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*Original article*

# ISOLATION, IDENTIFICATION, MOLECULAR DETECTION AND SENSITIVITY TO ANTIBIOTICS OF *SALMONELLA* FROM CATTLE FAECES

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**Summary**

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The present study was designed with the aim of isolation and identification of *Salmonella* by conventional culture method and their confirmation by polymerase chain reaction (PCR). Antibacterial sensitivity study of isolated *Salmonella* from cattle faeces was also performed. During the study period of July 2017 to June 2018, a total of 200 faecal samples were collected from different government and private farms in Sylhet district of Bangladesh. Out of 200 samples, 24 (12%) were found to be positive for *Salmonella* by conventional culture methods. Among the twenty four suspected colonies of *Salmonella,* seventeen were confirmed by biochemical test and same number was detected by PCR estimating a prevalence of 8.5% (17/200). The prevalence was higher in calves under 1 year of age (16%) compared with older animals (11.25% of 1–2 years; 10% of above 2 years of age) but without statistically significant differences ($\chi^2$=4.835, P=0.089). Moreover, in diarrhoeic animals the prevalence was significantly higher (32.14%, $\chi^2$=49.414, P<0.01) than in apparently healthy animals (8.72%). The antibiotic sensitivity test showed that highest number of *Salmonella* isolates were sensitive to ciprofloxacin (100%), gentamicin (100%) and neomycin (100%). On the other hand, significantly high resistance of *Salmonella* isolates was detected to erythromycin (100%), amoxicillin (100%), cotrimoxazole (81.48%), streptomycin (62.96%) followed by tetracycline (55.56%).

**Key words**: antibacterial sensitivity, cattle isolation, PCR detection, *Salmonella*

## INTRODUCTION

Salmonellosis in cattle is worldwide distributed and is considered the most important animal related zoonotic disease nowadays (Anderson *et al.,* 1999). There are above 2,668 different serotypes under the genus *Salmonella* responsible for large numbers of infections in both humans and animals (Keusch, 2002). *Salmonella*

strains are not detectable in clinical samples containing few organisms. However, the number of *Salmonella* in faeces of infected individuals is substantial (Aida *et al.,* 2012). Excretion level is maintained through several weeks before falling gradually until the individual no longer excretes. Up to 5% of patients, upon recovery from salmonellosis, may become carriers by shedding the bacterium in their faeces after its disappearance from the intestinal tract (Jay, 2005). Therefore, this is not only important to detect *Salmonella* strains in faecal samples and diagnose the *Salmonella* infection but also essential to identify the carriers of this organism. Most of the cases of human salmonellosis is caused by the consumption of contaminated eggs, chickens, pork, beef and milk products (Geimba *et al.,* 2004). *Salmonella* in cattle has been widely reported (Hollinger *et al.,* 1998; McDonough *et al.,* 1999). The *Salmonella* infection in calves is still a major problem worldwide. The enormous economic losses due to salmonellosis were manifested through mortality and poor growth of infected animals as well as the hazard of transmitting food poisoning to humans. *S.* Typhimurium*, S.* Enteritidis*, S.* Anatum, *S.* Newport, *S.* Cerro, *S.* Montevideo, *S.* Agona and *S.* Dublin are considered the most important host-adapted *Salmonella* from cattle (Ritchie *et al.,* 2001; Veling *et al.,* 2002). The most common serovars isolated from diarrhoeic patients is *S.* Typhimurium; Choleraesuis, Dublin, and Enteritidis are often isolated from patients with bacteraemia (Guiney, 1995). In many cattle farms, the *Salmonella* is endemic and outbreaks of the disease are relatively infrequent and typically reflect a combination of environmental conditions and farm management (House & Smith*,* 2004). The conventional laboratory culture methods require a long time to give a positive or negative result. Molecular techniques such as polymerase chain reaction give rapid, sensitive and specific detection of pathogens in the environment (Josephson *et al*., 1991). Without showing any clinical signs of salmonellosis, the infected cattle may shed the *Salmonella* organism in their faeces. Therefore, a rapid, specific and sensitive detection method for *Salmonella* is important for animal and human health and for the diagnostic industry (Gouws, 1998). Detection of *Salmonella* serovars in clinical samples from pigs, horses and cattle by PCR is more rapid than conventional culture techniques. The sensitivity and specificity of this assay are 100% compared with 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,* both in diarrhoeic and visually healthy cattle and confirmation by PCR detection.

## MATERIALS AND METHODS

### *Sample collection*

Fresh faecal samples were collected directly from the rectum of 200 cattle in different farms. All procedures have been carried out in accordance with the guidelines laid down by the Sylhet Agricultural University Ethics Committee and in accordance with local laws and regulations. Information regarding health history, identification, age, sex and breed of the animals was recorded. The animals were divided according to their age, sex and breed. After collection, the samples were kept in icebox and brought to the laboratory of the Department of Medicine in Sylhet Agricultural University, Bangladesh for further testing the presence of *Salmonella*. All faecal samples were col-

lected in separately labelled clean plastic specimen containers. To prevent potential cross contamination between faecal samples, separate disposable gloves were worn for each collected sample. Faecal samples collected in the field were kept at 4 $^{o}$C until processed for nucleic acid purification within 24 h after collection.

