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ISOLATION, MOLECULAR IDENTIFICATION AND ANTIBIOTIC SENSITIVITY OF *SALMONELLA* FROM BUFFALO FECES IN SYLHET DISTRICT OF BANGLADESH

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Abstract

Salmonella is a widely distributed foodborne pathogen affecting humans and animals around the globe. This cross-sectional bacteriological study was aimed at isolation of *Salmonella* from fecal samples of buffalos in Sylhet district (Upazilas Jaintapur and Fenchuganj) of Bangladesh, their molecular confirmation, and learning their antibiotic sensitivity patterns. A total of 334 fecal samples were collected using a simple random sampling technique. Standard conventional bacteriological culture and biochemical tests were done to isolate and confirm *Salmonella*. The isolates were confirmed with polymerase chain reaction (PCR). In this study, *Salmonella* was isolated from 56 samples (16.77%) using bacteriological culture methods and biochemical tests, and all isolates were confirmed in PCR tests. The prevalence of *Salmonella* was estimated to be 17.57% and 13.93% in Jaintapur and Fenchuganj Upazilas, respectively. Buffalo calves under one year of age had a higher prevalence (24.32%) than older animals aged 1 - 2 (18.62%), 2 - 4 (13.25%), and > 4 years (10.67%). Furthermore, the prevalence was substantially higher in diarrheic animals (72.22%) than in their healthy counterparts (13.60%). It did not vary significantly ($p > 0.05$) between animals from Jaintapur and Fenchuganj Upazilas. Likewise, no significant difference ($p > 0.05$) in *Salmonella* isolates was detected between different age groups. The results revealed that the isolation rate of *Salmonella* was significantly ($p < 0.05$) higher in female and diarrheic animals.

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The most effective antibiotics against the majority of *Salmonella* isolates were gentamycin (100%), levofloxacin (100%), and ciprofloxacin (76.67%). On the other hand, *Salmonella* isolates were highly resistant to tetracycline (100%), ampicillin (87.5%), and streptomycin (78.6%). Pathogenic microorganisms in feces are a potential risk to public health due to environmental and animal food contamination. Therefore, infection control and establishing strategic antibiotic therapy should be a priority.

Key words: Bangladesh, buffalo feces, *Salmonella*, *inv*A gene, antibiotics sensitivity

IZOLACIJA, MOLEKULARNA IDENTIFIKACIJA I ANTIBIOTSKA OSETLJIVOST *SALMONELLA* IZ BIVOLJEG IZMETA U OKRUGU SILHET, BANGLADEŠ

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Kratak sadržaj

Salmonella je široko rasprostranjen patogen koji se prenosi hranom i pogađa ljude i životinje širom sveta. Ova bakteriološka studija poprečnog preseka imala je za cilj izolaciju *Salmonella* iz uzoraka fecesa bivola u okrugu Silhet (distrikti Jaintapur i Fenchuganj) u Bangladešu, njihovu molekularnu potvrdu i osetljivost na antibiotike. Ukupno 334 uzorka fecesa prikupljena su jednostavnom tehnikom slučajnog uzorkovanja. Standardne konvencionalne bakteriološke metode i biohemijski testovi su urađeni da bi se izolovala i potvrdila *Salmonella*. Izolati su potvrđeni lančanom reakcijom polimeraze (PCR). U ovoj studiji *Salmonella* je izolovana iz 56 uzoraka (16,77%) metodom kultivacije i biohemijskim testovima, a svi izolati su potvrđeni i PCR testovima. Prevalencija *Salmonella* je iznosila 17,57% i 13,93% u Jaintapuru i Fenchuganj distriktu. Mladunčad bivola mlađih od jedne godine imala

su veću prevalenciju (24,32%) od starijih životinja uzrasta 1 - 2 (18,62%), 2 - 4 godine (13,25%) i > 4 godine (10,67%). Osim toga, prevalencija je bila znatno veća kod životinja sa dijarejom (72,22%) nego kod zdravih životinja (13,60%). Prevalencija nije značajno varirala ($p > 0,05$) između životinja iz Jaintapura i Fenchuganj distrikta. Isto tako, nije otkrivena značajna razlika ($p > 0,05$) u izolatima *Salmonella* između različitih starosnih grupa. Rezultati su otkrili da je stopa izolacije *Salmonella* značajno ($p < 0,05$) veća kod ženki i životinja sa dijarejom. Najefikasniji antibiotici protiv većine izolata *Salmonella* bili su gentamicin (100%), levofloksacin (100%) i ciprofloksacin (76,67%). S druge strane, izolati *Salmonella* su bili visoko otporni na tetraciklin (100%), ampicilin (87,5%) i streptomicin (78,6%). Patogeni mikroorganizmi u fecesu predstavljaju potencijalni rizik za javno zdravlje zbog kontaminacije životne sredine i hrane za životinje. Stoga, kontrola infekcije i uspostavljanje strateške antibiotske terapije treba da budu prioritet.

