

Project ID: 349

Competitive Research Grant (CRG)

Sub-Project Completion Report

on

Identification of the causes of calf mortality in buffalo and their mitigation measures in Bangladesh

Project Duration

July 2017 to September 2018

Department of Pathology
Faculty of Veterinary, Animal and Biomedical Sciences
Sylhet Agricultural University, Sylhet 3100



Submitted to
Project Implementation Unit-BARC, NATP 2
Bangladesh Agricultural Research Council
Farmgate, Dhaka-1215



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Citation

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Acronyms

<i>E. coli</i>	=	<i>Escherichia coli</i>
SS	=	<i>Salmonella Shigella</i>
OIE	=	Office International des Epizooties
WHO	=	World Health Organization
FAO	=	Food and Agriculture Organization of the United Nations
NCCLS	=	National Committee for Clinical Laboratory Standards
CLSI	=	Clinical and Laboratory Standards Institute
HL	=	Heat Labile
HS	=	Heat Stable
DLS	=	Department of Livestock Services
FDA		Food and Drug Administration
EMB	=	Eosin Methylene Blue
NB	=	Nutrient Broth
<i>T_m</i>	=	Temperature
ANOVA	=	Analysis of Variance
SAU	=	Sylhet Agricultural University
Hrs	=	Hours

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Executive Summary

Calf mortality in a buffalo farms has been identified to be one of the major limiting factors in buffalo production in Bangladesh. There is little or no information available on the causes of buffalo calf mortality and their mitigation measures. Therefore, the research was conducted to identify the causes of buffalo calf mortality and associated risk factors and thereby to develop effective mitigation measures of the problem in Bangladesh. Three divisions from different geographical and climatic conditions namely Sylhet, Khulna and Barsial were selected for the study. From each of the divisions, two districts were selected based on distribution of concentration of buffalo population. To identify the risk factors of buffalo calf sickness and mortality, data from 600 buffalo farmers (100 small holder buffalo farmers from each district of Sylhet, Moulvibazar, Bagerhat, Jhenaidah, Bhola and Patuakhali) were collected using structured questionnaire. A total of 240 feces samples, 120 nasal swabs and 195 blood samples were collected, with the history of recent calf mortality, from clinically sick buffalo calves from different locations of the study areas. Feces and swab samples were subjected to bacteriological analysis using standard procedures. Feces were analyzed to identify parasitic causes of illness. Blood samples were analyzed for blood protozoa. Sera were used to determine the prevalence of viral diseases using specific ELISA kits. Blood and fecal samples were also used for confirmation of selected viral diseases by PCR. A total of 240 fecal samples were tested for identification of bacterial causes of which, *E. coli* was isolated from 80 cases (33.33%) and *Salmonella* was isolated from 28 cases (11.67%). Similarly, 120 swab samples were also tested for bacteria of which 37 (30.83%) were positive for *Pasteurella*, 16 (13.33%) for *E. coli* and 14 (11.67%) for *Salmonella*. All the 240 feces samples were also tested by direct smear and McMaster methods for the presence of gastrointestinal parasites where infestation of *Neoscaris vitulorum* was detected in 70 cases (33.33), *Trichostrongyles* were found in 11 cases (13.75%) and *Trichuris* were found in 06 cases (7.5%). A total of 45 blood samples were analyzed by direct smear method to detect blood protozoa of which 3 were positive only for *Babesia*. The presence of Rotavirus in diarrheic buffalo calves, detected by PCR, was found to be 15.56% and for BVD virus it was 12.22%. Out of 150 serum samples from diarrheic buffalo calves tested, 29 (19.33%) were positive for Rotavirus and 11 (17.33%) were positive for BVD virus. After post mortem examination of 10 dead calves followed by laboratory diagnosis, the cause of death for the five calves was found to be due to *Pasteurella* infection (20%), *Neoscaris vitulorum* infection (20%) and *E coli* infection (10%). The cause of death for the rest five dead calves could not be identified. Season of birth, herd size and quarantine measures were identified to be the potential risk factors of buffalo calf sickness and /or mortality. Levofloxacin, Gentamycin and Colistin were found sensitive to *E. coli*, *Salmonella* & *Pasteurella* and streptomycin was found sensitive to *Salmonella* & *Pasteurella*.

CRG Sub-Project Completion Report (PCR)

A. Sub-project Description

1. Title of the CRG sub-project:

Identification of the causes of calf mortality in buffalo and their mitigation measures in Bangladesh

2. Implementing organization:

Sylhet Agricultural University (Department of Pathology)

3. Name and full address with phone, cell and E-mail of PI/Co-PI (s)

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4. Sub-project budget (Tk):

4.1 Total: Tk 24,00,000.00

4.2 Revised (if any): N/A

5. Duration of the sub-project:

5.1 Start date (based on LoA signed): July 2017

5.2 End date: September 2018

6. Justification of undertaking the sub-project:

Buffaloes are highly social animals with strong instincts that play important roles in the livelihood and economy of small holders as well as landless people in the rural areas of Bangladesh (FAO, 1974). Buffaloes are being raised as a major contributor to the agriculture and livestock industry in many Asian countries through the production of good quality meat and farm yard manure (Singh, 2010). Buffaloes are also recognized as the world's second most important milk producing species (Bhatti *et al.*, 2009).

Calves are the future herd and keeping them in a fit and healthy condition not only increases the efficiency of a livestock farm but also contributes to the economy and production outputs in the years to come. Mortality in the calves renders economic losses as well as contributes to the low production of the herd when taking futuristic terms for the farming scenario (Ablaha *et al.*, 1995). Mother and young are closely bonded, and the buffalo calf usually becomes more stressed when separated from the dam than the calves of cattle (FAO, 1974). A large number of calves die during the first year of their life, causing heavy drain on the economics of livestock production. It is roughly estimated that a calf mortality of 20% can reduce net profit to 38% (Blood and Radostits, 1989). Neonatal calf mortality varied from 8.7% to 64% throughout the world and the mortality in the first months of age accounted to be 84% of the total mortality (Jenny *et al.*, 1981) where it is particularly high in third week of life (Umoh, 1982). Major causes of mortality of neonatal calves were detected to be diarrhea and pneumonia (Khan *et al.*, 2009; Shimizu and Nagatoma, 1978). Calf mortality is thought to be the major limiting factor in buffalo production in Bangladesh. There is little or no information available on the causes of buffalo calf mortality and their mitigation measures in the country. This alarming situation attracts the attention to assess the causes of calf mortality in buffaloes and their mitigation measures in Bangladesh.

7. Sub-project goal:

To increase buffalo production through control of buffalo calf mortality in Bangladesh

8. Sub-project objective (s)

- a) Identification of environmental and managerial risk factors associated with calf mortality in buffaloes
- b) Isolation and identification of causative organisms associated with buffalo calf mortality in Bangladesh
- c) Development of appropriate therapeutic measures and control strategies against buffalo calf diseases

9. Implementing location (s):

Department of Pathology; Faculty of Veterinary, Animal and Biomedical Sciences; Sylhet Agricultural University; Sylhet

10. Methodology in brief:

The handling of animals in the study was performed in accordance with the current Bangladesh legislation (Cruelty to Animals Act 1920, Act No. I of 1920 of the Government of the People's Republic of Bangladesh).

10.1. Identification of environmental and managerial risk factors associated with calf mortality in buffaloes

Selection of study area

Three divisions from different geographical and climatic conditions namely Sylhet, Khulna and Barisal (Figure 1) were selected for the study. From each of the divisions two districts (Sylhet and Moulvibazar

from Sylhet Division, Bagerhat and Jhenaidah from Khulna Division and Bhola and Patuakhali from Barisal Division) were selected based on distribution of concentration of buffalo population.

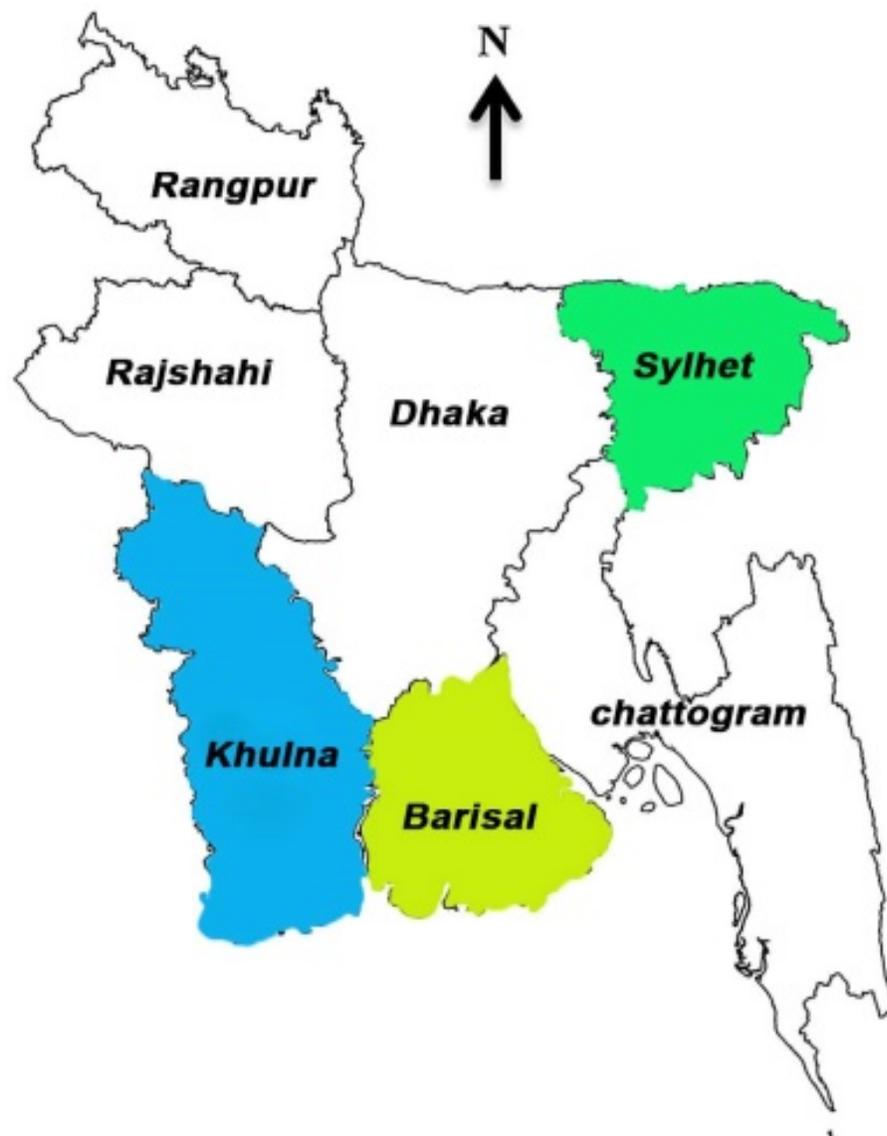


Figure 1: Map of Bangladesh showing the sampling locations in different colors

Questionnaire design and pre-testing of questionnaire

A questionnaire was designed and pre-tested to conform to the objectives targeted to obtain information from the selected farmers. Information like, date of birth of a calf, type of birth, season of birth, weight of calves at birth, colostrum feeding dam's number and number of abnormal calving (stillbirths and abortions) were included in the questionnaire. Other related information like age of dam at the time of parturition, the parity number of the dam, herd size, feeding regimes, husbandry practices, etc. were also included in the questionnaire (Annex-I).

Data collection

Data from 600 buffalo farmers from selected districts under Sylhet (Sylhet and Moulovibazar), Khulna (Bagerhat and Jhenaidah) and Barisal (Bhola and Patuakhali) division were collected using structured questionnaire. All collected data were statistically analyzed using International business machine Statistical Package for Social Science (SPSS) version 22, 2018 to identify risk factors associated with buffalo calf mortality. A p-value of < 0.05 was considered as statistically significant.

10.2. Isolation and identification of causative organisms associated with buffalo calf mortality

Sample collection

To identify causes of buffalo calf mortality, a total of 240 feces samples, 120 nasal swabs and 195 blood samples (with and without anti-coagulant) were collected from clinically sick buffalo calves from selected districts under Sylhet (Sylhet and Moulovibazar), Khulna (Bagerhat and Jhenaidah) and Barisal (Bhola and Patuakhali) division with the history of recent calf mortality (Figure 2 & Table 1). Representative samples were also collected from 10 dead calves after performing post mortem examination following standard procedure mentioned by FIVET, Postmortem Techniques in Livestock (Figure 3). Dead calves (10) were collected from Sylhet (n=6; 03 from Sylhet district and 03 from Moulovibazar district), Khulna (n=02; 01 each from Bagerhat and Jhenaidah districts) and Barisal (n=02; 01 each from Bhola and Patuakhali districts) divisions.



Figure 2: Collection of samples from clinically sick buffalo calves



Figure 3: Post mortem examination and sample collection from dead buffalo calves

Table 1: Plan of collection of samples from the clinically sick buffalo calves

Sampling sites		No. of feces samples collected	No. of nasal swabs collected	No. of blood samples collected
Divisions	Districts			
Sylhet	Sylhet	40	20	40
	Moulvibazar	40	20	35
Khulna	Bagerhat	40	20	30
	Jhenaidah	40	20	30
Barisal	Bhola	40	20	30
	Patuakhali	40	20	30
Total		240	120	195

Isolation and identification of bacterial agents

Isolation and identification of bacteria was performed as shown in the flow diagram in Figure 4.

