, when administration of antibiotics in label and extra label fashion and also not following of withholding period after treatment.The minimum withholding period for milk and egg is 7 days and for meat is 28 days after treated with antibiotics. Thedrug resistance has gained its importance due to the transmission of antibiotic resistance factorto other enteric organisms which have posed a serious public health concern. In general,harmful effects of drug and chemical residues on health, which may be mutagens,carcinogenic, teratogenic, reduction in reproductive performance, drug allergy and acute toxicity or poisoning in human. The present review forms on antibiotic resistance in animals and its impact on human health.

Antibiotic resistance (AMR) and multi-drug resistant (MDR)

According to the Centers for Disease Control and Prevention (CDC), **antibiotic resistance** or antimicrobial resistance (AMR) is the ability of microbes to resist the effects of drugs- that is, the germs are not killed, and their growth is not stopped. According to the Clinical Laboratory Standards Institute (CLSI), a strain of a bacterium is defined as **multi-drug resistant** (MDR) strain when it is found non-susceptible to at least one agent in three or more antimicrobial different classes of antimicrobial agents.

Why globe is concerned on antimicrobial resistance?

AMR is one of the biggest challenges facing global public health. Antibiotic resistance is a growing concern

that threatens the effective treatment of infectious diseases. Now-a-days antimicrobial resistance is a worldwide problem. The use and misuse of antimicrobial drugs in food producing animals accelerates the emergence of drug-resistant strains. Poor disease/infection control practices, inadequate sanitary conditions and inappropriate food-handling encourage the further spread of antimicrobial resistance. The selection and spread of resistant organisms in developing countries that can often be traced to complex socioeconomic and behavioral antecedents contribute to the escalating problem of antibiotic resistance in human.

Development of Antibiotic Resistance and Multi-drug Resistance:

Some bacteria are naturally resistant to certain types of antibiotics. However, bacteria may also become resistant in two ways: i) by a genetic mutation or ii) by acquiring resistance from another bacterium. Different genetic mutations yield different types of resistance. Some mutations enable the bacteria to produce potent chemicals or enzymes (eg. extended spectrum beta-lactamases-ESBL) that inactivate antibiotics, while other mutations eliminate the cell target that the antibiotic attacks. Bacteria can acquire antibiotic resistance genes from other bacteria in several ways- acquiring resistance genes through conjugation with resistant bacteria; acquiring resistance genes from bacteria through a virus; acquiring naked, "free" DNA directly from their environment.

Multidrug resistance in bacteria occurs by the accumulation of resistance (R) plasmids or transposons of genes, with each coding for resistance to a specific agent, and/or by the action of multidrug efflux pumps, each of which can pump out more than one drug type **(Fig. 1)**. Any bacteria that acquire resistance genes, whether by spontaneous mutation or genetic exchange with other bacteria, have the ability to resist one or more antibiotics. Because bacteria can collect multiple resistance traits over time, they can become resistant to many different families of antibiotics.

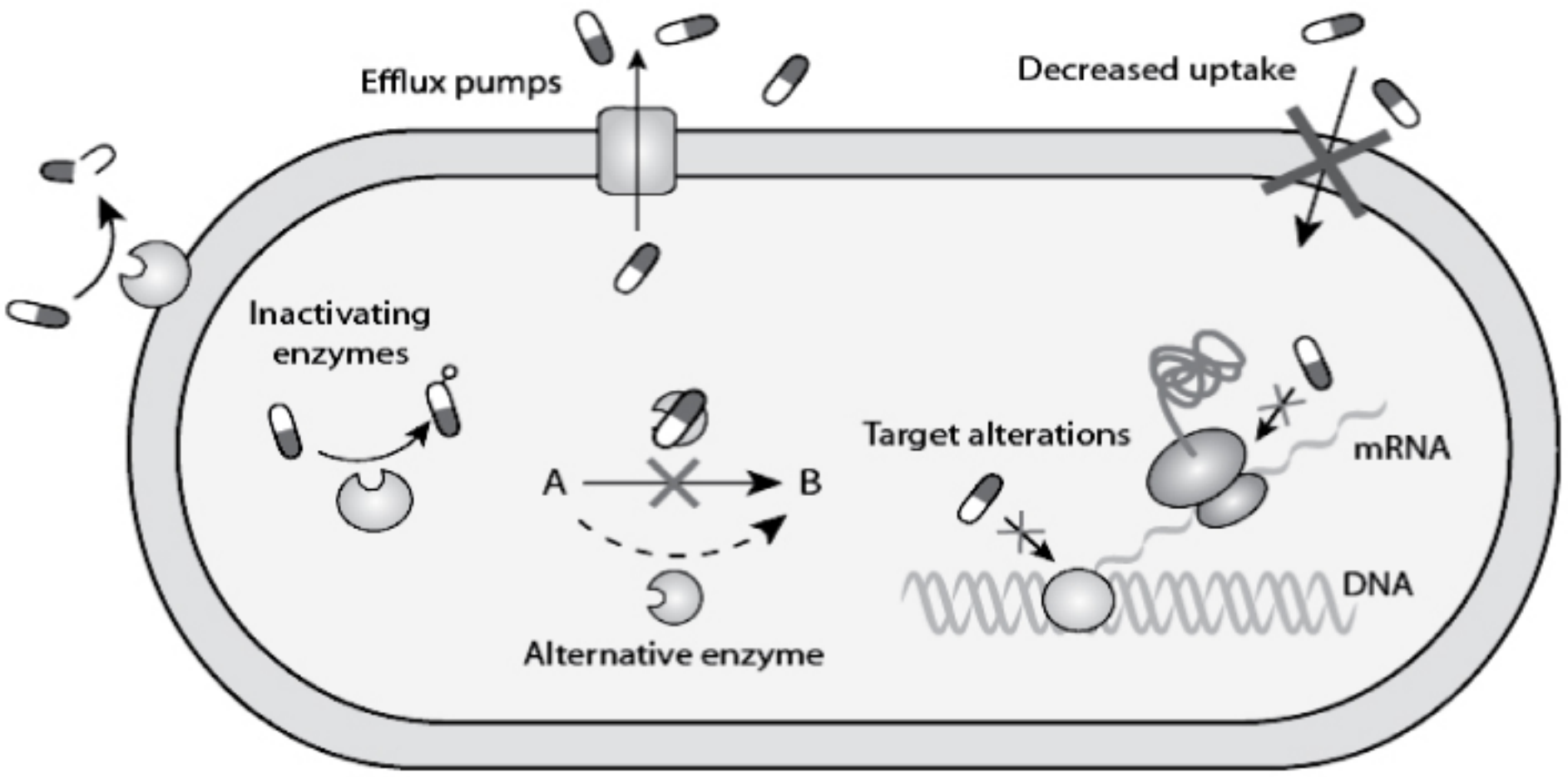

Fig. 1: Mechanism of development of antibiotic resistance in bacteria (Courtesy of Dr. E. Gullberg for providing the image).

Development of Resistant Bacteria in food producing animals and Transfer in Humans:

In many developing countries including Bangladesh, indiscriminant use of antibiotics is practiced for the treatment of animal diseases which may develop antibiotic resistant bacteria in those animals. Animals carry bacteria in their gut. Studies have shown that giving antibiotics to animals kill many bacteria, but resistant bacteria can survive and multiply in the gut **(Fig. 2)**. Scientists around the world have provided strong evidence that resistant bacteria in animals can enter into the human food chains through different ways and thus impose potential threat to human health around the world **(Fig. 3)**.

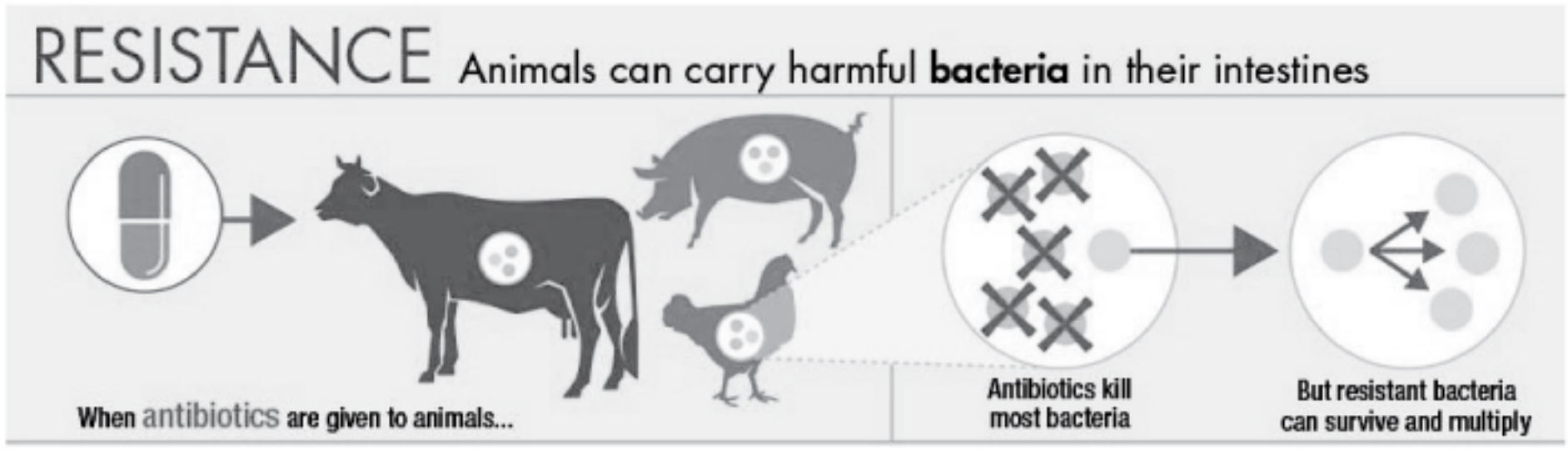

Fig. 2: Mechanism of development of resistant bacteria in food producing animals (Adapted from CDC spotlight image).

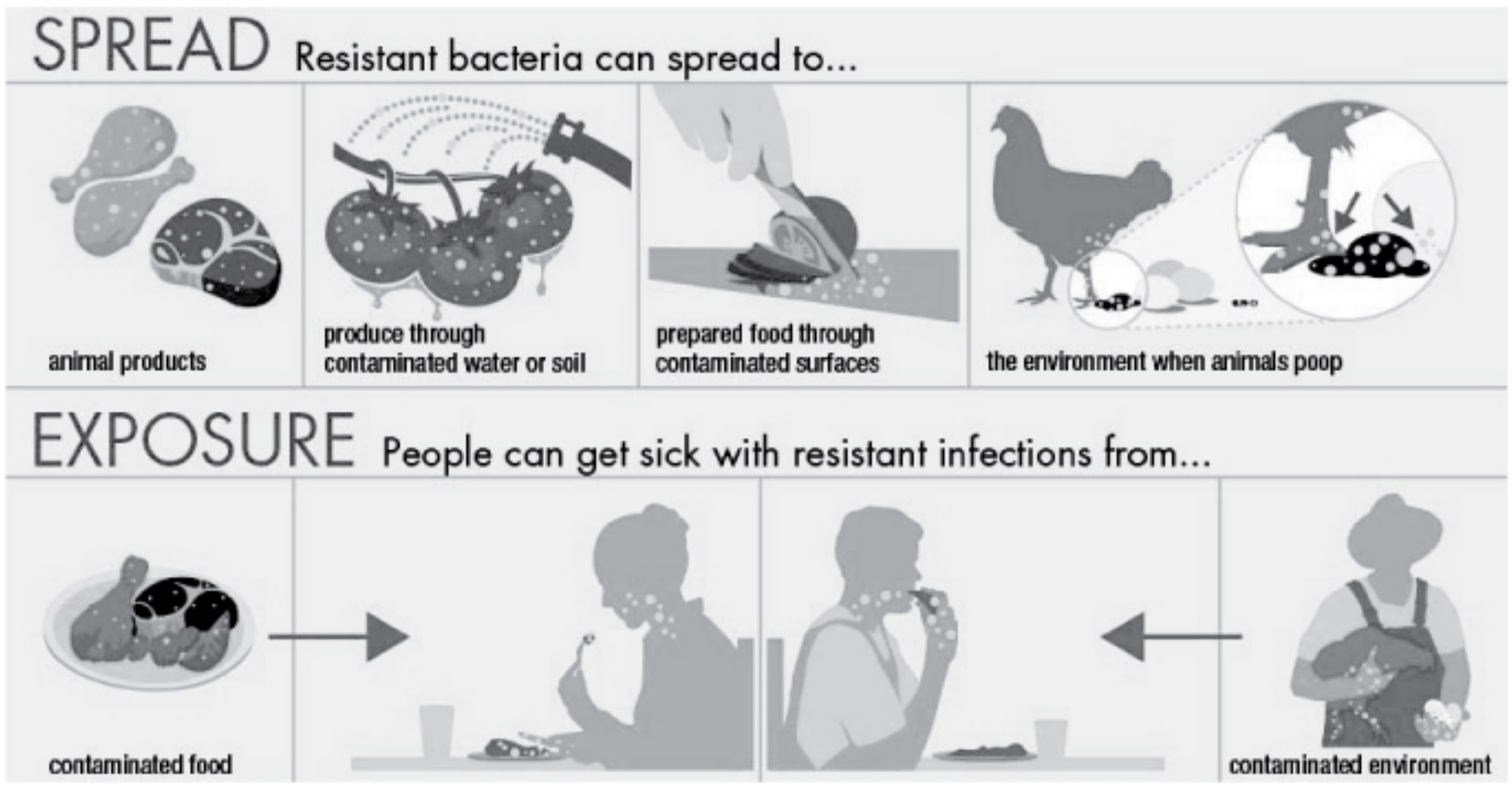

Fig. 3: Transfer of antibiotic resistance in humans: from the farm to the table (Adapted from CDC spotlight image).

Beginning of Antimicrobial Resistance:

Discovery	Emergence of Resistance
Penicillin-1943	1945
Vancomycin-1972	1988
Imipenem-1985	1998

Causes of emergence of antimicrobial resistant bacteria in food producing animals

In comparison to developed countries, the irrational use of antibiotics occurs more frequently in developing or least developed countries. Most of the developing countries anybody can buy any drug in any amount including antibiotics without prescription from the drug shops. In many cases, the sales man of the drug shops are prescribing and selling drug without any prescription from authorized physicians/veterinarians thus contributing finally to the development of resistance in microorganisms against commonly used antibiotics. Factors such as unregulated dispensing and manufacture of antimicrobials, truncated antimicrobial therapy, inadequate access to effective drugs and sometimes drugs of questionable quality and overall poverty are likely to be contributing to antimicrobial resistance.The widespread and inappropriate/irrational use of

antibiotic and also continuous use of antibiotics in feeds results in the development of a progressively antibiotic-resistant microbial ecosystem. Due to commercialization and introduction of exotic breeds of animals, more intensive animal keeping and natural resistance against diseases reduces which results in more use of antibiotics. Especially in developing countries, small and medium dairy farms have minimum biosecurity practices, which increase disease incidences and subsequent continuous use of antibiotics. Apart from therapy and prophylaxis, antibiotics are consumed to increase growth and feed efficiencies in poultry. This is clearly indicated by the high prevalence of antibiotic resistance among the community.

Antimicrobial Resistance in animals

Antimicrobial agents are widely administered in therapeutic treatment of animals worldwide. In most cases animal products & byproducts are marketed before the withdrawal period of antimicrobial drugs, even farmers sell their product during the treatment period with antibiotic. Antimicrobial drug residue in animal food chain values is a growing issue worldwide. Resistance of microorganism to antimicrobial drug is increasing day-by-day due to indiscriminate use of antibiotic for the treatment of bacterial diseases of animals. It is clear that use of antibiotics in adult dairy cows and other food-producing animals does contribute to increased antimicrobial resistance. Many feed companies and farmers use antibiotics to poultry feed as a growth promoter by preventing infection, which may also contribute to antimicrobial resistance. A recent estimate in the United States suggests that 24.6 million pounds of antibiotics are given to animals each year as growth promoters at sub-therapeutic amounts in their feed compared to 3 million pounds consumed by humans. Several studies shown antimicrobial drug resistance viz. oxytetracycline (80-85%), doxycycline (85-90%), chlortetracycline (70-80%), ciprofloxacin (40-50%), amoxicillin (50-60%), sulphar drugs (40-70%) and erythromycin (70-80%) is very common in poultry. In recent studies, it was detected large number of multi-drug resistant *E. coli* in broiler and layer meat among which 62% were resistant to three classes of antibiotics, 46% were resistant to four classes of antibiotics and 30% were resistant to five classes of antibiotics. Among all MDR *E. coli* isolates, 6.8 % were ESBL producing. The highest frequency of antibiotic resistance and selection pressure occurs in poultry processing. The average concentrations of amoxicillin residue in local milk, commercial milk, local egg, and commercial egg were 9.84 µg/ml, 56.16 µg/ml, 10.46 µg/g and 48.82 µg/g, respectively, in raw samples. This is dangerous not only for animals but also for human. If the situation continues, there will be no effective antibiotic for treatment until new generation of antibiotic comes.

Containment of Antimicrobial Resistance

To overcome the present situation, it should take the energetic measures to slow down the emergence and spread of antimicrobial resistance, should include programmes on surveillance, education and research on AMR, and regulation of use of antimicrobials in the animal sector. The efforts should include:

- Detection and awareness of problem of antimicrobial resistance by microbiology laboratory and public media.
- Research and surveillance (local, regional and international) on antimicrobial resistance in animals should be established in collaboration with international organizations.
- Advance development and use of rapid and innovative diagnostic tests for identification and characterization of resistant bacteria
- Antibiotic use guidelines and committee should be developed for each country.
- Antibiotic use in veterinary practices should be regulated and rationalized.
- Education on antibiotic resistance to manufacturers, prescribers, dispensers and consumers through continuing education and other means.
- Vaccines for preventing infectious diseases should be encouraged in all circumstances.
- Hygiene and sanitation should be improved and practiced.
- Value chain based standard practices to be formulated and practiced for different animal origin food products (milk, meat, egg and other value added products)
- Animal slaughter and processing system to be strengthened.
- Nutritional status of the animal population should be improved and maintained.
- One health approach considering human health, animal health and environmental health is required.

Conclusion:

Without any doubt, the introduction and use of antimicrobial agents in veterinary medicine had and still has an enormous impact on the health and welfare of animal species. But indiscriminant use of antibiotics in animals in developing countries might generate antibiotic resistant bacteria and transfer those resistant bacteria in humans through entering into human food chains. Therefore, strict guidelines for the use of antimicrobials in food producing animals and comprehensive antimicrobial drug administration monitoring systems should be urgently devised and implemented in developing countries to reduce the emergence of acquired multi-drug resistance in bacteria and to prevent the entrance of such bacteria into human food chain. Also, further research on alternative agents to antibiotics is needed.

characterization of *staphylococcus aureus* from milk and dairy products sold in some local markets of mymensingh district of Bangladesh

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Abstract

Staphylococcus aureus is an important foodborne pathogen which can be found in a good extent in milk as it is a nutrient enriched medium for their rapid growth. The main objective of this study was to determine the prevalence of *Staphylococcus aureus* in raw milk and some dairy products sold in the local markets of Mymensinghby using conventional and molecular techniques. Raw milk, pasteurized milk, curd, cheese, lassi, matha, milk shake, roshmalai, custard, faluda, pudding and borhani were sampled from the local markets of Mymensingh.The microbial load of *S. aureus*in the samples were determined by total viable count using pour plate technique method according to the International Commission on Microbiological Specifications for Foods (ICMSF, 1996) by using a surface plate technique. Out of 72 samples tested, all the samples were positive for *Staphylococcus spp.* and 57 isolates were coagulase positive *S. aureus*. The antimicrobial susceptibility of the pathogenic isolates was determined using 10 commercially available antimicrobial drugs by disk diffusion assay. The resistance pattern of coagulase positive *S. aureus* isolates of this study revealed that the most of theisolates (79.16%) were resistant to more than three antimicrobial agents. Some isolates (24.56%) showed resistance against both methicillin and oxacillin and intermediately resistant against vancomycin. In conclusion, our findings revealed that the milk and dairy product sold at local markets of Mymensingh are contaminated with multidrug resistant *S. aureus*which are very alarming for both human and animal health.

Key Words: Foodborne, Pathogen, *S. aureus*, Multidrug resistant

Introduction

Food is one of the most important basic needs of human being. Among various food items milk and dairy products are frequently consumed by the people as an ideal source of nutrients.Food is the good territory of proliferation and colonization of disease causing microorganisms (Rahman and Noor, 2012; Acharjee*et al.*, 2013; Ahmed *et al.*, 2013; Noor *et al.*, 2013; Ahmed *et al.*, 2014; Marjan*et al.*, 2014). In addition microorganisms are also essential for the production of food such as cheese, yogurt, bead, beer, wine and other fermented food. So it is very usual that there are so many chances of having diseases arising from food. The contamination of these food products with the pathogens and its persistence, growth, multiplication and/or toxin production is of great concern for public health. A number of pathogens has been responsible for food-borne diseases among them *Staphylococcus spp, Escherichia coli , Salmonella spp*tops the list.

