2021, Scienceline Publication
World's Veterinary Journal
World Vet J, 11(3): 395-401, September 25, 2021
ISSN 2322-4568
DOI: https://dx.doi.org/10.54203/scil.2021.wvj51

Molecular Detection and Antibiotic Sensitivity of *Salmonella* Species Isolated from Goat Feces in Sylhet District of Bangladesh

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ORIGINAL ARTICLE
pii: S232245682100051-11
Received: 27 June 2021
Accepted: 10 August 2021

ABSTRACT

The present study aimed at the molecular detection of *Salmonella* species from feces of goats and the characterization of the isolated *Salmonella* by biochemical and antimicrobial sensitivity techniques. A total of 220 goat feces samples were collected, of which 27 (12.27%) were positive for *Salmonella* by conventional culture methods and 20 (9.09%) by biochemical and PCR techniques. The prevalence was higher in goats under one year of age (20%), compared to older animals aged one to two years (7.8%) and more than two years of age (4.7%), respectively. Moreover, the prevalence of diarrheic goats was significantly higher (38.46%) than healthy animals (2.76%). DNA was extracted from *Salmonella* strains and amplified by PCR using the specific primers of *Salmonella* invasion gene (*invA gene*). The antibiotic sensitivity test indicated that Ciprofloxacin (100 percent sensitivity), Gentamycin (100 percent sensitivity), and Neomycin (100 percent sensitivity) were the most effective antibiotics for the majority of *Salmonella* isolates. On the other hand, *Salmonella* isolates were found to have substantially high resistance to Erythromycin (100%), Amoxicillin (100%), Trimethoprim-Sulfamethoxazole (81.48%), Streptomycin (62.96%), and Tetracycline (55.56 percent). Since the rate of *Salmonella* carriers was relatively high, eating goat meat could increase the risk of foodborne salmonellosis.

Keywords: Antibiotic sensitivity, Goat isolation, PCR detection, *Salmonella*

INTRODUCTION

Capra aegagrus hircus, popularly the black Bengal goat domesticated in Bangladesh, preliminary reared for chevon by millions of poor women and landless peoples to increase poverty (Rahmanet al., 2017). Salmonellosis is one of the most important foodborne zoonoses all over the world. The pathogen has been isolated from the feces, lungs, and liver of goats worldwide (Ziinoet al., 2009). *Salmonella* can infect a wide range of animals, including poultry and other birds, horses, cattle, pigs, sheep, goats, dogs, cats, and reptiles (Songer and Post, 2004). These Gram-negative bacteria are mainly transmitted through contaminated food and water and are clinically characterized by septicemia and enteritis. However, the possibility of the upper respiratory tract bacterial transmission in the animal has been reported (Garg and Sharma, 1979). Salmonellosis in goats could occur at all ages during the year in both males and females and is responsible for the considerable loss of kids and may even cause abortion in adults (Arrudaet al., 2004). Salmonellosis is the most frequent disease in goats among the most common bacterial zoonotic diseases characterized by diarrhea (Radostits et al., 2007; Kahn et al., 2010). Detection of these organisms could be a serious public health concern (Adesiji et al., 2011).

The conventional laboratory cultural methods take a long time to achieve a positive or negative result. Molecular techniques, such as genetic probes and Polymerase Chain Reaction (PCR) enable rapid, sensitive, and pathogen identification in the atmosphere with precision (Josephson et al., 1991). Without displaying any clinical symptoms of salmonellosis, the infected animals may shed the *Salmonella* organism in their feces. As a result, a rapid, specific, and responsive *Salmonella* detection method is critical for animal and human health and as well as the diagnostic industry. Polymerase chain reaction detection of Salmonella in clinical samples from animals is faster than traditional culture techniques, with a sensitivity and specificity of 100 percent as compared to culture techniques. The method could be applied for rapid routine diagnosis (Stone et al., 1994). The objective of the present study was to estimate the prevalence of *Salmonella* in both diarrheic and healthy goats and to confirm it by PCR detection.

