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MOLECULAR CHARACTERIZATION AND ANTIBIOGRAM STUDY OF BACTERIA ISOLATED FROM DIARRHOEIC CALVES

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A B S T R A C T
This study was conducted to investigate the causative agents of bacterial infections in diarrhoeic calves and to assess their antibiotic susceptibility patterns in Dinajpur Sadar, Bangladesh. A total of forty-five (45) fecal samples were collected and examined using conventional microbiological methods, including bacterial culture, biochemical tests, and antibiotic sensitivity assays. Molecular characterization of *Escherichia coli* and *Salmonella* spp. was performed using PCR with universal primers targeting the 16S rRNA and invA gene respectively. The bacterial isolates identified from the diarrhoeic samples were *E. coli* (53.33%), *Shigella* spp. (15.5%), *Salmonella* spp. (20%), *Enterobacter* spp. (8.88%), and *Bacillus* spp. (2.22%). The bacterial isolates showed resistance to amoxicillin, ampicillin, erythromycin, and cephalexin. *E. coli* exhibited sensitivity to azithromycin, cotrimoxazole, doxycycline, and levofloxacin. *Shigella* spp. was sensitive to cefixime, tetracycline, and gentamycin. *Enterobacter* spp. showed sensitivity to azithromycin and cefixime. *Salmonella* spp. was sensitive to tetracycline and streptomycin. *Bacillus* spp. exhibited susceptibility to streptomycin and bacitracin. Continued monitoring of antimicrobial resistance in livestock is essential to guide rational antibiotic use and protect public health in Bangladesh.

1. INTRODUCTION

Diarrhoea is a major health concern affecting both humans and animals. It can be caused by a wide range of infectious agents including bacteria (*E. coli, Salmonella, Campylobacter, Clostridium*),

viruses (e.g., Rotavirus, Coronavirus, BVDV, Bovine Norovirus), fungi, protozoa (*Coccidia, Cryptosporidium*), helminths, as well as chemical toxins, nutritional deficiencies, and poor management practices (Sharif et al., 2005). These factors may act individually or synergistically to trigger diarrhoeal disease. The prevalence of diarrhoea among cattle often varies with factors like herd size, geographical location, and farm management (Cho and Yoon, 2014). In Bangladesh, the livestock sector is significant, with an estimated 25.7 million cattle playing a vital role in food production and rural livelihoods (Mahedi et al., 2024; Uddin et al., 2022; M. Uddin et al., 2022). Cattle are commonly affected by microbial diseases including anthrax, mastitis, and diarrhoea, which significantly reduce productivity. Bacterial diarrhoeas such as colibacillosis, salmonellosis, campylobacteriosis, and clostridial infections are widely recognized both locally and globally. These infections contribute to substantial economic losses through reduced growth rates, treatment expenses, and high morbidity and mortality rates (Fulton et al., 2000; Malik et al., 2013; Cho and Yoon, 2014; Muktar et al., 2015). Diarrhoea is a leading cause of death in neonatal calves and a key contributor to reduced performance in the early stages of life (Radostits et al., 2000). Losses stem from direct calf mortality, treatment costs, labor, and long-term effects on weight gain and productivity (Bazeley, 2003). Contributing factors include overfeeding, lack of colostrum, poor hygiene, and environmental stressors such as cold temperatures. Ruminants, including cattle, are significant reservoirs of Shiga toxin-producing *E. coli* (STEC). Infected calves can become “super shedders,” releasing large quantities of bacteria into the environment and increasing the risk of transmission to humans. In people, STEC infections can cause severe conditions like hemorrhagic colitis and hemolytic uremic syndrome (Ferens et al., 2011). *Shigella* spp. also pose health risks, causing an estimated 164.7 million human infections annually (Perepelov et al., 2012). Though less studied in animals, shigellosis is commonly treated with antibiotics such as ciprofloxacin. However, rising antimicrobial resistance (AMR) complicates treatment (Pal et al., 2016). *Salmonella* spp. are also important diarrhoeal agent in Bangladeshi cattle, and their identification often involves cultural, biochemical, serological, and molecular diagnostic methods. *Bacillus cereus*, a spore-forming, Gram-positive foodborne pathogen, is another potential threat as its spores can withstand heat and cause illness if food is mishandled (Blackburn and McClure, 2009). Antibiotic resistance among different species is a major global threat to public health, which contributed between 1.27 and 4.95 million deaths globally in 2019 (Murray et al., 2022; Salam et al., 2023). The overuse and misuse of antibiotics in both human and veterinary medicine have led to the emergence of multidrug-resistant (MDR) organisms, reminiscent of the pre-antibiotic era (Marshall et al., 1990). *E. coli* is considered a sentinel organism for tracking AMR in animals (Hamzah et al., 2013). Resistance genes are often plasmid-borne, making them transferable between species and increasing the public health risk (Schwarz & Chaslus-Dancla, 2001; Ewers et al., 2012). Advanced molecular tools such as microarrays enable rapid bacterial genotyping and virulence gene detection (Bumgarner, 2013). Combating AMR requires rational antibiotic use, continuous monitoring, and the development of alternatives. This study aimed to isolate and identify bacteria from diarrhoeic calves, assess their antibiotic susceptibility, and characterize them at the molecular level.