Infections caused by *Staphylococcus aureus*, especially methicillin-resistant *Staphylococcus aureus* (MRSA) , are emerging as a major public health problem in hospital and community setting, causing a wide range of diseases. The emergence and spread of both health care and community associated MRSA has made infection control intervention and treatment challenging.

Antimicrobial agents are widely used not only in human and veterinary medicine, but also in animal husbandry and other agricultural activities. This has contributed to an alarming increase in antimicrobial resistance. Transmission of resistant bacteria from farm animals to humans can occur not only by contact with the animals but also through contact with, or ingestion of, food products of animal origin. However, in Bangladesh, the overall sanitary maintenances of the production, processing of milk and hygiene policies have unfortunately been reported not to be within the required standard (Khan *et al.*, 2008; Addo*et al.*, 2011; Marjan*et al.*, 2014). In addition to the proliferation of pathogens in milk and milk products, another important

concern has risen with the increase in antibiotic resistance which might pose a major clinical obstacle in diseases medication especially in the developing countries like Bangladesh (Tenover, 2006; Bennett, 2008; Canton, 2008, Jilani*et al*., 2008; Acharjee*et al*., 2013; Ahmed *et al*., 2013; Dutta*et al*., 2013; Noor *et al*., 2013). Several studies have indicated that the presence of *Staphylococcus aureus*in the raw milk sample is very common even in pasteurized milk because of their ability to produce heat-stable enterotoxin (Loir *et al*., 2003; Zinke*et al*., 2012; Shanebandi*et al*., 2014).

In Bangladesh a limited study has been conducted about the presenceof *S. aureus*in raw milk and cheese (Jahan*et al*., 2015, Nusrat*et al*., 2015,Kabir*et al*., 2017) and still no systematic study has been conducted to isolate and identify this organism from entirely the milk and dairy products and reports are very scanty on the microbial status of different milk and dairy products.

Along these lines, present study attempted todetermine the load as well as characterization of*S. aureus*in milk and dairy products available in and around Mymensingh townof Bangladesh.

Materials and methods

Sample Collection: **A** total of 72 samples of 12 different types of milk and dairy products were collected from different shops and restaurants available in and around Bangladesh Agricultural University and Mymensingh city corporation aseptically using sterile eppendorf tube, falcon tube and polyethylenebag and then immediately transferred to the laboratory at fresh condition .

Bacteria Culture and Enumeration:The samples were aseptically processed by homogenizer and diluted by using PBS for the identification and enumeration of *S. aureus*. An aliquot of 1 ml/ gm of each samples were homogenized with 9 ml/10 ml of phosphate buffer solution (PBS) (pH 7.2 ± 0.2) and diluted up to 10^{-4} according to the standard guideline (Cappuccino and Sherman, 1996). In order to determine the presence of *S. aureus*, 0.1ml of samples from each dilution was introduced onto the *Staphylococcus aureus* medium 110 (Oxoid Pvt. Ltd, England). The plates were incubated at 37 °C for 48 hours. The bacterial load of *S. aureus*in the samples was determined using the total viable count (TVC) method according to the International Commission on Microbiological Specifications for Foods (ICMSF, 1996) by using a surface plate technique (Fig. 1).

All the isolates were then subjected to sub-culturing on Mannitol Salt agar for obtaining pure colony (Fig. 2), Gram staining (Fig. 3) and culture on blood agar (Fig. 4) for morphological confirmation.Catalase (Fig. 5) and coagulase (Fig. 6) test were done for biochemical confirmation.

Antimicrobial Susceptibility Testing: All the coagulase positive isolates were evaluated for antimicrobial resistance using the Kirby–Bauer disk diffusion method according to the (Clinical Laboratory Standards Institute previously National Committee on Clinical Laboratory Standards., 2014). In this study ten commonly available antibiotics such as norfloxacin (NX, 30µg), ciprofloxacin (CIP, 5 µg), nalidixic acid (NA, 5µg), methicillin (MEC, 5 µg), oxacillin (OXA, 1 µg), tetracyclin (TE, 30 µg), Gentamycin (GEN, 10 µg), erythromycin (ERY, 15 µg), vancomycin (VAN, 30 µg) and penicillin (P, 10 units) were used. Suspensions of the test organisms were prepared using Muller-Hinton broth by adjusting the turbidity of the broth with normal saline to match the equivalent turbidity standard of McFarland (0.5 standards) and were incubated for 2 hours. Sterile cotton swabs were dipped into the suspensions and the swabs were then evenly spreaded over the entire surface of a Muller-Hinton agar plate to obtain uniform inoculums. Antibiotic discs of appropriate concentrations were applied aseptically over the surface of the inoculated plates at appropriate spatial arrangement by means of sterile forceps within a distance of 5 mm. Plates were then inverted and incubated at 37°C. After 24 hours, plates were examined and the diameters of the zones of complete inhibition were measured (Fig. 7) and interpreted as susceptible, or resistant (Ferraro *et al*., 2001, 2013-CLSIFDA).

Molecular characterization of S. aureus: Molecular identification and characterization of isolated Methicillin and Oxacillin resistant *S. aureus* strains were carried out by the detection of *mec*A gene (Fig. 8).

Polymerase Chain Reaction (PCR) were done following the method described elsewhere using Standard primers (Hamid *et al*., 2012)

Results and discussion

Out of 72 samples tested, all the samples were positive for *Staphylococcus spp.*that was confirmed by observing the cultural and morphological characteristics of the isolates (Table 1). According to Kabir*et al*., 2017 the growth of *S. aureus*on mannitol salt (MS) agar was confirmed by the fermentation of mannitol with change of color of media and formation of yellow color colonies and in Gram's staining, the organism revealed Gram positive, violet colored, cocci shaped and arranged in grapes like cluster under light microscope. A total of 47 raw milk samples were tested and *S. aureus*was isolated from 12 (25.53%) samples based on cultural and biochemical properties and all the 12 isolates showed β-hemolysis on blood agar media enriched with 5% sheep bloodwhich is similar to the findingsreported by byJahan*et al*., (2015) .

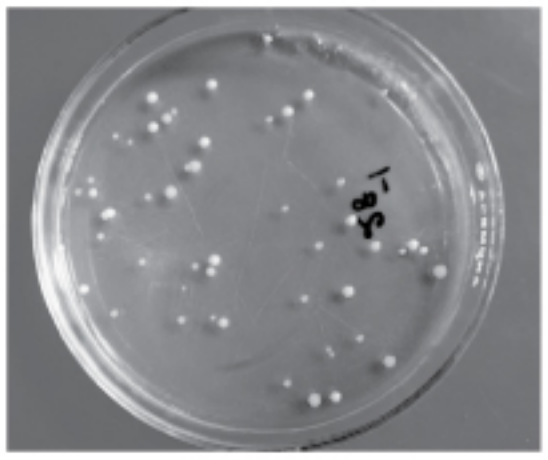

Fig. 1: CFU count after culturing on Staphylococcus Medium No. 110 agar plate

Fig. 2: Subculture on Mannitol Salt agar and obtaining pure colony of *S. aureus*

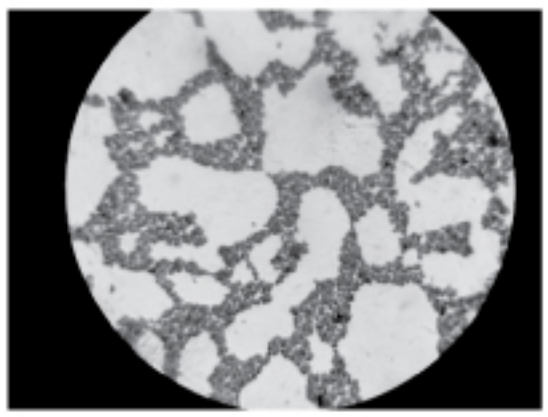

Fig. 3: Gram positive, purple colored, grapes like structure under Microscope

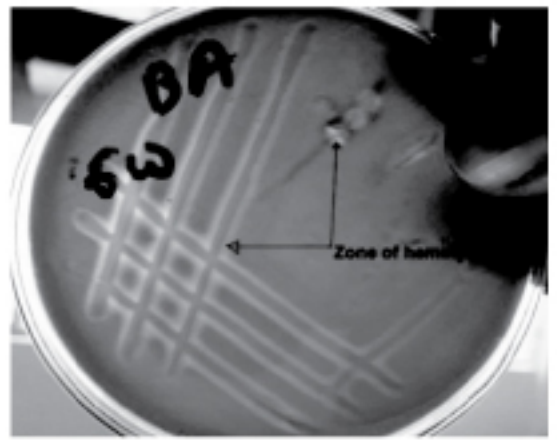

Fig. 4: Beta hemolysis on Blood agar

To confirm the initial pathogenicity of the isolated *S. aureus*, Catalase (fig. 5) and Coagulase test (Fig. 6) were performed as biochemical test that showed 57 isolates were coagulase positive out of 72(Table 2) and then those were subjected to further study by Antimicrobial Sensitivity test. Catalase and coagulase positive *S. aureus* were identified by observing the characteristics of bubble formation and formation of curd like clotting accordingly (Kabir*et al*., 2017).

The Total Viable Count of the food samples revealed the highest mcrobial load in Roshmalai, cheese and Yogurt accordingly and the lowest average CFU/g sample was found in Pudding (Table 3).

According to FDA *S. aureus* will not produce enterotoxin until $>10^5$ CFU/g is reached but the minimal infective dose is $<10^3$ CFU/g *S. aureus* in milk and other dairy products $\geq 10^4$ CFU/g indicates the product was produced under insanitary condition.Our findings revealed that milk and different dairy products tested in this study are heavily contaminated with *S. aureus*.However, to the best of our knowledge, this is the first

comprehensive report on the status of *S. aureus* load (CFU/gm) in different milk and dairy products available inMymensingh town of Bangladesh.

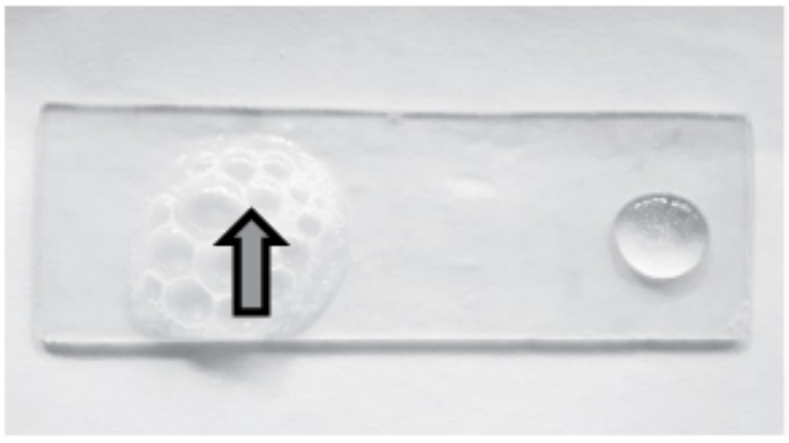

Fig. 5: Catalase test Positive

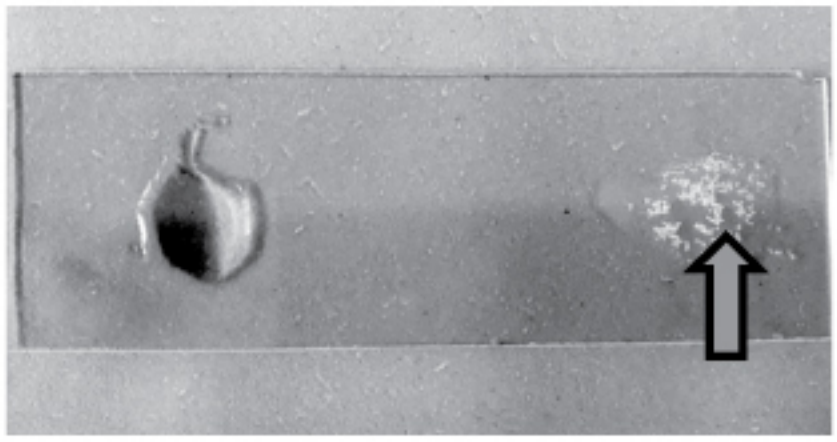

Fig. 6: Coagulase test Positive

The coagulase positive isolates were subjected to antibiotic sensitivity test by using 10 antimicrobials among which the highest resistance was observed against penicillin and lowest against vancomycin (Table 4).

The resistance pattern of coagulase positive *S. aureus* isolates of this study revealed that the most of theisolates (79.16%) were resistant to more than three antimicrobial agents. Some isolates (24.56%) showed resistance against both methicillin and oxacillin and intermediately resistant against vancomycin. The pathogenic isolates found in milk and cheese samples exhibited the multi-drug resistance trait against six commonly used antibiotics among which coagulase positive *Staphylococcus aureus*was found to be highly resistant against oxacillin (100%) and methicillin (58.82%) as reportedby Nusrat*et al*., (2015), which were significantly higher than the present study. Based on antibiotic sensitivity test, about 20-25% *S. aureus*isolates were found multidrug resistant and 100% were resistant to Penicillin which are very similar to the findings by Jahan*et al*., (2015).

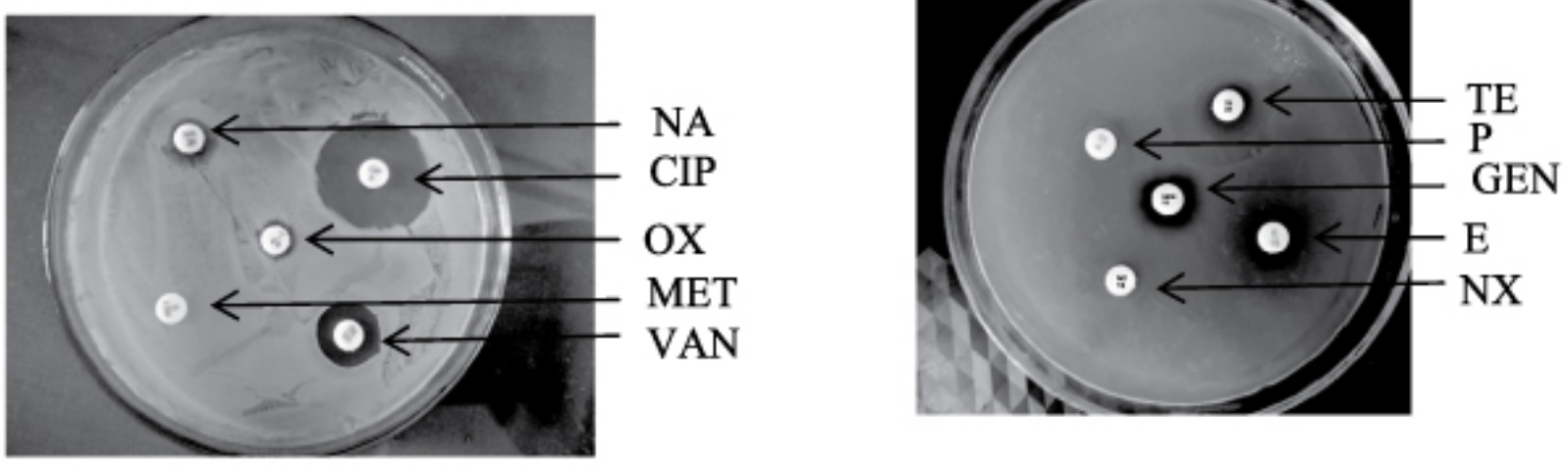

Fig. 7: Results of Antibiotic sensitivity test by disc diffusion method

For molecular characterization by PCR among the 14 isolates of methicillin and oxacillin resistant 5 isolates were found to be *mec*A positive (Table 5)and no isolates were *mecA* positive in a similar study reported byJahan*et al*., (2015).

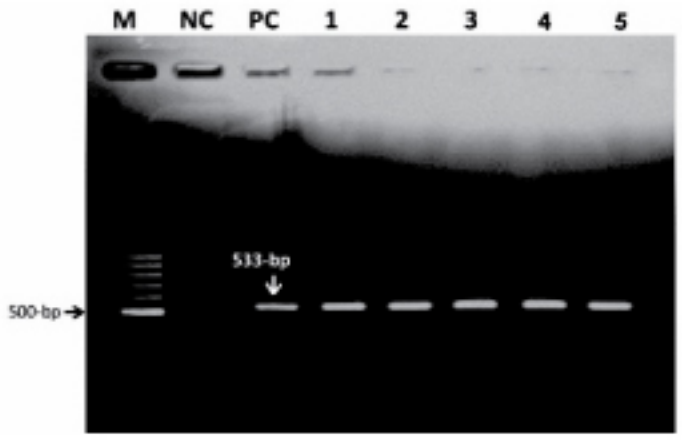

Fig. 8: PCR amplification of mecA gene of *S. aureus* M=Marker, NC=Negative control, PC=Positive control Lane ,1-5=Test samples

Traditional dairy products, especially those produced from raw milk under neglected hygienic conditions, are potential vehicles for the transmission of different foodborne pathogens including toxigenic *S. aureus* (Kadariya et al., 2014). MRSA, including animal-associated strains, have been frequently recovered from different dairy products such as raw milk or raw-milk cheeses worldwide (Peton and Le Loir, 2014). This study find out the status of the MRSA present in milk and dairy products available in Mymensingh district of Bangladesh. Considering the outcome of this study a control strategy may be developed to prevent and control the harmful effects of MRSA on public health in aspect of our country.

Table 1: Cultural, Morphological and Staining characteristics of the isolated *S. aureus*

Cultural characteristics				Morphological and Staining characteristics
Nutrient broth	Staphylococcus Medium No. 110	Mannitol salt agar	Blood agar	Gram positive, purple colored, small cocci shaped organisms arranged in grape like structure.
After overnight incubation the organisms showed turbidity	Smooth, circular Orange colony	Smooth, circular, yellowish to orange colonies with changing color of the media due to fermentation of mannitol.	Small round colonies with beta hemolysis.	

Table 2: Biochemical Characterization of the isolated *S. aureus*

Total No. of Sample	No. of *S. aureus*Positive Isolates	No. of Catalase Positive Isolates	No. of Coagulase Positive Isolates	No. of Isolates showing Hemolysis on Blood Agar
72	72	70	57	52

Table 3: Status of the *S. aureus*load in the Food Samples

Sl. No.	Types of Sample	No. of Sample	Minimum CFU/g	Maximum CFU/g	Average CFU/g
1	Raw milk	8	3×10^5	1.92×10^5	2.8×10^5
2	Pasteurized milk	2	2×10^5	3×10^5	2.5×10^5
3	Yogurt	23	9×10^4	19×10^6	11×10^6
4	Lassi	5	37×10^5	93×10^5	73×10^5
5	Matha	2	87×10^4	1.5×10^6	67×10^5
6	Borhani	5	52×10^5	64×10^6	98×10^5
7	Faluda	5	13×10^5	28×10^5	23×10^5
8	Cheese	3	37×10^5	21×10^6	13.9×10^6
9	Custard	3	4×10^5	13×10^5	8.5×10^5
10	Pudding	3	5×10^4	13×10^5	55×10^4
11	Roshmalai	10	5×10^6	26×10^6	16.2×10^6
12	Milk Shake	3	8×10^4	20×10^5	17.4×10^5

Table 4: Results of Antibiotic Sensitivity Test for the Coagulase positive *S. aureus*

Antimicrobial agents	Resistance Pattern of the Isolates		
	Resistant	Intermediate	Sensitive
Penicillin	57 (100%)	-	-
Erythromycin	42 (74%)	7 (12%)	8 (14%)
Tetracycline	43 (75%)	9 (16%)	5 (9%)
Gentamycin	21 (37%)	9 (16%)	27 (47%)
Nalidixic Acid	44 (78%)	2 (3%)	11 (19%)
Ciprofloxacin	29 (51%)	5 (9%)	23 (40%)
Norfloxacin	17 (29%)	3 (5%)	37 (66%)
Methicillin	19 (33%)	15 (26%)	23 (41%)
Oxacillin	16 (28%)	6 (11%)	35 (61%)
Vancomycin	-	14 (24%)	43 (76%)

Table 5: Molecular Characterization of *S. aureus*

No. of total sample	No. of coagulase positive sample	No. isolates resistant to Methicillin and Oxacillin in antibiotic sensitivity test	No. of isolates used for PCR targeting mecA gene	No. of positive isolates in PCR
72	57 (79.16%)	14 (24.56%)	14 (24.56%)	5 (35%)

Conclusion

The presence of methicillin-resistant *S. aureus*in milk and dairy products samples is indicative of treatment failure scenario during food borne disease outbreaks. Furthermore, the current findings may provide the proper guidelines of hygienic handling and processing of raw milk and milk products followed by proper pasteurization and fermentation.In conclusion, our findings revealed that the milk and dairy product sold in and around Mymensingh town are contaminated with multidrug resistant *S. aureus*which are very alarming for both human and animal health.

