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Isolation, molecular identification and antibiogram profiles of *Escherichia coli* and *Salmonella* spp. from diarrhoeic cattle reared in selected areas of Bangladesh

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Abstract: The present research work was undertaken to find out the passive causes of occurrences of diarrhoea in terms of age, sex, season and location differences through isolation and identification of the *E. coli* and *Salmonella* spp. using cultural, biochemical and molecular from the field samples of the diarrhoeic cattle and to study the antibiogram profiles of the isolated bacterial species. Considering above purposes, a total of 57 rectal swab samples were collected from the diarrhoeic cattle of Mymensingh sadar, Trishal, Valuka, Natore sadar and Gomostapur, Chapai Nawabganj. Different types of cultural media like Nutrient agar, MacConkey's (MC) agar, Eosin Methylene Blue (EMB) agar, Salmonella-Shigella (SS) agar, Xylose-Lysine-Deoxycholate (XLD) agar and Blood agar were used to isolate and to study the cultural properties of the *E. coli* and *Salmonella* spp. Finally Gram's staining and different biochemical tests were performed to identify those two bacterial species. Out of 57 samples, 27 were positive for *E. coli* and 8 were positive for *Salmonella* spp. On the basis of information from cattle owners and clinical signs the prevalence of diarrhoea was recorded as 30.99% and the *p*-value was calculated as 0.001 ($p < 0.01$) which was noted as highly significant. The prevalence percentages of the *E. coli* and *Salmonella* spp. were differed depending on different epidemiological parameters like age, sex, season and location. Moreover, the molecular identifications were further confirmed by means of PCR assay using specific primers for *E. coli* and *Salmonella* spp. This was done targeting 16S rRNA gene where they were found to be positive showing amplification of 585 bp for *E. coli* and 574 bp for *Salmonella* spp. From the study of the antibiogram profiles, it was revealed that *E. coli* were susceptible to ciprofloxacin, gentamicin and norfloxacin but resistant to tetracycline, erythromycin, amoxicillin and streptomycin whereas *Salmonella* spp. were susceptible to ciprofloxacin, gentamicin, amoxicillin and streptomycin but resistant to azithromycin, tetracycline and erythromycin. The findings of this research work would certainly help to select the proper antibiotics against diarrhoea in cattle of Bangladesh and to overcome the multi-drug resistant problem of the bacteria.

Keywords: diarrhoea; rectal swab; *E. coli*; *Salmonella*; PCR; prevalence; antibiogram

1. Introduction

Diarrhoea is one of the major community health hazards both for man and animal which is caused by bacteria (such as *Campylobacter*, *Escherichia coli*, *Salmonella* and *Clostridium*), virus (Rotavirus, coronavirus, Bovine viral diarrhoea virus, along with newly emerging enteric viruses such as bovine torovirus and caliciviruses like bovine norovirus and Nebovirus), fungus, protozoa (*Coccidia*, *Cryptosporidium*), helminths, chemical agents, clay, sands, nutritional deficiency factors, indigestion, managemental factors, hepatic cirrhosis and other toxic factors. These factors act singly or in combination for the production of diarrhoea complex (Sharif *et al.*, 2005).

The prevalence of each of the pathogen and disease incidence can vary by geographical location of the farms, farm management practices and herd size (Cho and Yoon, 2014).

Livestock population in Bangladesh is currently estimated to comprise 25.7 million cattle, 0.83 million buffaloes, 14.8 million goats, 1.9 million sheep, 118.7 million chicken and 34.1 million ducks (Banglapedia, 2015). Among livestock population cattle are considered as the most important animals in livestock sub-sector. Various microbial diseases are common in cattle like anthrax, haemorrhagic septicaemia, brucellosis, tuberculosis, mastitis, diarrhoea etc. that can act as alarming side for cattle production. Among these diseases, diarrhoea is very common and may be fatal if proper treatment is not given to the affected cattle. Diarrhoea in cattle due to bacterial species were detected in home and abroad as colibacillosis, pasteurellosis, salmonellosis, campylobacteriosis and clostridial infections where serious losses of income generation activities are being occurred either in terms of morbidity and mortality of cattle (Fulton *et al.*, 2000; Mailk *et al.*, 2013; Cho and Yoon, 2014; Muktar *et al.*, 2015). Out of these bacterial species colibacillosis and salmonellosis were being targeted as a primary step to reduce the diarrhoea in cattle population of Bangladesh. The etiological agents of diarrhoea due to colibacillosis and salmonellosis could be isolated and identified through cultural, biochemical, serological and molecular techniques.