2. MATERIALS AND METHODS

2.1 Study period and location

The present study was conducted during the period from January to December, 2023 in the Bacteriology laboratory of the Department of Microbiology, Hajee Mohammad Danesh Science and

Technology University (HSTU), Dinajpur.

2.2 Sample Collection Area

A total of forty-five (45) diarrhoeic fecal samples were collected from calves at five locations of Dinajpur district: Uttar Sadipur, Baserhat (n = 10); Fultoli Bazar, Birampur Road (n = 5); Upazila Livestock Office (n = 10); District Livestock Office (n = 10); and the Veterinary Teaching Hospital, HSTU (n = 10). Information regarding clinical history and environment was obtained through direct interviews with animal owners using a structured questionnaire.

2.3 Sample Transportation and Processing

Samples were collected aseptically in sterile, airtight containers and immediately transported in an insulated icebox (4°C) to the laboratory. Samples were processed within four hours of collection.

2.4 Experimental Design

The study was structured into three phases: (1) isolation and phenotypic identification of bacterial pathogens, (2) molecular characterization via PCR, and (3) comparative antibiotic susceptibility profiling.

2.5 Culture Media and Reagents

The following culture media were used for bacterial isolation and identification: Nutrient Agar, MacConkey Agar, Eosin Methylene Blue (EMB) Agar, Xylose Lysine Deoxycholate (XLD) Agar, Salmonella-Shigella (SS) Agar, and Mueller-Hinton Agar (MHA). Nutrient Broth and 1% Peptone Water were used for enrichment. All culture media were sourced from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), except for MHA and Peptone Water, which were obtained from Difco Laboratories (Detroit, MI, USA). Media were prepared and sterilized following the manufacturer's instructions.

2.6 Isolation and Identification of Bacteria

Primary cultures were obtained by inoculating fecal samples onto Nutrient Agar and incubating at 37°C for 24 hours. Secondary subcultures were performed on selective and differential media including MacConkey, EMB, SS, and XLD Agar. Bacterial colonies were assessed for morphology, pigment, margin, and elevation.

2.7 Gram Staining and Microscopy

Gram staining was performed according to the protocol by Merchant and Packer (1967). Smears were prepared, heat-fixed, and stained sequentially with crystal violet, Gram's iodine, decolorized with 95% ethanol, and counterstained with safranin. Morphology and Gram reaction were observed under 100x oil immersion objective.

2.8 Biochemical Characterization

The isolates were subjected to standard biochemical tests (Cheesbrough, 2000) including: Indole Test**,** Methyl Red (MR) Test**,** Voges-Proskauer (VP) Test**,** Citrate Utilization Test**,** Triple Sugar Iron (TSI) Agar**,** Motility Indole Urease (MIU) Test**,** Catalase and Oxidase Tests.

2.9 Molecular Identification by PCR

Genomic DNA from E. coli and Salmonella spp. was extracted using the boiling and snap-chilling method described by (Medici et al. 2003). PCR amplification of the **16S rRNA** gene from *E. coli* was performed using specific primers (Schippa et al., 2010). PCR amplification of the **invA** gene from *Salmonella* spp. was performed using specific primers (Li et al., 2012). PCR reaction mixtures (25

µL) included: 12.5 µL Go Taq Green Master Mix (Promega, USA), 1 µL forward primer, 1 µL reverse primer, 5 µL DNA template, 5.5 µL nuclease-free water. PCR conditions: Initial denaturation: 95°C for 5 min; 35 cycles of: Denaturation at 95°C for 1 min, Annealing at 55–56°C for 40 sec, Extension at 72°C for 1 min, Final extension at 72°C for 5 min. Amplified products were visualized using 1.5% agarose gel electrophoresis stained with Runsafe dye (Bio-Rad, USA) and viewed under a UV transilluminator.