Colibacillosis (*Escherichia coli*) was identified through isolation of the causative organism on nutrient agar at first which showed large, thick, grayish white, moist, smooth, opaque or translucent discs. Then they were cultured on MacConkey agar followed by eosin methylene blue agar and typical biochemical characteristics shown in a triple sugar iron slant. Isolates suspected to be *E. coli* were confirmed by the positive result of the indole test and MR test.

Salmonellosis (*Salmonella* spp.) was identified through isolation of the causative organism on nutrient agar at first which showed circular, smooth, opaque and translucent colonies followed by culturing on Salmonella-Shigella agar and typical biochemical characteristics shown in triple sugar iron agar slants. Salmonellosis was confirmed by the positive result of MR test and Citrate Utilization test.

Pasteurellosis (*Pasteurella* spp.) was identified through isolation of the causative organism on nutrient agar at first which showed Smooth, glistening and opalescent colony followed by culture on McConkey Agar and Blood agar. *Pasteurella* spp. was confirmed by the typical biochemical characteristics shown in

triple sugar iron agar slants and positive result for Indole and Catalase tests. Under microscopic examination of Giemsa stained blood smears presence of characteristic bipolar organisms were found.

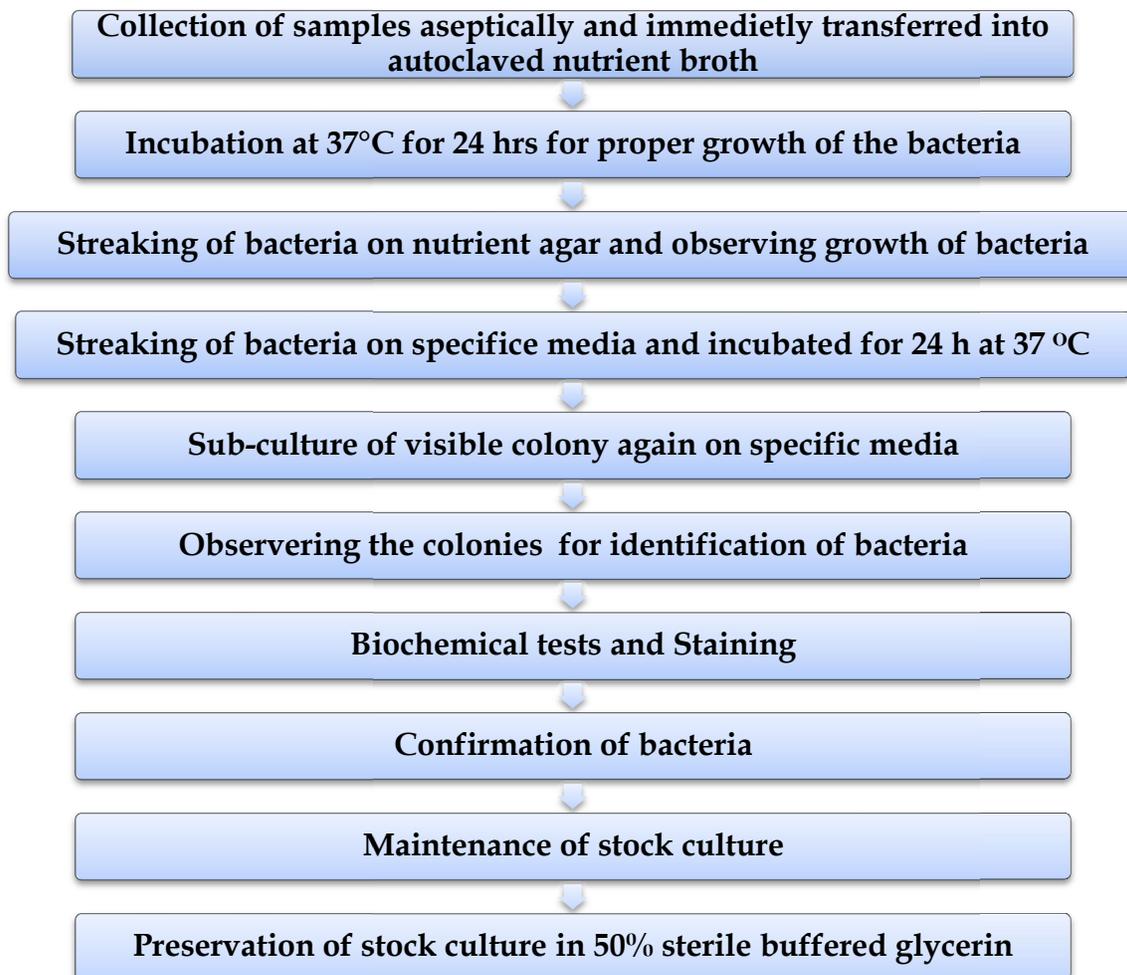


Figure 4: Flow chart for isolation and identification of specific bacteria

Isolation and identification of parasites (GI parasites)

Endo-parasitic diseases were diagnosed using fecal sample examinations (direct smear, flotation) following standard procedures of OIE.

Physical Examination

The fecal samples were examined first for their color, consistency, presence of blood, mucus, dead worms, etc. The physical examination was done with naked eye.

Microscopic examination

In microscopic examination, parasites were identified by observing morphology of their eggs using flotation technique and sedimentation technique. Positive cases were counted by Mc Master Technique.

Simple flotation technique (Qualitative method)

Approximately 3 g of feces was taken into container 1 with 1.50 ml of floatation fluid (saturated salt solution). The contents were thoroughly mixed with a stirring device (tongue blade fork). The resultant fecal suspension was poured through a tea strainer or a double-layer of cheesecloth into container 2. Then test tubes were filled with the fecal suspension that were then kept standing for 20 minutes in a test tube stand or rack covering with a cover slip on top. After 20 minutes, the cover slip was carefully lift off, together with the drop of fluid adhering to it, and immediately placed on a glass slide and examined under microscope (10x) for eggs/larvae identification.

Sedimentation technique (Qualitative method)

Approximately 3 g of feces was taken into container 1 with 40-50 ml of tap water and thoroughly mixed with a stirring device (fork, tongue blade). The fecal suspension was then poured through a tea strainer or double layer of cheese cloth into container 2. From where, the filtered material was poured into a test tube and allowed to sediment for 5 minutes. The supernatant was removed very carefully. The sediment was re-suspended in 5 ml of water. The sediment was allowed to stand for 5 minutes. The supernatant was discarded very carefully. The sediment was stained by adding one drop of methylene blue. Then it was transferred to a micro slide. With the help of a cover slip examination was done under microscope (10x).

McMaster technique (Quantitative method)

Four gram (4 g) of feces was weighed and placed into container 1 with 56 ml of a floatation fluid. Mixing was done thoroughly with a stirring device (fork, tongue blade). The fecal suspension was filtered through a tea strainer or a double layer of cheese cloth into container 2. While stirring the filtrate in container 2, a sub sample was taken with pasture pipette. The both slides of the McMaster counting chamber was filled with the sub samples. The counting chamber was allowed to stand for 5 minutes. Examination was done under a microscope at a 10 x 10 magnification. All the eggs and oocytes were counted within the engraved area of both chambers. The number of eggs per gram of feces was calculated as followed:

The egg count of the two chambers was added. Then the total was multiplied by 50. This gave the E.P.G. of feces.

Isolation and identification of protozoal agents (blood protoza)

Blood smears were prepared on glass slides from blood samples collected with anti-coagulant. The smears were air dried, fixed and stained using commercially available staining kit according to the manufacturer's instruction and finally examined under microscope to detect protozoal infections (e.g., *Babesia*, *Anaplasma*, etc.).

Identification of viral agents

Determination of sero-prevalence of Rota virus and Bovine Viral Diarrhea Virus by ELISA

Antibodies in serum against Rota virus and Bovine Viral Diarrhea (BVD) were detected by enzyme-linked immunosorbent assay (ELISA) using the Rota virus ELISA kit and Bovine Viral Diarrhea ELISA kit (My Biosource, Inc., San Diego, California, USA) according to manufacturer's instructions (Figure 5). All reagents were kept at 18-26°C before use. The reagents were mixed by shaking gently. Every test was also conducted with the positive and negative control sera supplied by the manufacturer. Results were expressed as a percent value of the test sample optical density (OD%) which was calculated as $OD\% = [(OD \text{ sample} - OD \text{ negative control}) / (OD \text{ positive control} - OD \text{ negative control}) \times 100]$.

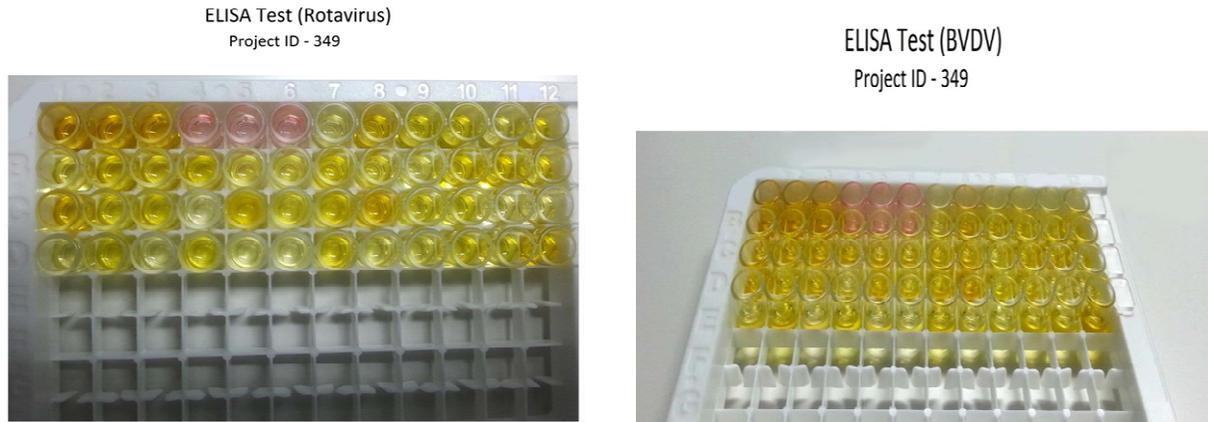


Figure 5: ELISA test to identify Rotavirus and BVDV

Detection of Rota virus and Bovine Viral Diarrhea Virus by PCR

Viral RNA of Rota virus and Bovine Viral Diarrhea were extracted from the fecal samples using a commercial PureLink™ Genomic RNA Mini Kit (Invitrogen, Van Allen Way Carlsbad, CA, USA), according to the manufacturer's instructions. The extracted RNA was reverse transcribed using specific primers and cDNA was stored at -20°C until use. The PCR was performed using specific primer pairs for Rota virus and Bovine Viral Diarrhea Virus as mentioned in Table 2.

For both the viruses, PCR reactions were performed in a final volume of 20 µl using the PCR Master Mix (Promega, Madison, USA). Target DNA was denatured by incubation for 10 min at 95°C before amplification for 30 cycles at 94°C for 1 min, annealing at specific temperatures (55°C for Rota virus and 62°C for Bovine Viral Diarrhea) for 1 min and extension at 72°C for 1 min followed by 72°C for 10 min (final extension) and holding at 4°C until collection. Amplified PCR products were run on a 1.5% agarose gel for electrophoresis under 100 volts for 1 h. Following electrophoresis, the gel was stained using ethidium bromide (0.5 µg/ml) and results were evaluated by UV transillumination.

Table2. Primers used to detect Rotavirus and BVDV by PCR

Virus	Primer Sequence	Amplicon Size (bp)	References
Rota Virus	F:5' CTATTCAGTGTGTCGTGAGAGG 3'	503 bp	Gouvea <i>et al.</i> , 1991
	R: 5' CGTGGCTTTGGAAAATTCTTG 3'		
BVDV	F: 5' AAGATCCACCCTTATGA(A/G)GC 3'	639 bp	Gilbert <i>et al.</i> , 1999
	R; 5' AAGAAGCCATCATC(A/C)CCACA 3'		

10.3. Development of appropriate therapeutic measures and control strategies against buffalo calf diseases

- Disease calendars and vaccination schedule were prepared both as poster and leaflets and distributed to the farmers of study area and to the regional livestock office.
- Deworming schedule along with some deworming medicine was provided to the farmers to keep their animal safe.
- Antibiotic sensitivity tests were performed to provide effective antibiotic treatment guide lines against specific bacterial diseases.