A course of antibiotic therapy is necessary to treat bacterial diarrhoea in cattle. Incomplete course of treatment and continuous indiscriminate use of antibacterial drugs against diarrhoeal infection of man and animal might have influenced to produce a new generation of virulent and resistant type of bacteria (Marshall *et al.*, 1990; Izumiya *et al.*, 2001).

Different parameters including prevalence, isolation, identification, antibiotic sensitivity testing, epidemiological investigation, plasmid profiling of *E. coli* of different species were studied by Paul *et al.*, 2010; Hossain *et al.*, 2013; Hasan *et al.*, 2015. Isolation, identification, serotypic characterization, pathogenicity testing, antibiogram study of *Salmonella* of different species were also studied by Sarker *et al.*, 2009; Nesa *et al.*, 2011; Islam *et al.*, 2013; Ansari *et al.*, 2014; Muktar *et al.*, 2015. However, isolation, molecular identification and antimicrobial susceptibility profiles of *E. coli* and *Salmonella* spp. from diarrhoeic cattle were not systematically and properly focused by the previous research works in context of Bangladesh. So, the present study was undertaken to survey the diarrhoeic cattle, to isolate and identify the *E. coli* and *Salmonella* spp. with their antibiogram profiles that will be helpful towards cattle diseases management and enhance dynamism of cattle farming which not only alleviate poverty but also boost up the national economy of Bangladesh.

2. Materials and Methods

2.1. Sampling areas and period

A total number of 57 rectal swab samples were aseptically collected using sterilized cotton buds from diarrhoeic cattle of the Mymensingh sadar (25), Trishal (8), Valuka (5), Natore sadar (9) and Gomostapur, Chapai Nawabganj (10) during the period of June 2015 to May 2016. After collection, samples were brought to the laboratory in the Department of Microbiology and Hygiene, Bangladesh Agricultural University (BAU), Mymensingh-2202, Bangladesh for the isolation, molecular identification and antibiogram profiles of the *E. coli* and *Salmonella* spp.

2.2. Isolation of *E. coli* and *Salmonella* spp.

The collected rectal swab samples were enriched into nutrient broth by incubation at 37°C for 24 hours. To isolate and to study the cultural properties of the *E. coli* and *Salmonella* spp., enriched cultures were streaked onto different types of differential and selective culture media like MC agar, EMB agar, SS agar, XLD agar and blood agar according to the method described by Cowan, 1985 where all of the media were brought from the Indian company, HiMedia.

2.3. Identification of *E. coli* and *Salmonella* spp. by conventional methods

For identification of isolated *E. coli* and *Salmonella* spp., Gram's staining and biochemical tests were performed. Gram's staining was performed according to the method described by Merchant and Packer, 1967 where all of the reagents like crystal violet, Gram's iodine, safranin, acetone alcohol, immersion oil were brought from the German company, Merck. Different types of biochemical tests like sugar fermentation test, MR-VP reaction, indole reaction, catalase test were performed according to the methods described by Douglas *et al.*, 1998 and OIE, 2000 where all of the reagents were brought from the German company, Merck. Motility test using MIU medium base was also performed according to the method described by Cowan, 1985 to

differentiate motile bacteria from the non-motile one where MIU medium base was brought from the Indian company, HiMedia.