*Isolation and identification of Salmonella*

The conventional bacteriological methods were used to isolate the *Salmonella* from faecal samples. The latter were subjected to initial pre-enrichment in buffered peptone water (Merck) and incubated for 24 hours at 37 ºC. Afterwards, 0.5 mL were transferred to MacConkey agar plates and incubated at 37 ºC for 24 h. Finally, a loopful was transferred to dishes containing XLD agar (Merck), a *Salmonella* selective medium, in triplicates, and incubated for 24 hours at 37 ºC. Presumptive *Salmonella* colonies were phenotypically confirmed by biochemical properties in differential agars, such as TSI, LIA, Simmons citrate and SIM (Merck) after incubation at 37 ºC for 24–48 h.

*Extraction of genomic DNA*

The DNA extraction was performed according to the manufacturer instructions using the AddPrep genomic DNA extraction kit (AddBio Inc. Ltd., Korea). Briefly, 200 µL overnight cultured cells were harvested by centrifugation at 13,000 rpm for 30 s, and the supernatant was discarded and 200 µL of lysis solution was added and suspended by pipetting. Proteinase K solution (20 mg/mL) was added and incubated at 56 ºC for complete lysis. After centrifugation and adding binding solution, the mixture was well homogenised and incubated at 56 ºC for 10 min. Then absolute ethanol was added and the lysate was carefully transferred to spin column tube, centrifuged, washed out using washing solutions. The spin column was dried by additional centrifugation at 13,000 rpm for 1 min to remove the residual ethanol. Thirty µL elution buffer was added to spin column and incubated 1 min at room temperature. The genomic DNA was eluted by centrifugation and eluted DNA samples were stored at –80 °C until further analysis.

*PCR amplification*

All *Salmonella* suspected cultures subjected to PCR amplification generated a product of approximate molecular size 100 bp (*invA* gene) according to the manufacturer instruction (AddBio Inc. Ltd., Korea). A 100 bp DNA marker was used as a molecular weight marker. PCR assay was conducted in a final reaction volume of 20 µL using 10 µL of Master Mix (2× with UDG – AddBio Inc. Ltd., Korea). For the detection of *Salmonella*, we used the primers invS-F (5'-TAA TGCCAGACGAAAGAGCGT-3') and invS-R (5'-GATATTGGTGTTTATGGG GTCGTT-3') (AddBio Inc. Ltd., Korea). Each reaction mixture contained 5 µL of extracted DNA, 10 µL of Master Mix, 5 µL (10 pmol/µL) of primers (forward and reverse). PCR assay performed in thermo cycler TC1000G PCR System® (DLAB Scientific Inc., USA) with a heated lid. The cycling conditions included 50 °C for 3 min (UDG Reaction), 95 °C for 10 min (initial denaturation), 35 cycles of 95 °C for 30 sec (denaturation), 68 °C for 45 sec (annealing) and 72 °C for 5 min for (final extension). The above PCR cycling conditions were chosen on the basis of preliminary experiments performed to optimise amplification of *Salmonella* target sequence. In each reaction, positive and negative control specimens were added and PCR amplified in parallel with all

specimens. PCR products were analysed on 1.8% agarose gels stained with RedSafe™ Nucleic Acid Staining Solution (20,000×), photographed, and stored as digital image.

*Antimicrobial susceptibility*

For the antibiotic sensitivity testing, the qualitative agar diffusion method (Kirby-Bauer method) was used employing Mueller Hinton agar. *In vitro* antibiotic sensitivity test was determined by the standard disc diffusion procedure according to the Clinical and Laboratory Standard Institute (CLSI 2009). The following antibiotic discs were used: erythromycin (15 µg), gentamicin (10 µg), streptomycin (10 µg), amoxicillin (30 µg), ciprofloxacin (5 µg), tetracycline (30 µg),) neomycin (30 µg) and cotrimoxazole (25 µg). The antimicrobial susceptibility was determined according to the standard diameter of inhibition (mm) for each antibiotic used (CLSI, 2009).

Minimum inhibitory concentrations (MIC) were determined by the serial dilution method in Mueller Hinton broth for 8 antibiotics used: erythromycin (ERY), gentamicin (GEN), streptomycin (STR), amoxicillin (AMX), ciprofloxacin (CIP), tetracycline (TET), neomycin (NM) and cotrimoxazole (CTX). The MICs ($MIC_{50}$ and $MIC_{90}$) were determined for each of the antibiotics used and the sensitivity or resistance was determined according to the protocol described (CLSI, 2009).