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Industrial

Use of phytobiotics to improve health in tilapia

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Abstract

Phytobiotics are immunostimulants and prebiotics modulating gut flora and leading to a better microvilli development. Natural compounds are able to improve animal health minimizing considerably the use of chemicals in animal production. The continuous inclusion of the phytobiotic in the whole cycle can improve significantly the production parameters reducing bacterial and parasites outbreaks. A trial in a commercial farm of tilapia in Mexico was made using a phytobiotic made with herbs, essential oils and organic acids. The study started with tilapia of 5 days after yolk sac absorption and were stocked in 4 tanks of 28 m^3 with 10,000 fingerlings each. Two tanks were treated with food enriched with Liptocitro Growth Plus (treatment group) and two others fed with commercial feed (control), for a period of 23 days in the open system, with a 0% water replacement during the first 5 days and approximately 7% daily for the remaining 18 days.The phytobiotic was top dressed at a dose of 5g/Kg on a commercial feed with 52% crude protein then mixed with vegetable oil and dried. Survival rate (SV) in the group treated with the phytobiotic was 13% higher than the control group. During the trial three tanks out of four suffered a bacterial outbreak for 8 days (one from the treatment group and two from the control group). Average body weight improved 23.07% compared with the control group. Parasite counts (*monogenean* and *trichodinas*) were done every four days in 20 fish from each tank. Results showed that treatment groups had less parasites decreasing their number as the culture time increased.

Keywords : Phytobiotics, Gut, Health, Immunostimulant, Prebiotics, Tilapia.

Introduction

Tilapia farming is growing very fast in recent years in Bangladesh. In 2015 Bangladesh was ranked the 4th country in production with 377.346 Mt after China, Indonesia and Egypt in this order of importance (FAO, 2016). Starting tilapia farming in 2009 with 16.237 MT Bangladesh has grown tremendously at a rate of 52% in 2010, 320% in 2011 and 210% in 2015.

To maintain this huge increase sustainably a lot of technical development and improvements in genetics, management, feed quality need to be done. A special attention to density and production increases considering diseases and outbreaks must be paid. The use of chemicals to increase growth and survival will not last long, fish will get resistant and contamination to the ponds, soil and water, and to neighbour farms and water banks will be a serious problem.

Preventive management through phytobiotics is a sustainable strategy for a better control production and free of chemicals.

Phytotherapy provides efficient solutions to the cultured animal helping them to fight against parasites, bacteria and/or fungal pathogens.Preventive strategies together with best management practices are vital for better profitability and sustainable farm production. The continuous inclusion of phytobiotics in the feed produces healthier animals, with better immune system and growth performance ratio.

Materials and methods

An experiment done in 2016 with a phytobiotic under the commercial name of Liptocitro Growth Plusfrom Liptoaqua-Liptosa, Spain, made with organic acids, plants and essential oils ina commercial tilapia farm in Mexico showed positive results in survival and growth in tilapia. The study started with 5 days after yolk sac absorption and were stocked in 4 tanks of 28 m3 with 10,000 fingerlings each. Two tanks were treated with food enriched with Liptocitro Growth Plus (Treatment group) and two others fed with commercial feed (Control), under normal growing conditions for a period of 23 days in the open system, with a 0% water replacement during the first 5 days and approximately 7% daily for the remaining 18 days.

The phytobiotic was top dressed at a dose of 5g/Kg on a commercial feed with 52% crude protein then mixed with vegetable oil and dried.

Results

Growth and survival

The survival rate (SV) in the group treated with Liptocitro Growth Plus was 13% higher than the control group (**Figure 1**). Also, three tanks out of four suffered a bacterial outbreak for 8 days after starting the trial (one from the treatment group and two from the control group).

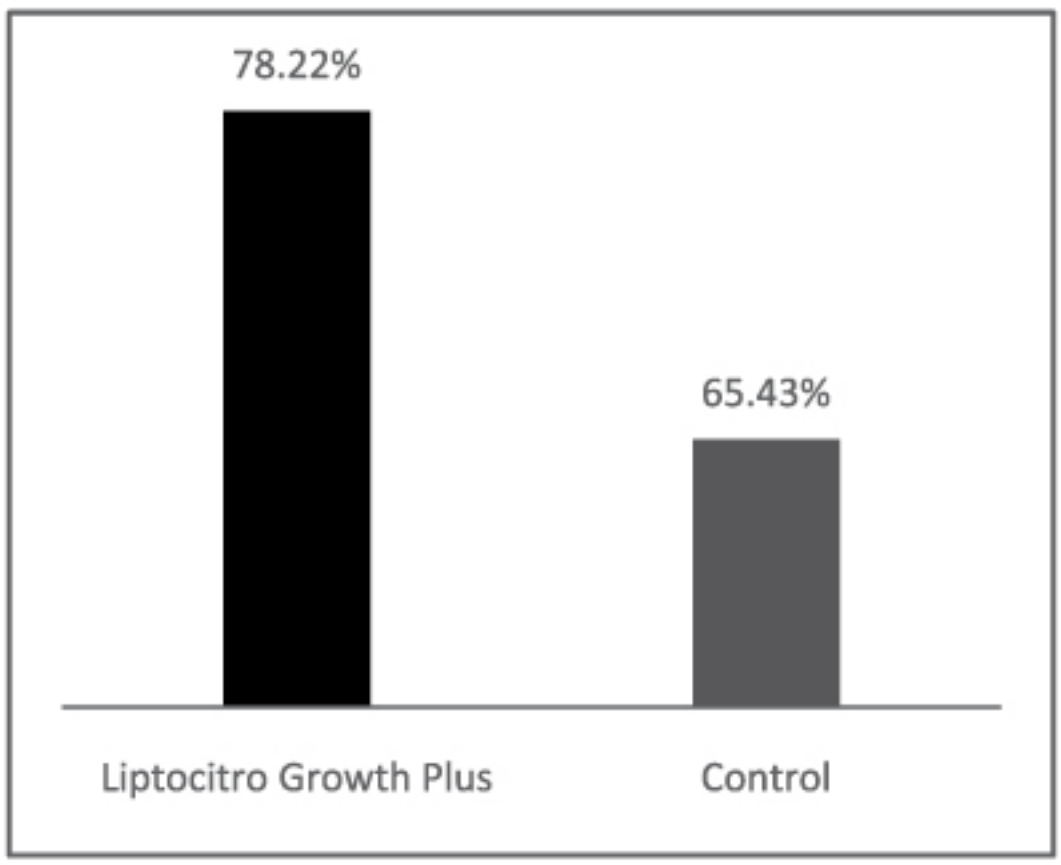

Figure 1.- Survival data obtained per tank after 24 days of treatment

The group with Liptocitro Growth Plus performed better than the control group with an average increase of 23.07% more in average weight (**Figure 2**).

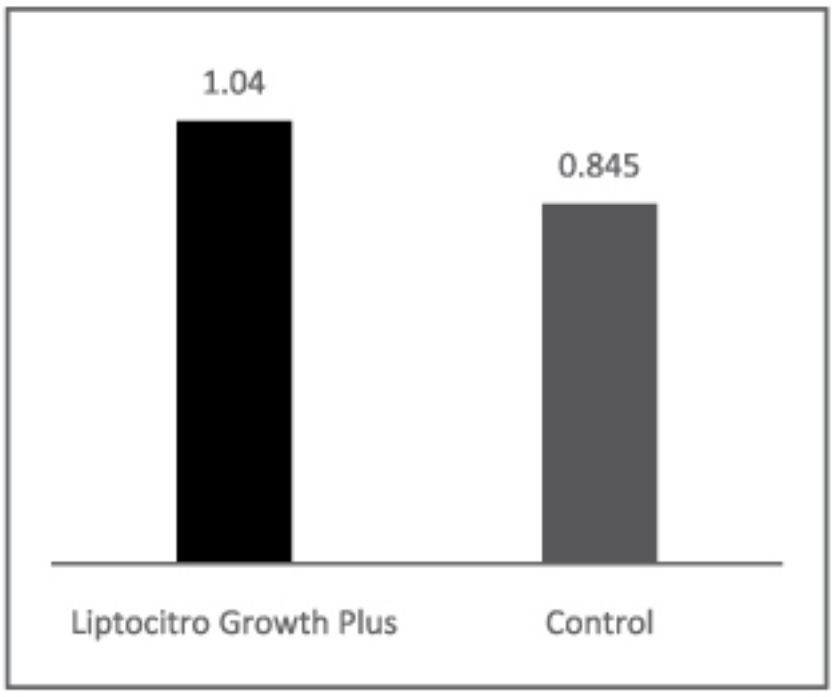

Figure 2.- Average body weight data after 24 days of treatment

Parasites infestation

Presence of ectoparasites (*monogeneas* and *trichodinas*) was monitored every 4 days, slaughtering 20 fish from each group. In both groups it was observed that the presence of parasites decreased as the culture time increased (**Figure 3 & 4**).

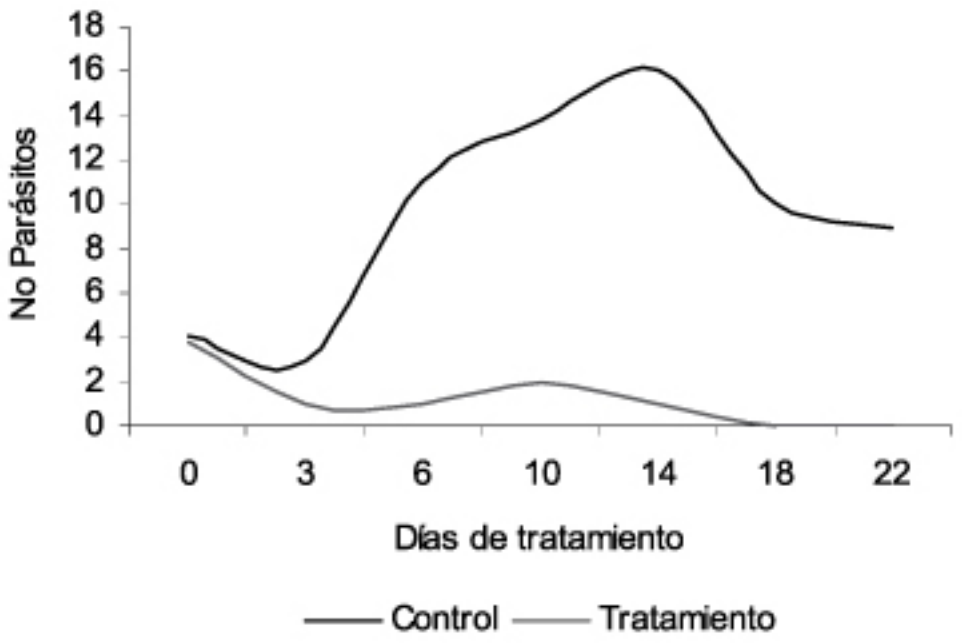

Figure 3.- Incidence of Trichodina sp. in each of the test groups during 18 days of treatment.

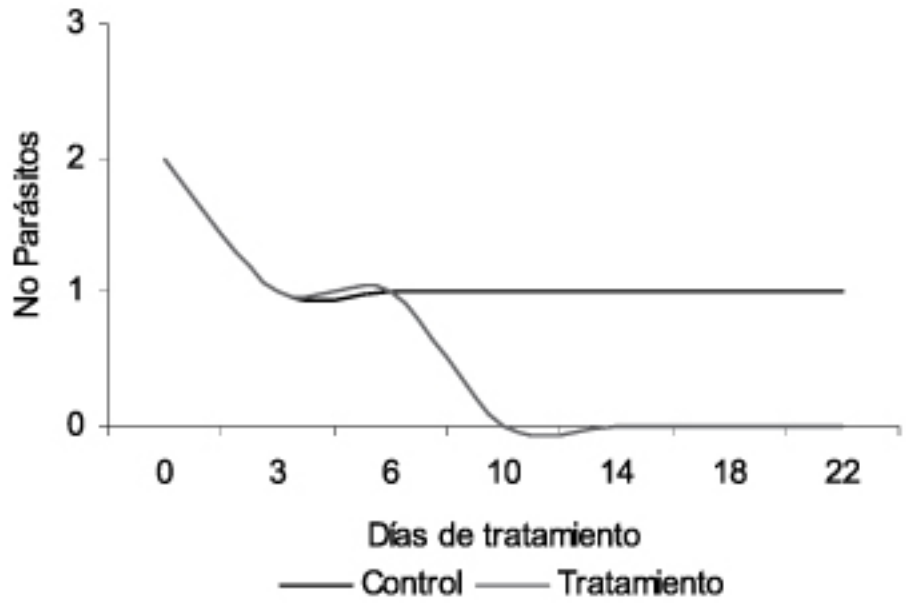

Figure 4.- Incidence of monogenean in each of the test groups during 18 days of treatment.

Discussion

The main risk in fish production is the occurrence of diseases that may adversely affect the overall operating costs and production durations, thus resulting in a loss in profitability. A direct consequence of high culture densities is the appearance of opportunistic pathogens which thrive in a crowded environment and affect negatively the immune response of the species cultivated. The inclusion of specific phytobiotics in feed leads to zootechnical parameter advantages, such as better survival rate and feed conversion ratio (FCR), and higher daily gain growth.

Natural products made from plants and their extracts provide efficient solutions to the cultured animal helping it to fight against parasites, bacteria and/or fungal pathogens. Preventive strategies as well as best management practices are recommended for better profitability and sustainable farm production. The continuous inclusion of phytobiotics in the feed given to fish and shrimp will produce healthier animals, with better immune system and growth performance ratio.

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Incidence of common diseases and disorders of cattle at Kotalipara Upazila, Gopalgonj, Bangladesh.

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ABSTRACT

The study was conducted at Kotalipara Upazilla, Gopalgonj, Bangladesh during the period from October to January 2017 to determine the incidence of common diseases and disorders in cattle. A total of 460 sick cattle were examined during the study where young 18.26%, growing 27.83% and adult 53.91%; male 29.78% and female 70.22%; local breed 35.87% and 64.13% cross breed cattle were recorded. Among the common diseases disorders parasitic infestation was recorded 21.74% (100/460). Similarly anorexia 10.43% (48/460), myiasis 6.52% (30/460), anoestrus 6.30% (29/460), mastitis 4.35% (20/460), malnutrition 6.52% (30/460), diarrhea 3.70% (17/460), pneumonia 3.70% (17/460) and wound 5.65% (26/460) were also commonly recorded. According to consideration of different system the diseases and disorders were more recorded in digestive system 50.22% (231/460) than other systems. Proper prevention and control measures should be taken to reduce the infection and infestation of diseases and disorders in cattle. Further extensive epidemiological approaches are suggested for the prevention and control measures of diseases and disorders in study area.

Key words: Incidence; Diseases; Disorders; Cattle.

INTRODUCTION

Bangladesh is considered as the most densely populated country in the world. Most of its population living in rural area and their per capita income is very low. With continuous growing of its population, the demand for food particularly animal sources are increasing rapidly. Rearing of livestock species can be the only way to fulfill this demand. About 25% of our population is directly and 50% is indirectly dependent on livestock for their income generation and livelihood improvement (Rahman *et al* 2014). In recent years, the purpose of rearing cattle has been changed from traditional use for draught power to milk and meat production. About 36% of total animal protein comes from the livestock products in our everyday life (Hoque and Samad, 2010). Moreover, livestock plays a vital role to national GDP through producing organic manure, supplying fuel, leather, horns, hoofs, generating cash income and earning foreign remittance. However, current domestic milk and meat production is insufficient compared to our daily requirement.

In recent years, the purpose of rearing cattle has been changed from traditional use for meat to milk production. About 36% of total animal protein comes from the livestock products in our everyday life. However, current domestic milk and meat production is insufficient compared to our daily requirement. Livestock diseases and disorders of animals are the most important hindrance towards these production and livestock development in our country. Most of our animals are weak, unhealthy, emaciated and their productive performances are not satisfactory due to various diseases and disorders. The prevalence of diseases and disorders of cattle were reported in various regions of Bangladesh.

Sarker *et al*. (2013) reported 73.95% and 17.16% diseases and disorders in cows and calves respectively. It has also been reported that among the clinical diseases and disorders, general and systemic 58.42%, infectious diseases 23.56%, production diseases 12.48%, and surgical and reproductive diseases 5.54%.The prevalence

of clinical diseases and disorders in cattle reported by Pallab *et al* (2012) as 47.05%. Roy *et al.* (2012) reported 55% gastro-intestinal parasitic infestation. The common surgical disorders include myiasis 1.21%, tympani 1.13%, navel ill 0.99%, wound 0.79%, dog bite 0.73%, foot diseases 0.59%, abscess 0.32%, urolithiasis 0.27%, fracture 0.23% and upward patellar fixation 0.11% were recorded by Arju (2013). Rahman *et al.* (2010) showed that 29.2% parasitic infestation in cattle. But, there is no study available on prevalence of diseases and disorders of cattle in study area. So, present study was undertaken with a view to fulfill the following objectives.

- To know the overall incidence of disease and disorder in cattle.
- To find out the present status of disease and disorder in relation to sex (male vs. female), age(young, growing and adult), breed (local vs. cross breed) and the affected system of the cattle.

MATERIALS AND METHODS

The study was conducted at Upazila Livestock Office, Kotalipara, Gopalgonj from October to January, 2017. A total of 460 animals were examined during the study. All information of cattle includes age, sex, breed, type, body weight etc. were recorded for clinical examination. The presumptive diagnosis of different diseases and disorders were performed on the basis of owner's complaints, anamnesis, general clinical examination, physical examination and clinical findings.

1. Owner's complaints

Complaints of the owners or attendant were considered during examination of cattle.

2. Anamnesis

Information's of animals were recorded from owner or attendant. The cases were recorded by asking question to the owners or attendant directly or indirect way.

3. Physical examination

Examination of different parts and system of the body of each of the sick animals were examined by using the procedures of palpation, percussion, auscultation, needle exploration, extension and flexion of limbs and walking of animals as per methods described by Kelly (1979) and Samad (1988a).

4. General clinical examination

Clinical examination of all cattle were conducted on the basis of diseases history and owner's complaint, clinical findings and techniques used by Rosenberger (1979) and Samad (1988a) to diagnose the following diseases and disorder.

4.1. Parasitic infestation

Gastrointestinal parasitic diseases were diagnosed by fecal examination in the laboratory. Fecal samples were collected from the rectum of all the diarrheic and suspected parasitic cases to detect the gastro-intestinal parasitic infestation. Collected feces were examined by a) Direct smear, b) Simple sedimentation and c) Centrifugation–floatation method as described by Samad (1988a) to detect the parasitic eggs and oocysts.

4.2. Anorexia: Anorexia with the history of partial and complete absence of appetite with varying degrees of decreased food intake.

4.3. Myiasis : Swelling of naval cord or vagina with presence of larva.

4.4. Pneumonia

Pneumonia was diagnosed on the basis of owner's complaint with recorded abnormal functions of respiratory system like polypnea, dyspnea, coughing, sneezing etc. and examined the entire respiratory tracts as described by Rodostits *et al.* (2007)

4.5. Fever

The rectal temperature when recorded ≥ 103°F considered as fever (Radostits *et al*., 2007).

4.6. Babesiosis

Babesiosis was presumptibly diagnosed on the basis of tick biting and clinical findings include increase body temperature, pallor of mucus membrane and coffee color urine.

4.7. Skin diseases

Different types of discrete and diffuse skin lesions were diagnosed clinically by visual examination, parting of the hairs using magnifying glass and skin scraping examination by Rosenberger, (1979).

4.8. Foot and Mouth disease (FMD)

Presence of fever, vesicular eruption, erosion, ulceration in the mouth and on the feet with the history of rapid spread of the disease was regarded as FMD.

4.9. Retained placenta

Placenta retained or unable to expel after parturition.

4.10. Diarrhea

Profuse watery feces, dehydration, loss of body weight, shrunken face and eye ball reduce in the socket was the principal clinical manifestation for diarrhea.

4.11. Black quarter

Black quarter was diagnosed from the owner's complain and from the clinical examination from the affected muscle area, lameness condition and the age of the cattle below 2 years of age.

4.12. Bloat: Abnormal distention of abdomen

4.13. Abscess: Swelling of the area with presence of pus in the cavity.

4.14. Anemia: Paleness of the mucous membrane with malnutrition and sickness.

RESULTS AND DISCUSSION

1. Parasitic Infestation

Parasitic infestation was recorded in cattle 21.74% (Table 1). In cattle it was also recorded 4.34% in calf, 6.73% in growing, 10.65% in adult; 6.74% in male, 15% in female; 8.69% in local, 13.04% in cross breed (Table 2,3,4). Ahmed *et al* (2015) recorded the incidence of gastrointestinal helminths 65.2% in cattle. Rashid *et al* (2015) reported 84.8% infestation in Brahman cross breed cattle of Bangladesh. Rahman (2012) also reported prevalence of parasitic infestation 50.4% which was higher than recorded study.

2. Anorexia/Inappetence

Complete or partial loss of appetite was diagnosed 10.34% on the basis of history obtained from the owner (Table 1). Relatively higher occurrence was reported by Sarker *et al*. (2013), who found anorexia 17.55%. Oral lesion, indigestion, fever, toxemia, stress, poisoning and a lot of others may be responsible for anorexia/ inappetance which should be confirmed by clinical and laboratory examination.