Antibiotic sensitivity test

In vitro antibiotic sensitivity test was done according to the guidelines of Clinical and Laboratory Standard Institute (CLSI guidelines, 2014) using disc diffusion method. A flow diagram of antibiotic sensitivity test by disc diffusion method is shown in Figure 6 and the antibiotics used with disc concentration are shown in Table 3. A flow chart of antibiotic sensitivity test by disc diffusion method is given below:

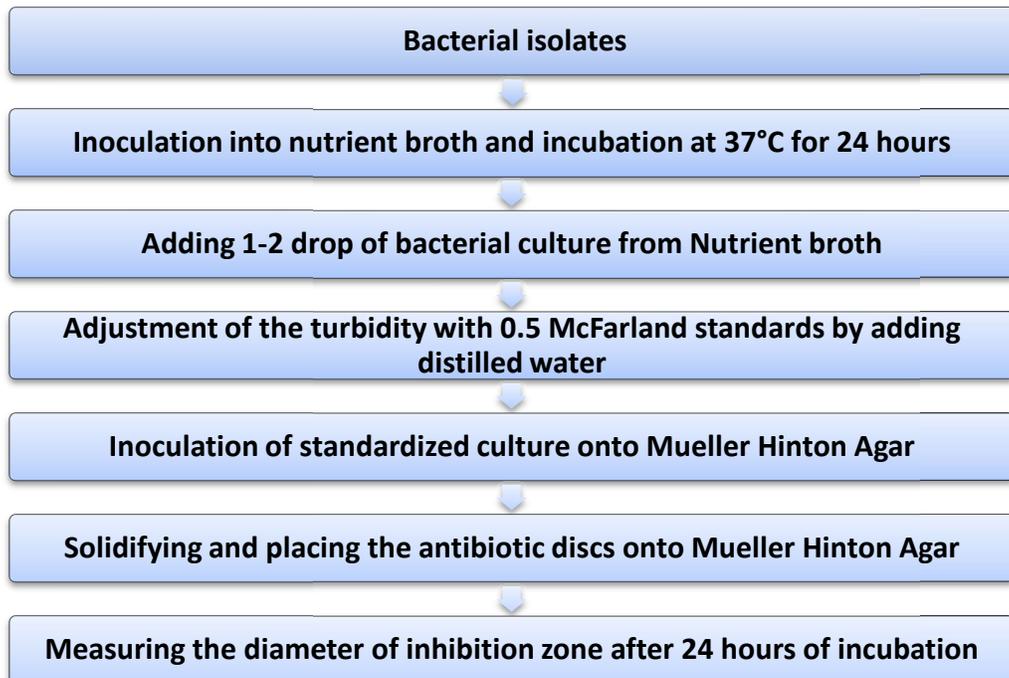


Figure 6: Flow chart of antibiotic sensitivity test by disc diffusion method

Table 3. Antimicrobial discs for antibiotic sensitivity test (CLSI guidelines, 2014)

Name of Antibiotic (Concentration per disc)	Symbol	Resistant*	Intermediate*	Sensitive*
Erythromycin (15µg)	E	≤13	14-22	≥23
Colistin (10µg)	CL	≤10	0	≥11
Gentamycin (10µg)	GEN	≤12	13-14	≥15
Streptomycin (10µg)	S	≤11	12-14	≥15
Ciprofloxacin (5µg)	CIP	≤20	21-30	≥31
Levofloxacin (5µg)	LE	≤13	14-16	≥17
Ampicillin (25µg)	AMP	≤13	14-16	≥16
Amoxicillin (30µg)	AMX	≤13	14-15	≥16
Chloramphenicol (30µg)	C	≤12	13-17	≥18
Salpha/Trimethoprim (23.75/1.25 µg)	COT	≤10	11–15	≥16
Tetracycline (30µg)	TE	≤11	12-14	≥15

* Diameter of zone of inhibition in millimeter

Statistical Analysis

A two way analysis of variance (ANOVA) without replication was used to determine significant difference between different bacterial organisms and among the three divisions using Microsoft Office Excel 2010. A p-value of < 0.05 was considered statistically significant.

11. Results and discussion

11.1 Results

11.1.1. Identification of environmental and managerial risk factors associated with calf mortality in buffalos

Analysis of data in relation to farm management

A total of 600 farmers from three sites (200 from each of Sylhet, Khulna and Barisal) were selected for data collection using the structured questionnaire. Around 43.2% ($p=0.65$) of the farmers responded to have livestock other than buffaloes. Out of the farms surveyed around half (51.33%) of them had medium size herd (10-30 animals) ($p=0.906$). The disease frequency was found significantly high ($p=0.003$) in summer season (51.89%) followed by winter (30.10%) and rainy season (18.01%). Most of the farmers (59.5%) disposed off the dead calves in the river. All of the farmers (100%) fed the calves with colostrum after birth. Among the farmers who introduced new animals to the herd, only 10.86% maintained quarantine and 67.5% farmers responded to vaccinate their animals. Of the parameters studied, there was no significant difference between three divisions. Summary of data analysis in relation to farm

management as responded by the owners of buffalo farms in the selected areas of Bangladesh to identify the risk factors for buffalo calf sickness and /or mortality is shown in Table 4.

Table 4. Analysis of data in relation to buffalo farm management

Parameters	Response of farmers	Study areas			Total (n=600)	P value
		Sylhet (n=200)	Barisal (n=200)	Khulna (n=200)		
Presence of other livestock	Yes	138 (69%)	51 (25.5%)	70 (35%)	43.17%	0.65 (NS) [#] 1 (NS) ^{##}
	No	62 (31%)	149 (74.5%)	130 (65%)	56.83%	
Herd Size	Small (3-10)	175 (87.5%)	50 (25%)	51 (25.5%)	276 (46%)	0.906 (NS) [#] 0.99 (NS) ^{##}
	Medium (10-30)	25 (12.5%)	139(69.5%)	144 (72%)	308 (51.33%)	
	Large (30-100)	0	11 (5.5%)	5 (2.5%)	16 (2.67%)	
Colostrum Feeding	Yes	100%	100%	100%	100%	
	No	0	0	0	0	
Quarantine of new animals	Yes	5 (13.16%)	2 (5.88%)	5 (12.82%)	12 (10.81%)	0.003 (**) [#] 0.99 (NS) ^{##}
	No	33 (86.84%)	32 (94.12%)	34 (87.18%)	99 (89.19%)	
Disposal of dead calves	Buried	31 (15.5%)	35 (17.5%)	37 (18.5%)	103 (17.17%)	0.0001 (***) [#] 1 (NS) ^{##}
	Water Disposal	119 (59.5%)	129 (64.5%)	122 (61%)	370 (61.67%)	
	Field Disposal	50 (25%)	36 (18%)	41 (20.5%)	127 (21.16%)	
Frequency of disease according to season	Summer	110 (47.62%)	109 (49.55%)	124 (59.05%)	343 (51.89%)	0.003(*) [#] 1 (NS) ^{##}
	Winter	75 (32.47%)	71 (32.27%)	53(25.24%)	199 (30.10%)	
	Rainy	46 (19.91%)	40 (18.18%)	33 (15.71%)	119 (18.01%)	
Vaccination	Yes	145 (72.5%)	124 (62%)	136 (68%)	405 (67.5%)	0.02 (*) [#] 1 (NS) ^{##}
	No	55 (27.5%)	76 (38%)	64 (32%)	195 (32.5)	

p<0.05 is significant, #variance between different traits on the rows, ##variance among different division, NS = not significant

Risk factor analysis in relation to buffalo calf mortality

Analysis of risk factors in relation to buffalo calf mortality is shown in Table 5. The overall mortality rate was 14% among which the mortality rate of female calves was higher (8.1%) than male calves (5.9%) ($p=0.054$). Summer born calves died significantly ($p= <0.001$) more in number (7.1%) than the calves born in winter (3.9%) and rainy (3.0%) season. Significantly ($p = <0.001$) more animal died in medium herd size (11.5%) farms than small (1.9%) and large (0.6%) herd size farms. Mortality was found to be high (11.3%) among the birth weight group of 21-25 kg ($p=0.696$). River type buffalo calves died significantly more in number (11.5%) than swamp type (2.5%) buffalo calves ($p =0.001$). After introduction of new animals significantly ($p= <0.001$) more animals (4.8%) died in the farms which didn't maintain quarantine.

Table 5. Association of various risk factors with buffalo calf mortality

Parameters		Frequency of mortality				x ²	df	p value
		Sylhet (n=494)	Barisal (n=660)	Khulna (n=730)	Overall mortality (14%) n=1884			
Sex	Male (n=899)	17 (3.4%)	49 (7.4%)	40 (5.5%)	111(5.9%)	3.723	1	0.054 (NS)
	Female (n=985)	30 (6.1%)	60 (9.1%)	67 (9.2%)	152 (8.1%)			
Birth Session	Summer	28 (5.7%)	61(9.2%)	52(7.1%)	133 (7.1%)	32.21	2	<0.001**
	Winter	14 (2.8%)	25(3.8%)	34 (4.7%)	73 (3.9%)			
	Rainy	5 (1.1%)	23 (3.5%)	21(2.9%)	57 (3.0%)			
Herd size	Small (1-10 animals)	15 (3.1%)	9 (1.4%)	11(1.5%)	35 (1.9%)	33.86	2	<0.001**
	Medium (11-30 animals)	32 (6.5%)	92(13.9%)	93(12.7%)	217 (11.5%)			
	Large (30-50 animals)	0	8(1.2%)	3(0.4%)	11 (0.6%)			
Birth weight	16-20 kg	19(3.8%)	25(3.8%)	16(2.2%)	60 (5.0%)	1.442	3	0.696 (NS)
	21-25 kg	22(4.5%)	58(8.8%)	55(7.5%)	135 (11.3%)			
	26-30 kg	5(1.1%)	20(3%)	16(2.2%)	41 (3.4%)			
	31-35 kg	1(0.2%)	6(0.9%)	20(2.7%)	27 (2.3%)			
Vaccination	Yes	13(2.63%)	42(6.4%)	37(5.1%)	92 (4.9%)	0.452	1	0.501 (NS)
	No	34(6.9%)	67(10.1%)	70(9.6%)	171 (9.1%)			
Breed	Swamp	47(9.51%)	0	0	47 (2.5%)	11.01	1	0.001**
	River	0	109(16.51%)	107(14.65%)	216 (11.5%)			
	Number of animals	202	344	342	888			
	Number of animals	202	344	342	888	31.80	1	<0.001**
	Yes	1 (0.5%)	3 (0.9%)	1 (0.2%)	5 (0.6%)			
	No	8 (3.9%)	19 (5.5%)	16 (4.7%)	43 (4.8%)			

p<0.05 is significant, #variance between different traits on the rows, ##variance among different divisions, NS = not significant

11.1.2. Isolation and identification of causative organisms associated with buffalo calf mortality

Isolation and Identification of Bacterial agents

Isolation and identification of different bacterial organisms were done by cultural and biochemical properties and morphological characteristics (staining characteristics) of bacteria which are shown in figures 7, 8, 9, 10 and 11.

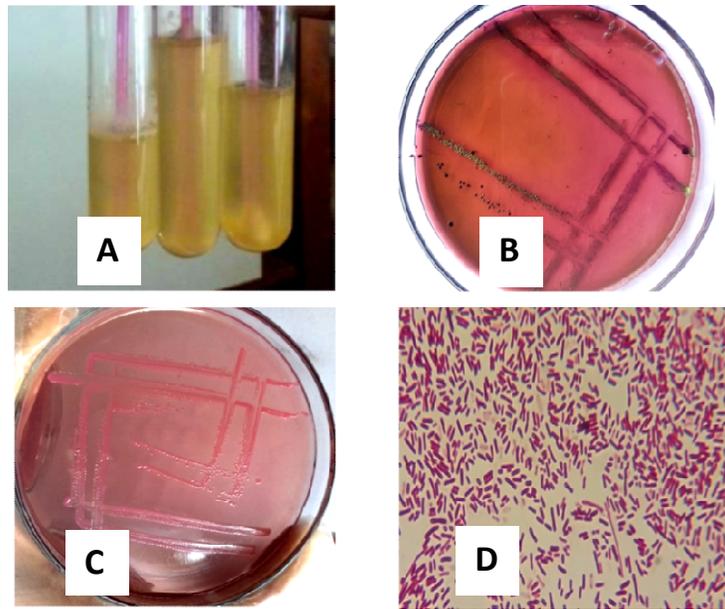


Figure 7: Results of different cultural tests and gram staining for isolation of *E. coli*
 A) Turbidity on Nutrient agar, B) Metallic sheen on EMB agar, C) Pink color colony on McConkey agar, D) Gram negative, pink color, rod shape *E. coli* in Gram's staining under microscope.

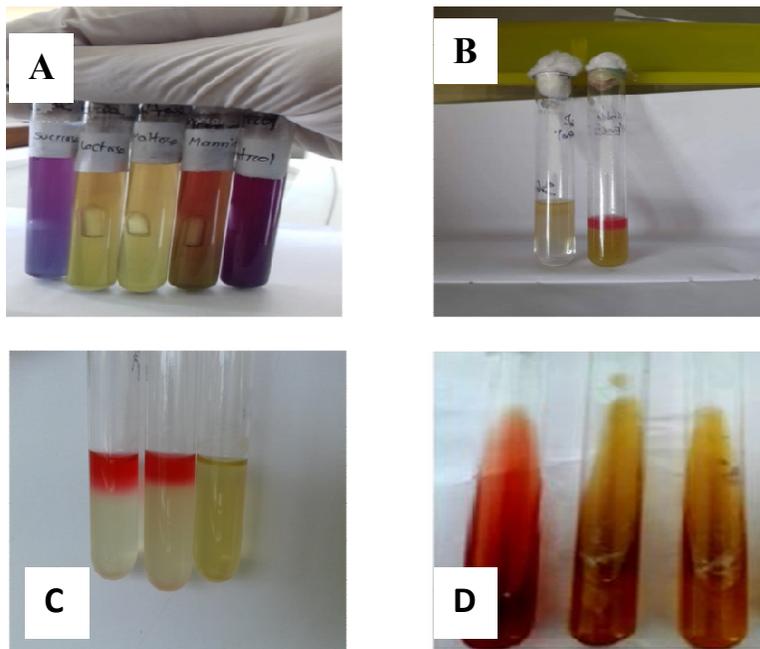


Figure 8: Results of different biochemical tests for isolation of *E. coli*
 A) Acid and Gas production in Sugar fermentation test; B) In Indole test *E. coli* produced cherry red color ring; C) Cherry color ring produced in MR test; D) In TSI agar slant *E. coli* produced yellow color butt and slant

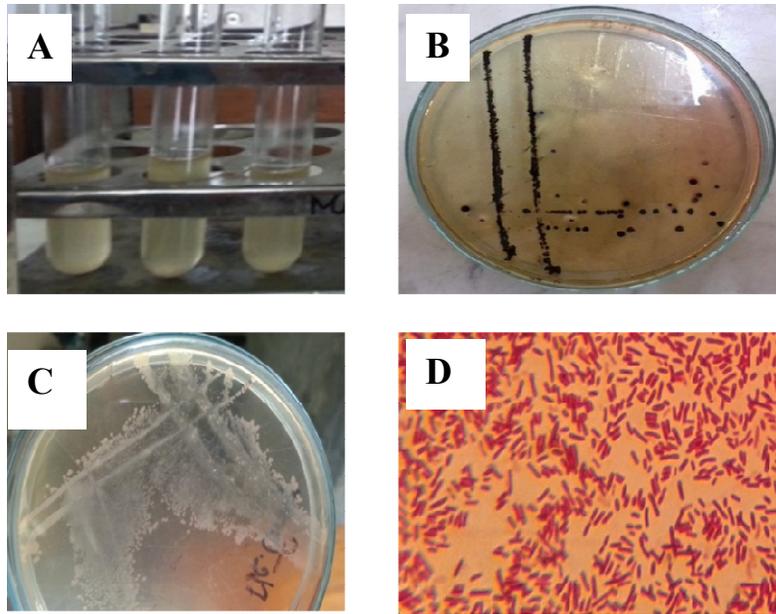


Figure 9: Results of different cultural tests and gram staining for isolation of *Salmonella*
 A) On nutrient broth *Salmonella* produced turbidity; B) Black centered colony on Salmonella-Shigella (SS) agar; C) Colorless colonies on MacConkey agar; D) In Gram's staining under microscope revealed gram-negative, small rod shaped and arranged in single or paired or in clusters characteristics.