2.10 Antibiotic Susceptibility Testing

Bacterial susceptibility to anti-microbial agent was determined in vitro by using the standardized agar disc-diffusion method. The covers of each of the agar plates were labeled with name of the test organisms were inoculated. A sterile cotton swab was dipped into a well-mixed saline test culture and removed excess inoculums by pressing the saturated swab against the inner wall of the culture tube. The swab was streaked in the entire agar surface horizontally, vertically, and around the outer edge of the plate to ensure a heavy growth over the entire surface. All culture plates were allowed to dry for about 5 minutes. The individual antibiotic discs were distributed at equal distance with forceps dipped in alcohol and flamed. The discs were gently pressed down to ensure that the discs adhered to the surface of the agar. The plates were then inverted and incubated at 37^0 C for 24 hours. After incubation, the plates were examined, and the diameter of the zones of complete inhibition was measured. Antibiotic sensitivity and resistance was determined according to (CLSI 2013).

3. RESULTS AND DISCUSSIONS

3.1 Frequency of different Bacteria

In our study, bacterial isolates were found in all the collected faecal samples from diarrhoeic calves. Out of 45 samples, 24 were *E. coli, 7 were Shigella* spp, 4 were *Enterobacter* spp, 9 were *Salmonella* spp and 1 was *Bacillus* spp. (Table 1)

Table 1: Frequency of bacteria from collected sample

Bacterial species	Total Sample	Positive	% (Percentage)
1. *E. coli*	45	24	53.33
2. *Shigella* spp		7	15.55
3. *Enterobacter* spp		4	8.88
4. *Salmonella* spp		9	20.0
5. *Bacillus* spp		1	2.22

3.2 Prevalence of Bacteria on the basis of sex, age and season

In this study, Prevalence of *E coli*, *Shigella* spp., *Enterobacter* spp and *Samonella* spp. were found higher in female calves than male calves (Table 2). These bacterial isolates were also more prevalent in calves under 3 months of age (Table 3). The bacterial diarrhea cases were found higher in the rainy season (Table 4).

Table 2: Prevalence of isolated bacteria on the basis of sex

Bacterial species	Male (n=16)	Female (n=29)	Total (n=45)
1. *E. coli*	9 (37.50)%	15 (62.50)%	24
2. *Shigella* spp	2 (28.57)%	5 (71.42)%	7
3. *Enterobacter* spp	1 (25.0) %	3 (75.0)%	4
4. *Salmonella* spp	3 (33.3)%	6 (66.6) %	9

5. *Bacillus* spp	1 (100)%	0 (0) %	1

Table 3: Prevalence of isolated bacteria on the basis of age

Age	Total Sample	Bacteria	Positive Sample	Percentage
Under 3 Months	25	1. *E. coli*	13	52%
		2. *Shigella* spp	4	16%
		3. *Enterobacter* spp	2	8%
		4. *Salmonella* spp	6	24%
		5. *Bacillus* spp	0	0%
3-6 Months	20	1. *E. coli*	11	55%
		2. *Shigella* spp	3	15%
		3. *Enterobacter* spp	2	10%
		4. *Salmonella* spp	3	15%
		5. *Bacillus* spp	1	5%

Table 4: Prevalence of bacteria on the basis of season

Season	Total Sample	Bacteria	Number	Percentage
Rainy season	29	*E. coli*	14	48.27%
		Shigella spp	5	17.24%
		Enterobacter spp	4	13.79%
		Salmonella spp	6	20.68%
		Bacillus spp	0	0%
Autumn season	16	*E. coli*	10	62.5%
		Shigella spp	2	12.5%
		Enterobacter spp	0	0%
		Salmonella spp	3	18.75%
		Bacillus spp	1	6.25%

3.3 Results of cultural examination

Cultural and morphological biochemical properties of isolated *E coli*, *Shigella* spp., *Enterobacter* spp., *Samonella* spp. and *Bacillus* spp., are shown in Table 5.

Table 5: The result of cultural characteristics of bacteria which were isolated from faecal sample of diarrhoeic calves

Name of bacteria	Staining characteristics	Media for cultivation	Colony characteristics
1. *E coli*	Gram negative Rod shape pink Color	Nutrient agar	Large, mucoid, white gray colonies
		Mac conkey agar	Mac Conkey Pink color smooth transparent raised colonies
		EMB agar	Greenish colonies with metallic sheen.