2.4. Preparation of DNA templates

Extraction of DNA from the *E. coli* and *Salmonella* spp. was carried out by conventional boiling and rapid cooling method (Medici *et al.*, 2003). In brief, 200 μ l deionized water was taken into an eppendorf tube, a pure bacterial colony from nutrient agar was mixed with the deionized water. The tube then transferred to boiling water and boiled for 10 minutes then immediately to the icebox for cold shock about 10 minutes and then centrifuged at 10,000 rpm for 10 minutes. Supernatant were collected and used as DNA template during PCR.

2.5. Identification of *E. coli* and *Salmonella* spp. by PCR assay

Details of the oligonucleotide primers used for the amplification of 16S rRNA gene of the *E. coli* and *Salmonella* spp. are summarized in Table 1. PCR reaction mixture (25 μ l) for *E. coli* was prepared using 12.5 μ l master mixture (Promega, USA), 10 pmol primer (Bioneer, South Korea) of each, 5 μ l DNA template and 5.5 μ l nuclease free water. PCR reaction mixture (20 μ l) for *Salmonella* spp. was prepared using 10 μ l master mixture (Promega, USA), 10 pmol primer (Bioneer, South Korea) of each, 3 μ l DNA template and 5 μ l Nuclease free water. For the amplification of 16S rRNA gene of *E. coli*, the cycling conditions consisted of initial denaturation for 5 minutes at 95°C, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 1 minute, extension at 72°C for 1 minute and final extension at 72°C for 10 minutes where the cycling conditions for the amplification of 16S rRNA gene of *Salmonella* spp. consisted of initial denaturation for 5 minutes at 94°C, followed by 35 cycles of denaturation at 94°C for 20 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 30 seconds and the final extension at 72°C for 5 minutes. Amplification was performed in a thermal cycler (Eppendorf, Germany). The amplified products were electrophoresed into 1.5% agarose (Sigma-Aldrich, USA) gel at 100 volt visualized under Gel doc/UV trans-illuminator (BioRad). 100 bp (for *Salmonella* spp.) and 1 kb (for *E. coli*) DNA size marker (Promega, USA) were used.

2.6. Antibiogram profiles

Antibiogram was performed by employing the Kirby-Bauer disc diffusion method (Bauer *et al.*, 1959) using eight different commercially available antibiotic discs (HiMedia, India and Oxoid Ltd., England) on Mueller-Hinton agar (HiMedia, India) to assess the susceptibility and resistance pattern of the isolates. The selected antibiotics used were ciprofloxacin (5 μ g/disc), azithromycin (30 μ g/disc), amoxicillin (30 μ g/disc), gentamicin (10 μ g/disc), norfloxacin (10 μ g/disc), erythromycin (30 μ g/disc), streptomycin (10 μ g/disc), and tetracycline (30 μ g/disc). The interpretation on susceptibility was done according to the guidelines of Clinical and Laboratory Standards Institute (CLSI, 2012).

2.7. Statistical analysis

The experimental data were analysed by SPSS software (version 20) where the prevalence of diarrhoea based on questionnaire survey was calculated by ANOVA table method and the prevalence percentages of the isolated *E. coli* and *Salmonella* spp. based on different epidemiological parameters like age, sex, season and location were calculated by Chi-square (χ^2) test method.

3. Results

3.1. Prevalence of diarrhoea in cattle as per collected data from study areas

For this study, data were collected through a developed questionnaire from study areas of Bangladesh. Prevalence was recorded as 22.92% at farm level and 39.06% at household level. Overall prevalence was recorded as 30.99%. The *p*-value was calculated as 0.001 (*p*<0.01) which was noted as highly significant. The prevalence percentages of diarrhoea from different locations are shown in Table 2.