3. Myiasis

Myiasis or maggot infestation was recorded 6.52% in (Table 1). In cattle it was recorded 5.44% in young 1.08% in adult; 1.08% female 5.44% in male; 2.83% in local and 3.69% in cross breed (Table 2,3,4). Arju (2013) reported myiasis 1.21% under hospital conditions in cattle which is lower than present study. Soyelu & Masika (2009) reported that a great majority of the interviewed farmers reported incidences of wounds and myiasis 84.3% and 92.5% respectively in their herds.

4. Pneumonia

Pneumonia was recorded 3.69% (Table 1). It was also recorded 2.17% in young, 1.08% in growing and 0.43% in adult; 0.86% in male cattle, 2.83% in female; 2.17% in cross breed and 1.53% in local (Table 2,3,4). Rahman *et al.* (2010) reported respiratory disorders 5.5%, which was higher than recorded study. Young are more susceptible to pneumonia due to low immunity level for insufficient colostrum feeding and sometime lack of post parturition care of calves. Inadequate ventilation of calf barns increases the risk of pneumonia due to humidity of environment, noxious gases, dust and bacterial content (Lorenz *et al*., 2011).

5. Mastitis

Mastitis, an economically important disease of milch animal, was diagnosed on the basis of history and physical abnormalities of udder (Radostits, *et al*. 2007). Clinical mastitis was recorded 4.34 % in cattle (Table 1) during the period of study. It was also recorded 0.00% in growing and 4.34 % in adult; 4.34 % in female; 0.65% in local and 3.69% in cross breed cattle (Table 2,3,4). Kabir *et al.* (2010) reported clinical mastitis (1.14%) which was lower than recorded study.

6. Black quarter

Black quarter was recorded in 0.65% of infectious diseases (Table 1). It was recorded 0.65% in growing; 0.22% in male 0.43% in female and 0.65% cross breed (Table 2,3,4). Kabir *et al.* (2010) reported black quarter 0.57%, which is lower from this study. It is mostly confined to young stock between 6 months to 2 years but occasionally it may occur in younger animals and cattle up to 3 years (Radostits *et al.,* 2007). In performed study the highest percentage of diseases are recorded in growing cattle which are in support of Kabir *et al.* (2010).

7. Bovine Ephemeral Fever

Bovine Ephemeral Fever was recorded 1.53% (Table 1) in cattle. It was recorded 0.65% in growing, 0.86% in adult; 0.65% in male 0.86% in female and 0.65% in local 0.86% in cross (Table 2,3,4).

8. Foot and Mouth Disease

FMD was recorded 1.30% among the diseases & disorders ((Table 1). It was also recorded 0.65% in growing and 0.65% in adult; 0.22% in male cattle, 1.08% in female and 0.22% in local cattle and 1.08% in cross breed (Table 2,3,4) during the study period. Sarker *et al.* (2013) reported FMD 7.02% which is higher than the study.

9. Rabies

Occurrence of rabies was recorded in cattle 0.87% (Table 1). It was also recorded 0.65% in young, 0.22% in growing; 0.65% in female 0.22% in male and 0.22% in local 0.65% cross breed (Table 2,3,4).The recorded case of rabies in cattle due to dog bite. However Uddin *et al.* (2015) reported the occurrence of 10.3% suspected case of rabies due to dog bite in cattle, which is lower than the observed study. The prevalence of suspected rabies was recorded 38% and 3.5% in goat and horse, respectively. Incidence of rabies in non-vaccinated cattle were recorded 84.6% where female had higher suspected cases as 24.1% than male 14.7% (Uddin *et al* 2015).

10. Babesiosis

Babesiosis was recorded in cattle 0.65% (Table 1). In cattle it was recorded 0.65% in adult; 0.65% in female and 0.65% in cross breed (Table 2,3,4). Samad and Shahidullah (1984) reported 1.19% clinical incidence of bovine babesiosis in Mymenshing district.

11. Enteritis/Diarrhoea

Diarrhoea is the increased frequency of defecation accompanied by feces containing an increased concentration of water and decreased in dry matter content. In this study 3.69% (Table 1) cattle was suffering from diarrhoea whereas 8.99% and 25.97% diarrhoea were described in cattle in Bangladesh by Hoque and Samad (1996) and Samad (2001), respectively. In cattle it was also recorded 1.08% in young, 0.86% in growing, 1.74% in adult; 1.53% in male, 2.17% female; 1.53% in local and 2.17% cross-breed (Table 2,3,4). Bushen & Guerrant, (2003) reported diarrhea 11%, which is higher than recorded study.

12. Acidosis

Bloat was recorded 1.74% in (Table 1). It was recorded 0.65% in adult 1.08% in growing; 0.65% in male, 1.08% in female; 1.08% in local and 0.65% in cross breed (Table 2,3,4).

13. Anoestrus

It was recorded (6.30%) in (Table 1). In cattle it was recorded (6.30%) in adult (Table-4); (6.30%) in female (Table-3) and (1.96%) in local 20(4.34%) in cross breed (Table-2). Sarker *et al.* (2013) reported anestrous 11.44%in bovine which is higher than the recorded data. It has been reported that most of the anoestrus cases were due to malnutrition, vitamin and mineral deficiency.

14. Bloat

Bloat is clinically characterized by marked distension of abdomen with severe distress and dyspnea. About 1.52% cases were suffering from bloat in this study (Table 1). In cattle it was recorded 0.22% in young, 0.65% in growing, 0.65% in adult; 1.08% in female, 0.43% in male; 0.43% in local and 1.08% in cross breed (Table 2,3,4). Arju (2013) reported tympany 1.13% which is lower than this study. It was mainly dietary origin and occurs most frequently in ruminants in Bangladesh (Sutradhar, *et al*., 2000, Samad, 2001) who reported 1.73% bloat in cattle. Bloat if not treated early it is fatal. If some controllable risk factors of bloat can be identified, it might be possible to reduce the extent of this problem.

15. Retained placenta

Retained placenta was recorded 0.43% in (Table 1). In cattle it is recorded 0.43% in adult; 0.43% in female; 0.43% in local (Table 2,3,4). Sarker *et al.* (2013) reported 14.27% retention of placenta in bovine which is much higher than the recorded data.

16. Wound

It was recorded 5.65% (Table 1). In cattle it was recorded in male 1.96%, 3.69% in female; young 1.30%, growing 2.17% adult 2.17% (Table 2,3,4). Sarker *et al* (2013) reported wound 20.19% which is much higher than the recorded data. Soyelu & Masika (2009) reported that a great majority of the interviewed farmers reported incidences of wounds and myiasis 84.3% and 92.5%, respectively in their herds.

Table 1: Overall incidence of diseases and disorders in cattle

SL. No.	Diseases and disorders	Percentage N (%)
01	Parasitic Infestation	100(21.74)
02	Anorexia	48(10.43)
03	Myiasis	30(6.52)
04	Pneumonia	17(3.70)
05	Infectious Bovine Keratoconjunctivitis	4(0.87)
06	Mastitis	20(4.35)
07	Black quarter	3(0.65)
08	FMD	6(1.30)
09	Bovine Ephemeral fever	7(1.52)
10	Rabies	4(0.87)
11	Babesiosis	3(0.65)
12	Ectoparasitic infestation	21(4.57)
13	Dermatitis	8(1.74)
14	Indigestion	10(2.17)
15	Bloat	7(1.52)
16	Acidosis	8(1.74)
17	Retention of placenta	2(0.43)
18	Milk fever	19(4.13)
19	Diarrhoea/Enteritis	17(3.70)
20	Wound	26(5.65)
21	Anestrous	29(6.30)
22	Alopecia	3(0.65)
23	Malnutrition	30(6.52)
24	Pyometra	7(1.52)
25	Papillomatosis	5(1.09)
26	Anemia	3(0.65)
27	Dermayophytosis	2(0.43)
28	Poisoning	3(0.65)
29	Dystocia	5(1.09)
30	Abscess	5(1.09)
31	Uterine Prolapse	2(0.43)
32	Simple Fracture	3(0.65)
33	Abortion	2(0.43)
34	Dermoid Cyst	1(0.22)
Total		**460(100)**

Table 2: Incidence of common diseases and disorders in cattle according to breed.

SL. No.	Diseases and disorders	Breed	
		Local N (%)	Cross N (%)
01	Parasitic Infestation	40 (8.70)	60(13.04)
02	Anorexia	17(3.70)	31(6.74)
03	Myiasis	13 (2.83)	17(3.70)
04	Pneumonia	7(1.52)	10(2.17)

05	Infectious Bovine Keratoconjunctivitis	1 (0.22)	3 (0.65)
06	Mastitis	3 (0.65)	17(3.70)
07	Black quarter	0(0.00)	3 (0.65)
08	FMD	1 (0.22)	5 (1.09)
09	Bovine Ephemeral fever	3(0.65)	4(0.87)
10	Rabies	1 (0.22)	3(0.65)
11	Babesiosis	0(0.00)	3(0.65)
12	Ectoparasitic infestation	11 (2.39)	10(2.17)
13	Dermatitis	3 (0.65)	5(1.09)
14	Indigestion	5 (1.09)	5 (1.09)
15	Bloat	2(0.43)	5 (1.09)
16	Acidosis	5 (1.09)	3(0.65)
17	Retention of placenta	2(0.43)	0(0.00)
18	Milk fever	2(0.43)	17(3.70)
19	Diarrhoea/Enteritis	7(1.52)	10(2.17)
20	Wound	9(1.96)	17(3.70)
21	Anestrous	9(1.96)	20(4.35)
22	Alopecia	0(0.00)	3 (0.65)
23	Malnutrition	10(2.17)	20(4.35)
24	Pyometra	2(0.43)	5 (1.09)
25	Papillomatosis	2(0.43)	3 (0.65)
26	Anemia	2(0.43)	1 (0.22)
27	Dermayophytosis	2(0.43)	0(0.00)
28	Poisoning	1 (0.22)	2(0.43)
29	Dystocia	4(0.87)	1 (0.22)
30	Abscess	1 (0.22)	4(0.87)
31	Uterine Prolapse	0(0.00)	2(0.43)
32	Simple Fracture	0(0.00)	3 (0.65)
33	Abortion	0(0.00)	2(0.43)
34	Dermoid Cyst	0(0.00)	1 (0.22)
Total		**165(35.87)**	**295(64.13)**

Table 3: Incidence of common diseases and disorders in cattle according to sex.

SL. No.	Diseases and disorders	Sex	
		Male N (%)	Female N (%)
01	Parasitic Infestation	31(6.74)	69(15.00
02	Anorexia	23(5.00)	25(5.43)
03	Myiasis	25(5.43)	5 (1.09)
04	Pneumonia	4(0.87)	13(2.83)
05	Infectious Bovine Keratoconjunctivitis	1 (0.22)	3 (0.65)
06	Mastitis	0(0.00)	20(4.35)
07	Black quarter	1 (0.22)	2(0.43)

08	FMD	1 (0.22)	5 (1.09)
09	Bovine Ephemeral fever	3(0.65)	4(0.87)
10	Rabies	1 (0.22)	3(0.65)
11	Babesiosis	0(0.00)	3(0.65)
12	Ectoparasitic infestation	11 (2.39)	10(2.17)
13	Dermatitis	2(0.43)	6(1.30)
14	Indigestion	2(0.43)	8(1.74)
15	Bloat	2(0.43)	5 (1.09)
16	Acidosis	3(0.65)	5 (1.09)
17	Retention of placenta	0(0.00)	2(0.43)
18	Milk fever	0(0.00)	19(4.13)
19	Diarrhoea/Enteritis	7(1.52)	10(2.17)
20	Wound	9(1.96)	17(3.70)
21	Anestrous	0(0.00)	29(6.30)
22	Alopecia	1 (0.22)	2(0.43)
23	Malnutrition	5 (1.09)	25(5.43)
24	Pyometra	0(0.00)	7(1.52)
25	Papillomatosis	2(0.43)	3 (0.65)
26	Anemia	0(0.00)	3 (0.65)
27	Dermayophytosis	0(0.00)	2(0.43)
28	Poisoning	0(0.00)	3 (0.65)
29	Dystocia	0(0.00)	5(1.09)
30	Abscess	1 (0.22)	4(0.87)
31	Uterine Prolapse	0(0.00)	2(0.43)
32	Simple Fracture	1 (0.22)	2(0.43)
33	Abortion	0(0.00)	2(0.43)
34	Dermoid Cyst	1 (0.22)	0(0.00)
TOTAL		**137(29.78)**	**323(70.22)**

Table 4: Incidence of common diseases and disorders in cattle according to age.

SL. No.	Diseases and disorders	Age		
		Calf (< 1year)	Growing (1-3 year)	Adult (>3 year)
01	Parasitic Infestation	20(4.35)	31(6.74)	49(10.65)
02	Anorexia		17(3.70)	31(6.74)
03	Myiasis	25(5.44)	1 (0.22)	4(0.86)
04	Pneumonia	10(2.17)	5(1.09)	2(0.43)
05	Infectious Bovine Keratoconjunctivitis	1 (0.22)	3 (0.65)	0(0.00)
06	Mastitis	0(0.00)	0(0.00)	20(4.35)
07	Black quarter	0(0.00)	3(0.65)	0(0.00)
08	FMD	0(0.00)	3 (0.65)	3 (0.65)
09	Bovine Ephemeral fever	0(0.00)	3(0.65)	4(0.87)
10	Rabies	3 (0.65)	1 (0.22)	0(0.00)

11	Babesiosis	0(0.00)	0(0.00)	3 (0.65)
12	Ectoparasitic infestation	0(0.00)	11 (2.39)	10(2.17)
13	Dermatitis	0(0.00)	3 (0.65)	5(1.09)
14	Indigestion	1 (0.22)	4(0.87)	5 (1.09)
15	Bloat	1 (0.22)	3(0.65)	3(0.65)
16	Acidosis	0(0.00)	5 (1.09)	3(0.65)
17	Retention of placenta	0(0.00)	0(0.00)	2(0.43)
18	Milk fever	0(0.00)	0(0.00)	19(4.13)
19	Diarrhoea/Enteritis	5 (1.09)	4(0.87)	8(1.74)
20	Wound	6(1.30)	10(2.17)	10(2.17)
21	Anestrous	0(0.00)	0(0.00)	29(6.30)
22	Alopecia	0(0.00)	0(0.00)	3(0.65)
23	Malnutrition	8(1.74)	12(2.61)	10(2.17)
24	Pyometra	0(0.00)	0(0.00)	7(1.52)
25	Papillomatosis	1 (0.22)	2(0.43)	2(0.43)
26	Anemia	0(0.00)	1 (0.22)	2(0.43)
27	Dermayophytosis	0(0.00)	1 (0.22)	1 (0.22)
28	Poisoning	0(0.00)	1 (0.22)	2(0.43)
29	Dystocia	0(0.00)	0(0.00)	5(1.09)
30	Abscess	1 (0.22)	2(0.43)	2(0.43)
31	Uterine Prolapse	0(0.00)	0(0.00)	2(0.43)
32	Simple Fracture	1 (0.22)	2(0.43)	0(0.00)
33	Abortion	0(0.00)	0(0.00)	2(0.43)
34	Dermoid Cyst	1 (0.22)	0(0.00)	0(0.00)
Total		**84(18.26)**	**128(27.83)**	**248(53.91)**

Table 5: Incidence of common diseases and disorders in cattle in relation to affected system of the body

System affected	**Digestive system**	**Respiratoy system**	**Urinary system**	**Nervous system**	**Musculo Skeletal system**	**Integumentary system**	**Reproductive System**
N (%)	231(50.22)	17 (3.70)	3 (0.65)	4 (0.87)	32 (6.96)	101 (21.96)	72 (15.65)

Table 6: Incidence of diseases and disorders in cattle according to therapeutic management.

Therapeutic management		
Medicinal cases N (%)	Surgical cases N (%)	Gyne-obstetrical cases N (%)
342 (74.35)	71 (15.43)	47(10.22)

CONCLUSIONS

It is concluded that incidence of parasitic infestation cases are very common in cattle as compared to other diseases and disorders in study area. So, regular anthelmintic treatment should be performed to control the parasitic diseases. On the other hand, proper hygienic management, feeding, nutrition, housing and vaccination should be effective tools to reduce the infectious and noninfectious diseases in cattle. So, further extensive epidemiological investigation should be performed to determine the accurate strategies to curb the identified diseases and disorders in cattle at study area.

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PRESENT STATUS AND PROBLEMS OF FISH SEED MARKETING IN SYLHET DISTRICT, BANGLADESH

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Abstract:

This study was conducted to explore the present status and problems of fish seed marketing system in Sylhet district, Bangladesh. Data were collected through questionnaire interview from the selected areas during April to September 2016. Brood fishes were collected from wild sources as well as hatchery produced brood fishes also used for seed production. Good length, weight and age of brood fishes were selected for spawning and induced breeding. In nursery, hatchlings were reared for 30-40 days and 37.7% nursery owner practiced single cycle production/year where the average stocking density of seed was found 24.65±3.94 g/decimal (mean±SD). Six different fish seed marketing channels were identified where hatchery owners, nursery owners, *forias* (retailer) and fish farmers were main stakeholders. The highest (6520 Tk/day) and lowest (355 Tk/day) average income were found in hatchery owner and fish farmer, respectively. Oxygenated bag, big aluminum bowl/container and plastic barrels with continuously agitate the water were used for seed transportation. Maximum 17.67% seed mortality was noticed in hatchery owner and minimum 5.67% in fish farmers. Late breeding season, lack of capital, lack of technical knowledge on hatchery operation and management, poor transport facilities, high transportation cost, high labor cost, lack of training and high price of spawn are identified as some major problems.

Key words: Fish seed, Marketing, Income, Transportation, Mortality.

INTRODUCTION

Bangladesh is a riverine country having lots of rivers, canals, floodplains, ponds, beels, haors, reservoir, manmade lakes and a long coastline etc. These waterbodies are valuable sources of fish. Both capture and culture fisheries play an important role for animal protein supply in Bangladesh. Aquaculture sector of Bangladesh is expanded rapidly with respect to both the quantity and variety of species. In the year of 2014-2015, total fish production of the country was 3.68 million MT of which inland aquaculture sector provides 2.06 million MT (DoF, 2016). Development of aquaculture is influenced by good quality seed and good quality feed. In the year of 1985, spawn production from natural and hatchery sources were 19,362 kg and 4,962 kg (Hussain and Mazid, 2001). The demand of substantial supply of quality fish fry and fingerlings are increased day by day. At present private fish hatcheries and Government Fish Seed Multiplication Farm (FSMFs) with hatchery facilities contributed 5,36,983 kg and 10,566 kg hatchlings respectively (FRSS, 2016). Department of fisheries (DoF) of Bangladesh is encouraging people to enhance the fish seed production by setting up hatcheries and nurseries. On the other hand 800 private hatcheries all over the country mostly help fish farmer to reduce the dependency on nature for fish seed. This large numbers of hatcheries created a new marketing system all over the country to reach their fish seed to fish farmer.

A market system is the network of buyers, sellers and other actors that come together to trade in a given product or service. In this marketing system there are different actors such as nursery owners, foria, hawkers, wholesalers, fry traders etc. The marketing channels are the alternative courses of item flows from producers

to consumers (Kohls and Uls, 1980). Marketing channel may be small or long for a particular product depending on the type and quality of the relevant factors. Fish seed marketing of the country largely depends on private sector and a large number of people are associated with this system for their livelihoods. But, stress from transportation caused 6200 MT fish seed mortality in a year over the country (Hasan and Brat, 2006). Besides, lack of capital, lack of technical knowledge, high lease value, high price of production inputs, violence, intense market competition, the lack of policy support etc. have impact on stakeholders on this marketing system. Sylhet (north western district of Bangladesh) district is endowed with numerous natural inland water bodies such as rivers, canals, ponds, beels etc. A special wetland ecosystem of the country is found in sylhet region named haor having huge possibility of aquaculture. There are about 5 fish hatcheries and 152 fish nurseries in the Sylhet region. In 2014, two government fish seed multiplication farm, one government nursery, private hatcheries and nurseries are produces 950 kg hatchling and 81.94 million fry and fingerling (DoF, 2005). But, the marketing channel and problems associated with marketing of fish seed in Sylhet region has not been yet quantified. The objectives of the study were to know about the existing fish seed marketing system and identify the problems of the fish seed marketing system in Sylhet region.

MATERIALS AND METHODS

Selection of study area, Target groups and Period of the study

Four upazillas of Sylhet district were selected for this study and the target groups were hatchery and nursery owners, fish seed hawkers as well as fish farmers. Production season of fish seed generally started from 1st week of April and ends up by 1st week of September every year. However, the survey was carried out for six months from April to September 2016.