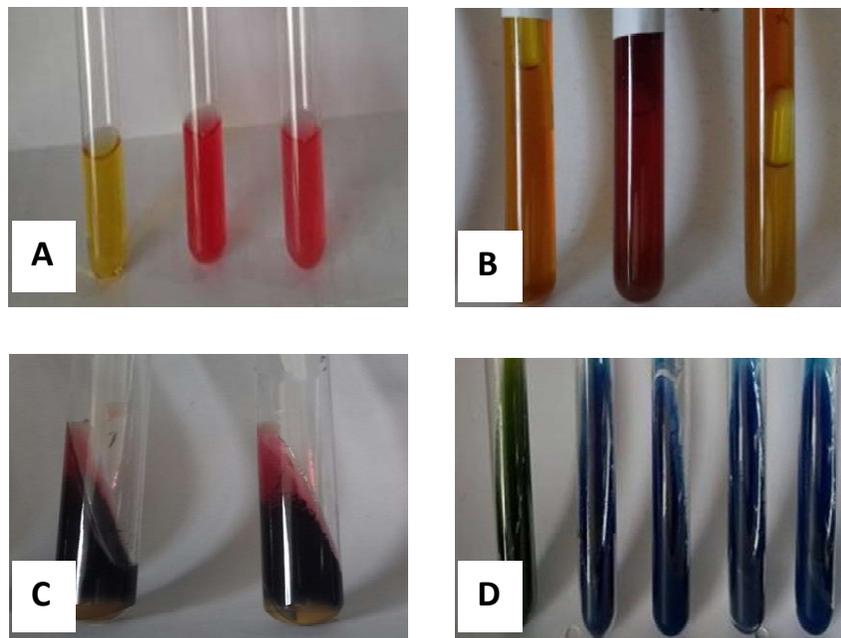


Figure 10: Results of different biochemical tests for isolation of *Salmonella*
 A) MR Test (positive, red color); B) Sugar fermentation (Glucose and Mannitol +ve with the production of acid and gas, Sucrose -ve); C) TSI test (positive, Slant : red, butt: black); D) Bluish color of slant in citrate utilization test;

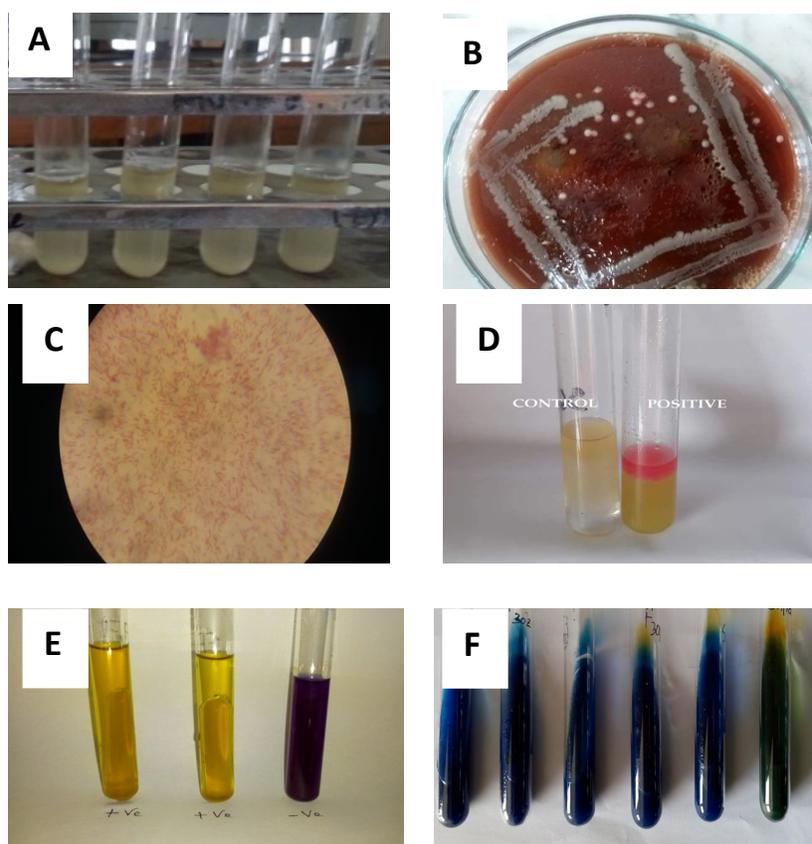


Figure 11: Results of different cultural, morphological and biochemical tests for *Pasteurella*
 A) On Nutrient broth *Pasteurella* produced turbidity; B) Dewdrop like colonies on sheep blood agar (SS) agar; C) In Gram's staining under microscope revealed coccobacillary arranged singly or in pair; D) In Indole test *Pasteurella* produced cherry red color ring E) Sugar fermentation (positive result); F) Bluish color of slant in citrate utilization test.

Division wise prevalence of bacteria involved in buffalo calf sickness due to pneumonia

In the present study it has been found that among the pneumonic buffalo calves (n=120), the prevalence of *Pasterulla* at Sylhet, Barishal and Khulna divisions was 32.5%, 32.5% and 27.5% respectively ($p>0.05$). The prevalence of *Salmonella* was 7.5%, 12.5% and 15% and in case of *E. coli* the prevalence was 15%, 15% and 10% respectively at the three divisions ($p>0.05$) (Table 6). There was no significant difference among the districts. On the other hand there was significantly higher percentage of *Pasteurella* in pneumonic buffalo calves than *E. coli* and *Salmonella* ($p<0.05$).

Table 6. Identification of bacterial causes of buffalo calf sickness associated with pneumonia

Sample type	Division	Districts	Number of samples	<i>Pasteurella</i> n (%)	<i>Salmonella</i> n (%)	<i>E. coli</i> n (%)	p value
Nasal swabs from pneumonic calves	Sylhet	Sylhet	20	8 (40%)	2 (10%)	4 (20%)	0.03 (*) [#] 0.07 (NS) ^{##}
		Moulavibazar	20	5 (25%)	1(5%)	2(10)	
		Sub-total	40	13 (32.5%)	3 (7.5%)	6 (15%)	
	Barisal	Bhola	20	6 (30%)	1 (5%)	4 (20%)	0.02 (*) [#] 0.69 (NS) ^{##} 0.025 (*) [#] 0.422 (NS) ^{##}
		Patuakhali	20	7 (35%)	4 (20%)	2 (10%)	
		Sub-total	40	13 (32.5%)	5 (12.5%)	6 (15%)	
	Khulna	Bagerhat	20	6 (30%)	3 (15%)	2 (10%)	
		Jhenaidah	20	5 (25%)	3 (15%)	2 (10%)	
		Sub-total	40	11 (27.5%)	6 (15%)	4 (10%)	
Total			120	37 (30.83%)	14 (11.67%)	16 (13.33%)	

p<0.05 is significant, #variance among different bacteria, ## variance among different districts, NS = not significant

Prevalence of bacteria involved in buffalo calf sickness due to pneumonia

Considering all divisions under study (n=120), the presence of *Pasteurella* in pneumonic buffalo calves was found to be 30.83% which was significantly (*p*=0.005) higher than that of *Salmonella* (11.67%) and *E. coli* (13.33%) (Figure 12).

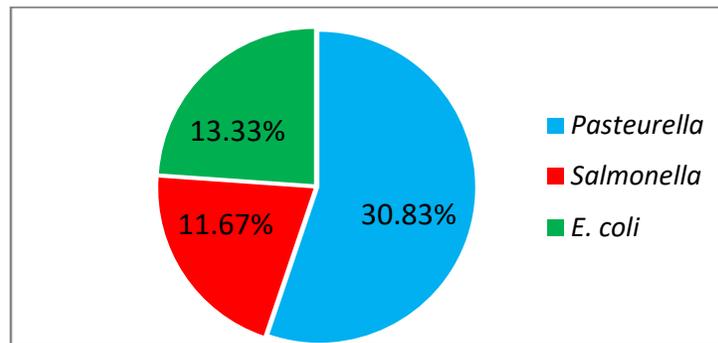


Figure 12: Prevalence of bacteria involved in buffalo calf sickness due to pneumonia

Division wise prevalence of bacteria involved in buffalo calf sickness due to diarrhea

In the present study it was found that among the buffalo calves suffering from diarrhea (n=240), the prevalence of *E. coli* at Sylhet, Barishal and Khulna divisions was 43.75% 23.75% and 32.5% respectively (*p*>0.05). The prevalence of *Salmonella* was 12.5%, 8.75% and 13.75 (*p*>0.05) respectively. The difference was not found to be significant among the districts studied (Table 7).

Table 7. Identification of bacterial causes of buffalo calf sickness associated with diarrhea

Sample type	Division	Districts	Number of samples	<i>E. coli</i> n (%)	<i>Salmonella</i> n (%)	p value
Feces/Rectal Swabs from diarrheic calves	Sylhet	Sylhet	40	20 (50%)	6 (15%)	0.03 (*) [#]
		Moulavi bazar	40	15 (37.5%)	4 (10%)	0.25 (NS) ^{##}
		Sub-total	80	35 (43.75%)	10 (12.5%)	0.04 (*) [#]
	Barisal	Bhola	40	10 (25%)	3 (7.5%)	0.69 (NS) ^{##}
		Patuakhali	40	9 (22.5%)	4 (10%)	0.02 (*) [#] 0.5
		Sub-total	80	19 (23.75%)	7 (8.75%)	(NS) ^{z#}
	Khulna	Bagerhat	40	13 (32.5%)	6 (15%)	
		Jhenaidah	40	13 (32.5%)	5 (12.5%)	
		Sub-total	80	26 (32.5%)	11 (13.75%)	
Total			240	80 (33.33%)	28 (11.67%)	

p<0.05 is significant, #variance between different bacteria organism, NS = not significant

Prevalence of bacteria involved in buffalo calf sickness due to diarrhea

Considering all the divisions under study (n=240) the percentage of *E. coli* in diarrheic buffalo calves was found to be 33.33% which was significantly (*p*=0.001) higher than the percentage of *Salmonella* (11.67%) (Figure 13).

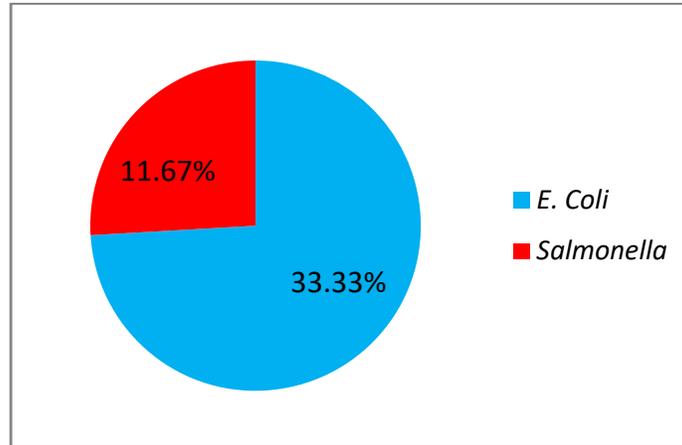


Figure 13: Prevalence of bacteria involved in buffalo calf sickness due to diarrhea

Identification of parasitic causes (GI parasites) of buffalo calf sickness

In the present study, it has been found that among the diarrheic buffalo calves (n=240), the prevalence of *Neoscaris vitulorum* at Sylhet, Barishal and Khulna divisions was 21.25%, 45% and 33.75% respectively (*p*>0.05). The prevalence of *Trichostrongylus* was 5%, 6.5% and 2.5% and in case of *Trichuris* the prevalence was 5%, 1.25% and 1.25% respectively at the three divisions (*p*>0.05) (Table 8). There was no significant difference among the districts.