3.2. Isolation and cultural characterization of *E. coli* and *Salmonella* spp.

The growth of *E. coli* and *Salmonella* spp. was indicated by the presence of turbidity in the nutrient broth after overnight incubation at 37°C. On nutrient agar, *Salmonella* spp. produced circular, smooth, opaque, translucent colonies and *E. coli* produced circular, smooth, colorless colonies. On EMB agar, *E. coli* produced greenish-black colonies with metallic sheen. On MC agar, *E. coli* produced circular raised, bright pink colored colonies while *Salmonella* spp. produced colorless, smooth, pale and transparent, raised colonies. On SS agar, *E. coli* produced slight pinkish colored colonies while *Salmonella* spp. produced pinhead or lentil sized, raised, round or circular, smooth, glistening, opaque, black, transparent or translucent colonies. On blood agar, *E. coli*

produced colorless colony without hemolysis while *Salmonella* spp. produced white, round, raised colonies with no hemolysis. On XLD agar, *Salmonella* spp. produced blackish centered colonies while *E. coli* produced yellow colored colonies.

3.3. Identification of *E. coli* and *Salmonella* spp. by conventional methods

In Gram's staining, the isolated *E. coli* revealed Gram negative, pink colored, short plump rod shaped appearance arranged as single, paired or in short chain and *Salmonella* spp. revealed Gram negative, pink colored, very short plump rod shaped appearance arranged as single or paired. All of the isolates of *E. coli* and *Salmonella* spp. were found as positive to motility test.

Fermentation of five basic sugars with the production of acid and gas indicated that the isolates were *E. coli* positive while *Salmonella* spp. fermented dextrose, maltose and mannitol with the production of acid and gas but did not ferment lactose and sucrose. *E. coli* were found as indole positive where *Salmonella* spp. were found as indole negative. Both organisms (*E. coli* and *Salmonella* spp.) were found as MR test positive, VP test negative and catalase test positive.

3.4. Identification of *E. coli* and *Salmonella* spp. by PCR assay

DNA extracted from all of the isolates (27 *E. coli* and 8 *Salmonella* spp.) were used in PCR assay. Polymerase chain reaction with the primers ECO-1 and ECO-2 (for *E. coli*) and Sal 16S rRNA (for *Salmonella* spp.) identified all of those isolates as positive for *E. coli* and *Salmonella* spp. showing amplification of 585 bp and 574 bp respectively as presented in Figures 1 and 2.

3.5. Prevalence of the isolated *E. coli* and *Salmonella* spp. at different epidemiological parameters (age, sex, season and location)

Prevalence of *E. coli* was 52.00% (n=13/25) in Mymensingh sadar, 12.50% (n=1/8) in Trishal, 20.00% (n=1/5) in Valuka, 33.33% (n=3/9) in Natore sadar and 90.00% (n=9/10) in Gomostapur, Chapai Nawabganj whereas prevalence of *Salmonella* spp. was 12.00% (n=3/25) in Mymensingh sadar, 37.50% (n=3/8) in Trishal, 40.00% (n=2/5) in Valuka and no samples were detected as positive for *Salmonella* spp. in Natore sadar and in Gomostapur, Chapai Nawabganj. The *p*-value was recorded as 0.009 ($p < 0.01$) for *E. coli* and 0.047 ($p < 0.05$) for *Salmonella* spp. that meant the results were highly significant for *E. coli* and significant for *Salmonella* spp. The prevalence of the isolated *E. coli* was 64.00% (n=16/25) in 1-12 month aged cattle and 34.37% (n=11/32) in above 12 month aged cattle. The *p*-value was calculated as 0.026 ($p < 0.05$) that meant the results were significant. The prevalence percentages of *E. coli* were 26.31% (n=5/19) in male cattle and 57.89% (n=22/38) in female cattle. The *p*-value was recorded as 0.024 ($p < 0.05$) that meant the results were significant. The prevalence percentages of *E. coli* were 56.75% (n=21/37) in summer season, 71.42% (n=5/7) in rainy season and 7.69% (n=1/13) in winter season. The *p*-value was calculated as 0.004 ($p < 0.01$) that meant the results were highly significant. The prevalence of the isolated *Salmonella* spp. was found as 28.00% (n=7/25) in 1-12 month aged cattle and 3.12% (n=1/32) in above 12 month aged cattle. The *p*-value was recorded as 0.007 ($p < 0.01$) that meant the results were highly significant. The prevalence of the isolated *Salmonella* spp. was found as 21.05% (n=8/38) in female cattle and there was no prevalence of *Salmonella* spp. in male cattle. The *p*-value was calculated as 0.038 ($p < 0.05$) that meant the results were significant. The prevalence percentages of the isolated *Salmonella* spp. were found as 13.51% (n=5/37) in summer season, 42.85% (n=3/7) in rainy season and out of 13 samples no samples were detected as positive for *Salmonella* spp. in winter season. The *p*-value was recorded as 0.031 ($p < 0.05$) that meant the results were significant. The prevalence percentages of the isolated *E. coli* and *Salmonella* spp. at different epidemiological parameters (age, sex, season and location) are shown in Tables 3 and 4.