Ključne reči: Bangladeš, bivolji feces, *Salmonella*, *inv*A gen, antibiotska osetljivost

INTRODUCTION

Salmonella, a genus of the family *Enterobacteriaceae*, is a major cause of foodborne disease of public health significance both in developed and developing countries (Gebeyehu et al., 2022). All across the world, pathogens have been isolated from the feces of animals (Sabur et al., 2021). Salmonellosis, the clinical form of *Salmonella* infection, is a costly disease to livestock producers on account of mortality, treatment expenses, reduced milk yield, and weight loss/decreased weight gain within the herd (Callaway et al., 2005). By producing high-quality milk, meat, and farmyard waste, buffalo ranching contributes significantly to the agriculture and cattle sectors in many Asian nations (Khongsai, 2020). Bangladesh has only 1.485 million indigenous buffaloes, with a more significant concentration (40%) in coastal areas and certain buffalo pockets. Smallholder farmers in Bangladesh raise a small number of imported Nili-Ravi and Murrah buffaloes and their crosses on a few fields (Samad, 2020). Pathogens that infect buffaloes and their offspring or are transferred via their products are significant because they impact milk production and total livestock performance. In buffaloes, *Salmonella* is a major pathogen causing calf diarrhea (Khongsai, 2020; Ribeiro et al., 2000), leading to early-age

calf mortality (Rana et al., 2012) and a foremost pathogen transmitted through animal products. *Salmonella enterica* subsp. *enterica* can cause infections with a variety of clinical signs, as well as systemic infections characterized by diarrhea and septicemia, and, in extreme cases, death.

Antimicrobial resistance (AMR) is now recognized as one of the most serious global threats to human health in the 21st century (Morehead and Scarbrough, 2018; Tacconelli et al., 2018a; Tacconelli et al., 2018b) and a decline in the rate of new antibiotic development (Luepke and Mohr, 2017). It causes a considerable risk of death and economic hardship all over the world. However, impoverished nations are more harmed due to extensive antibiotic abuse, non-human antibiotic usage, poor medication quality, and insufficient surveillance (Ayukekbong et al., 2017; Van Boeckel et al., 2019). Bangladesh, a Southeast Asian developing country with a high level of AMR, poses a regional and global concern. Several studies have found irrational antibiotic prescribing by doctors and veterinarians, a patient habit of self-medication, and indiscriminate antibiotic usage in agriculture and farming in various sections of the nation (Biswas et al., 2014; Sutradhar, 2014).

Salmonella species with single and multidrug resistance are becoming more common (Liu et al., 2010). Salmonellosis is treated with a variety of antibiotics. Fluoroquinolones and third generation cephalosporins are the most often prescribed antibiotics to treat salmonellosis. Chloramphenicol, Ampicillin, Amoxicillin, and trimethoprim-sulfamethoxazole are some of the older antibiotics occasionally used as alternatives (Ayukekbong et al., 2017). Antimicrobial resistance in *Salmonella* has been observed to be on the rise in several developing and developed nations (Schmidt et al., 2018).

In some upazilas of the Sylhet region, the farmers depend upon the buffaloes primarily for draught and meat purposes, an integral part of the farming system in this area. Buffalo is economically significant in this region and could be considered a "small tractor" for farmers, and its meat and milk are nutritionally rich. Despite their great contribution to the socio-economy, they are still undervalued. Diseases are one of the most significant constraints for buffalo production, which decrease milk and meat production and reduce farmer's income. In addition, the status of *Salmonella* in buffalo populations in Sylhet district is unknown. Therefore, this study aimed to isolate and identify *Salmonella* from buffalo feces by conventional bacteriological, biochemical, and molecular methods and to determine the antibiotic sensitivity of *Salmonella* isolates.