2. *Shigella* spp.	Gram negative Rod shape, single Or pairs	Nutrient agar	Circular grayish or coloress smooth and translucent colonies.
		Mac conkey agar	Colorless colony
		SS agar	Pale colony
		Hektoen Enteric agar	Greenish Blue
		XLD agar	Bright pink or red appearance
3. *Enterobacter* spp	Gram negative Bacilli shape	Nutrient agar	white gray Colony form
		Mac conkey agar	Pink color , smooth, transparent raised colony
		EMB agar	Large mucoid colony pink to purple.
4. *Samonella* spp	Gram negative Rod shape	XLD agar	Bright pink or red appearance
		SS agar	Opaque smooth round colony
		Mac conkey agar	Non lactose farmenter colorless colony
		BGA agar	Reddish pink color colony
5. Bacillus spp	Gram positive Rod shape	Nutrient agar (petridish)	Medusa head like growth
		Nutrient agar (stab culture)	Inverted fir tree like colony

3.4 Results of Biochemical test

Biochemical properties of isolated *E coli*, *Shigella* spp., *Enterobacter* spp., *Samonella* spp. and *Bacillus* spp. are shown in Table 6.

Table 6: Biochemical test for *E. coli, Enterobacter* spp *and Shigella* spp, *bacillus* spp and *Salmonella* spp

BACTERIA	INDOLE	MR	VP	TSI	SCU	MIU	OXIDASE	CATALASE
E. coli	+	+	-	A/A	-	+	-	+
Enterobacter spp	-	-	+	A/A	-	+	-	+
Shigella spp	+	+	+	ALK/A	-	Non motile	-	+
Bacillus spp	+	+	+	A/A	-	Non motile	+	+
Salmonella spp	+	+	-	ALK/A	-	Motile	-	+

Legends: + = Positive; - = Negative; MR =Methyl –Red; VP = Voges-Proskauer; TSI = Triple Sugar iron, MIU= Motility Indole Urea

3.5 Result of PCR amplification of *E. coli and Salmonella spp* DNA gene with primers

Molecular characterization of *E. coli* and *Salmonella spp* isolated from fecal sample were performed by polymerase chain reaction (PCR). The PCR assay was able to amplify 585 bp and 284 bp fragments of the targeted gene from the genomic DNA of E. coli (Fig-1)and *Salmonella spp* (Fig-2)

respectively.

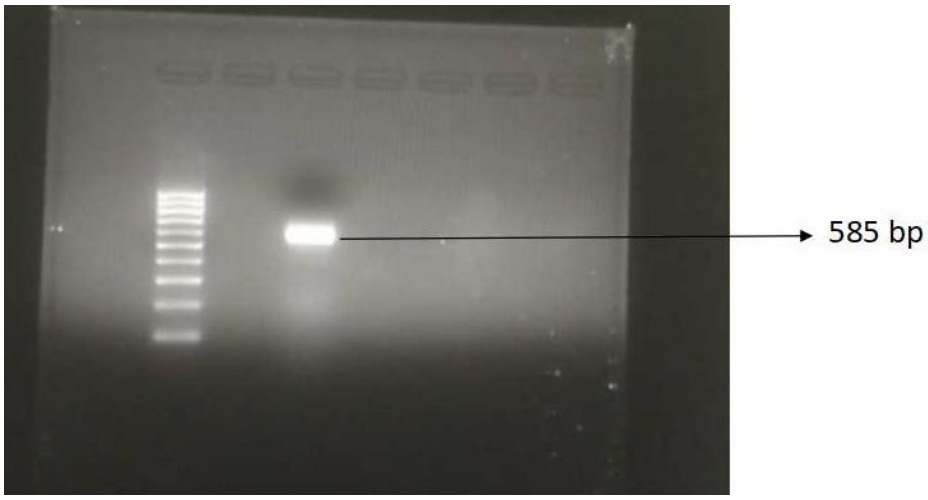

Figure 1: PCR amplification of 16SrRNA gene from *E coli* isolates, L=100bp; positive *E. coli* showing band at 585 bp