3.6. Antibiogram profiles

E. coli were susceptible to ciprofloxacin, gentamicin and norfloxacin; intermediate to azithromycin but resistant to tetracycline, erythromycin, amoxicillin and streptomycin. *Salmonella* spp. were susceptible to ciprofloxacin, gentamicin, amoxicillin and streptomycin; intermediate to norfloxacin but resistant to azithromycin, tetracycline and erythromycin as shown in Table 5.

Table 1. List of primers used in this study.

Primer	Sequence (5'-3')	Target	Amplicon size (bp)	Reference
ECO-1	GACCTCGGTTTAGTTCACAGA	16S rRNA gene of <i>E. coli</i>	585	Schippa <i>et al.</i> , 2010
ECO-2	CACACGCTGACGCTGACCA			
Sal 16S rRNA	F: TGTTGTGGTTAATAACCGCA R: CACAAATCCATCTCTGGA	16S rRNA gene of <i>Salmonella</i> spp.	574	Adamu <i>et al.</i> , 2014

Table 2. Results of prevalence of diarrhoea in cattle as per collected data based on questionnaire survey.

Location	No. of visited farm (s)	Total No. of cattle	No. of diarrhoeic cattle	Prevalence (%)	Overall prevalence (%)	No. of household (s)	Total No. of cattle	No. of diarrhoeic cattle	Prevalence (%)	Overall prevalence (%)
Mymensingh sadar	3	96	18	18.75	22.92	2	16	7	43.75	39.06
Trishal	1	15	4	26.66		1	9	4	44.44	
Valuka	1	17	4	23.52		1	5	2	40.00	
Natore sadar	2	29	6	20.68		2	13	4	30.76	
Gomostapur, Chapai Nawabganj	2	32	8	25.00		2	11	4	36.36	
Overall prevalence (%)					30.99					
p-value					0.001**					

**means 1% level of sig. ($p < 0.01$)

Table 3. Results of prevalence of *E. coli* and *Salmonella* spp. at epidemiological parameter (location).

Location	No. of collected sample (s)	<i>E. coli</i>		<i>Salmonella</i> spp.	
		No. of positive	Prevalence (%)	No. of positive	Prevalence (%)
Mymensingh sadar	25	13	52.00	3	12.00
Trishal	8	1	12.50	3	37.50
Valuka	5	1	20.00	2	40.00
Natore sadar	9	3	33.33	0	0.00
Gomostapur, Chapai Nawabganj	10	9	90.00	0	0.00
Overall prevalence (%)		41.56		17.90	
p-value		0.009**		0.047**	

* means 5% level of sig. ($p < 0.05$) ** means 1% level of sig. ($p < 0.01$)

Table 4. Results of prevalence of *E. coli* and *Salmonella* spp. at different epidemiological parameters (age, sex, season).

Epidemiological parameters	Level of patterns	No. of animal examined	<i>E. coli</i>			<i>Salmonella</i> spp.		
			No. of Positive	Prevalence (%)	p-value	No. of Positive	Prevalence (%)	p-value
Age	1-12 month (s)	25	16	64.00	0.026*	7	28.00	0.007**
	Above 12 months	32	11	34.37		1	3.12	
Sex	Male	19	5	26.31	0.024*	0	0.00	0.038*
	Female	38	22	57.89		8	21.05	
Season	Summer	37	21	56.75	0.004**	5	13.51	0.031*
	Rainy	7	5	71.42		3	42.85	
	Winter	13	1	7.69		0	0.00	

*means 5% level of sig. ($p < 0.05$) ** means 1% level of sig. ($p < 0.01$)

Table 5. Results of antibiogram profiles of the isolated *E. coli* and *Salmonella* spp.