MATERIAL AND METHODS

Study Area and Sample Collection

For this study, Fenchuganj and Jaintapur Upazilas of Sylhet District of Bangladesh were purposively selected due to their relativeness of larger buffalo population and milk production. The sampling units for the study were buffalo of different ages. A total of 334 fecal samples of buffaloes (112 from Fenchuganj and 222 from Jaintapur) of Sylhet District constituted the study population, which was selected by using a simple random sampling method. The study was conducted for a period of one year (July 2021 to June 2022). Feces samples were collected directly from the rectum and freshly voided feces from buffalo. The samples were placed in a labeled clean plastic container and immediately transferred to the laboratory for further analysis.

Isolation and identification of Salmonella

Conventional bacteriological methods were used to isolate the *Salmonella* from feces samples. The samples were inoculated into buffered peptone water (BPW), incubated at 37°C for 24 hours, and 0.5 mL was transferred to 10 mL Tetrathionate Broth (Merck) and incubated at 37°C for 24 hours. Following enrichment, a loopful of samples were streaked on xylose-lysine deoxycholate (XLD) agar and *Salmonella – Shigella* (SS) agar plates and incubated at 37 °C for 24 h. Biochemical confirmation of *Salmonella* was performed using TSI agar, Urea agar, Simmon's citrate agar, methyl red, and Voges Proskauer broth.

Bacterial genomic DNA Extraction and PCR assay

The DNA extraction was performed according to the manufacturer's instructions 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 instructions (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.

Determination of Antibiotic sensitivity patterns

The *in vitro* antibiotic sensitivity test was determined by the standard disc diffusion method according to the Clinical and Laboratory Standard Institute (CLSI, 2020). The antibiotic discs used in the present study were Ampicillin (AMP, 10 µg), Amoxicillin/Clavulanic Acid (AMC, 30 µg), Gentamycin (CN, 10 µg), Streptomycin (S, 10 µg), Cefixime (CFM, 5 µg), Ceftriaxone (CRO, 30 µg), Azithromycin (AZM, 15 µg), Ciprofloxacin (CIP, 5 µg), Levofloxacin (LEV, 5 µg), Colistin sulfate (CT, 10 µg), Tetracycline (TE, 10 µg), Sulfamethoxazole-trimethoprim (SXT, 25 µg) all from Oxoid company, UK. After incubation, each disc's diameter of the clear zones produced by antibacterial inhibition of bacterial growth was measured to the nearest millimeter and then classified as resistant, intermediate, or susceptible using a published interpretive chart of Clinical and Laboratory Standards Institute (CLSI,2020).

Statistical analysis

Microsoft Excel Office 2010 was used for 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).

RESULTS

Salmonella was isolated from 56 (16.77%) out of 334 examined samples, of which 39 (17.57%) and 17 (13.93%) were from feces of Jaintapur and Fenchuganj upazila, respectively (Table 1). The prevalence of *Salmonella* was higher in buffalo calves aged less than one year (24.32%); although the difference was not statistically significant ($p = 0.11$) (Table 2). Furthermore, the isolation rate was relatively higher in animals aged 1 - 2 years (18.62%) than in the groups of 4 years and above four years (Table 2).

Table 1: Overall prevalence of *Salmonella* in buffalo feces and its association with study area

Study area	Number of samples tested	Tests name					
		Culture positive	Prevalence	Biochemical Test positive	Prevalence	PCR positive	Prevalence
Jaintapur	222	45	20.27%	39	17.57%	39	17.57%
Fenchuganj	122	19	15.57%	17	13.93%	17	13.93%
Total	334	64	19.16%	56	16.77%	56	16.77%

Table 2: Association of the prevalence of *Salmonella* with age of buffalo populations

Age of animals	No. of animals examined	No. of positive fecal samples	Prevalence	P-value
Up to 1 year	74	18	24.32%	0.11
1 – 2 years	102	19	18.62%	
2 - 4 years	83	11	13.25%	
> 4 years	75	8	10.67%	
Total	334	56	16.77%	

Level of significance $p < 0.05$

It was observed that out of the total 334 feces samples collected from two different upazilas, the female buffaloes showed a higher prevalence (21.56%) of *Salmonella* in their fecal samples (Table 3). All suspected *Salmonella* colonies underwent specific biochemical tests such as indole formation, methyl red and Voges Proskauer reaction, citrate utilization, and triple sugar iron agar due to their cultural and morphological characteristics. Sixty-four of the suspected *Salmonella* colonies had 56 confirmed colonies by biochemical test results. Most of the *Salmonella* isolates showed fermentation of glucose, gas

production from glucose, H_2S formation, but none of the isolates showed either lactose or sucrose. The results showed that the prevalence rate of *Salmonella* was statistically significant (p = 0.01). In this study, the *Salmonella* isolation rate was categorized based on the health condition of buffalo populations. The isolation rate of *Salmonella* was higher in diarrheic buffalo fecal samples (72.22%) compared with apparently healthy animals (13.60%). The result revealed statistically significant differences (p = 0.01) (Table 4).