To cite this paper: Sabur MA, Das MR, Uddin MB, Rahman MM, Islam MR, Chowdhury MSR and Hossain MM (2021). Molecular Detection and Antibiotic Sensitivity of *Salmonella* Species Isolated from Goat Feces in Sylhet District of Bangladesh. *World Vet. J.*, 11 (3): 395-401. DOI: https://dx.doi.org/10.54203/scil.2021.wvj51

MATERIALS AND METHODS

Ethical statement

This study was approved by the Ethical Committee of Sylhet Agricultural University, Sylhet-3100, Bangladesh, as well as by mutual (verbal) understanding of the respective farm owners.

Geo-location of the study area

The present study was conducted in the Sylhet district of Bangladesh, which is located in the northeastern part of Bangladesh. The average maximum and minimum temperatures were 38°C and 7°C, respectively. The present study was conducted over a 12-month period from January to December 2018 and the data regarding climatic conditions were retrieved from Regional Meteorological Centre, Sylhet, Bangladesh.

Animals and samples

A total of 180 black Bengal goats of different ages from the Government Goat Development Farm, Sylhet, and 40 goats from individual farmers in Sadar Upazilla, Sylhet, constituted the study population. Fecal samples were collected directly from the rectum of apparently healthy goats and stored refrigerated at 4°C until processed for nucleic acid purification within 24 hours of collection. A cross-sectional study was conducted to isolate and identify the *Salmonella* from the feces of goats.

Salmonella isolation and identification

The conventional bacteriological methods were used to isolate the *Salmonella* from the samples as described (Khan et al., 2021). The samples were inoculated into the nutrient broth and incubated at 37°C for 24 hours. Fecal samples were subjected to initial nutrient pre-enrichment and incubated, and 0.5 mL was transferred to 10 mL Tetrathionate Broth (Merck) and incubated at 37°C for 24 hours. Pre-incubated brilliant green and Xylose Lysine Deoxycholate (XLD) agar was streaked with a loop of each enrichment broth and incubated. The plates were then analyzed for the existence of *Salmonella* colonies. Suspected colonies were inoculated into TSI (Triple Sugar Iron agar), peptone water, Simmon's Citrate, Urea medium, and MR-VP.

DNA extraction and PCR amplification

The DNA extraction was performed according to the manufacturer's instruction using the Addprep genomic DNA extraction kit (Addbio Inc. Ltd., Korea). These eluted DNA samples were stored at -80°C until further analysis. The PCR analysis was performed to detect the *Salmonella* invasion gene (*invA* gene) according to the manufacturer's instruction (Addbio Inc. Ltd., Korea). PCR assay performed in the thermal cycler TC1000G PCR System® (DLAB Scientific Inc., USA) with a heated lid. The cycling conditions included 50°C for 3 minutes (UDG Reaction), 95°C for 10 minutes (Initial Denaturation), 35 cycles of 95°C for 30 seconds (denaturation), 68°C for 45 seconds (annealing), and 72°C for 5 minutes for final extension (Khan et al., 2021). For the detection of *Salmonella*, the primers of invS-F (5'-TAA TGCCAGACGAAAGAGCGT-3') and invS-R (5'-GATATTGGTGTTTATGGG GTCGTT-3') were used (Khan et al., 2021). All reaction mixtures, including the negative control and *Salmonella* positive DNA, were tested in duplicate in the same run of PCR assay. PCR products were analyzed on 1.8% agarose gels stained with RedSafe™ (iNtRON Biotechnology, Korea) Nucleic Acid Staining Solution (20,000×), photographed, and stored as a digital image.

Antimicrobial sensitivity testing

The qualitative agar diffusion method (Kirby-Bauer method) was used for antibiotic sensitivity testing employing Mueller Hinton agar. The *in vitro* antibiotic sensitivity test was determined by the standard disc diffusion procedure according to the Clinical and Laboratory Standard Institute (CLSI, 2012). The antibiotic discs used in the present study were Erythromycin (ERY), Gentamycin (GEN), Streptomycin (STR), Amoxicillin (AMX), Ciprofloxacin (CIP), Tetracycline (TET), Neomycin (NM), and Trimethoprim-Sulfamethoxazole (SXT). The MIC (MIC50 and MIC90) was determined for each of the antibiotics used and the sensitivity or resistance was determined according to the protocol described (CLSI, 2012).

Statistical analysis

Microsoft Excel was used for the descriptive statistics. Chi-square tests were used to assess the significance of differences in prevalence between age, sex, and health status of the animals. P values less than 0.05 were considered significant using Chi-square tests (SPSS Inc., Chicago, IL, USA).