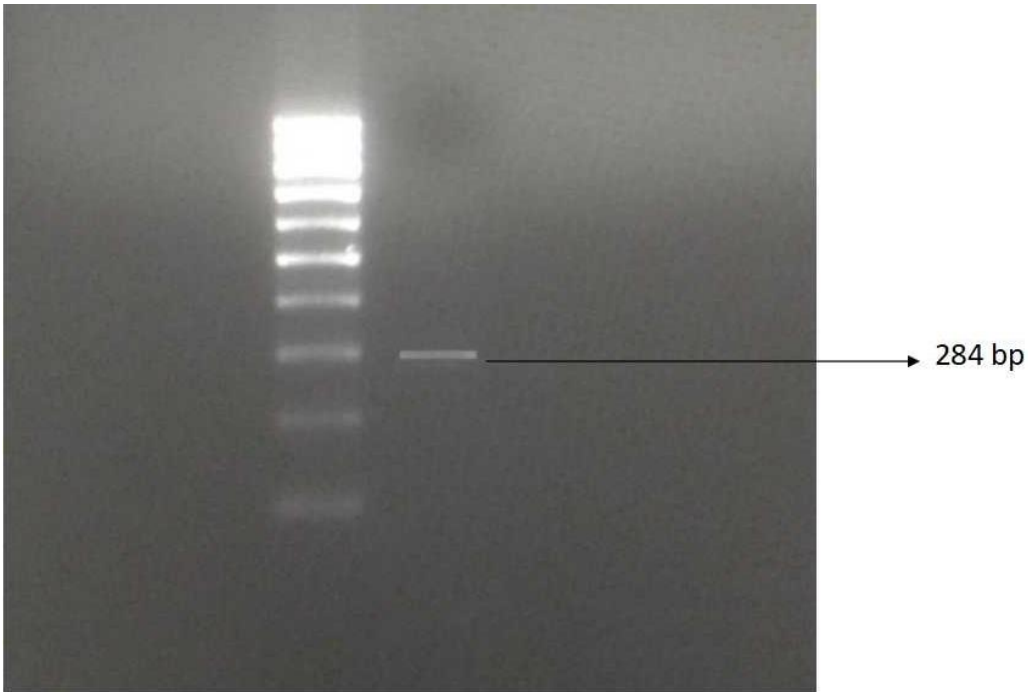

Figure 2: PCR amplification of invA gene from *Salmonella spp* isolates, L=100bp; positive *Salmonella* spp showing band at 284 bp

3.6 Antibiotic sensitivity test

Antibiotic sensitivity pattern of *E coli*, *Shigella* spp., *Enterobacter* spp., *Samonella* spp. and *Bacillus* spp. are shown in Table 7.

3.6.1 Antibiotic sensitivity pattern of *E.coli*

Antibiotic sensitivity test revealed that *E. coli* isolates were resistance to amoxicillin, ampicillin erythromycin, and cephalexin but was sensitive to cotrimoxazole, penicillin, tetracycline, doxycycline, azithromycin, gentamycin and levofloxacin.

3.6.2 Antibiotic Sensitivity pattern of *Shigella* spp

The isolates of Shigella spp. were resistant to ampicillin, cloxacillin and cephalexin but showed sensitivity to tetracycline and gentamycin, penicillin and cefixime.

3.6.3 Antibiotic sensitivity pattern of *Enterobacter* spp

The isolates of Enterobacter spp. were resistant to ampicillin, amoxycillin, and cephalexin, but sensitive to tetracycline, azithromycin, gentamycin and cefixime.

3.6.4 Antibiotic sensitivity pattern of *Salmonella* spp

The isolates of Salmonella spp. showed resistance to amoxicillin, ampicillin, erythromycin and cefalexin but exhibited sensitivity to tetracycline and streptomycin.

3.6.5 Antibiotic sensitivity pattern of *Bacillus* spp

The isolates of Bacillus spp. exhibited resistance to amoxicillin, ampicillin, erythromycin, cephalexin and bacitracin but showed susceptibility to penicillin, streptomycin, azythromycin, tetracycline and gentamycin.

Table 7: Antibiotic sensitivity pattern of *E coli*, *Shigella* spp., *Enterobacter* spp., *Samonella* spp. and *Bacillus* spp.