Table 8. Division wise prevalence of parasites causing diarrhea in buffalo calves

Sample type	Divisions	Districts	Number of samples	<i>Neoscaris vitulorum</i>	<i>Trichostrongylus</i>	<i>Trichuris</i>	P value
Feces/ Rectal Swabs from diarrheic calves	Sylhet	Sylhet	40	5 (12.5%)	1 (2.5%)	3 (7.5%)	0.02 (*) [#] 0.46 ^{##}
		Moulavi bazar	40	12 (30%)	3 (7.5%)	1 (2.5%)	
		Sub-total	80	17 (21.25%)	4 (5%)	4 (5%)	
	Barisal	Bhola	40	19 (47.5%)	2 (5%)	0	0.008 (**) [#] 1 ^{##}
		Patuakhali	40	17 (42.5%)	3 (7.5%)	1 (2.5%)	
		Sub-total	80	36 (45%)	5 (6.25%)	1 (1.25%)	
	Khulna	Bagerhat	40	13 (32.5%)	1 (2.5%)	1 (2.5%)	0.004 (**) [#] 1 ^{##}
		Jhenaidah	40	14 (35%)	1 (2.5%)	0	
		Sub-total	80	17 (33.75%)	2 (2.5%)	1 (1.25%)	
Total			240	70 (33.33%)	11 (13.75%)	6 (7.50%)	

p<0.05 is significant, #variance between different traits on the rows, ##variance among different divisions, NS = not significant

Prevalence of parasite involved in buffalo calf sickness due to diarrhea

Considering all divisions under the study (n=240) the percentage of *Neoscaris vitulorum* in diarrheic buffalo calves was found to be 33.33% which was significantly (*p*=0.01) higher than *Trichostrongylus* (13.75%) and *Trichuris* (7.5%) (Figure 14).

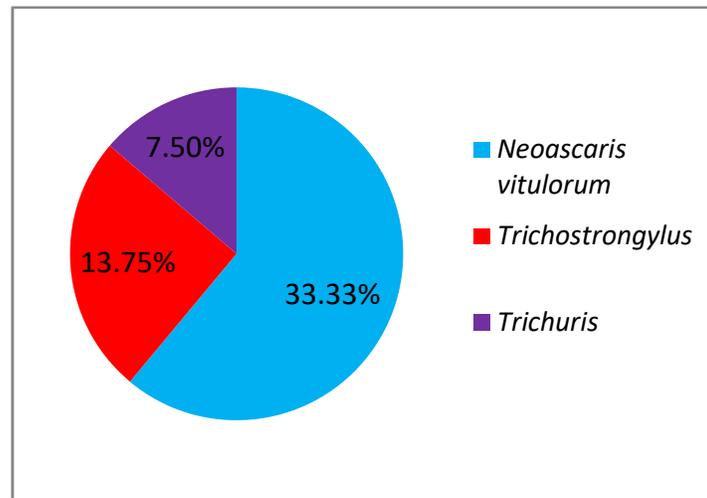


Figure 14: Prevalence of parasite involved in diarrhea in buffalo calf

Identification of protozoal causes (blood protozoa) of buffalo calf sickness

In the present study, Babesia was identified in sylhet (2) and in Barisal (1) but there were no presence of Anaplasma or any other protozoal organisms in any division (Table 9).

Table 9. Prevalence of protozoa in sick buffalo calves

Division	No. of blood sample	Anaplasma	Babesia	others
Sylhet	15	0	2 (13.33%)	0
Barisal	15	0	1 (6.67%)	0
Khulna	15	0	0	0
Total	45	0	3 (6.67%)	0

Identification of parasitic causes of buffalo calf sickness

Parasitic diseases were diagnosed using fecal and blood sample examinations following standard procedures of OIE and shown in Figure 15.

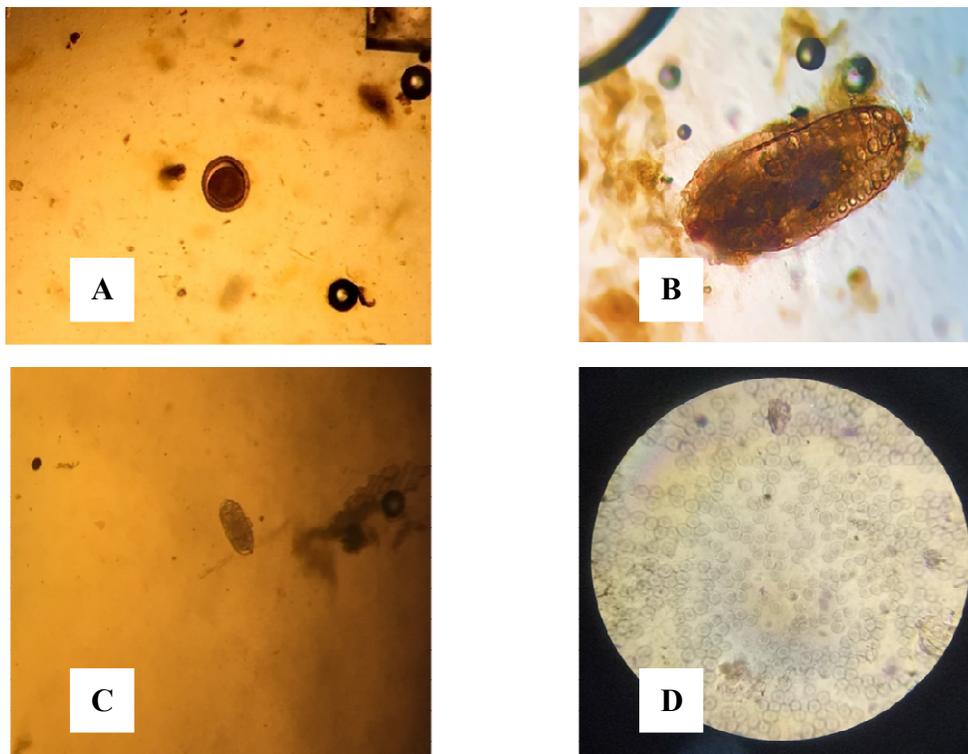


Figure 15: Identification of parasitic causes of buffalo calf sickness

A) Egg of *Neoscaris vitulorum*, B) Egg of *Trichostrongylus* spp, C) Egg of *Trichuris* spp. and D) *Babesia* spp. (blood smear).

Post mortem examination and identification of causal agents of buffalo calf mortality

A total of 10 dead calves were considered for post mortem examination. Dead calves were collected from Sylhet (n=6; 03 each from Sylhet and Moulvibazar districts), Khulna (n=02; 01 each from Bagerhat and Jhenaidah districts) and Barisal (n=02; 01 each from Bhola and Patuakhali districts) division. After post mortem examination and laboratory diagnosis it was found that 20% of the buffalo calves died due to *Pasteurella* infection, 20% died due to *Neoscaris vitulorum* infection, 10% died due to *E coli* infection and for the rest five dead calves the causal agents could not be unidentified (Table 10).

Table 10. Identification of causal agents from dead calves

Cause of death	No of Dead calf	<i>E. Coli</i>	<i>Pasteuralla</i>	<i>Salmonella</i>	<i>Neoscaris vitulorum</i>	unidentified
Pneumonia	05	0	02 (40%)	0	-	03 (60%)
Diarrhea	05	01 (20%)	0	0	2 (40%)	02 (40%)
Total	10	01 (10%)	02 (20%)	0	02 (20%)	05 (50%)

Determination of sero-prevalence of Rota virus and Bovine Viral Diarrhea Virus by ELISA

Serum from blood samples were collected and subjected to ELISA test to determine the sero-prevalence of Rota virus and bovine viral diarrhoea virus (BVD) among the clinically sick diarrhetic calves. In the present study, it was found that among the diarrhetic buffalo calves (n=150), the sero-prevalence of Rota virus at Sylhet, Barishal and Khulna divisions was 24%, 16% and 18% respectively and the sero-prevalence of BVDV was 18%, 22% and 12% respectively (Table 11). Overall, the sero-prevalence of Rotavirus in diarrhetic buffalo calves was found to be 19.33% which was higher than the percentage of BVD (17.33%).

Table 11. Determination of sero-prevalence of Rota virus and Bovine Viral Diarrhea Virus by ELISA

Sample type	Division	Number of samples	Rotavirus n (%)	BVDV n (%)	p value
Blood Samples from diarrhetic calves	Sylhet	50	12 (24%)	9 (18%)	0.6 (NS) [#] 0.7 (NS) ^{##}
	Barisal	50	8 (16%)	11 (22%)	
	Khulna	50	9 (18%)	6 (12%)	
	Total	150	29 (19.33%)	11 (17.33%)	

p<0.05 is significant, #variance between different traits on the rows, ##variance among different divisions, NS = not significant

Detection of Rota virus and Bovine Viral Diarrhea Virus by PCR

Viral genome extraction was done from 90 fecal samples collected from diarrhetic calves and was subjected to PCR for molecular detection of Rota virus and Bovine Viral Diarrhoea, the two common causes of calf diarrhoea in Bangladesh (Figure 16).

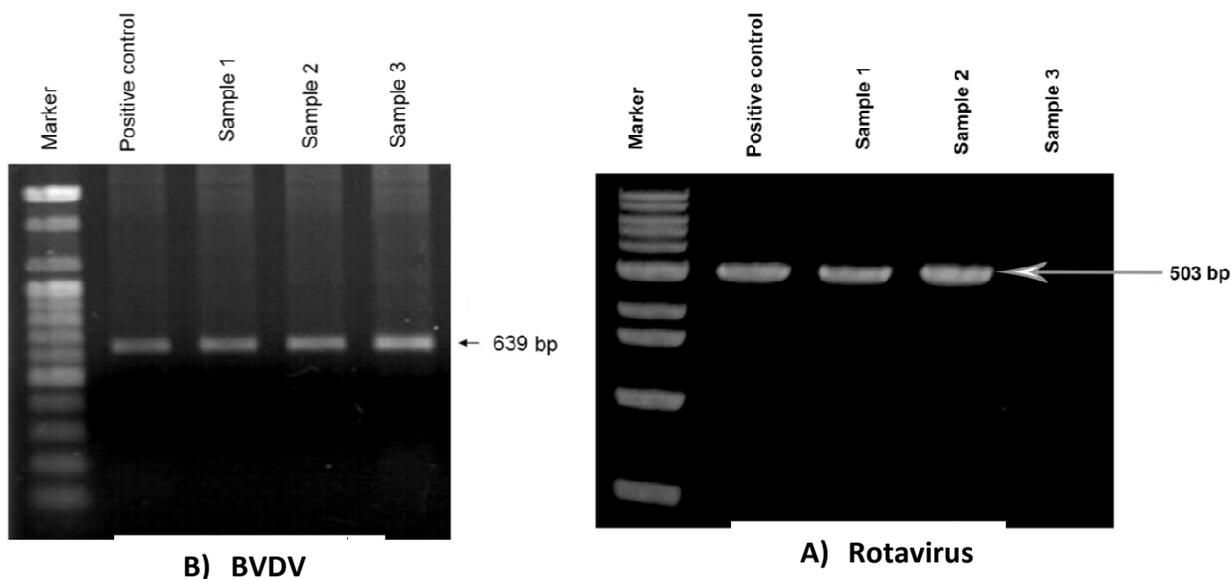


Figure 16: Detection of BVDV and Rotavirus from fecal samples of buffalo calves by PCR

In the present study, it was found that among the diarrheic buffalo calves (n=90), the prevalence of Rotavirus at Sylhet, Barishal and Khulna divisions was 10%, 23.33% and 13.33% respectively. The prevalence of BVDV at Sylhet, Barishal and Khulna divisions was 13.33%, 16.67% and 6.67% respectively (Table 12). Overall, the percentage of Rotavirus in diarrheic buffalo calves was found to be 15.56% which was higher than the percentage of BVD virus (12.22%).

Table 12. Detection of Rota virus and Bovine Viral Diarrhea Virus by PCR

Sample type	Division	Number of samples	Rotavirus n (%)	BVDV n (%)	p value
DNA extracted from Fecal Samples (diarrheic calves)	Sylhet	30	3 (10%)	4 (13.33%)	0.2 (NS) [#] 0.4 (NS) ^{##}
	Barisal	30	7 (23.33%)	5 (16.67%)	
	Khulna	30	4 (13.33%)	2 (6.67%)	
	Total	90	14 (15.56%)	11 (12.22%)	

p<0.05 is significant, [#]variance between different traits on the rows, ^{##}variance among different divisions, NS = not significant

11.1.3. Development of appropriate therapeutic measures and control strategies against buffalo calf diseases

Preparation of guidelines as control strategies against buffalo calf diseases

As guidelines to control buffalo calf diseases following posters and leaflets were prepared (Figure 17, 18, 19) and distributed among the farmers and local livestock offices of the study areas.