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RESULTS

Prevalence of *Salmonella* in goats

From January to December 2018, a total of 220 goat feces samples (180 from Government Goat Development Farm, Sylhet, Bangladesh, and 40 from different private goat farms in Sadar Upazilla in Sylhet) were collected for isolation and identification and molecular detection of *Salmonella* and antibiotic sensitivity testing. Among 220 samples, *Salmonella* isolates were detected in 27 (12.27%) samples using the conventional cultural method. The isolates were followed by biochemical identification and PCR assays in which 20 (9.09%) samples indicated *Salmonella* positive (Table 1). Furthermore, the prevalence of *Salmonella* in the personal private farm was lower (3.89%) than the Goat development farm, Sylhet, which was estimated at 7.20% (Table 2).

Cultural and morphological characterization

All *Salmonella* colonies in Mac Conkey agar plates appeared as non-lactose fermenters, colorless and transparent (Figure 1C), and *Salmonella* turbidity produced on nutrient broth (Figure1A). The isolates of *Salmonella* grown in *Salmonella-Shigella* (SS) agar plates indicated characteristic black-centered colonies (Figure 1B), but isolates were absent in the black dot. In microscopic analysis, the thin smears prepared with the colony from SS agar for Gram's staining showed Gram-negative, pink-colored, tiny rod-shaped appearance arranged in single or paired or short-chain (Figure 1D).

Biochemical characterization

Due to the cultural and morphological properties, all suspected *Salmonella* colonies were subjected to selected biochemical tests, including indole formation (Figure 2E), methyl red (Figure2B), and Voges Proskauer reaction (Figure 2C), citrate utilization, and triple sugar iron agar. Of the 27 suspected *Salmonella* colonies, 20 were confirmed by biochemical test results. On TSI slants, most of the *Salmonella* isolates indicated fermentation of glucose (Figure 2A), gas production from glucose, H_2S formation, but none of the isolates fermented either lactose or sucrose (Figure 2D).

Antimicrobial susceptibility

The antibiotic sensitivity test indicated that the highest number of *Salmonella* isolates were sensitive to Ciprofloxacin (100%), Gentamycin (100%), and Neomycin (100%). Alternatively, there was significantly high resistance in *Salmonella* isolates to Erythromycin (100%), Amoxicillin (100%), Trimethoprim-Sulfamethoxazole (81.48%), Streptomycin (62.96%) followed by Tetracycline (55.56%, Table 3).

Detection of *Salmonella* by the polymerase chain reaction

All *Salmonella* suspected cultures subjected to PCR amplification generated a product of approximate molecular size 100 base per (bp) (*invA* gene) according to the manufacturer's instruction (AddBio Inc., Korea). A 100 bp DNA marker was used as a molecular weight marker (AddBio Inc., Korea). The band size detected in isolated *Salmonella* was consistent as analyzed by agarose gel electrophoresis (Figure3). The estimated prevalence of *Salmonella* by using PCR was 9.09% (20/220) in goat feces.

Table 1. Overall prevalence of *Salmonella* isolates in goats according to biochemical identification at Sylhet district of Bangladesh

Factors	Animal	Number of examined animals	Positive fecal samples	Prevalence	p value
Age	Up to 1 year	45	9	20%	< 0.01
	1 - 2 years	90	7	7.8%	
	> 2 years	85	4	4.7%	
Total		220	20	9.09%	
Sex	Male	150	9	6.0%	0.321
	Female	70	11	15.71%	
Total		220	20	9.09%	
Health status	Apparentlyhealthy	181	5	2.76%	< 0.01
	Diarrheic	39	15	38.46%	
Total		220	20	9.09%	

Level of significance $p < 0.05$

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Table 2. Farm-level prevalence of *Salmonella* isolates in goats according to conventional cultural methods and biochemical identification in Goat development farm, Sylhet

Farm type	Total number of animal	Number of positive by conventional culture methods	Number of positive by biochemical tests	Prevalence	
				Conventional methods	Biochemical /PCR tests
SGDF*	180	16	13	8.89%	7.20%
IPF	40	11	07	27.5%	3.89%
Total	220	27	20	12.27%	9.09%