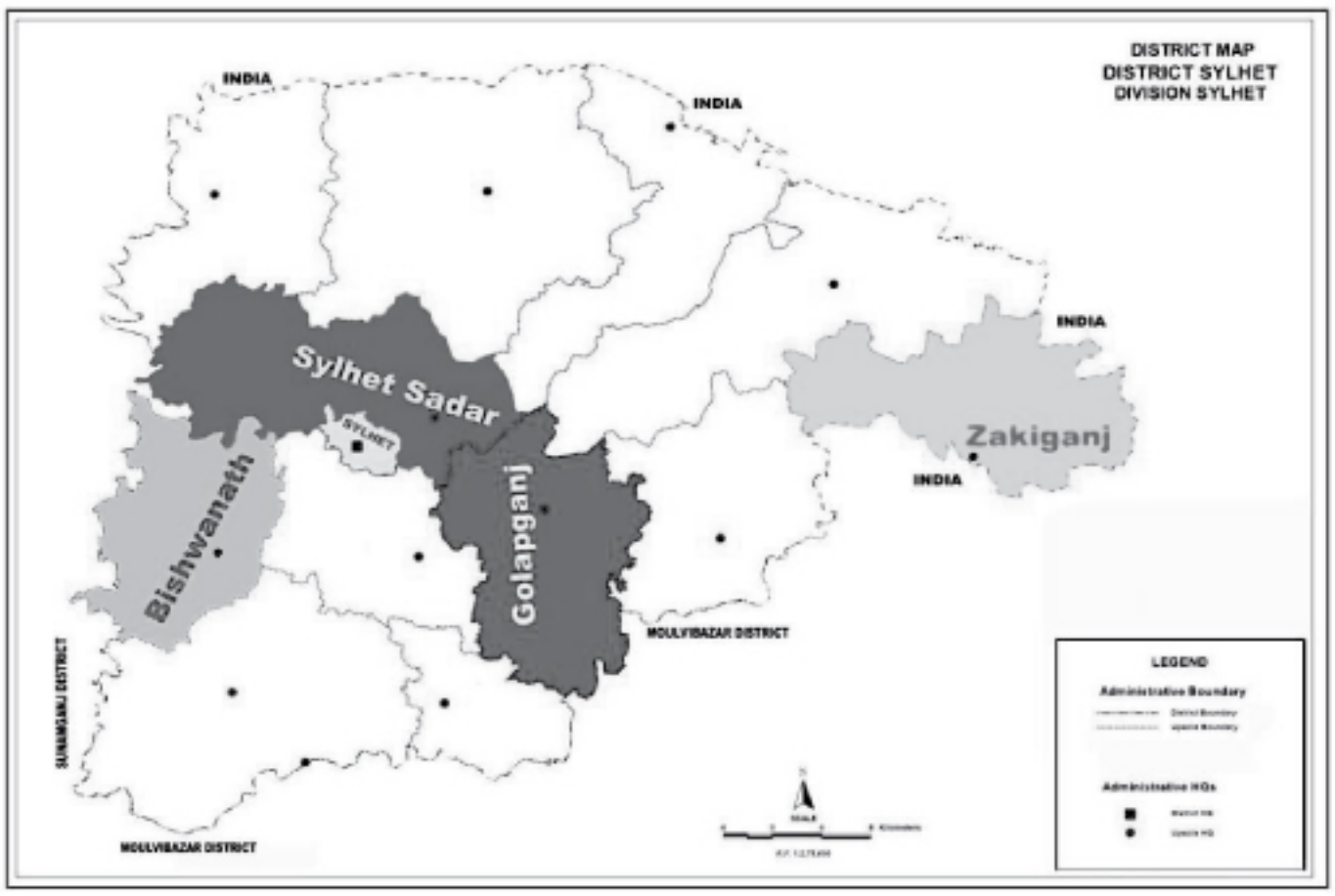

Figure 1.A map of selected study areas of Sylhet District. (*source: www.lged.gov.bd*)

Selection of sample and sampling techniques

In order to meet the objectives of the study, different types of fish seed and fingerling producers and hawker were selected. The sample size for the present study is given in Table 1.

Table 1. Sample size of the present study

Types of Stakeholders	Sylhet District				Total
	SylhetSadar	Golapganj	Zakiganj	Bishwanath	
Hatchery owner	1	2	2	2	7
Nursery owner	10	10	10	10	40
Fish seed hawker	10	10	10	10	40
Fish Farmer	10	10	10	10	40
Total					127

Collection of data, Data processing and analysis

Data were collected from the respondents by using the pre-prepared questionnaire interview. All the collected data were summarized and scrutinized carefully and recorded. Data were presented mostly in the tabular form, because it is simple in calculation, widely used and easy to understand.

RESULTS AND DISCUSSION

Hatchery Status

At present, there are only 5 private fish hatcheries and 2 Governments Fish Seed Multiplication Farm (FSMFs) in Sylhet district. The hatcheries are situated at SylhetSadar, Golapganj, Bishwanath, Osmaninagar and Zakiganj. Among 5 private hatcheries, one was Catfish hatchery, three was Carp hatchery and other one was Tilapia hatchery. During the study period spawn production was 431 kg carp fishes, 220 kg catfishes and 567 kg Tilapia (Figure 2). In the present study it was found that the carp hatchery activities start from 1st week of April and continued up to 1st week September. Peak seasons of seed production were late-April to mid-June. It was also found that the Catfish hatchery activities start from 1st week of April and continued up to 1st week September. Peak seasons of seed production were late-April to mid-June. The only one Tilapia hatchery in Golapganj produced only tilapia fry throughout the year. After 4 days hatchling was fed feed treated with 17α-Methyltestosterone. The brood fishes were produced in the on hatchery

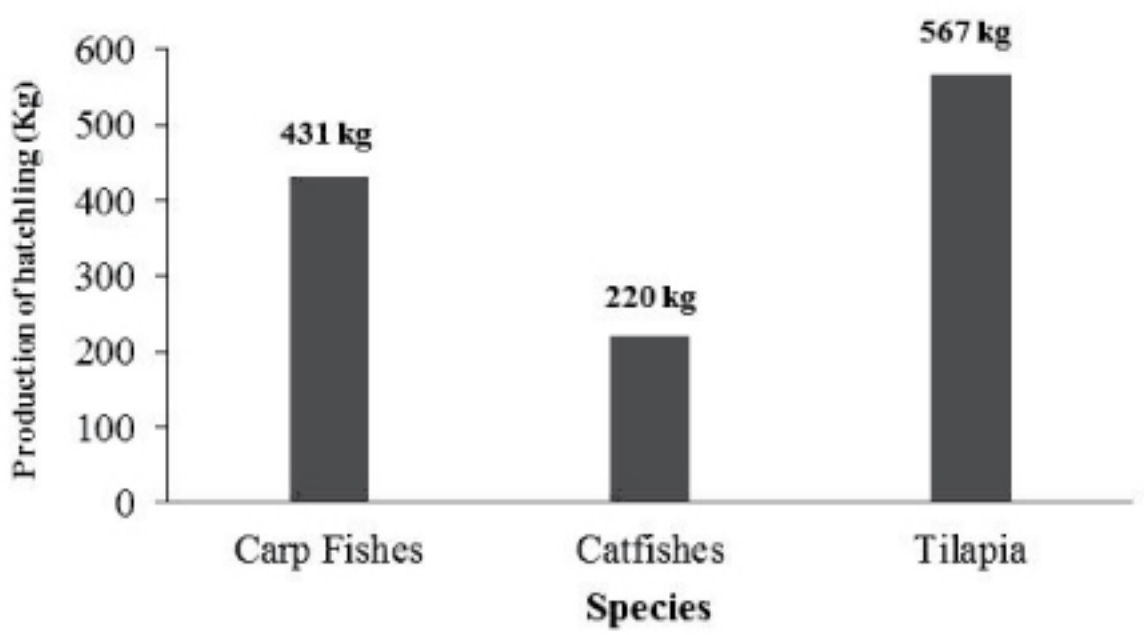

Figure 2. Hatchling production in Sylhet District

Above result was relevant with the Sharif and Asif (2015) reported that hatchlings production of Indian major carps were 24,720 kg, Exotic carps were 21754 kg and other species were 2966 kg respectively in 2013 at Jessoresadar. Rahaman (2007) also got that 85 hatcheries in Jessore area were produced 4211.5 kg hatchling

at study period of which 80% were reared in the same area.

Facilities used in Hatchery

Smooth operation of hatchery mainly dependent on facilities of hatchery. Good production of hatcheries depends on appropriate number of brood ponds, breeding tanks with other facilities, cisterns, incubators, fry rearing tanks etc. Besides, there is difference between capabilities used in Carps Hatchery, Catfish Hatchery and Tilapia Hatchery. A short list of facilities used in hatcheries was shown in the Table 2.

Table 2. Lists of facilities used in hatchery

Facilities		Fish Hatchery	Tilapia Hatchery	Catfishes Hatchery
Brood fish pond		5-1	5	3
Breeding Tank		7-1	1	5
Cistern		8-1	-	-
Incubator	Bottle	13-8	-	-
	Tray	-	60	-
Hormone		Ovaprim, PG, HCG	17α-Methyltestosterone	Ovaprim, Ovatide
Brood Transport materials		Plastic drum, AluminumPatil	Aluminum patil	Aluminum patil

Brood management

A good number of spawn productions from hatchery mainly depends on the selection and successful management of brood fishes. Best quality and higher number of spawns can be obtained from the good quality brood fishes. The criteria and management activities for brood fishes were followed by the hatchery owner show in the Table 3.

Table 3. Criteria and management of brood fishes are maintained by the hatchery owner

Criteria		Indian Major Carps (Rui, Mrigal, Kalibaus, Gonia)	Catla	Exotic Carp	Tilapia	Catfishes (Shing&Magur)
Average Weight	**Male**	500 gm to 2kg	1200 gm to 3 kg	500 gm to 2 kg	400-500 gm	160 gm
	Female	700 gm to 2 kg	1400 gm to 3 kg	700 gm to 2 kg	250-500 gm	200 gm
Age	**Male**	2-3 years	3-4 years	2-3 years	2 years	3 years
	Female	2-3 years	3-4 years	2-3 years	2 years	3 years
Source of Brood		River, Own farm, Other farm		Own or Other farm	Own farm	Own farm
Feed for brood		24 hour composted mustard oil cake with rice bran and wheat bran or commercial feed				Commercial feed
Feeding rate		1-2 times in a day				3 times/night

This result supported the result of Dwivedi and Zaidi (1983) who reported that brood stock is a prerequisite for all types of hatchery production and proper brood stock management will lead to better breeding responses and increased fecundity, fertilization, hatching and larval survival rates and more viable fish seed. Sultan (2008) also identified that maximum hatchery owners (80%) in Chachra collected broods from the Halda and the Jamunarivers to avoid inbreeding and all of the hatchery owners practiced induced breeding in their hatcheries.

Nursery operation

About 61.18% (93 of total 152 nurseries) nursery of Sylhet district are situated in study areas. Nursery of Sylhet district mainly produces fry or fingerlings of Rui, Catla, Mrigal, Bata, Kalbasu, Sarpunti, Bighead carp, Silver carp, Grass carp, Tilapia, Pangas etc. The size of the nursery depends on capital for nursery operation, land, knowledge about nursery operation etc. The highest 42.5% of nursery was found in a range of 0.5- 1.0 acre size while lowest 17.5% nursery was found in a range of 1.5-above acre (Figure 3).

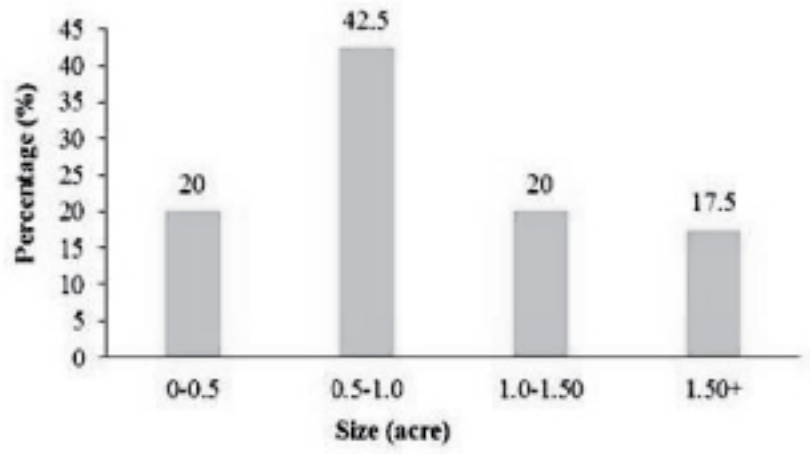

Figure 3. Size of nursery in acre

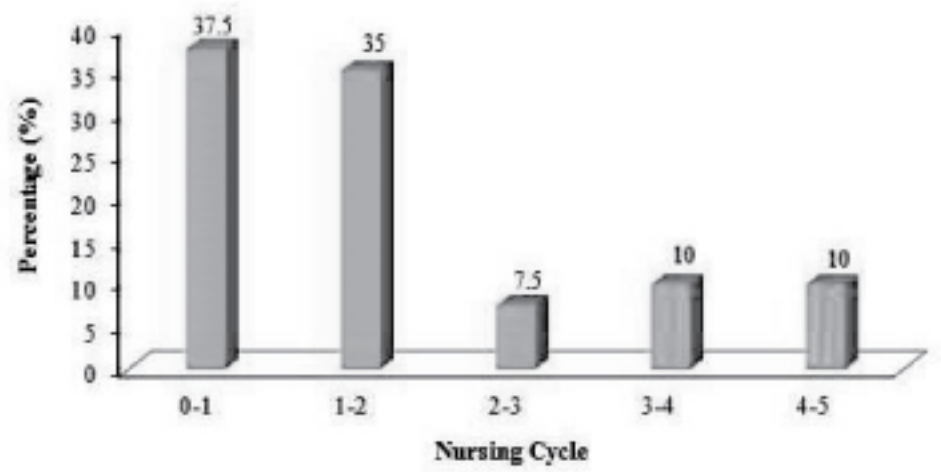

Figure 4. Number of nursery cycle by nursery owner per year

In this region nursing cycle varies from highest 4-5 cycle/year due to collection of spawn from outside of the district, high price of spawn than other region and nursing mostly for grow out of fish. It was observed that 37.7% farmers completed single cycle/year, 35% farmers completed 1-2 cycle/year and only 10% farmer completed 4-5 cycle/year (Figure 4).

Fish Farm

Fish hatchling, fry or fingerling are reared up to table size for selling in the market or home consumption. In the fish farm of Sylhet region mostly cultured species are Tilapia, Koi and carps like Rui, Catla, Kalbasu, Mrigal etc. The size of the farms depends on the economic condition of the farmer. The highest number of farms was covered area 2-4 acre (Figure 5).

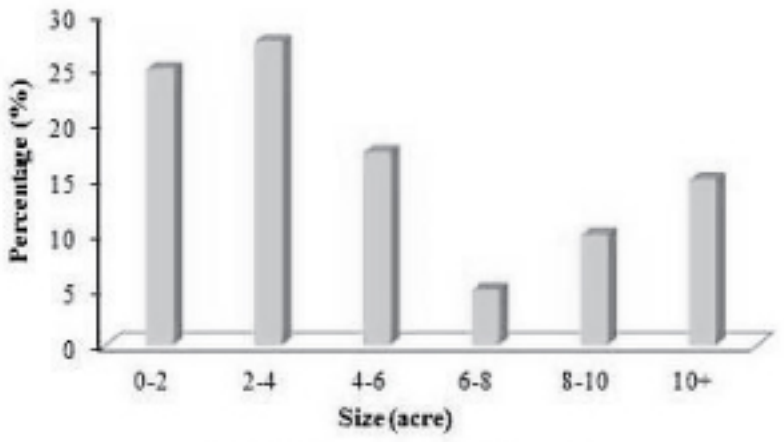

Figure 5. Size of fish farm in acre

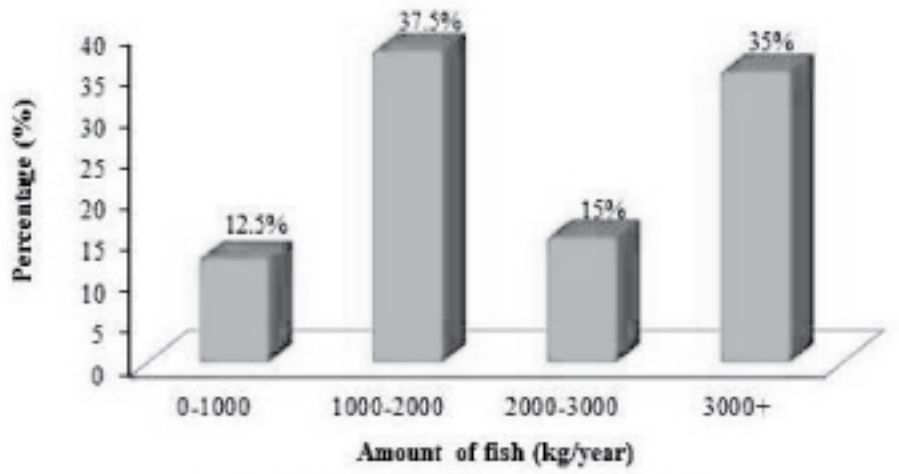

Figure 6. Total fish production in fish farms

Islam (2010) revealed that average pond size was 0.11 ha in Moulvibazar district. The most of the farmer (55%) found to operate nursery in their farm for fry or fingerlings to grow-out. The productions of farms relay on quality of fish seeds, management activities followed by the farmers, the size of the farms and knowledge of farmer on fish culture. The highest number of farm 37.5% produced fish between 1000-2000 kg/year (Figure 6).

Fish seed marketing channel

The marketing channels are the alternative routes of product flows from producers to consumers (Kohls and Uhl, 1980). Fish seed marketing channel starts from hatchery owners, passes through some intermediaries and ends with the fish farmers who culture table fish. In the present study, fish seed marketing channels lunched with the brood fish collection to fish farmers through proper channels (Figure 7).

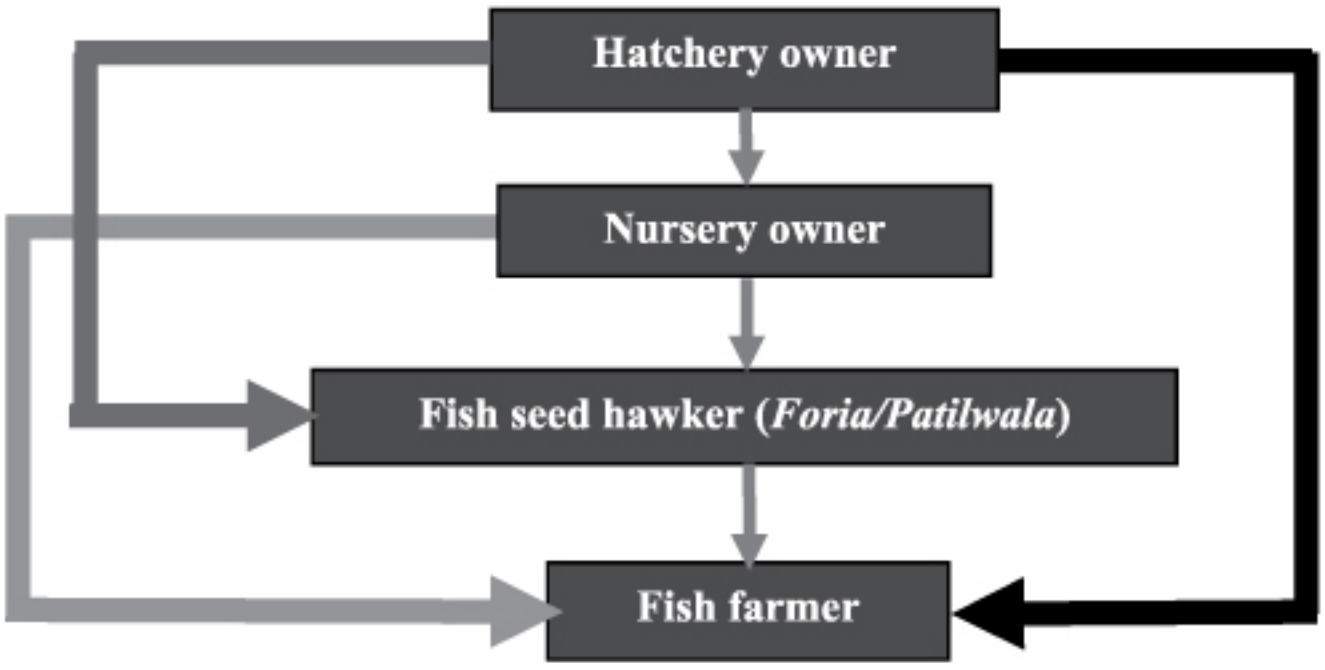

Figure 7. Flow chart of fish seed marketing channels in Sylhet district

Channels of commercial fish seed marketing in Sylhet area

About Six different fish seed marketing channels were identified in Sylhet area. Among them, channel-3was best. Because, there is no middleman between ha farmers can buy their quality seeds in low price. Abdulla-Al-Asif et al., (2015) reported that marketing channelof fish fry and fingerling is start with brood pond and continues with hatchery, nursery, fry and fingerling traders, intermediates, buyer, farmer, then farming pond or rearing pond. Malek (2007) identified seven marketing channels where hatchery and nursery owners, wholesalers, forias and fish farmers were major stakeholders. Similar findings were reported by Sharif and Asif (2015).