Antibiotics disc with concentration (µg/disc)	*E. coli* (4)		*Shigella* spp (4)		*Enterobacter* spp (4)		*Salmonella* spp (4)		*Bacillus* spp (4)	
	%R	%S	%R	%S	%R	%S	%R	%S	%R	%S
Cotrimoxazole (25)	0(0)	4(100)	NT	NT	NT	NT	NT	NT	NT	NT
Erythromycin(15)	4(100)	0(0)	4(100)	0(0)	4(100)	0(0)	4(100)	0(0)	4(100)	0(0)
Penicillin(10)	1(25)	3(75)	2(50)	2(50)	NT	NT	NT	NT	1(25)	3(75)
Tetracycline(10)	1(25)	3(75)	1(25)	3(75)	2(50)	2(50)	1(25)	3(75)	0	100
Amoxycillin(11)	4(100)	0(0)	4(100)	0(0)	4(100)	0(0)	4(100)	0(0)	4(100)	0(0)
Ampicillin(10)	4(100)	0(0)	4(100)	0(0)	4(100)	0(0)	4(100)	0(0)	4(100)	0(0)
Cephalexin(10)	4(100)	0(0)	4(100)	0(0)	4(100)	0(0)	3(75)	1(25)	4(100)	0(0)
Doxycycline(30)	0(0)	4(100)	NT	NT	NT	NT	NT	NT	NT	NT
Azythromycin(12)	0(0)	4(100)	NT	NT	0(0)	4(100)	NT	NT	1(25)	3(75)
Gentamycin(35)	1(25)	3(75)	1(25)	3(75)	2(50)	2(50)	NT	NT	1(25)	3(75)
Levofloxacin(5)	0(0)	4(100)	NT	NT	NT	NT	NT	NT	NT	NT
Cefixime(5)	NT	NT	0(0)	4(100)	2(50)	2(50)	NT	NT	NT	NT
Cloxacillin(5)	NT	NT	4(100)	0(0)	NT	NT	NT	NT	NT	NT
Streptomycin(10)	NT	NT	NT	NT	NT	NT	1(25)	3(75)	0(0)	4(100)
Bacitracin(5)	NT	NT	NT	NT	NT	NT	NT	NT	4(100)	0(0)

Legends: S = Sensitive, R = Resistance, % = Percentage and NT = Not Tested.

In our study, the prevalence of *E. coli in* diarrhoeic calf was 53.3% lower than the findings by other researchers Hemashenpagam et al. (2009) and Valdivia-Andy et al., (2000), who reported 75% and

63.7% respectively. The prevalence *Shigella* spp. in our study was in line with the study of Meshref et al., (2021) who reported 16% prevalence of *Shigella* spp. The lower prevalence was reported by Livio et al. (2014), who detected *Shigella flexneri* (1.63%), *Shigella sonnei* (1.32%), and *Shigella dysenteriae* (1.14%). In this study, we found *Salmonella* spp. in 20% of cases whereas lower prevalence was reported by de Vasconcelos et al. (2021) and Anju et al. (2024). While higher incidences of Salmonella isolated from diarrhoeic calves were also described by Sohidullah et al. (2016). We found *Enterobacter* spp. in 8.8% of samples, lower than the findings by Okela et al. (2010) who reported 26.1% prevalence *of Proteus mirabilis* and other Enterobacteriaceae. In this study, *Bacillus* spp. was found in only 2.2% of samples. In contrast, Samad et al. (2004) reported *Bacillus* spp. as most prevalent (87%), followed by *E. coli* (37%) and *Salmonella* spp. (5%). In this study, female calves were found to be more affected and diarrhoea cases rose during the rainy season. These results are similar with the study of Sohidullah et al. (2016). In our experiment, calves under six months were more susceptible, which differs from the study of Sohidullah et al. (2016) who reported that older calves were more vulnerable. Molecular identification by PCR confirmed the presence of *E. coli* with 585 band and *Salmonella* spp. with 284 band supported by the results of Schippa et al. (2010).

The antibiotic sensitivity test findings are in agreement with the studies by Srivani et al. (2017), and Shahrani et al. (2014). Antibiogram testing using disc diffusion method by Jahan et al. (2013), highlights the increasing issue of antimicrobial resistance in Bangladesh. This study indicates that *E. coli*, *Salmonella*, and *Shigella* as major bacterial agents of calf diarrhea. Effective surveillance, rational antibiotic use, and improved farm management are urgently needed to combat this issue.

4. CONCLUSIONS

The isolation of *E. coli*, *Shigella* spp., *Enterobacter* spp., *Salmonella* spp., and *Bacillus* spp. poses a significant public health concern due to their zoonotic potential and multidrug resistance (MDR) profiles. Proper selection of antibiotics can reduce treatment costs and shorten illness duration. Overall, the study provides valuable insights for veterinarians in selecting effective antibiotics and for policymakers aiming to control antimicrobial resistance in livestock.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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