*****Goat development farm, Sylhet, IPF**:** Individual private farm

Table 3. Overall susceptibility and resistance patterns of *Salmonella* isolates to selected antibiotics according to clinical and laboratory standard institute

Antimicrobial drug	Antimicrobial class	Abbreviation	Susceptible	Resistant*
Amoxicillin	β-lactams	AMX	-	100%
Ciprofloxacin	Quinolones	CIP	100%	-
Trimethoprim-Sulfamethoxazole	Folate pathway inhibitors	SXT	18.52%	81.48%
Erythromycin	Macrolids	ERY	-	100%
Gentamicin	Aminoglycosides	GEN	100%	-
Neomycin	Aminoglycosides	NM	100%	-
Streptomycin	Aminoglycosides	STR	37.04%	62.96%
Tetracycline	Tetracyclines	TET	44.44%	55.56%

*****_Salmonella_ isolates that indicate moderate resistance to some antibiotics were considered resistant according to the CLSI recommendations.

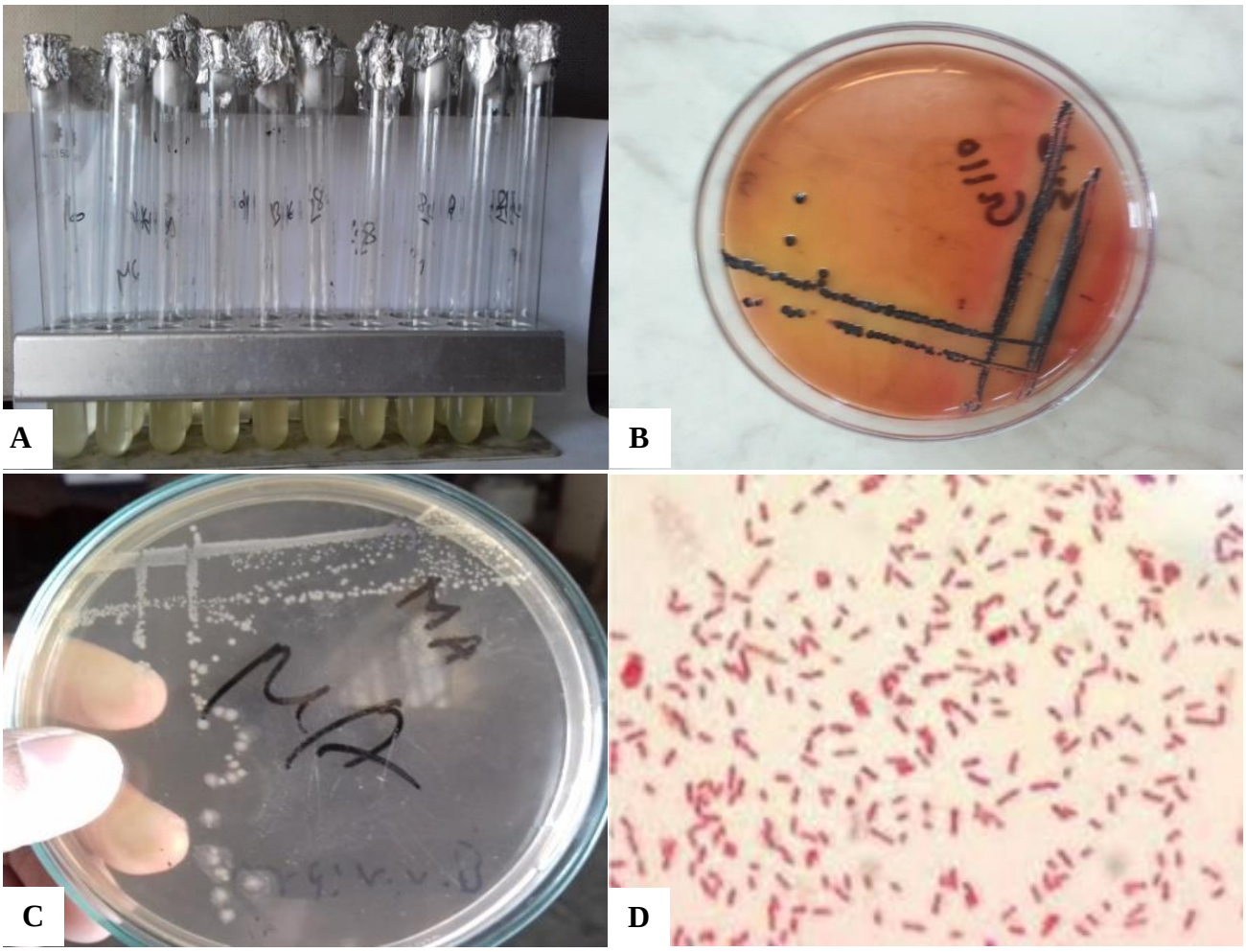

Figure1. A: Nutrient broth *Salmonella* produced turbidity, **B:** Black centered colony on *Salmonella-Shigella* (SS) agar, **C:** Colorless colonies on MacConkey agar, **D:** Gram staining under the microscope revealed Gram-negative, small rods arranged in single, paired, or clustered characteristics

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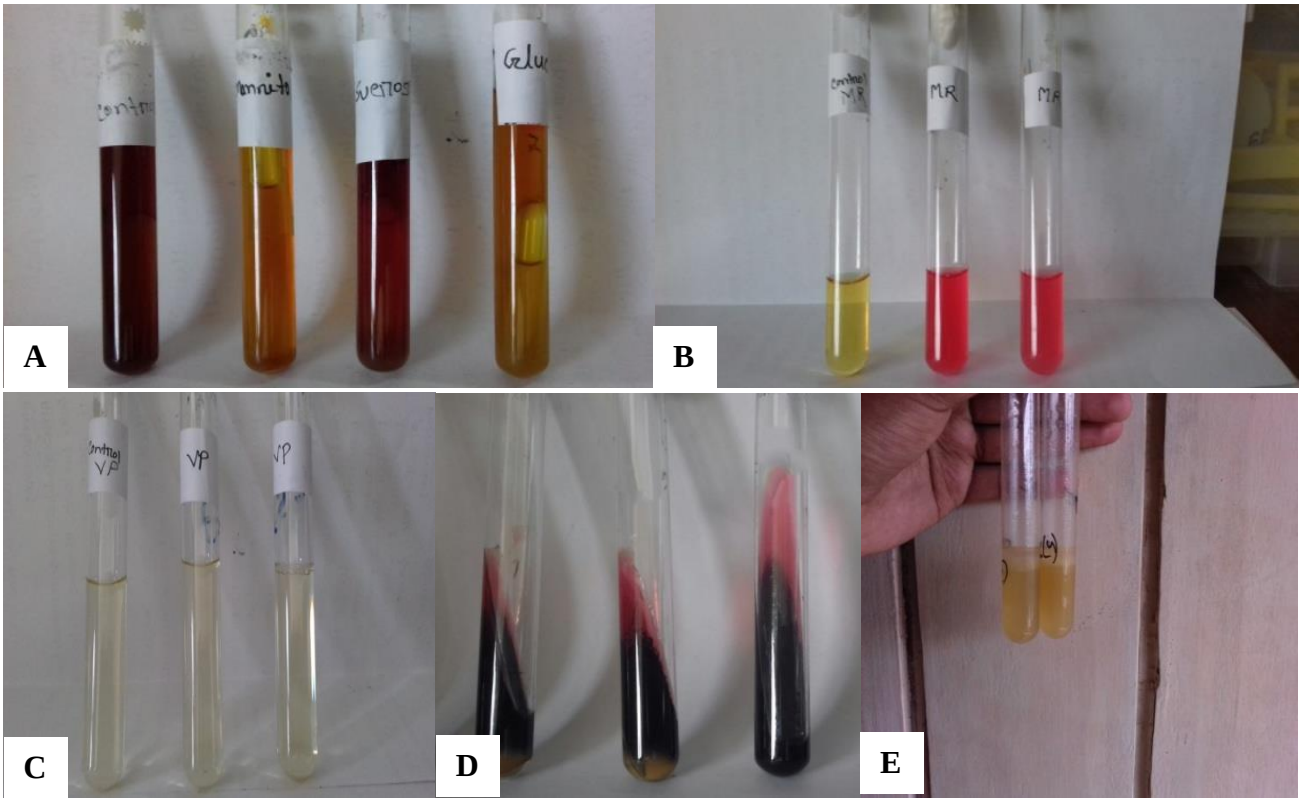