Figure 17: Poster on Disease Calendar



Figure 18: Leaflet on symptoms of common buffalo calf diseases

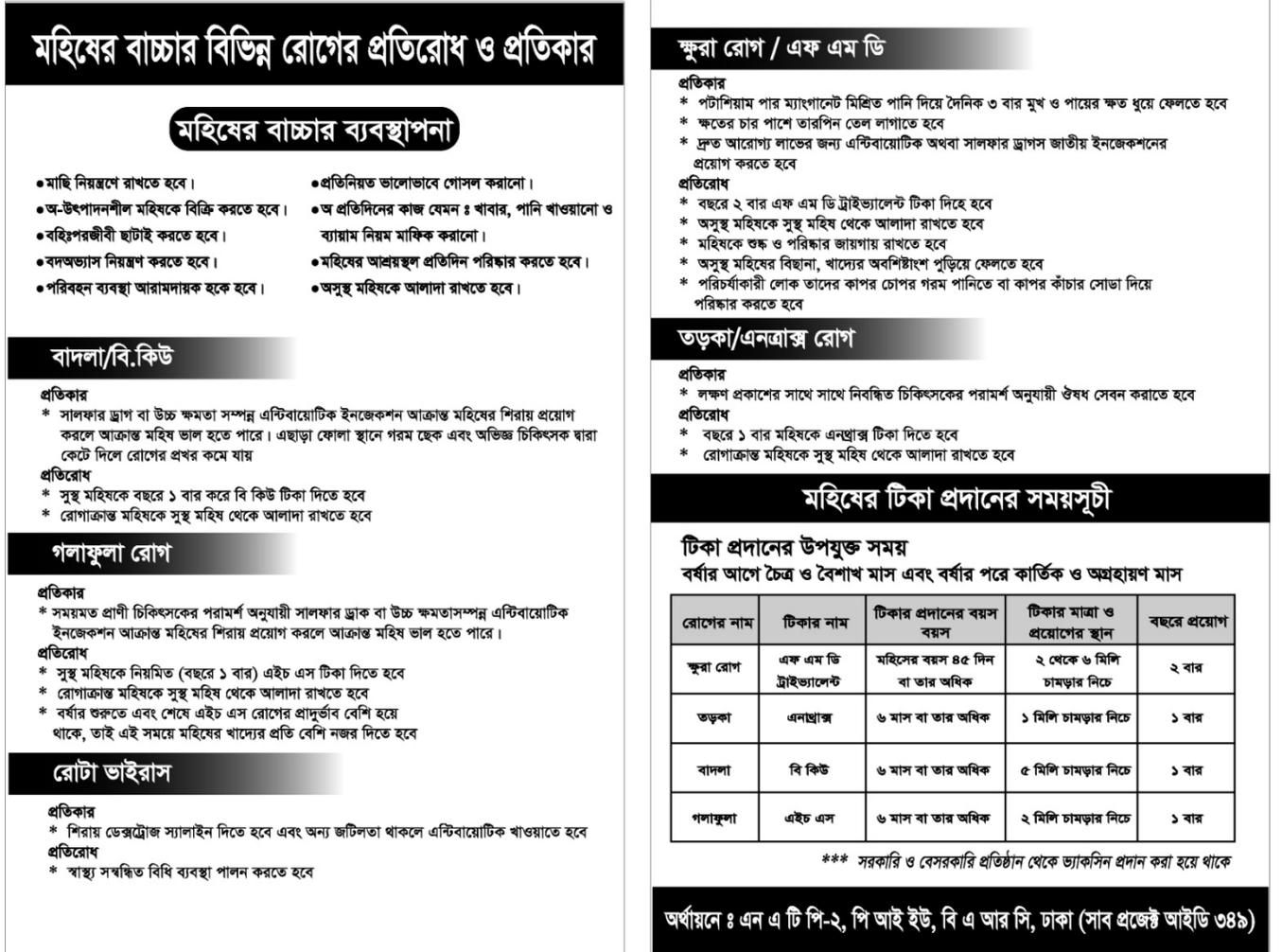


Figure 19: Leaflet on management and preventive measures of various diseases of buffalo calves

Selection of effective antibiotics for treatment of sick buffalo calves using antibiotic sensitivity test to provide guidelines on effective treatment to mitigate buffalo calf mortality

Sensitivity tests were done in case of identified bacteria (*E coli*, *Salmonella sp* and *Pasteurella sp*) to identify the susceptibility pattern against specific antibiotic from which efficient treatments can be provided in case of these organisms.

Susceptibility and resistance patterns of *E. coli* isolates to selected antibiotics

Isolated *E. coli* were examined against 11 most commonly used commercial antibiotics (Figure 20). Antibigram studies were performed by disc diffusion method and the results were determined by measuring diameter of inhibition zone (Figure 21). The highest resistance was found against amoxicillin (92.41%) whereas Colistin demonstrated 0% resistance against isolated *E. coli* bacteria.

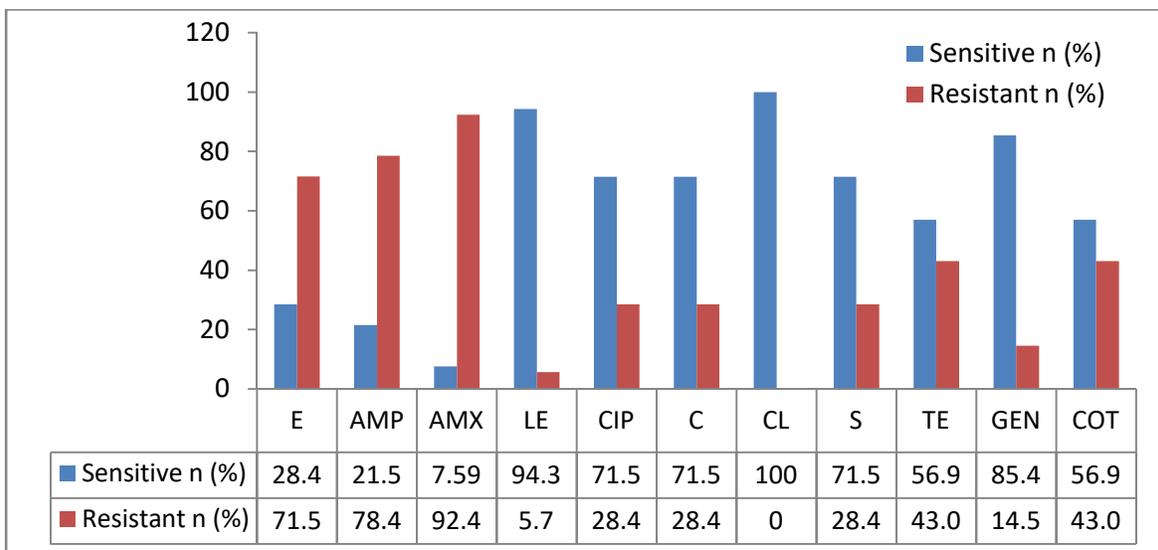


Figure 20: Susceptibility and resistance patterns of *E. coli* isolates to selected antibiotics

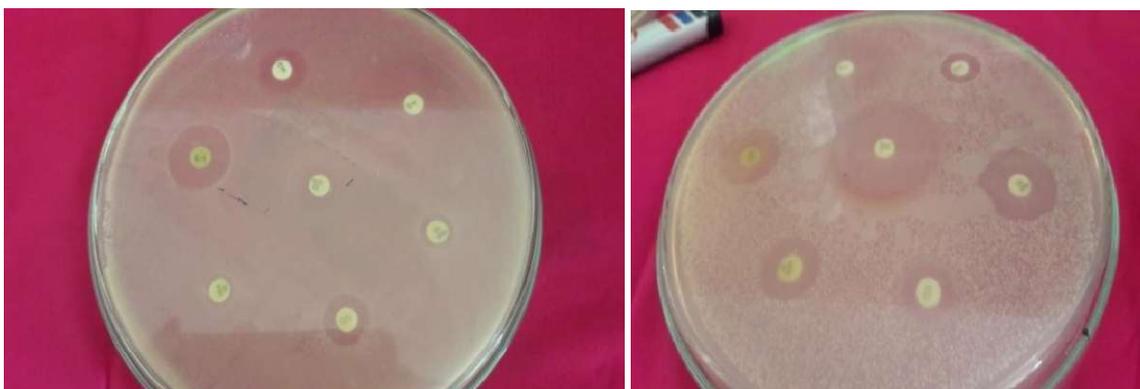


Figure 21: Antimicrobial susceptibility test for *E. coli* by disc diffusion method

11.1.15.2. Susceptibility and resistance patterns of *Salmonella* isolates to selected antibiotics

Isolated *Salmonella* were examined against 11 most commonly used commercial antibiotics (Figure 22). Antibiogram studies were performed by disc diffusion method and the results were determined by measuring diameter of inhibition zone (Figure 23). The highest resistance was found against amoxicillin as 88.46% (resistant 34.61%, intermediate resistant 53.85%) where *Salmonella* showed 100% sensitivity to gentamycin, levofloxacin and colistin. Again, isolated *Salmonella* demonstrated only intermediate range of resistance to ciprofloxacin, erythromycin, salpha/trimethoprim, chloramphenicol and streptomycin as 44.23%, 34.62%, 7.69%, 15.38% and 3.85% respectively. On the other hand, susceptibility to tetracycline and ampicillin was found to be 82.69% and 63.46% respectively.

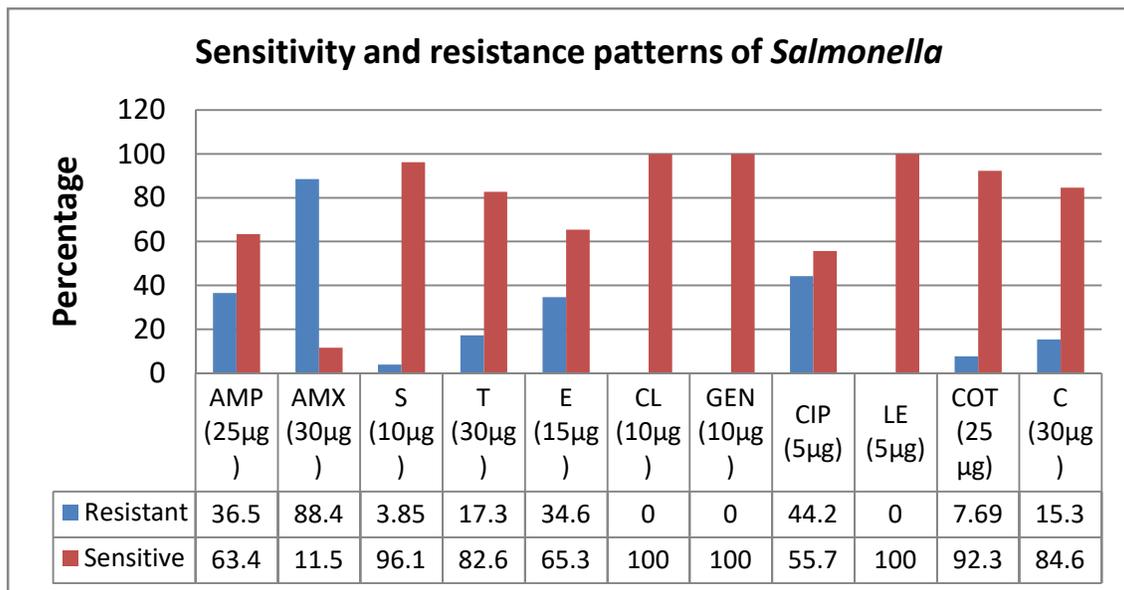


Figure 22: Susceptibility and resistance patterns of *Salmonella* isolates to selected antibiotics

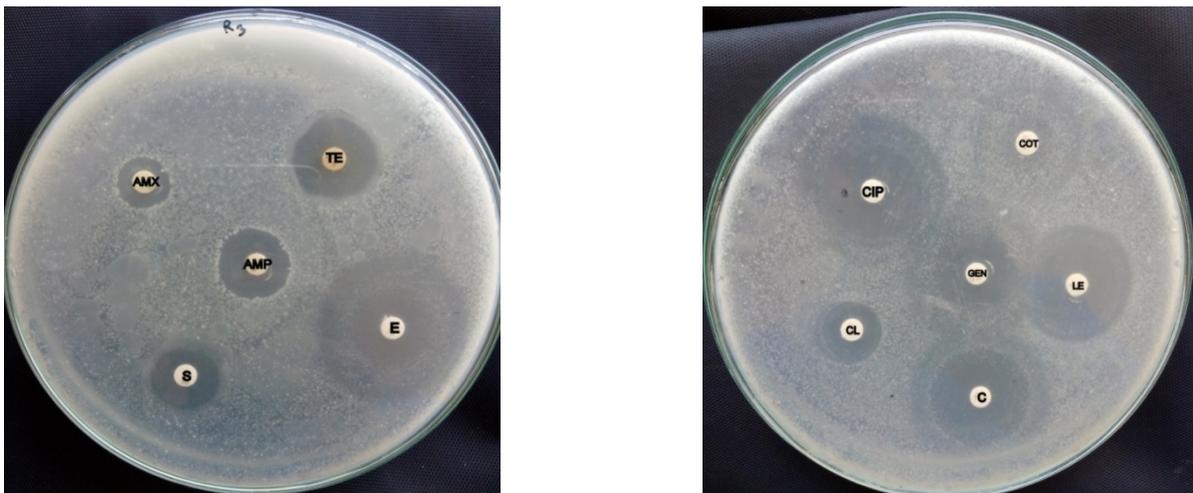


Figure 23: Antimicrobial susceptibility test for *Salmonella* by disc diffusion method

Susceptibility and resistance patterns of *Pasteurella* isolates to selected antibiotics

The result of antimicrobial susceptibility test of 23 *Pasteurella* isolates against 11 commercially available antimicrobial agents is summarized in Table 13. The antibiogram result of *Pasteurella* isolated from nasal swab revealed a varying degree of susceptibility and resistance to the different antimicrobial agents tested (Figure 24). Antibiotic studies were performed by disc diffusion method and it was determined through measuring diameter of zone of inhibition. The degree of susceptibility ranged from 0 % up to 100 %. Of the isolates, 100% was resistant to amoxicillin followed by ampicillin (91.3%) and chloramphenicol (91.29%). The resistance of colistin was 0 %. The susceptibility was 100 % in colistin, followed by gentamycin (91.30%) and levofloxacin (86.95%) as shown in Table 13.