Name of the antibiotic discs	<i>E. coli</i>		<i>Salmonella</i> spp.	
	Zone of inhibition diameter (mm)	Interpretation	Zone of inhibition diameter (mm)	Interpretation
Ciprofloxacin (CIP)	24	S	25	S
Azithromycin (AZM)	15	I	10	R
Tetracycline (TE)	10	R	0	R
Gentamicin (GEN)	20	S	21	S
Erythromycin (E)	10	R	0	R
Amoxicillin (AMX)	0	R	21	S
Norfloxacin (NOR)	21	S	16	I
Streptomycin (S)	0	R	15	S

Legends: S = Susceptible; I = Intermediate; R = Resistant

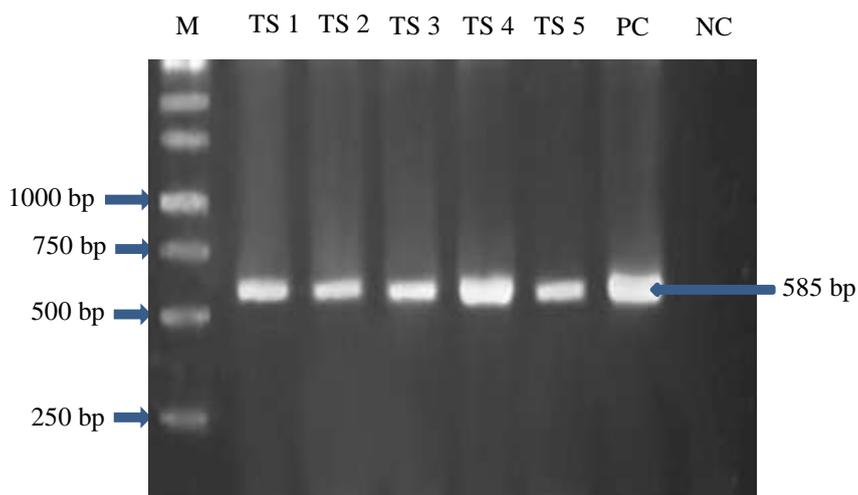


Figure 1. PCR assay to amplify 16S rRNA gene of *E. coli* isolates recovered from diarrhoeic cattle (Legends: M= 1kb size DNA marker; TS1-TS5= Test samples; PC= Positive control; NC= Negative control without DNA).

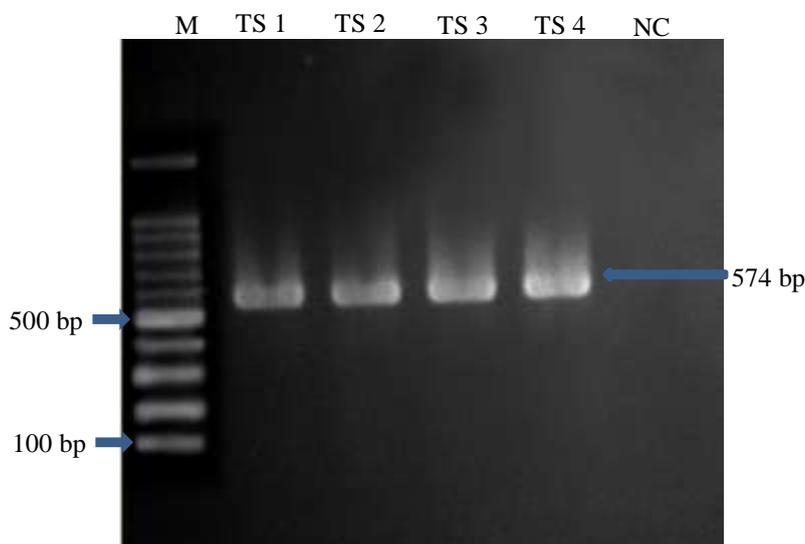


Figure 2. PCR assay to amplify 16S rRNA gene of *Salmonella* spp. isolates recovered from diarrhoeic cattle (Legends: M= 100 bp size DNA marker; TS1-TS4= Test samples; NC= Negative control without DNA)