Figure 2. A: Sugar fermentation (Glucose and Mannitol +ve with the production of acid and gas, Sucrose –ve), **B:** MR Test (positive, red color), **C:** VP Test (Negative, no color change), **D:** TSI test (positive, Slant: red, butt: black); **E:** Indole Test (Negative, no color change)

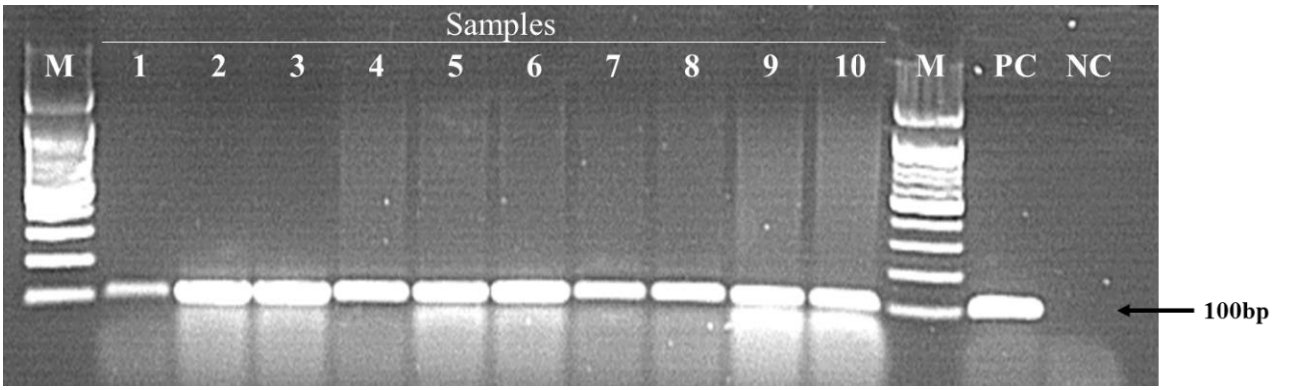

Figure 3. Results of PCR under UV illuminator (bands corresponding to the 100 bases per marker line indicated representative of *Salmonella* positive samples (1~10). M: Marker, NC: Negative control, PC: Positive control

DISCUSSION

The isolation and identification procedure included cultural examination, morphological examination, staining properties, biochemical tests, and molecular detection by PCR technique. In the present study, a total number of 220 feces samples from goats were collected by Sylhet Goat Development Farm (SGDF) and Individual Private Farm (IPF) in Sylhet. The bacteriological examination revealed the isolation of *Salmonella* organisms from goats with an estimated prevalence of 12.27% (27/220). Several studies reported a similar prevalence of *Salmonella* in goat feces (Teklu and Negussie, 2011; Saha et al., 2013; Bosilevac et al., 2015). The prevalence of *Salmonella* was higher in young (up to 1 year) and sick (diarrheic) animals, estimated to be 20% and 38.46%, respectively. The age-related result of the present study is consistent with the findings of other researchers (Saha et al., 2013; Mahmood et al., 2014). The findings also coincide with the results of Hunduma et al. (2010),who stated diarrhea as the major problem in goats with a higher prevalence of 42.2%.

A total of 20 (9.09%) fecal samples were PCR positive for *Salmonella*, which is higher than others who reported only 1.05% PCR positive cases (Esmaeili and Rahmani, 2016). Similar studies were conducted on cattle in Bangladesh and found 8.50% positive by biochemical and PCR (Khan et al., 2021). In contrast, Teklu and Negussie (2011)found a higher prevalence of *Salmonella* in sheep and goats in an export abattoir in Ethiopia. They reported that 7.7% and 11.7%

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of the sheep and goats were positive for *Salmonella*. Gallegos-Robles et al. (2009) isolated and detected the *Salmonella* from cattle feces using microbiological and PCR methods, estimated the prevalence at 55% and was much higher than in the present study. For the detection of *Salmonella* genus using the PCR technique, it was found that all PCR products of the isolated positive control resulted in 100 bases per amplified fragment. The *invA* gene has been reported to be present in all strains and clinical isolates of *Salmonella* (Dahshan et al., 2010). The detection of *Salmonella* in clinical samples by PCR results in a faster than conventional culture methods (Stone et al., 1994). The conventional method of isolating *Salmonella* is more laborious and requires more manpower (Van der Zee and Veld, 2000). There are a very small number of viable organisms in the feces that may fail to grow in artificial laboratory media. Molecular tests were most successful than conventional micro-biological techniques (Jungkind, 2001).