Table 13: Susceptibility and resistance patterns of *Pasteurella* isolates for selected antibiotics

Antibiotics	Total no of isolates tested	Resistant n (%)	Intermediate resistant n (%)	Sensitive n (%)
Erythromycin (15µg)	23	2 (8.69)	3 (13.04)	18 (78.26)
Colistin (10µg)	23	0 (0)	0 (0)	23 (100)
Gentamycin (10µg)	23	0 (0)	2 (8.69)	21 (91.30)
Streptomycin (10µg)	23	1 (4.34)	2 (8.69)	20 (86.95)
Ciprofloxacin (5µg)	23	2 (8.69)	9 (39.13)	12 (52.17)
Levofloxacin (5µg)	23	0 (0)	3 (13.04)	20 (86.95)
Ampicillin (25µg)	23	18 (78.26)	3 (13.04)	2 (8.69)
Amoxicillin (30µg)	23	22 (95.65)	1 (4.34)	0 (0)
Chloramphenicol (30µg)	23	5 (21.73)	16 (69.56)	2 (8.69)
Salpha/Trimethoprim (23.75/1.25µg)	23	4 (17.39)	6 (26.08)	13 (56.52)
Tetracycline (30µg)	23	0 (0)	3 (13.04)	20 (86.95)

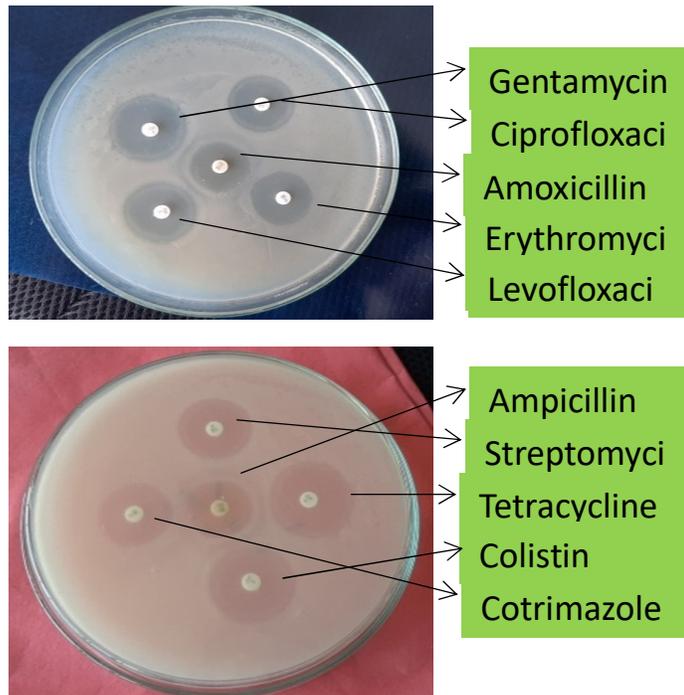


Figure 24: Antimicrobial sensitivity test for *Pasteurella* by disc diffusion method

11.2. Discussion

Calves play an important role in the development of the dairy sector in Bangladesh, as the future of the dairy herd solely depends upon the successful raising of young calves. Survival of neonatal calves is imperative for livestock propagation. Buffalo calf mortality is a great concern during last few decades. A large number of calves die during the first year of their life causing heavy drain on the economics of livestock production. Calf mortality has been reported to be very high in cow and buffalo neonates (Khan *et al.*, 1991). Martin and Wiggin (1973) estimated that 20% calf mortality resulted in reduction of 38% profit of a livestock farm. The average calf mortality rate in cattle was 29.1% while in buffaloes; it was 38.85% in Jabalpur, Madhya Pradesh, India (Jain *et al.*, 2008). Mortality of neonatal calves was attributed to conditions like diarrhea and pneumonia. However, environmental and managerial factors hasten the occurrence of such conditions.

In the present study swab (n=120) and fecal (n=240) samples were taken from both the clinically sick calves (suffering from diarrhea or pneumonia) and dead calves to identify the bacterial causes of buffalo calf mortality.

From the present study it has been seen that the percentage of *Pasteurella* isolated from nasal swab samples of pneumonic buffalo calves was higher in Sylhet (32.5%) and Barisal divisions (32.5%) followed by Khulna division (27.5%) which was higher than the findings (20.7%) reported by Noura *et al.* (2016). In case of *E. coli* the prevalence was found higher in Sylhet and Barisal division which was 15% followed by Khulna division which was 10%. More or less similar prevalence (3-51%) was reported by Reynolds *et al.*, 1986; Simeonov *et al.*, 1981; Snodgrass *et al.*, 1986). In this present study the percentage of *Salmonella* isolated from nasal swab samples was more in Khulna division (15%) followed by Barisal (12.5%) and Sylhet division (7.50%). The average percentage of *Salmonella* in the three divisions was 11.67% which was slightly lower than the percentage (13.64%) reported by Khan *et al.* (2009). Observed variation in prevalence among studies could be attributed to difference in sampling and isolation procedures, variability in sampled populations, diverse geographical origins of animals, numbers of animals, study design, season, sanitation and treatment during the process.

From the present study it has been seen that the percentage of *E. coli* isolated from fecal samples of diarrheic buffalo calves was higher in Sylhet division (43.75%) followed by Khulna division (32.75%) and lowest percentage was in Barisal division (23.75%). The average percentage of *E. coli* in the three divisions was 33.33% which agreed with the similar findings reported by Anwarullah *et al.* (2014) which was 33.3%. Another study in Pakistan reported that the percentage of *E. coli* in diarrheic buffalo calves was 15.3% (Khan *et al.*, 2009) which was lower than our study findings. In this present study the percentage of *Salmonella* isolated from fecal samples was more in Khulna division (13.75%) followed by Sylhet division (12.5%) and lowest percentage was in Barisal division (8.50%). The average percentage of *Salmonella* in the three divisions was 11.67% which was lower than the findings (16.3%) reported by Khan *et al.* (2009). Another study in Pakistan also reported higher percentage (18.6%) of *Salmonella* in diarrheic buffalo calves than our study findings (Anwarullah *et al.*, 2014). Observed variation in prevalence among studies could be attributed to difference in sampling and isolation procedures,

variability in sampled populations, diverse geographical origins of animals, numbers of animals, study design, season, sanitation and treatment during the process.

A total of 10 buffalo calves were found dead during the study. After post mortem examination and laboratory diagnosis it has been found that 20% of the buffalo calves died due to *Pasteurella* infection; 10% died due to *E coli* infection and rest were unidentified. Data were collected from 600 farmers from three selected divisions (200 from each of Sylhet, Khulna and Barisal) using the structured questionnaire. There was presence of other livestock in 41.5% farms ($p=0.65$). Around half (51.33%) of the selected farmers had medium size herd (10-30) ($p=0.906$). Comparatively more calves (54.75%) were born in summer season ($p=0.03$). The disease frequency was also found significantly higher in summer season (51.89%) ($p=0.003$). Most of the farmers disposed calves after death in the river (59.5%). All of the farmers fed the calves colostrum after birth. Among the famers who introduced new animals to the herd, only 10.86% farmers maintained quarantine. The mortality in buffalo calves was 14% which is slightly higher than the findings (13%) reported by Sharma et al. (1984) in India. Another study also reported lower percentage of calf mortality (9.4%) in India (Zaman et al., 2006). Mortality was seen significantly more in female calves (8.1%) than the male calves (5.9%) ($p=0.014$). Sharma et al., 1984 also reported higher mortality in female than male. Another study in Haryana, India reported that mortality was higher in male (25.0%) than in female (13.9%) neonatal calves (Kaushik et al., 1980). Significantly more calves died in summer season (7.1%) than the winter and rainy season ($p=0.004$) which was lower than the findings (11.9%) reported by Zaman et al. (2006) in Pakistan. Another study in India by Sharma et al. (1984) reported higher mortality rates in winter (15.36%) than in summer (5.97%). Around 81.5% farmers did not disposed off dead calf properly which has a significant effect on buffalo calf mortality ($p=0.0001$). Mansour et al. (2014) reported that 75% farmers didn't dispose dead calves off properly which might have been a risk factors for buffalo calf mortality. More calves died when their birth weight was within 21-25kg (11.3%) ($p=0.696$). Calf of river type buffalo died significantly more (11.5%) than swamp type buffalo ($p=0.001$). Calf died significantly more in herds (4.8%) which didn't maintained quarantine after introduction of new animals to herd than which maintained quarantine ($p=<0.001$). Calf mortality was significantly higher in medium size herd (11.5%) than small and large size herd ($p=<0.001$), but from Gujarat, India Patbandha et al. (2017) reported that incidence of calf mortality was significantly higher in smaller herds (25.0%), followed by medium (15.09%) and lower incidence was in the herd with larger size (6.51%). Observed variation in prevalence among studies could be attributed to difference in sampling procedures, variability in sampled populations, diverse geographical origins of animals, numbers of animals, study design, season, sanitation and treatment during the process.

Antibiotic resistance has become a great concern of the current world. Day by day the antimicrobial resistance has been increasing. To provide efficient treatment strategy and to reduce AMR, sensitivity test were performed. In the present study, in case of *E coli* it was found that amoxicillin was highly resistant (85.44% was resistant and 6.96% was intermediate resistant) which is higher than the study of Abd-Elrahman (2011) who found 78.43% resistance of amoxicillin from buffalo calves and lower than the findings reported by Ansari et al. (2014) who found 100% resistance of amoxicillin from diarrheic calves. On the other hand, the antibiotic sensitivity test showed that highest number of *E. coli* isolates were sensitive to Colistin (100%). Other antibiotics showed sensitivity were Levofloxacin (94.30%), Gentamicin

(85.44%), Ciprofloxacin (71.51%), Chloramphenicol (71.51%), Streptomycin (71.51%), Tetracycline (56.96%) and Sulpha/Trimethoprim (56.96%). In the present study the sensitivity of Gentamicin (85.44%) was higher than the findings of Malik et al. (2013) who reported 51.21% sensitivity from cattle and buffalo Calves. The finding of the study was in contrast to the findings of Tripathi and Soni (1982) who observed 69.69% *E. coli* sensitive for ampicillin (21.51%) but similar in case of Chloramphenicol (71.51%) where their finding was 69.70%. In case of *Salmonella* isolates the highest resistance was detected to amoxicillin (88.46%). This result is lower than the study of Hasan et al. (2018) who found 100% resistance of *Salmonella* to amoxicillin from buffalo meat. *Salmonella* was found sensitive to ciprofloxacin as 55.77% where Rahman et al. (2012) presented 100% sensitivity of *Salmonella* to ciprofloxacin in buffalo in Bangladesh. In case of gentamycin, *Salmonella* isolates was found to show 100% sensitivity. This study was entirely supported by Hasan et al. (2018) who also reported 100% sensitivity by *Salmonella* from buffalo meat to gentamycin in different areas of Bangladesh. Again, Khanal et al. (2007) demonstrated 98% sensitivity of *Salmonella* to gentamycin in Nepal which is also closely similar to the result of present study. *Salmonella* showed resistance to chloramphenicol as 15.38% in the study areas. Nguyen et al. (2016) found the chloramphenicol resistant *Salmonella* and trimethoprim/sulfamethoxazole resistant *Salmonella* as 37.5% and 31.3% respectively from meat sample in China. *Salmonella* were found as 100% susceptible to colistin and levofloxacin in all of the study areas. However, sensitivity to colistin by *Salmonella* isolates of poultry origin was recorded as 50% in Chittagong district of Bangladesh (Hassan et al., 2014). Again the present study revealed the erythromycin resistant *Salmonella* in buffalo calves as 34.62%. This result is significantly lower than the report of Hasan et al. (2018) who found 100% resistance of *Salmonella* to erythromycin from buffalo meat. Susceptibility of *Salmonella* to ampicillin was found as 63.46% from present study. This result is higher than the study of Kalambhe et al. (2016) who found the overall susceptibility of *Salmonella* to ampicillin as 40% from food animals (cattle, buffalo and pigs) in India. Sensitivity of *Salmonella* to streptomycin (96.15%) was slightly lower than the result of Hasan et al. (2018) who found susceptibility of *Salmonella* to Streptomycin as 100%. The findings of tetracycline resistant *Salmonella* were 7.69% which was significantly lower than the record of Hasan et al. (2018) (50%). In case of *Pasteurella* isolates the highest resistance rate were found to ampicillin (91.3%), followed by amoxicillin (100%) and chloramphenicol (91.29%). These results are in agreement with the previous study in which 81.11% (14/22), 86.36% (18/22) and 68.18% (15/22) resistance were found to Ampicillin, amoxicillin and chloramphenicol respectively (Hajikolaei, 2017). Another studies showed that the dominant type of resistance was for ampicillin followed by erythromycin (21.73%), Gentamycin (8.69%), streptomycin (13.03%) and salpha/ trimethoprim (43.47%) which are supported by Kamran et al., (2014) where this document highlighted of erythromycin (27.27%), gentamycin (27.27%), Streptomycin (15.4%) and salpha/ trimethoprim (63.64%). On the other hand, the antibiotic sensitivity test showed that highest number of *Pasteurella* isolates were sensitive to colistin (100%), levofloxacin (86.95%), streptomycin (86.95%), erythromycin (78.26%), salpha/ trimethoprim (56.52%) and tetracycline (86.95%). Similar studies by Kamran et al. (2014) showed *Pasteurella multocida* sensitive pattern was for erythromycin (72.72%), gentamycin (91.30%), streptomycin (61.5%) and salpha/trimethoprim (36.36%), while Kumar et al. (2009) found that *Pasteurella* sensitivity pattern was in ciprofloxacin (52.17%) and tetrycline (86%). Twenty three *Pasteurella* isolates showed 8 different patterns of antibiotic resistance to the agents used in this study.