4. Discussion

The cultural properties of the isolated *E. coli* were the production of metallic sheen on the EMB agar and rose pink colored colony on the MC agar which agreed with the findings of Ali *et al.*, 1998; Sharada *et al.*, 1999. In this study the colonies of *Salmonella* spp. on SS agar were opaque, translucent with black centers which were similar to the findings of Cheesbrough, 1985; Hossain, 2002. In Gram's staining, the morphology of the isolated *E. coli* was exhibited as Gram negative, short plump rod arranged as single, paired or in short chain which was reported by Merchant and Packer, 1967; Buxton and Fraser, 1977; Freeman, 1985; Sharada *et al.*, 1999 whereas the morphology of the isolated *Salmonella* spp. was Gram negative, very short plump rod arranged as single or paired and those properties of *Salmonella* spp. were supported by Freeman, 1985 and Hossain, 2002. Motility test revealed that the isolated *E. coli* and *Salmonella* spp. were motile which was correlated with the results of Merchant and Packer, 1967; Buxton and Fraser, 1977. *E. coli* isolates were able to ferment the five basic sugars by producing both acid and gas which was supported by Beutin *et al.*, 1997; Thomas, 1998 whereas isolated *Salmonella* spp. were able to ferment dextrose, maltose and mannitol with the production of both acid and gas but did not ferment lactose and sucrose and those characteristics of *Salmonella* spp. were satisfied the statement of Hossain, 2002; Han *et al.*, 2011. The isolated *E. coli* were found MR and indole test positive but VP test negative which were reported by Merchant and Packer, 1967; Ali *et al.*, 1998; Mishra *et al.*, 2002. The isolated *Salmonella* spp. were found MR test positive but indole and VP test negative that satisfied the statement of Douglas *et al.*, 1998; OIE, 2000. The results of prevalence of the isolated *E. coli* and *Salmonella* spp. calculated on the basis of age, sex, season and location were more or less similar with the findings of Baule *et al.*, 1997; Khan *et al.*, 2009. On the basis of information from the cattle owners and clinical signs, the prevalence of diarrhoea was recorded as 30.99% which showed more or less similar results with the findings of Gulliksen *et al.*, 2009; Drillich *et al.*, 2015. Molecular identifications of the isolated *E. coli* and *Salmonella* spp. were done by means of PCR assay that was similar to the findings of Ziemer and Steadham, 2003; Schippa *et al.*, 2010; Adamu *et al.*, 2014. In this study, 8 different antibiotics available in the market were used to study antibiogram profiles of the *E. coli* and *Salmonella* spp. isolated from diarrhoeic cattle by disc diffusion method and the results were supported by Akond *et al.*, 2009; Wouafo *et al.*, 2010; Hyeon *et al.*, 2011; De *et al.*, 2012; Jeyasanta *et al.*, 2012; Jahan *et al.*, 2013.

Due to financial crisis and limited time following further studies like collection of more samples covering many areas and farms of Bangladesh to get more precise information, molecular detection of shiga toxin producing *E. coli*, molecular characterization of the isolated bacterial species by pulsed field gel electrophoresis (PFGE), 16S rRNA gene sequencing of the isolated *E. coli* and *Salmonella* spp., serotyping, multiplex PCR of the isolated *Salmonella* spp., pathogenicity study would not be possible.

5. Conclusions

The findings of this research work would certainly help the veterinary practitioners to select the proper antibiotics against diarrhoea in cattle of Bangladesh. If the prescribers prescribe suitable antibiotics against diarrhoea in cattle then it would be possible to overcome the multi-drug resistant problem of bacteria, otherwise successful antibiotic therapy against diarrhoea in cattle would not be possible.

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Conflict of interest

None to declare.

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