Channel-1: Hatchery owners → Nursery owners → Forias → Fish Farmer

Channel-2: Hatchery owners → Nursery owners → Fish Farmer

Channel-3: Hatchery owners → Fish Farmer

Channel-4: Nursery owners → Forias → Fish Farmer

Channel-5: Nursery owners → Fish Farmer

Channel-6: Forias → Fish Farmer

Transportation

Fish seed or fingerlings were found to transport by vehicle like pick-up, auto-rickshaw, bus, truck, van etc. About 80% foria (retailer) used bus to transport fry or fingerlings to the district and sell the fingerlings to the fish farmers door to door on foot hanging the aluminums bowl/container on their shoulder. Pickups and auto-rickshaws were found to be the main means of transportation of fry to the distance places. Fish spawns were transported in polythene bag by using auto-rickshaw, pickup and truck. Brood fish were transported by aluminum patil and plastic drum by pickup or vans. Similar result was showed by Haque*et al.* (1991), Abdulla-Al-Asif*et al.* (2015) and Sultan (2008).

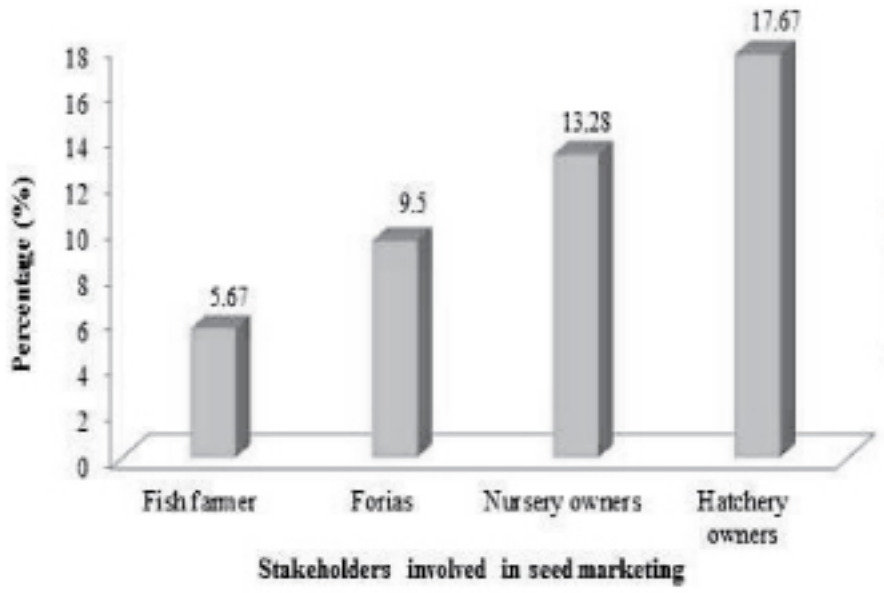

Figure 8. Mortality of fish seed of different stakeholders in marketing channel

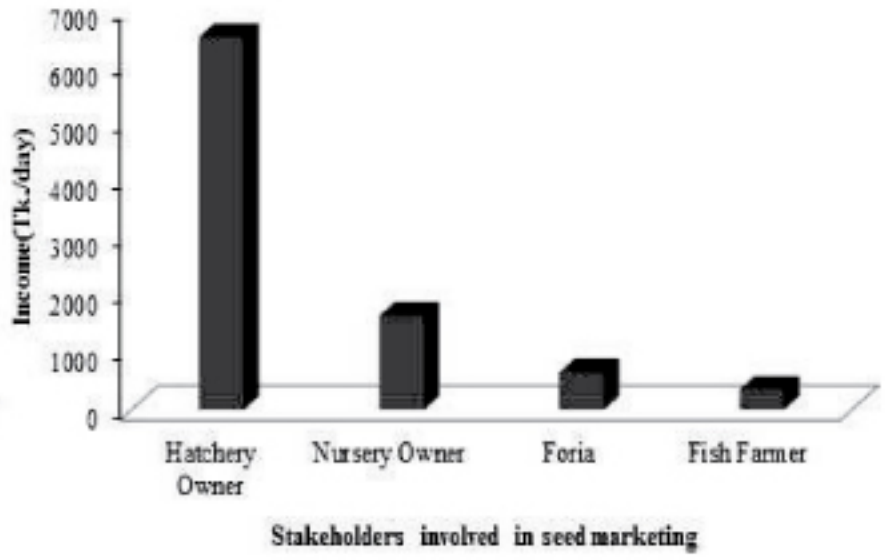

Figure 9. Average income (Tk/day) of Stakeholders involved in fish seed marketing

Mortality of fry due to transportation

It was observed that fries were transported by polythene bag and aluminum patils. Polythene bag were filled with oxygenated. Fries in aluminum patils were transported with agitation by hand and change water. Sometimes due to high density, longer duration and improper transportation method caused mass mortality which result into loss of overall business. In the study area average mortality was 17.67%, 13.28%, 9.5% and 5.67% for hatchery owners, nursery owners, forias and fish farmer respectively (Figure 8). Malek (2007) found average mortality was 15.67%, 11.25%, 8.5%, and 4.67% for hatchery owners, nursery owners, wholesalers and forias respectively in the seed marketing channel in Mymensingh area.

Income of different stakeholders in fish seed marketing channel

The income of hatchery and nursery owners in fish seed marketing system is more than the income of the fry traders, wholesalers and fish farms. The average income of hatchery owners were found at Tk.6,520 day/hatchery owner, average net profit of nursery owners were estimated at Tk. 1,646/day, average income of forias were estimated Tk. 655/day and average profit of fish farmers were estimated Tk. 355/day (Figure 9). Malek (2007) found similar result that the average income of hatchery owners were Tk.8,375 day/hatchery owner, nursery owners were Tk. 1,660/day, Tk. 1,362.17/day for wholesalers and forias were Tk. 490/day in Mymensingh area.

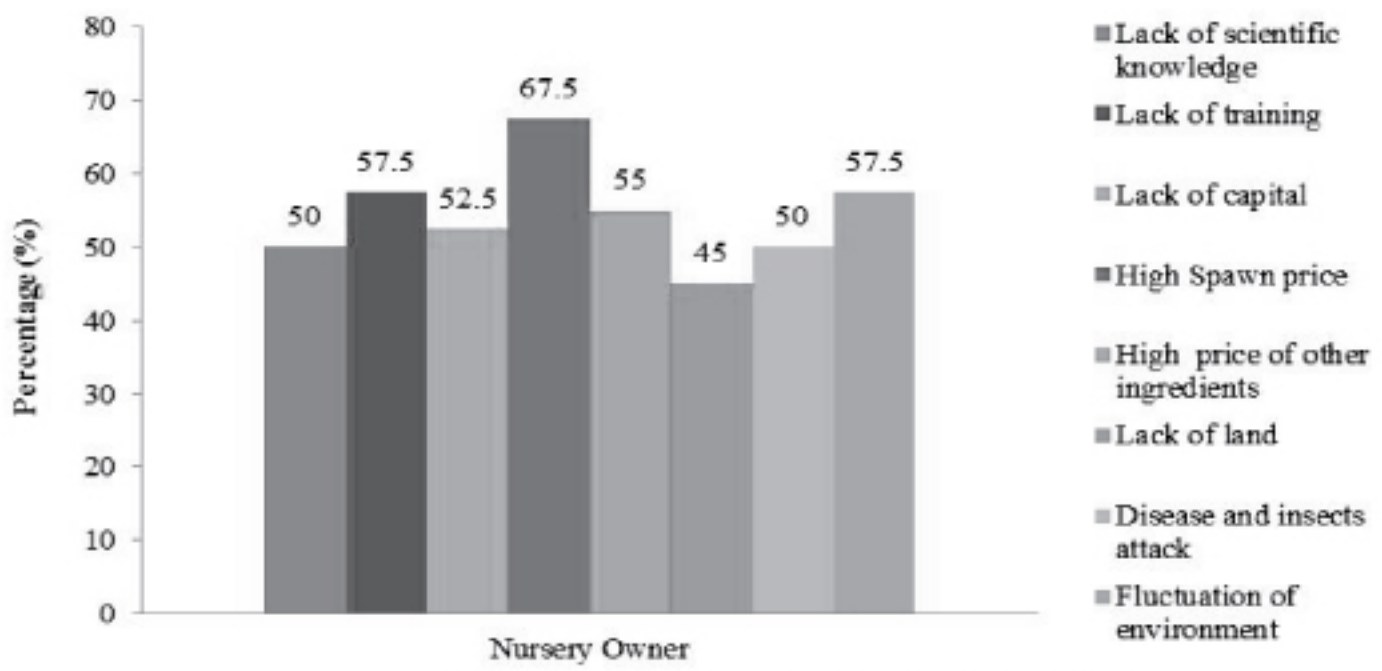

Figure 10. Problems faced by nursery owners

Problems and constraints of production and marketing of fish seed

Major problems of nursery owners includes- high price of spawn, lack of training on nursery operation, fluctuation of environmental condition, diseases of fingerlings, price fluctuation etc. Among the problems,

high price of spawn was highest (67.5%), followed by lack of training (57.5%) and fluctuation of environment (57.5%) were crucial constrains for nursery operation (Figure 10)

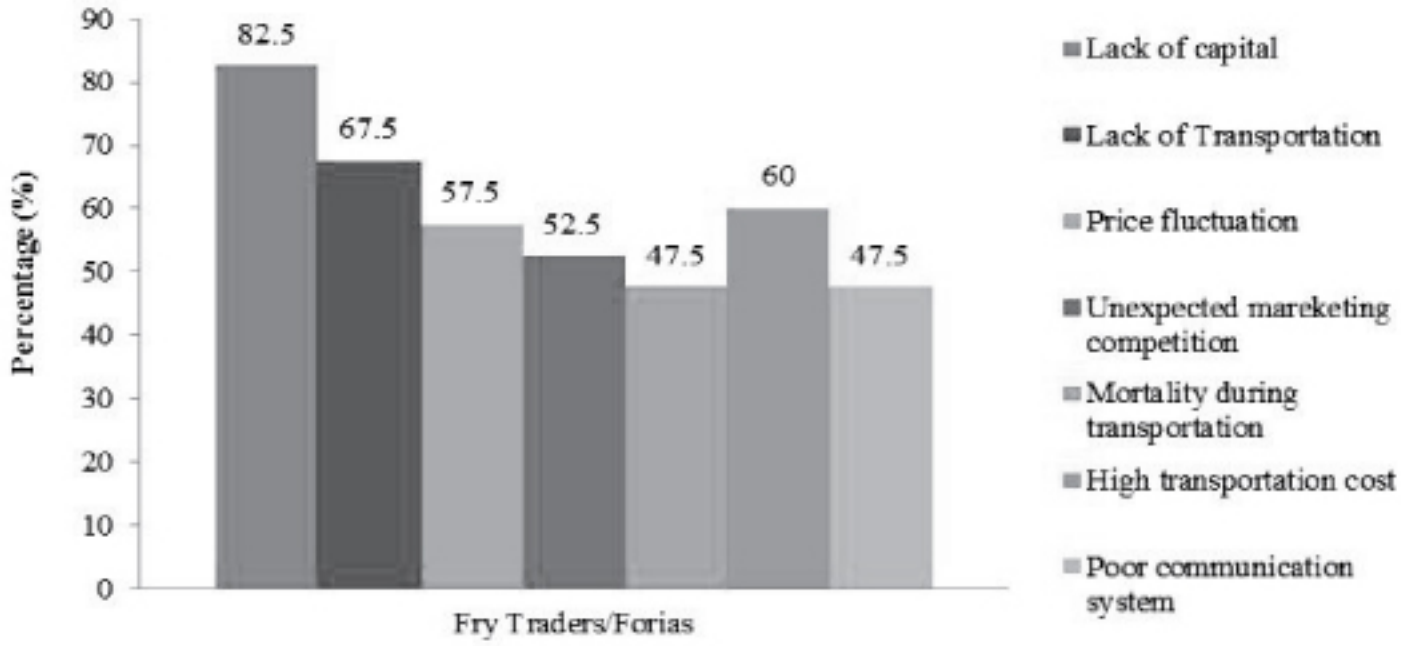

Figure 11. Problems faced by fry traders/retailers

Fry retailers maintained link between the producer (Hatchery owner, Nursery owner) and Fish farmer. The problems which are faced by the fry retailers/forias include lack of capital (82.5%) was most important. Among others lack of transport facilities (67.5%) and high transportation cost (60%) could be mentioned (Figure 11).

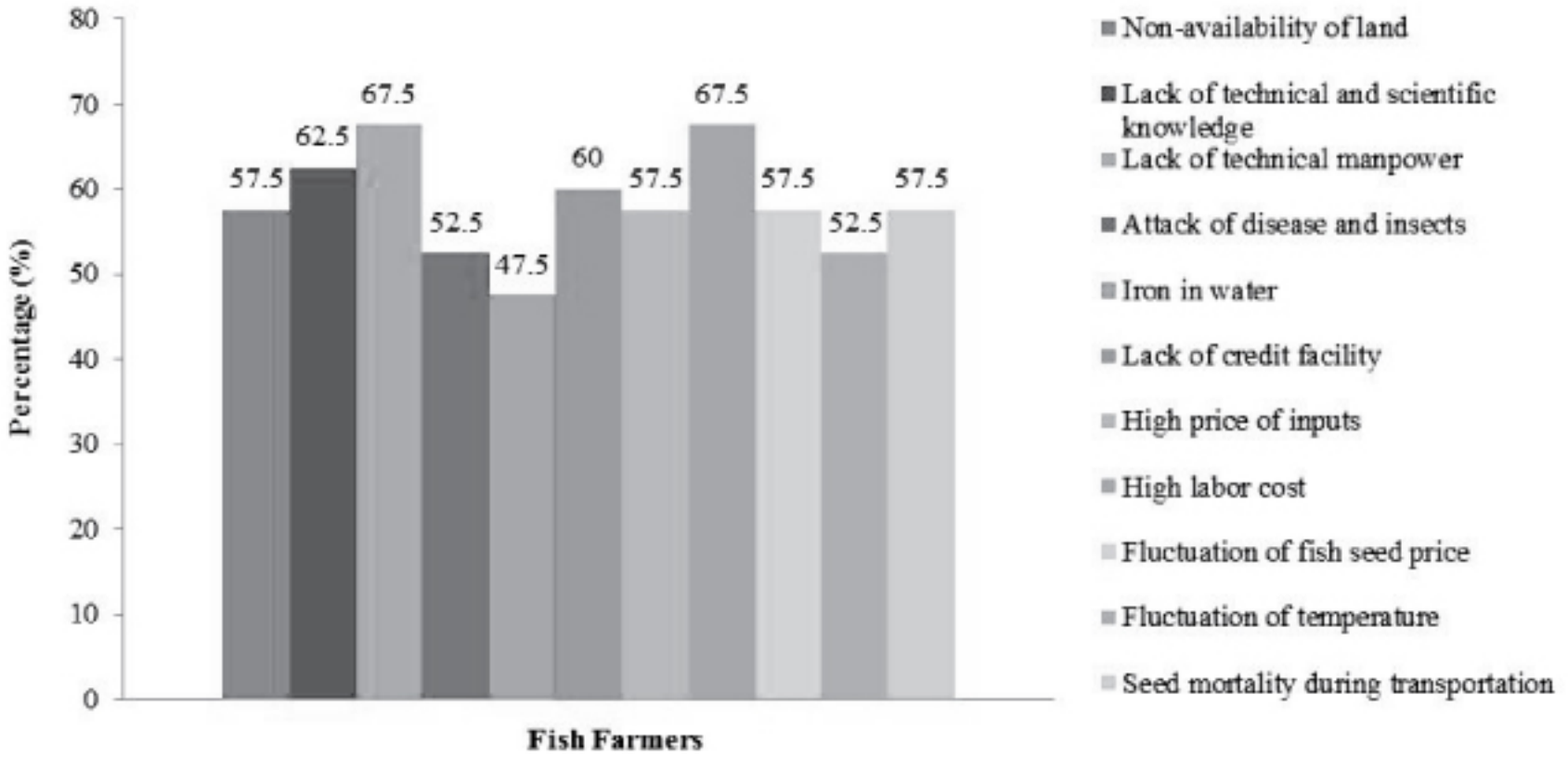

Figure 12. Problems faced by fish farmers

In this region 55% fish farmer collected seed directly from hatchery. Most of the farmer collected seed from outside of the district. In our study, it was observed that fish farmers faced a lots of problems. Among them, lack of technical man-powers (67.5%) and high labor cost (67.5%) were noticeable problems (Figure 12). Similarity was found by Malek (2007) stated that problems faced by hatchery owners in Mymensingh area included lack of disease tolerant brood, inbreeding problems, lack of technical knowledge, price fluctuation and unexpected market competition. On the other hand, the lack of capital, lack of transportation facilities was the main problems for nursery owners. Rahaman et al., (2007) also stated that the main constrains were lack of capital (35%) followed by lack of technical knows-how (27%) faced by fry traders. The nursery operators faced the problems like lack of capital (31%), high lease value (25%), high price of production inputs (17%), violence (12%), intense market competition (9%) and the lack of policy support (6%) in Jessore. Sharif and Asif (2015) also claimed *Argulus* disease as the main problem, 95% of hatchlings mortality is caused by Argulus disease in Jessore.

CONCLUSION AND RECOMMENDATION

Aquaculture activities are rapidly increased in the Sylhet region. The supply of good quality fry and fingerlings for successful aquaculture activities depends on a good seed marketing system. But, some problems are evident in the marketing channel and transportation system of fry. Traditional transportation system, involvement of multiple middlemen, high fry mortality, lack of technical knowledge of hatchery and nursery operators are major problems. For developing an efficient fish seed marketing the following recommendations should be followed through participatory approaches with hatchery owners, farmers, traders, government agencies, and NGO stakeholders. In this regard, quality brood should provide to the hatchery owners by government arrangement through establishment of live brood banks. Formal fry and fingerling trading networks should be developed locally and regionally by the government and other developing partners so that fry and fingerling producers and farmers can get their actual benefit. To produce quality brood stock, quality seed and an effective seed marketing system, institute-industry research partnership should establish along with hatchery and nursery operators.

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Biological efficacy of the mineral combination, Extramin™ on enhancing the growth by the regulation of molting and controlling disorders arising due to mineral deficiency in Pacific white leg shrimp, *Litopenaeus vannamei*

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Abstract

The research work elucidates the efficacy of the essential mineral formulation, Extramin in maintaining water quality for optimal plankton development and enhancing growth of shrimps by regulating the molting process in low saline cultures. In addition, efficacy of the product in recovering the shrimps affected by mineral deficiency disorders including White Muscle Disease (WMD) and Muscle Cramp Syndrome (MCS) were also explicated. Physico-chemical parameters of the pond water say, salinity, temperature, transparency, dissolved oxygen, and phytoplankton population dynamics were examined in the control and test ponds. Salinity ranged from 8 to 11 psu in the control and test ponds and no significant difference ($p < 0.05$) was observed between the two experimental groups. pH in control and test ponds ranged from 7.9 to 8.4. Test ponds administered with Extramin soon developed optimal and stable levels. In addition, no bloom crash was reported in the test treated ponds. In contrast, control ponds encountered bloom crash thrice during the culture period on the 43rd day, 78th day and on 97th day. Transparency level varied across weeks symbolizing the unstable bloom development. The variations between the experimental groups were observed to be statistically significant ($p<0.05$). Phytoplankton diversity and abundance exhibited thirteen species of phytoplankton in Extramin treated ponds and eleven species in control ponds. 100% recovery was observed in test ponds from muscular cramps and white muscles upon administration of Extramin on water and dietary administration, whereas, control ponds were observed to be unrecovered. Number of animals in molting crossed the margin of 60% in test ponds with margin of 40% in control ponds. Overall, administration of Extramin in diet and water enhanced the growth and ensured successful culture.

Keywords: Extramin, *Litopenaeus vannamei*, Molting, Phytoplanktons, Physico-chemical Parameters, White Muscle Disease and Muscular Cramp Syndrome

Introduction

Litopenaeus vannamei shrimp farming has become one of the lucrative businesses involving foreign currency and is now being done in various parts of India and Bangladesh successfully. Salinity in the culture area ranged from low saline, mid saline and high saline waters. Shrimps were cultured in low saline (0 to 13 psu) water using irrigation drains and rivers possess different ionic composition. In order to improve survival, achieve the desired productivity and maintain appropriate water quality in the shrimp pond, supplementation of minerals has become an essential act in shrimp aquaculture.

Roy, Davis, Saoud and Henry (2007a) emphasizes that *L. vannamei* is capable of tolerating a wide range of salinities, however, proper acclimation to low saline waters is the first step in the production process for successful rearing at low salinity. When shrimp are properly acclimatized, aquaculturists' have been quite successful at rearing shrimp using variety of different low saline water sources which vary greatly both in salinity and ionic profile (Lazartigue and Aralar 2009). Despite the success of many farmer's in rearing shrimp in low salinity, variable growth and survival among ponds are still being reported on a regular basis

(Roy *et al.* 2009a). The problem is compounded by the fact that no low saline water is the same with large variations in source, salinity, and ionic profile being reported (Boyd and Thunjai 2003).