12. Research highlight/findings:

- Three bacterial organisms (*Pasteurella*, *E coli* and *Salmonella*) were identified as the pneumonic cause of buffalo calf sickness among which *Pasteurella* was identified as the most frequent cause of buffalo calf pneumonia. The overall prevalence of *Pasteurella*, *E coli* and *Salmonella* causing pneumonia in buffalo calf was 30.83%, 13.33% and 11.67% respectively.
- Among the bacterial causes of buffalo calf diarrhea, *E coli* were more frequently identified than *Salmonella*. The overall prevalence of *E coli* and *Salmonella* causing diarrhea in buffalo calf was 33.33% and 11.67% respectively.
- *Neoscaris vitulorum* was the major parasite causing diarrhea in buffalo calf leading to death. The overall prevalence of *Neoscaris vitulorum*, *Trichostrongylus* and *Trichuris* was 33.33%, 13.75% and 7.50% respectively. Blood parasite (*Babesia*) was also identified in case of 3 sick buffalo calves.
- After post mortem examination and laboratory diagnosis it was found that 20% of the buffalo calves died due to *Pasteurella* infection, 20% died due to *Neoscaris vitulorum* infection, 10% died due to *E coli* infection and rest were unidentified which might be due to viral or managerial causes.
- Sero-prevalence of Rotavirus and BVDV in diarrheic buffalo calves were 19.33% and 17.33% respectively.
- The percentage of Rotavirus and BVDV infection in diarrheic buffalo calves detected by PCR were 15.56% and 12.22% respectively.
- Significantly more calves died in summer season (7.1%) than in winter and rainy season. Calf died significantly more (4.8%) in herds which didn't maintain quarantine after introduction of new animals to herd than which maintain quarantine.
- *E coli* isolates were highly resistant (92.41%) to amoxicillin and highly sensitive to Colistin (100%), Levofloxacin (94.30%) and Gentamycin (85.44%). *Salmonella* isolates were highly resistant to amoxicillin (88.46%) and highly sensitive to gentamycin (100%), colistin (100%) and levofloxacin (100%). *Pasteurella* isolates were highly resistant to ampicillin (78.26%) and amoxicillin (95.65%). *Pasteurella* isolates were highly sensitive to colistin (100%), levofloxacin (86.95%) and streptomycin (86.95%). Treatments are suggested according to sensitivity of bacteria causing buffalo calf sickness as a mitigation measure.

B. Implementation Position

1. Procurement:

Description of equipment and capital items	PP Target		Achievement		Remarks
	Phy (#)	Fin (Tk)	Phy (#)	Fin (Tk)	
(a) Office equipment	GD5 Package (RFQ) : Computer, Printer	60000.00	Computer, Printer	60000.00	100 % Achievement
	Wooden shelf (DPM)	10000.00	Wooden shelf	10000.00	
	Computer table (DPM)	4000.00	Computer table	4000.00	
	Computer chair (DPM)	3500.00	Computer chair	3500.00	
(b) Lab &field equipment	GD1 Package (RFQ) : Chemicals and apparatus (Cryovials, Eppendorf tube, Glassware: Screw cap bottle, Falcon tube, ELISA Kits)	200000.00	Procurement of chemicals and apparatus (Cryovials, Eppendorf tube, Glassware: Screwcap bottle, Falcon tube, ELISA Kits)	200000.00	
	GD2 Package (RFQ) : Chemicals and apparatus (Nutrient agar, Nutrient broth, SS agar, MacConkey Agar, EMB Agar, DNA extraction kits, PCR kit, PCR Primers, Agarose gel, TAE buffer, Ethedium bromide, RNase free water, KOH, Phenol, SDS, PBS, Ethanol, Xyline, Hematoxelin, Eosin)	423000.00	Procurement of chemicals and apparatus (Nutrient agar, Nutrient broth, SS agar, MacConkey Agar, EMB Agar, DNA extraction kits, PCR kit, PCR Primers, Agarose gel, TAE buffer, Ethedium bromide, RNase free water, KOH, Phenol, SDS, PBS, Ethanol, Xyline, Hematoxelin, Eosin)	423000.00	
(c) Capital Expense	GD3 Package (RFQ): Thermo cycler	350000.00	Thermo cycler	350000.00	
	GD4 Package (RFQ): Gel documentation, Thermo-mixture	277000.00	Gel documentation, Thermo-mixture	277000.00	
(c) Other capital items	Camera (DPM)	25000.00	Camera	25000.00	

2. Establishment/renovation facilities: N/A

Description of facilities	Newly established		Upgraded/refurbished		Remarks
	PP Target	Achievement	PP Target	Achievement	

3. Training/study tour/ seminar/workshop/conference organized: N/A

Description	Number of participant			Duration (Days/weeks/ months)	Remarks
	Male	Female	Total		
(a) Training					
(b) Workshop					

C. Financial and physical progress

Fig in Tk

Items of expenditure/activities	Total approved budget	Fund received	Actual expenditure	Balance/ unspent*	Physical progress (%)	Reasons for deviation
A. Contractual staff salary	306400	306400	301078	5322	100%	-
B. Field research/lab expenses and supplies	926800	844163	835500	8663	100%	-
C. Operating expenses	199700	186794	170031	16763	100%	-
D. Vehicle hire and fuel, oil & maintenance	120000	113000	112000	1000	100%	-
E. Training/workshop/seminar etc.	0	0	0	0	-	-
F. Publications and printing	171500	75687	82000	-6313	90%	-
G. Miscellaneous	25100	21275	18000	3275	100%	-
H. Capital expenses	650500	727071	727500	-429	100%	-

* Tk 44022.00 (from RPA) was refunded to Director, PIU-BARC, NATP-2 through cross cheque (No. CDC 54655; 30.09.2018). Tk 28041.00 (GoB) needs to be released to complete final adjustment.

D. Achievement of Sub-project by objectives: (Tangible form)

Specific objectives of the sub-project	Major technical activities performed in respect of the set objectives	Output (i.e. product obtained, visible, measurable)	Outcome (short term effect of the research)
(i) To identify the risk factors associated with buffalo calf mortality	Development questionnaires and field survey Analysis of risk factors	Significantly more calves died in summer season (7.1%) than in winter and rainy season. Calf died significantly more (4.8%) in herds which didn't maintain quarantine after introduction of new animals in a herd than which maintained quarantine.	Knowledge of risk factors associated with buffalo calf sickness will help planning for better farm management.
(ii) To isolate and identify the causative organisms associated with buffalo calf mortality in Bangladesh	Cultural, Biochemical and morphological examination to identify specific bacteria Microscopic examination of Fecal and blood samples to identify different parasite ELISA (Sera) and PCR (fecal samples) for sero-prevalence and confirmation of rotavirus and BVDV	Prevalence of <i>Pasteurella</i> , <i>E coli</i> and <i>Salmonella</i> causing pneumonia in buffalo calf was 30.83%, 13.33% and 11.67% respectively. Among the bacterial causes of buffalo calf diarrhea, <i>E. coli</i> and <i>Salmonella</i> causing diarrhea in buffalo calf was 33.33% and 11.67% respectively. Among the parasites causing diarrhea in buffalo calf leading to death prevalence of <i>Neoscaris vitulorum</i> , <i>Trichostrongylus</i> and <i>Trichuris</i> was 33.33%, 13.75% and 7.50% respectively. The percentage of Rotavirus and BVDV infection in diarrheic buffalo calves detected by PCR were 15.56% and 12.22% respectively.	Knowledge of different causative agents of buffalo calf sickness will help planning better health management in the buffalo farms.
(iii) To develop appropriate therapeutic measures and control strategies against buffalo calf diseases	Development and distribution of Disease calendar and leaflets (Symptoms of common buffalo calf diseases and Management and preventive measures of various diseases of buffalo calves) Distribution of deworming schedule and deworming medicine in the survey area. Sensitivity test of different bacteria.	200 posters and 200 leaflets were distributed among the farmers. <i>E coli</i> isolates were highly resistant (92.41%) to amoxicillin and highly sensitive to Colistin (100%), Levofloxacin (94.30%) and Gentamycin (85.44%). <i>Salmonella</i> isolates were highly resistant to amoxicillin (88.46%) and highly sensitive to gentamycin (100%), colistin (100%) and levofloxacin (100%). <i>Pasteurella</i> isolates were highly resistant to ampicillin (78.26%) and amoxicillin (95.65%). <i>Pasteurella</i> isolates were highly sensitive to colistin (100%), levofloxacin (86.95%) and streptomycin (86.95%).	The knowledge will help suggesting effective treatments according to sensitivity of bacteria as a mitigation measure of buffalo calf sickness.

E. Materials Development/Publication made under the Sub-project:

Publication	Number of publication		Remarks (e.g. paper title, name of journal, conference name, etc.)
	Under preparation	Completed and published	
Technology bulletin/ booklet/leaflet/flyer etc.	-	Poster (200 copies); Leaflet (2000 copies)	Distributed among the farmers
Journal publication	05	-	To be submitted
Information development	-	-	-
Conference Proceedings	-	1	Identification of bacterial causes of buffalo calf mortality in selected areas of Bangladesh. Proceedings of the 25 th Annual Scientific Conference 2019 of BSVER.
Other publications	-	05	MS Thesis*
<p>*Thesis Title:</p> <ol style="list-style-type: none"> 1. Identification of bacterial causes and associated risk factors of buffalo calf mortality in selected areas of Bangladesh 2. Prevalence of multi-drug resistant ESBL producing <i>Escherichia coli</i> isolated from buffalo calves in Sylhet and Barisal division of Bangladesh. 3. Prevalence of multi-drug resistant ESBL producing <i>Salmonella</i> isolated from buffalo calves in Sylhet and Barisal division of Bangladesh 4. Prevalence of multi-drug resistant ESBL producing <i>Pasteurella multocida</i> isolated from buffalo calves in Sylhet and Barisal division of Bangladesh 5. Assessment of risk factors associated with the prevalence and severity of gastro-intestinal parasites of buffalo calves in Sylhet division of Bangladesh 			

F. Technology/Knowledge generation/Policy Support (as applied):

i. Generation of technology (Commodity & Non-commodity)

Not Applicable

ii. Generation of new knowledge that help in developing more technology in future

New knowledge on causes of buffalo calf mortality with associated risk factors and their mitigation measure were generated that will help in developing new technologies in future.

iii. Technology transferred that help increased agricultural productivity and farmers' income

Not Applicable

iv. Policy Support

Results of the current study will help policy maker to undertake appropriate strategies to improve buffalo farming in Bangladesh

G. Information regarding Desk and Field Monitoring

i) Desk Monitoring:

Date of Workshop/Meeting	Description of Workshop/Meeting	Output
21.12.2017	Research Review Workshop on CGR Sub-projects at BARC Auditorium	Discuss the implementation progress of CRG activities
24.04.2018-25.04.2018	Progress Workshop of CRG Sub-projects under Livestock Division, BARC	Discuss the progress of CRG activities
15.05.2018-16.05.2018	Monitoring Workshop on CRG Sub-projects	Discuss the monitoring report of implementation and progress of CRG activities by Technical subdivision, BARC
22.09.2018-23.09.2018	Annual Workshop on CRG Sub-projects	Discuss the annual progress of CRG activities

ii) Field Monitoring (time& No. of visit, Team visit and output):

Date of Visit	No. of Visit	Team Visit	Output
05.04.2018	01	Technical Division/Unit, BARC	
07.04.2018	01	PIU-BARC, NATP-2	
04.06.2018	01	Internal Monitoring	

H. Lesson Learned

- Limitation of time: Lots of data had to be generated within very short time.

I. Challenges (if any)

- Sample collection from dead calves from remote areas was challenging.

Signature of the Principal Investigator
Date
Seal

Counter signature of the Head of the
organization/authorized representative
Date
Seal

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ANNEX – I

Questionnaire for the identification of the causes of calf mortality in buffalo and their mitigation measures in Bangladesh

- 1. Name and Address of the Farmer :
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- 2. Total Number of buffalo in the Farm :
- 3. Number of buffalo calf in the Farm :
- 4. Breed/type: :
- 5. Sex (calf) :
- 6. Age / Date of birth :
- 7. Type of birth: :
- 8. Season of birth: :
- 9. Weight of calves at birth:
- 10. Colostrum feeding practices :
- 11. Number of abnormal calving/ stillbirths and abortions :
- 12. Age of dam at the time of parturition: :
- 13. Parity number of the dam :
- 14. Buffalo Rearing System :
- 15. Feeding of Buffalo:
- 16. Introduction of New buffalo in the Farm : Yes / No (if yes; answer Q. No. # 17)
- 17. Quarantine
- 18. Number of Dead calf :
- 19. Disease History :
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.....
.....
- 20. Frequency of Disease :
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.....
- 21. History Described by the Owner (Clinical Manifestations of the Disease) :
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.....
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- 22. Vaccination Status :
- 23. Therapeutic Measures Taken :
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