Table 3: Association of *Salmonella* isolation rate with the sex of animals

Sex of animals	No. of animals examined	No. of positive fecal samples	Prevalence	*p*-value
Male	153	17	11.11%	0.01
Female	181	39	21.55%	
Total	334	56	16.77%	

Level of significance $p < 0.05$

Table 4: The association of *Salmonella* isolation rate with the health status of buffalo populations

Health status of animals	No. of animals examined	No. of positive fecal samples	Prevalence	*p*-value
Apparently healthy	316	43	13.60%	< 0.01
Diarrheic	18	13	72.22%	
Total	334	56	16.77%	

Level of significance $p < 0.05$

A total of 56 isolates of *Salmonella* were tested for commonly used 12 antibiotics from 8 groups. All the *Salmonella* isolates were found resistant to one or more antibiotics used. This antibiotic susceptibility study revealed that *Salmonella* isolates were 100%, 93.33%, and 76.67% sensitive to Gentamycin, Levofloxacin, Colistin sulfate, and Ciprofloxacin. On the other hand, isolates were 100%, 87.5%, and 78.6% resistant to Tetracycline, Ampicillin, and Streptomycin, respectively (Table 5.). All of the biochemically positive *Salmonella* isolates

(n = 56) were submitted to a PCR test using a particular primer set to identify the *invA* gene of *Salmonella*. After agarose gel electrophoresis under UV transilluminator, all the biochemically identified 56 isolates were confirmed positive for *Salmonella* and revealed *an invA* gene-specific band at 100bp (Figure 1.).

Table 5. Antibiotic susceptibility profile of *Salmonella* isolated (n = 56) from buffalo feces

Antibiotic tested	**Susceptible (%)**	**Intermediate (%)**	**Resistance (%)**
Penicillin			
Ampicillin (AMP, 10 µg)	-	7 (12.5)	49 (87.5)
Amoxicillin/clavulanic Acid (AMC, 30 µg)	30 (53.33)	15 (26.67)	11 (20)
Aminoglycosides			
Gentamicin (CN, 10 µg)	56 (100)	-	-
Streptomycin (S, 10 µg)	-	12 (21.4)	44 (78.6)
Cephalosporins			
Cefixime (CFM, 5 µg)	41 (73.21)	4 (7.14)	11 (20)
Ceftriaxone (CRO, 30 µg)	39 (70)	4 (7.14)	13 (23.21)
Macrolides			
Azithromycin (AZM, 15 µg)	39 (70)	-	17 (30)
Fluoroquinolones			
Ciprofloxacin (CIP, 5 µg)	43 (76.67)	13 (23.21)	-
Levofloxacin (LEV, 5 µg	56 (100)	-	-
Polymyxins			
Colistin Sulphate (CT, 10 µg)	52 (92.86)	-	4 (7.14)
Tetracyclines			
Tetracycline (TE, 10 µg)	-	-	56 (100)
Folate Pathway Inhibitors			
Sulfamethoxazole -Trimethoprim (SXT, 25 µg)	35 (62.5)	-	21 (37.5)