In order to develop a successful culture system, levels of minerals in pond water has to be more or less similar to the levels in seawater diluted to the same salinity. However, pond water characteristics of even the very closely located ponds will not be exactly the same (Edward Gnana Jothi George et al., 2017). The ratio of Na (Sodium) to K (Potassium) and Mg (Magnesium) to Ca (Calcium) in water appears to be more significant than pond water salinity. Improper ratios of these minerals in water lead to osmotic stress which may influence growth and survival of shrimp negatively (Zhu et al., 2004). Hence, to make it more acceptable for shrimp production, minerals that promote osmoregulation at low salinity need to be administered to the pond water and animal diet.

Mineral deficiency in shrimp ponds and poor feeding of the shrimps are supposed to be the causes for White Muscle Disease (WMD) and Muscle Cramp Syndrome (MCS), which remain a major threat and potential source of mortality (Gunalan et al., 2014). The present study illuminates the efficacy of the mineral consortium, Extramin on improving water quality, regulating molting and recovering the shrimps from disorders caused due to mineral deficiency.

Materials and methods

Study area

The study was conducted during July to September 2017 in Muarmalla (16.674° N, 82.167° E), East Godavari district, Andhra Pradesh, (Fig. 1). Four shrimp ponds of equal size (1 acre each) were selected for the study.

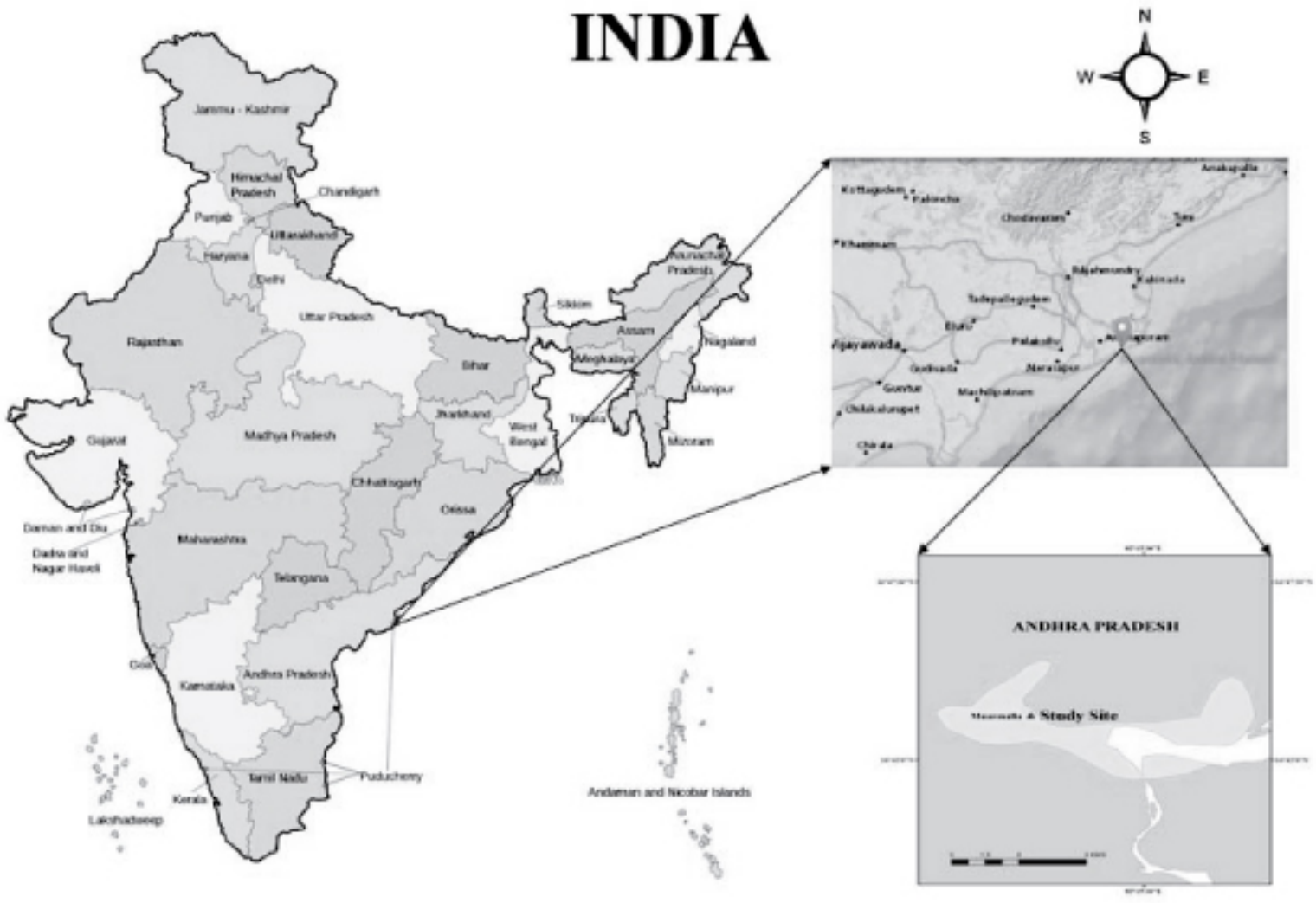

Fig. 1: Map showing the study area Muarmalla, East Godavari district, Andhra Pradesh

Product composition

Extramin is a commercial mineral product from the R&D facility of Tablets (India) Limited, Chennai, India. It is a perfect blend of macro and micro minerals in appropriate level to balance the ionic profile of the water. The composition of Extramin per 1000gms comprises of calcium, phosphorus, magnesium, manganese, potassium, ferrous, copper, cobalt, sulphur, zinc, selenium, chromium, iodine with lysine hydrochloride in a balanced proportion.

Experimental design

Proper pond preparation was carried out in all the selected ponds. Bore water with salinity ranging from 8 to 13 psu was pumped onto the shrimp ponds. Post larvae 12 were stocked at a density of 30/m^2 with the water depth of 1.5m. Two test ponds were treated with Extramin and two ponds were considered as control. 1.5 kgs/acre of Extramin for each pond was mixed thoroughly with same pond water and was broadcasted across the pond between 4 to 7 pm. No water exchange was carried out in any ponds. Physico-chemical parameters of the water and plankton analysis were carried out once in a week. Product response parameters including recovery from white muscles, muscular cramps and improper molting was calculated on control and test ponds.

Sampling analysis

Physico-chemical analysis

The physicochemical parameters of water were analysed in the control and test ponds pre and post usage of Extramin once in a week at three different points/spots. Water samples were collected between 07.00 and 08.00 hrs for *in situ* examination and laboratory analysis. Water samples were filtered using 0.45 µm membrane filter prior to analyses in order to remove any suspended particles and materials. The salinity in the ponds was recorded *in situ* by means of a portable hand-held optical refractometer (Atago, Japan) and cross-checked in laboratory using Mohr-Knudsen method as salinity is a key factor in this research work. pH was measured using electronic pH pen (Erma, Japan), and temperature was measured using standard Celsius Thermometer. The dissolved oxygen was estimated by modified Winkler's method as described by Strickland and Parsons (1972). Alkalinity was measured as per APHA (1998). Transparency was measured based on the penetration of light using a Secchi disc.

Plankton analysis

Plankton collection net of size 1 m length, 25 cm mouth diameter, and mesh size of 20 µm were used and were then rinsed into collection vessels. The samples were preserved in 4% neutral formaldehyde (final concentration) in polyethylene bottles for plankton abundance and identification. Samples were observed and morphologically identified with ZEISS research microscope coupled with an image analysing system using keys and illustrations by Prescott (1962), Patrick and Reimer (1966), Round (1990), Tomas (1997), and other taxonomic literature.

Phytoplankton abundance and diversity (standing drop) present in a litre of pond water sample was calculated using the formula: $N = nv/V$

Where,
N = Total number of phytoplankton cells per litre of water filtered
n = average number of plankton cells in 1mL of plankton sample
v = volume of plankton concentrate (mL)
V = volume of total water filtered (l)
The units of standing drop are N/l or $N\times10^3$ /m^3

Product response parameters

Cast net of known diameter (3.2 m) were used to enumerate the number of animals affected with white muscles, muscular cramps and improper molting. Recovery percentage was calculated post administration of 1.5 kg/acre of Extramin on water and 15 gm/kg over diet

Statistical analysis

The data were presented as mean ± SE. Student's t-test was applied to determine the significant difference ($p<0.05$) between the control and test ponds. All statistical calculations were performed using SPSS for Windows version 11.5 (SPSS Inc, Chicago, IL, USA). All column charts were plotted using Origin 6.1(OriginLab Corporation, Massachussets, USA).

Results and Discussion

The research study aims at studying the efficacy of the mineral blend, Extramin in improving the phytoplankton dynamics in pond water, regulating molting process and recovering the shrimps affected with disorders associated with mineral supplement. It is vital to supply shrimps with healthy water and nutrition. Nutrition is vital and it encompasses the chemical and physiological process which provides nutrients to an animal for normal function, increase in immunity, disease resistance, maintenance and growth. It involves ingestion, digestion, absorption and transport of nutrients and removal of waste. Poor nutrition and feeding affects the shrimp in various ways and may lead to irregular metabolism consequently leading to emergence of diseases opening up the system for pathogens and nutritional imbalance thus deteriorating the dynamics (Edward Gnana Jothi George et al., 2017).

Nutrients including minerals are very significant for a successful crop. The growth of the shrimps remains unaffected until equilibrium is maintained between the internal and external system of the animal. However, this equilibrium is distressed when there is impairment in the water quality parameters, which consequently makes the animal vulnerable to diseases due to stress and thus stunts growth and survival (Edward Gnana Jothi George et al., 2016). Hence, water quality plays a key role in shrimp farming.

In this research work, Salinity ranged from 8 to 11 psu (Fig. 2), pH ranged from 7.9 to 8.4 (Fig. 3) and alkalinity ranged 138 to 167 ppm (Fig. 5) in the control and test ponds with no significant difference ($p < 0.05$) between the two experimental groups. As the ponds were located one next to the other, received the water from the same source wherein no major differences were observed until it reached the pond. Ramanathan et al (2005), reported that, the optimum range of pH required for maximum growth and production of penaeid species is 6.8 to 8.7.

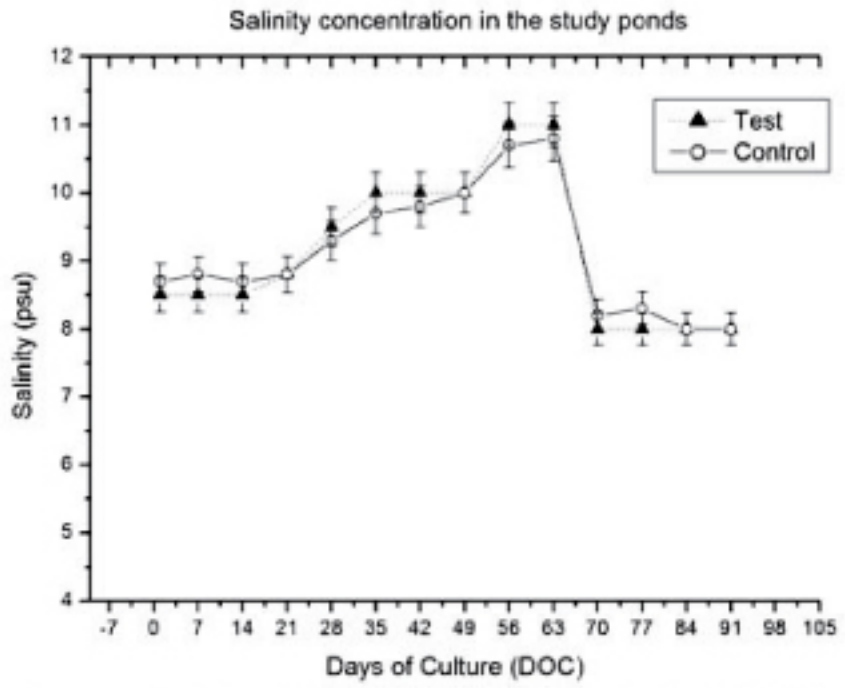

Fig. 2: Values of salinity recorded on control and test ponds for a period of 119 days at regular intervals of 7 days. *Results are presented as means with standard errors of two replicates for each experimental group (mean ± SE; n = 2)

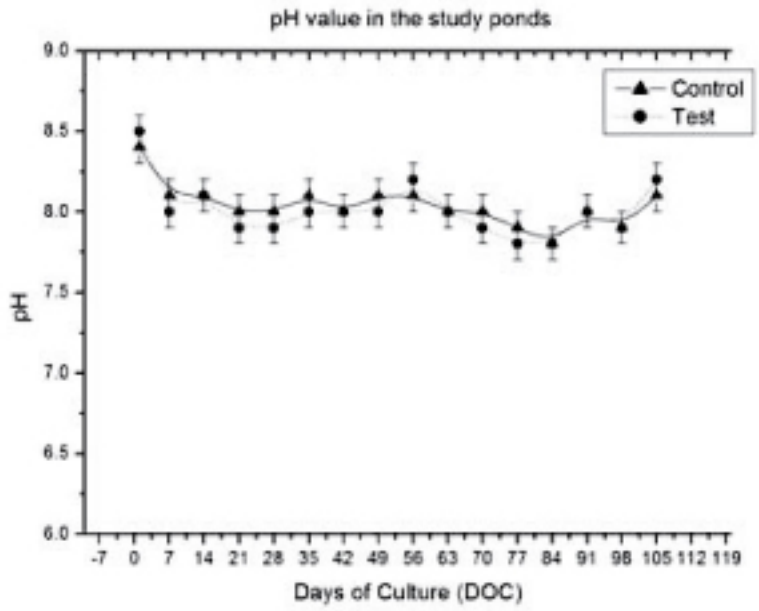

Fig. 3: Values of pH recorded on control and test ponds for a period of 119 days at regular intervals of 7 days. *Results are presented as means with standard errors of two replicates for each experimental group (mean ± SE; n = 2)

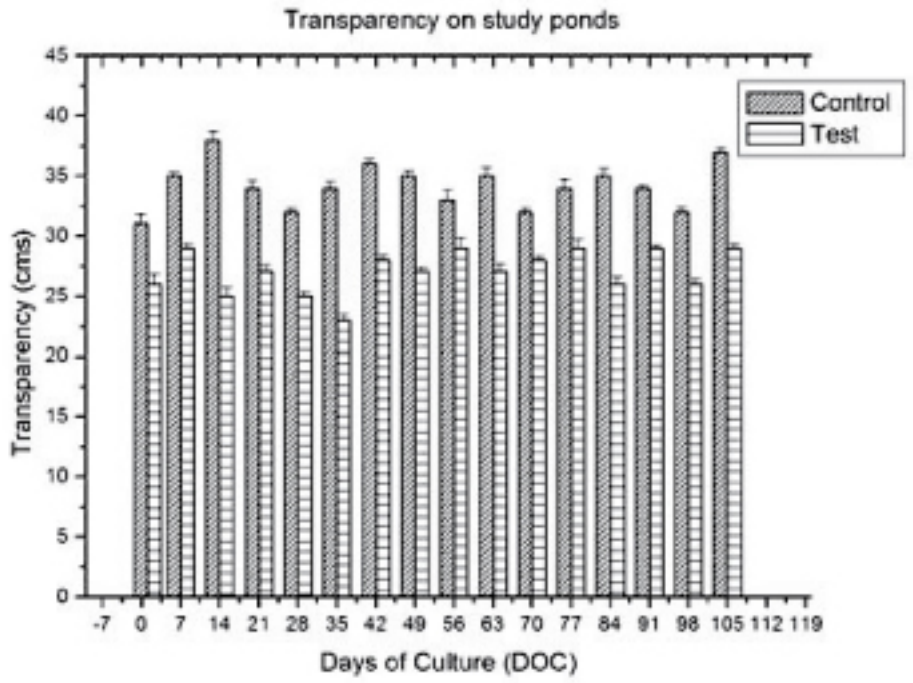

Fig. 4: Values of transparency recorded on control and test ponds for a period of 119 days at regular intervals of 7 days. *Results are presented as means with standard errors of two replicates for each experimental group (mean ± SE; n = 2)

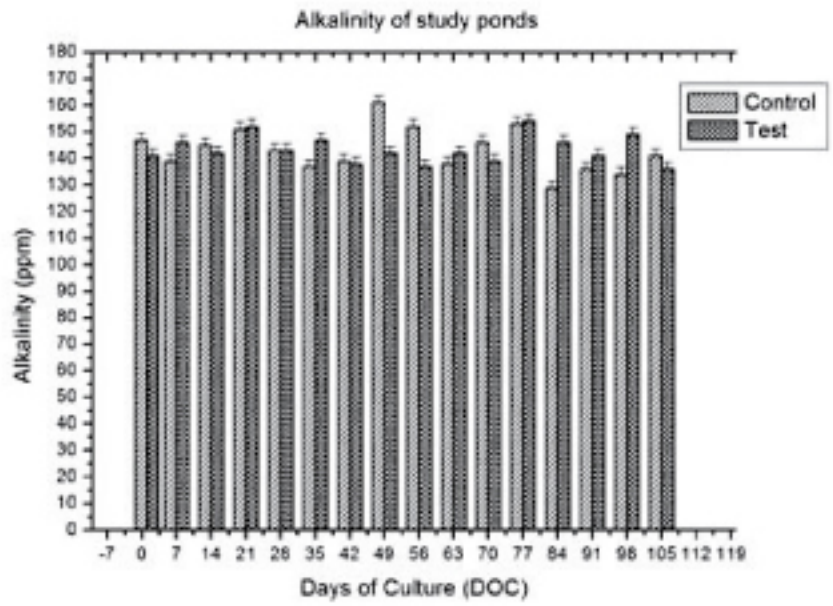

Fig. 5: Values of total alkalinity recorded on control and test ponds for a period of 119 days at regular intervals of 7 days. *Results are presented as means with standard errors of two replicates for each experimental group (mean ± SE; n = 2)

Phytoplankton plays a significant role in the pond ecosystem and minimizes the water quality fluctuations thereby enriches the water quality and suppress the bacterial growth (Yuvaraj and Karthik, 2015). Transparency was reported to be high symbolizing weaker bloom on both test and control ponds at the initial days of the culture. Test ponds administered with Extramin soon developed optimal and stable levels, thus favouring the growth of the post larvae leading to improved survival rate. In addition, no bloom crash was reported in the test treated ponds. In contrast, control ponds encountered bloom crash thrice during the culture period on the 43rd day, 78th day and on 97th day. The transparency level varied across week's symbolizing the unstable bloom development (Fig. 4). The means of the two experimental groups were notable and the variation was observed to be statistically significant ($p < 0.05$) between the control and test groups with p value of 6.5265E-12. The dissolved oxygen level was observed to be in the range of 3.9 to 4.4 ppm and 4.1 to 4.5 ppm in the control ponds and test groups respectively with significant difference ($p< 0.05$) between the two groups and p value of 3.42845E-5 (Fig. 6). Decreased oxygen level also interferes in the metabolic performances of the shrimp and can reduce growth and moulting, consequently leading to increased mortality (Edward Gnana Jothi George et al., 2016).