The antibiotic disk diffusion indicated that some isolates were resistant to Streptomycin (62.96%), Amoxicillin (100%), Erythromycin (100%), trimethoprim/sulfamethoxazole (81.48%), and Tetracycline (55.56%). On the other hand, the highest number of *Salmonella* isolates was sensitive to Ciprofloxacin (100%), Gentamycin (100%), and Neomycin (100%). Isolation of *Salmonella* from food and water to antibiotic resistance is of great importance in the case of public health. While these infections are caused by animal feces, it is also essential to identify the antibacterial agents that are used to treat or prevent infections, as well as the promoters that are created (Graham et al., 2007). It has been reported that a high percentage of *Salmonella* isolates from healthy and diseased animals were resistant to two or more antimicrobial agents (Esaki et al., 2004), which explains the high spread of these organisms through feces of apparently healthy animals and their wide dissemination in the environment. There have been reports of the release of *S. Typhimurium* in a pig plant (Tanaka et al., 2014). Although the animals were found to be healthy, the release was very high several days after inoculation; therefore even normal feces could be a source within the herd infection. It has been reported that multi-resistant S. *Enteritidis* (resistant to two or more antimicrobial agents) with different patterns could reach up to 51.6% (de Oliveira et al., 2005).

The most common patterns of resistance were sulfamethoxazole, streptomycin, and tetracycline while ciprofloxacin resistance was the least common. In Japan, *S. Typhimurium* isolated from various animal species indicated that 20% of the isolates were resistant to ampicillin and 24% to tetracyclines (Esaki et al., 2004). In 2008, 31 Salmonella strains were isolatedfrom 12 different serovars forms in cattle, and the transmission of microbial resistance from *S. Heidelberg* to *S. Typhimurium* and bacteriophages that are resistant to several beta-lactam antibiotics and tetracycline blaCMY-2, tet (A), and tet (B), was demonstrated (Zhang and LeJeune, 2008). Later, 58% resistance to trimethoprim/sulfamethoxazole and 56% to tetracycline, followed by ampicillin and amoxicillin were reported (Yang et al., 2010). In Chile, in a preliminary study, 20.5% of the *Salmonella* strains isolated mainly from pigs, indicated multidrug resistance (MDR),with oxytetracycline being the drug with the highest resistance (69.1%, Junodet al., 2013).

CONCLUSION

From the findings of the present study, it could be concluded that *Salmonella* is an important cause of diarrhea in goats with salmonellosis in Bangladesh. Therefore, a rapid and proper diagnosis could prevent harm inflicted on the livestock industry. In this regard, the most accurate and quick diagnostic methods are required. The molecular basis of *Salmonella* identification techniques, such as the use of the *invA* gene-specific PCR method, could be useful in diagnostic and research laboratories. Ciprofloxacin, Gentamycin, and Neomycin might be the best choice among the antibiotics available on the market. The variation in antibacterial susceptibility or resistance pattern was also observed in the present study. This study suggests the strategic use of antibiotics for the control of *Salmonella* infections in animals.

DECLARATIONS

Acknowledgment

The authors acknowledge the Sylhet Agricultural University Research System (SAURES) for funding this research, and the respective farm owners for data support.

Competing interests

The authors have declared no conflict of interest regarding the publication of this research.

Authors' contribution

This manuscript is from the master's degree thesis of Md. Abdus Sabur. Md. Mukter Hossain designed the study. Md. Abdus Sabur and Mouri Rani Das conducted the experiments. All authors were involved in data interpretation, write up and final approval of the manuscript.

Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by the authors.

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To cite this paper: Sabur MA, Das MR, Uddin MB, Rahman MM, Islam MR, Chowdhury MSR and Hossain MM (2021). Molecular Detection and Antibiotic Sensitivity of *Salmonella* Species Isolated from Goat Feces in Sylhet District of Bangladesh. *World Vet. J.*, 11 (3): 395-401. DOI: https://dx.doi.org/10.54203/scil.2021.wvj51