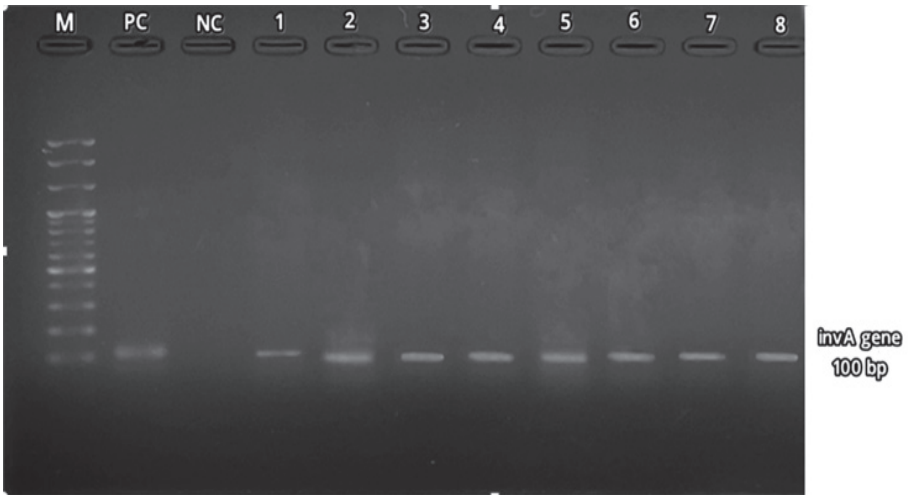

Figure 1. PCR amplification of biochemically identified *Salmonella*

DISCUSSION

In the present study, 334 buffalo fecal samples were collected from Jaintapur (222) and Fenchuganj (112) Upazilas of Sylhet district. Bacteriological examination revealed the *Salmonella* organisms from 56 of 334 buffalo fecal samples with a prevalence of 16.77% (Table 1), which is close to 11.2% (Zahran and Elbehiry, 2014). *Salmonella* in the buffalo population is a health risk for people who consume the products (meat, milk), as it has been reported that 25% of meat samples are contaminated with *Salmonella* (Abd-Elghany et al., 2022). The prevalence of *Salmonella* was higher in young (up to 1 year) and sick (diarrheic) animals, estimated to be 24.32% and 72.22%, 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 that diarrhea is a major problem in animals with a higher prevalence.

For detection of *Salmonella* genus using PCR, it was found that all PCR products, including positive control, resulted in 100 bp amplified fragment. Figure 1 revealed no amplified DNA fragments obtained from the non-*Salmonella* genus. *Salmonella* isolates produced a white band at the level of marker DNA (Figure 1). The PCR method is recommended for detecting *Salmonella* in feces samples because it is faster than culture methods (Stone et al., 1994; van der Zee and Huis in´t Veld, 2000).

According to Vella and Cuschieri (1995), *Salmonella* isolates were most frequently resistant to trimethoprim. *Salmonella* isolated from feces of dairy cattle were resistant to ampicillin (100%), streptomycin (66.7%), and tetracycline (55.56%) but susceptible to gentamicin (100%) and ciprofloxacin (91.7%) as reported by others (Khan et al., 2021). According to Wieczorek and Osek (2013), sulfamethizole resistance was found in various strains of *S.* Dublin and *S.* Enteritidis. *Salmonella* isolates are resistant to multiple antibiotics (ampicillin, streptomycin, tetracycline, and sulfamethizole). The isolates were susceptible to gentamycin (100%), levofloxacin (100%), and ciprofloxacin (76.67%) but have shown higher resistance to tetracycline, ampicillin, and streptomycin (100%, 87.5%, and 78.6%, respectively). According to Esaki et al. (2004), many *Salmonella* isolates from both healthy and ill animals were resistant to two or more antimicrobials. These microorganisms are disseminated in the environment and spread through the excrement of animals that appear to be in good health. According to reports, *S.* Typhimurium was released in a pig farm (Tanaka et al., 2014). The release was relatively high for several days following vaccination; therefore, the animals appeared to be in good health. As a result, herd infection could come even from the excrement of clinically healthy animals. Furthermore, a range of serotypes of antibiotic-resistant bacteria may exist. *Salmonella* isolates were shown to be resistant to numerous drugs in the current investigation. Tetracycline resistance was found in all *Salmonella* isolates. The results of this study revealed that *Salmonella*-infected buffalo could be effectively treated with gentamycin, Levofloxacin, and Ciprofloxacin.

CONCLUSION

It could be concluded that *Salmonella* is a significant cause of diarrhea in buffaloes with salmonellosis in Bangladesh. Therefore, a rapid and proper diagnosis could prevent harm inflicted on the livestock industry. The molecular basis of *Salmonella* identification techniques, such as using the *invA* gene-specific PCR method, could be helpful in diagnostic and research laboratories. This study suggests the strategic use of antibiotics to control *Salmonella* infections in animals.

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Author's Contribution:

MMH and MMR: Supervised and contributed to study design; BDG and RB: Carried out the laboratory experiments; SA and MSRC: Performed statistical analysis; and BDG and MMH: Wrote the manuscript. All authors read and approved the final manuscript.

Competing interest

The authors declare that they have no competing interests.

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