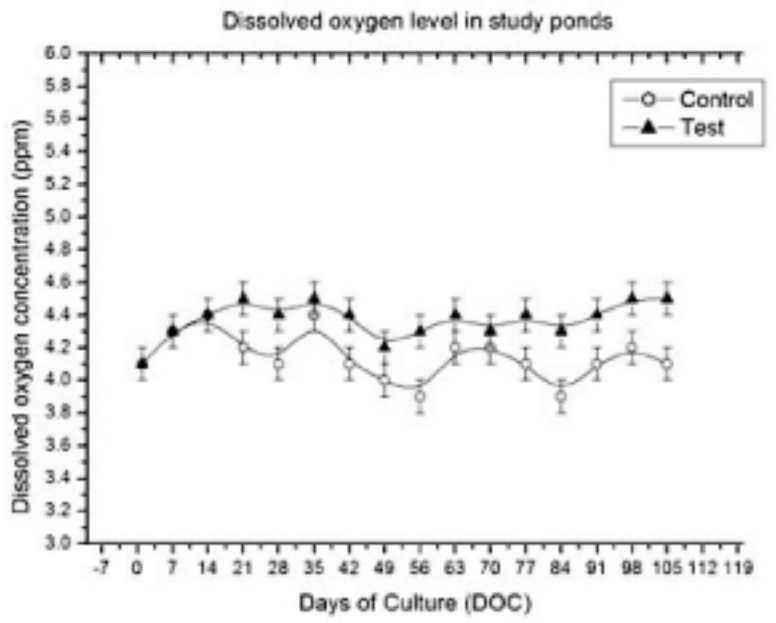

Fig. 6: Values of dissolved oxygen recorded on control and test ponds for a period of 119 days at regular intervals of 7 days. *Results are presented as means with standard errors of two replicates for each experimental group (mean ± SE; n = 2)

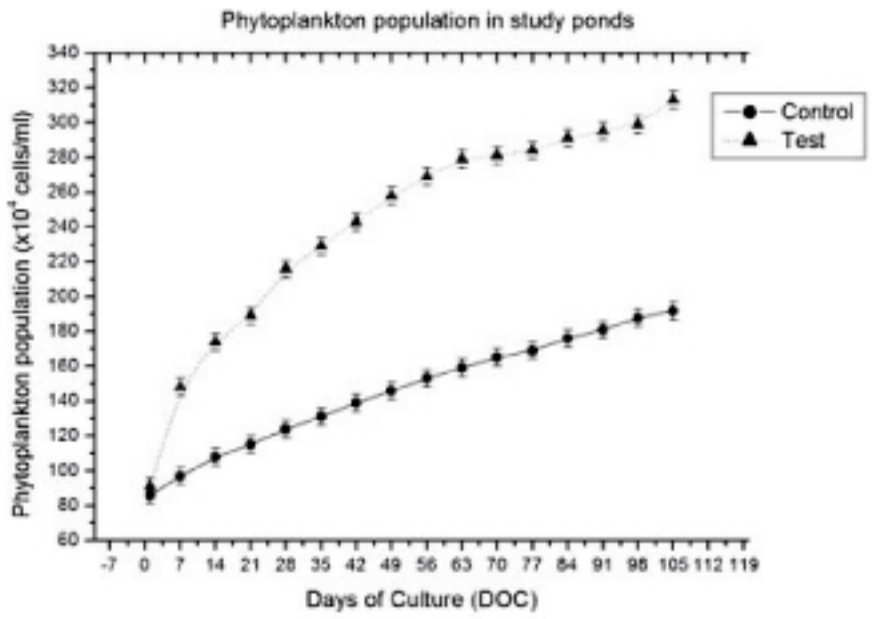

Fig. 7: Values of phytoplankton population recorded on control and test ponds for a period of 119 days at regular intervals of 7 days. *Results are presented as means with standard errors of two replicates for each experimental group (mean ± SE; n = 2)

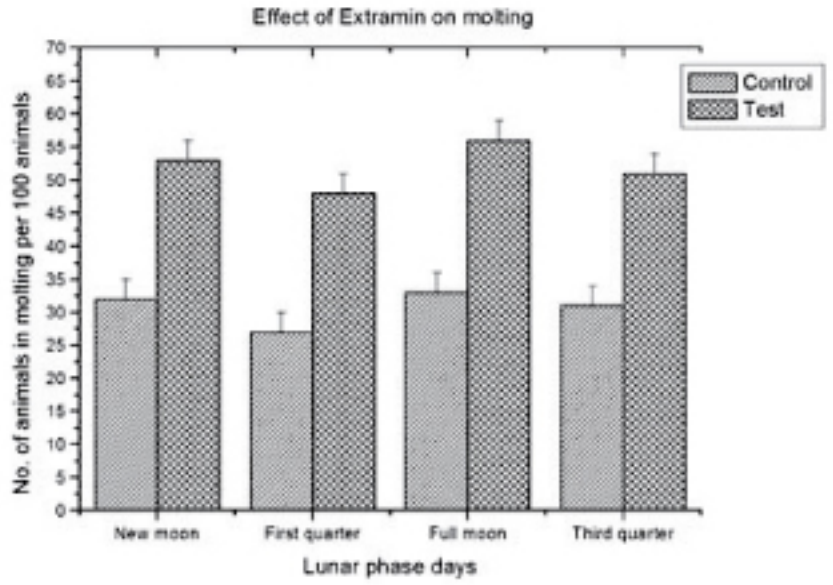

Fig. 8: Figure showing the effect of Extramin on controlling improper molting in shrimps. *Results are presented as means with standard errors of two replicates for each experimental group (mean ± SE; n = 2)

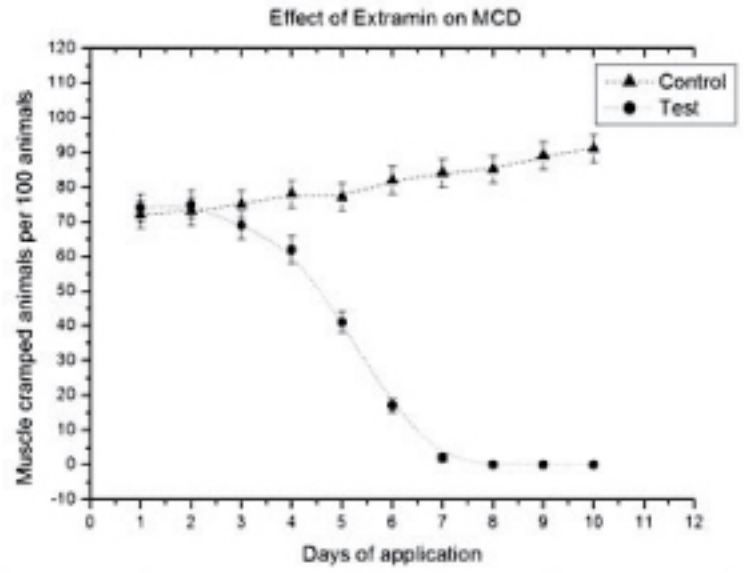

Fig. 9: Figure showing the effect of Extramin on controlling muscular cramps in shrimps. *Results are presented as means with standard errors of two replicates for each experimental group (mean ± SE; n = 2)

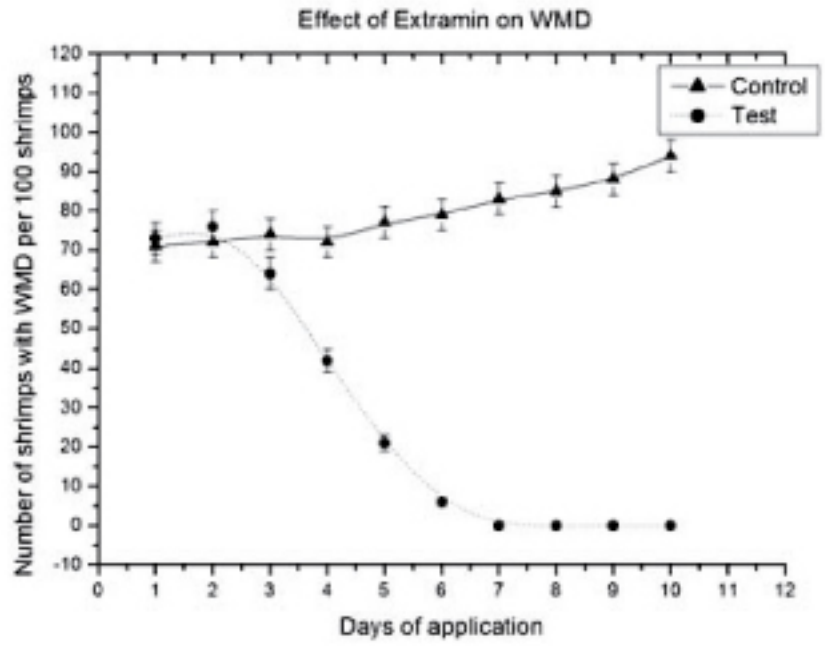

Fig. 10: Figure showing the effect of Extramin on controlling white muscles in shrimps. *Results are presented as means with standard errors of two replicates for each experimental group (mean ± SE; n = 2)

Phytoplankton diversity and abundance exhibited thirteen species of phytoplankton in Extramin treated ponds and eleven species in control ponds. Test ponds demonstrated density of 259-349×10^4 cells/ml and control ponds with 203-271×10^4 cells/ml with significant difference ($p < 0.05$) between the two groups and p value of 7.57325E-5 (Fig. 7). The plankton diversity showed that water quality parameters in optimum range and nutrient rich water influenced the growth of both phyto and zoo plankton groups. Similar observation was reported by Hossain et al. (2007). Margalef (1964) also reported that the phytoplankton population in nutrient rich waters is more diverse than those in nutrient deficient waters.

Number of animals in molting crossed the margin of 56% in test ponds with margin of 27% in control ponds (Fig. 8). A higher growth rate implies an increase in the molting frequency (Gauquelin et al., 2007) and high molting frequency might not only increase energy expenditure for exuviations, but also alter the animal's entire energy allocation strategy (Cockroft and Woolridge, 1985). 100% recovery was observed in test ponds with muscular cramps (Fig. 9) and white muscles (Fig. 10) upon administration of Extramin on water and dietary administration, whereas, control ponds were found to be unrecovered. Application of Extramin in water, followed by feed has helped shrimp recover white muscles and muscle cramps very effectively. Several authors reported white muscle disease in shrimps and prawns (Sudhakaran et al, 2006).

The test study pond with optimal and stable bloom is shown in the fig. 11. This study showed that Bacillariophyta, Cyanophyta and Chlorophyta constituted the major composition of the microalgal population in the shrimp pond. Few species in the shrimp ponds were presented in Fig. 12. When shrimps were introduced into the pond, diatoms started to occur and the diatoms became the dominant species during shrimp culture period. These results were in agreement with Asma Liyanna Shaari (2011) findings. Pulle and Khan (2003) showed that variation in plankton densities is influenced not only by temperature and other factors such as pH and alkalinity, also nutrients are responsible for the organic production.

Fig. 11. Figure showing the Extramin used test study pond water with optimal and stable bloom

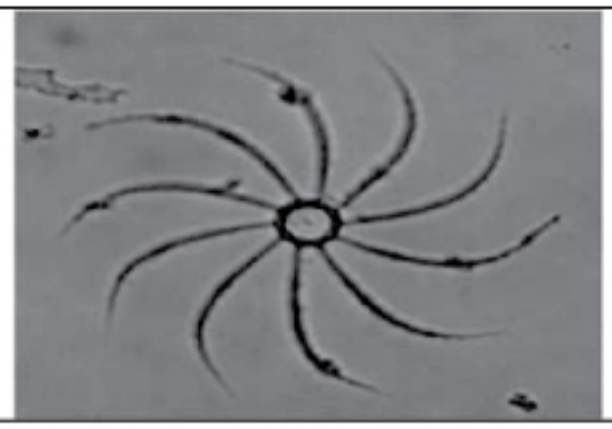

Fig. 12.1 *Bacteriastrum comosum*

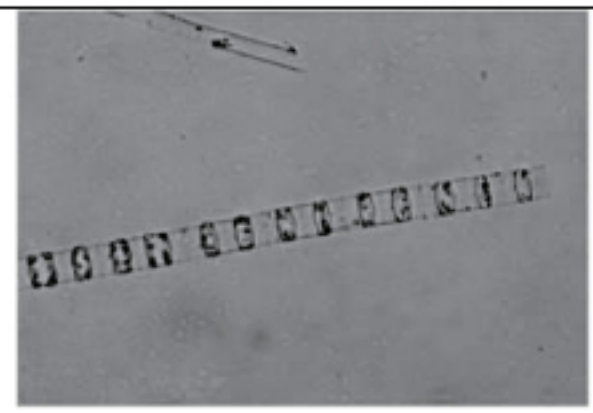

Fig. 12.2 *Skeletonema costatum*

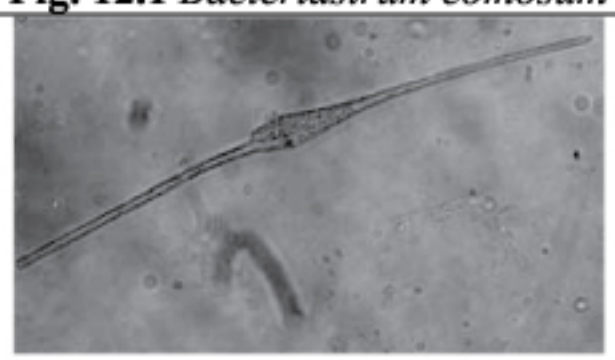

Fig. 12.3 *Ceratium extensum*

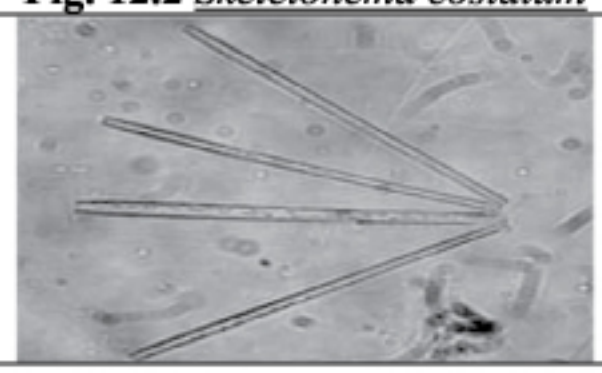

Fig. 12.4 *Thalassiothrix frauenfeldii*

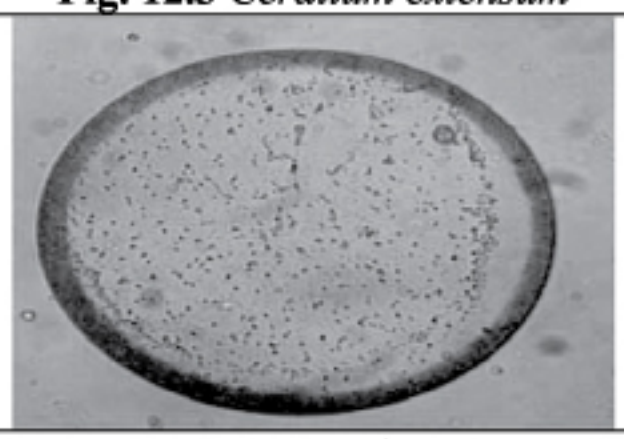

Fig. 12.5 *Coscinodiscus gigas*

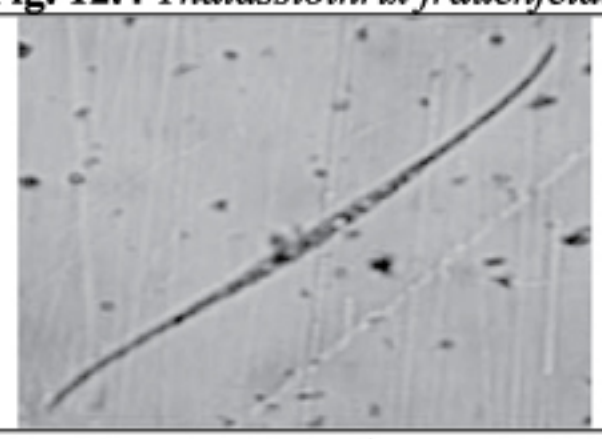

Fig. 12.6 *Nitzschia sigma*

Fig. 12.7 *Odentella sinensis*

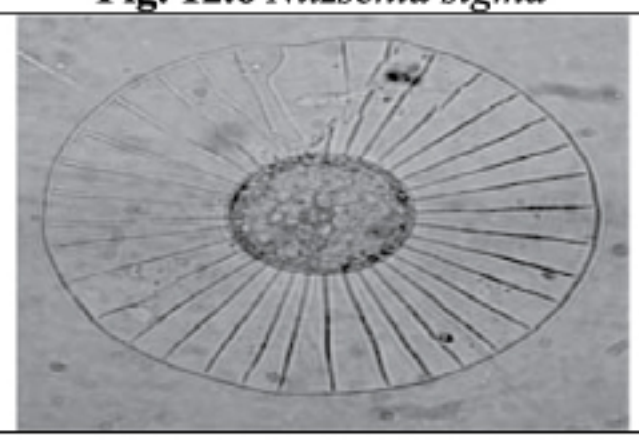

Fig. 12.8 *Planktoniella sol*

Fig. 12.9 *Pleurosigma elongatum*

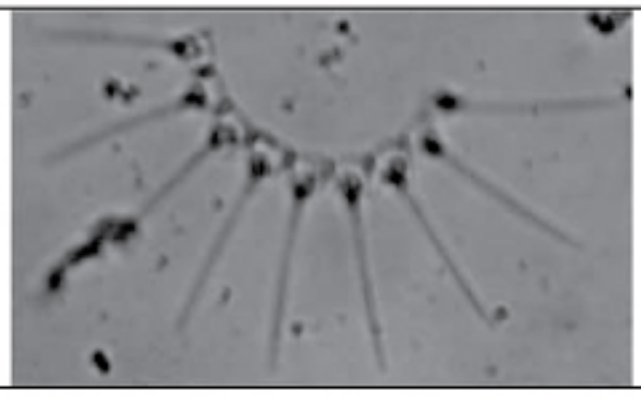

Fig. 12.10 *Asterionella glacialis*

Fig. 12: Figures showing the phytoplankton distribution in the shrimp ponds (12.1 – 12.10)

Toxic mineral contamination which involves the accumulative elements copper, lead, cadmium, mercury, arsenic, fluorine, selenium, molybdenum and vanadium in the dietary feed ingredients remains as a major threat. For example, contamination with copper may arise from products fermented within copper lined vessels (ie. brewery by-products). However, Extramin is a purified combination of essential minerals with lysine hydrochloride for improved meat conversion with zero toxic minerals, antibiotics and steroids and was tested and proven with standard testing laboratories.

Conclusion

From the study result, it is concluded that Extramin is a potential formulation of mineral mixture which facilitates the growth of beneficial planktons by adding nutrients to the water and also when administered through feed meets the mineral requirement of the shrimps. As a result, the shrimp ponds were observed to develop optimal and stable bloom which enhances the production of required concentration of dissolved oxygen in the pond. Also, optimal bloom kept the pond without bloom crash round the culture duration which enhanced the growth and survival of the shrimps thus leading to higher productivity. Above all, as the entire mineral requirement of the pond water and shrimps were met properly, molting cycle were positively regulated and no symptoms of mineral deficiency disorders like MCD and WMD were ever noticed in the ponds. Overall, Extramin is a perfect mineral product of choice for developing successful and organic shrimp farming.

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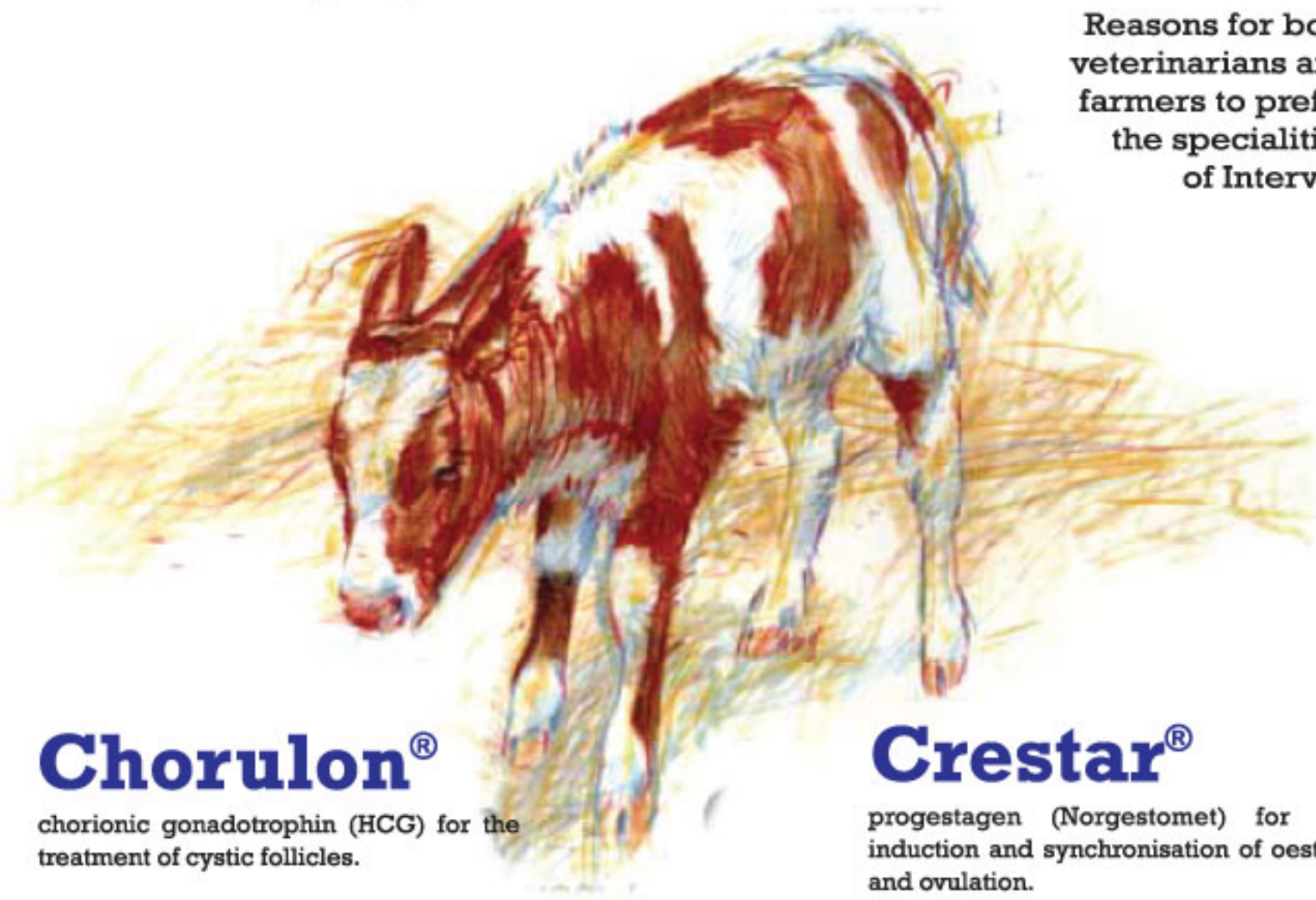

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