*Statistical analysis*

Microsoft Excel was used for the descriptive statistics. Chi-square tests were used to assess the significance of differences in prevalence among age, sex, breed and health status. P values of <0.05 were considered to be significant using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

## RESULTS

*Cultural and morphological characterisation*

All *Salmonella* colonies in MacConkey agar plates appeared non-lactose fermenting, colourless and transparent. The isolates of *Salmonella* grown in Salmonella-Shigella (SS) agar plates showed characteristic black centered colonies but in the black dot the isolates were lacking. The thin smears prepared with the colony from SS agar for Gram staining revealed Gram-negative, pink coloured, small rod-shaped organisms, single or paired or arranged in a short chain in the microscopic examination.

On TSI slants most of the *Salmonella* isolates showed fermentation of glucose, gas production from glucose, $H_2S$ formation but none of the isolates fermented either lactose or sucrose (Table 1).

*Prevalence of Salmonella in cattle*

Among the 200 collected samples, 24 (12%) were found to be positive for *Salmonella* by conventional isolation and identification methods in the laboratory recommended by ISO 6579:2002. The prevalence was higher in calves under 1 year of age (16%,) compared with older animals (11.25% of 1–2 years; 10% of above 2 years of age) but no statistically significant differences were found ($\chi^2$=4.835, P=0.089) (Table 2). Moreover, in diarrhoeic animals the prevalence was significantly higher (32.14%, $\chi^2$=49.414, P<0.01) than in apparently healthy animals (2.90%).

**Table 1.** Summary of the biochemical test results of *Salmonella* isolates

| Biochemical test | Samples | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | F-1 | F-2 | F-3 | F-4 | F-5 | F-6 | F-7 | F-8 | F-9 | F-10 |
| TSI acid from glucose | + | + | + | + | + | + | + | + | + | + |
| TSI gas from glucose | + | + | + | + | –* | + | + | + | + | + |
| TSI acid from lactose | – | – | – | – | – | – | – | – | – | – |
| TSI acid from sucrose | – | – | – | – | – | – | – | – | – | – |
| TSI $H_2S$ production | + | + | + | + | + | + | – | + | + | + |
| Indole formation | – | – | – | – | – | – | – | – | – | – |
| Methyl red test | + | + | + | + | + | + | + | + | + | + |
| Voges-Proskauer reaction | – | – | – | – | – | – | – | – | – | – |
| Citrate utilisation | + | + | – | + | + | + | –* | + | + | – |

F-1, -2, -3…… = Farm 1, 2, 3; * 2 samples were negative.

**Table 2.** Prevalence of *Salmonella* isolates in cattle according to biochemical identification

| Factors | Animals | Tested (n) | Positive | Prevalence | P-value |
|---|---|---|---|---|---|
| Age | < 1 year | 50 | 8 | 16.0% | 0.089 |
| | 1–2 years | 80 | 5 | 6.25% | |
| | > 2 years | 70 | 4 | 5.71% | |
| | Total | 200 | 17 | 8.50% | |
| Sex | Male | 112 | 8 | 7.14% | 0.438 |
| | Female | 88 | 9 | 10.22% | |
| | Total | 200 | 17 | 8.50% | |
| Breed | Local | 133 | 11 | 8.27% | 0.227 |
| | Crossbred | 67 | 6 | 8.96% | |
| | Total | 200 | 17 | 8.50% | |
| Health status | Apparently healthy | 172 | 5 | 2.90% | <0.01 |
| | Diarrhoeic | 28 | 12 | 42.86% | |
| | Total | 200 | 17 | 8.50% | |

*Biochemical characterisation*

On the basis of cultural and morphological properties all suspected colonies of *Salmonella* were subjected to selected biochemical tests including indole formation, methyl red and Voges-Proskauer reaction, citrate utilisation and triple sugar iron agar. Among the 24 suspected *Salmonella* colonies 17 were confirmed by biochemical test results analysis.

*PCR detection of Salmonella*

The estimated prevalence of *Salmonella* in cattle faeces by PCR was 8.5% (17/200). The band size detected in *Salmonella* isolates (100 bp amplified fragment) was consistent with that of *invA*

gene as shown by agarose gel electrophoresis (Fig. 1).

*Antibiotic sensitivity*

The antibiotic sensitivity test showed that *Salmonella* isolates were sensitive to ciprofloxacin (100%), gentamicin (100%) and neomycin (100%). On the other hand, significantly high resistance of *Salmonella* isolates was detected to erythromycin (100%), amoxicillin (100%), cotrimoxazole (81.48%), streptomycin (62.96%) followed by tetracycline (55.56%) (Table 3).

## DISCUSSION

In the present study, a total number of 200 cattle feces from different farms in Sylhet were collected. Bacteriological examination revealed the isolation of *Salmonella* organisms from cattle with a prevalence of 12% (24/200). Several studies reported

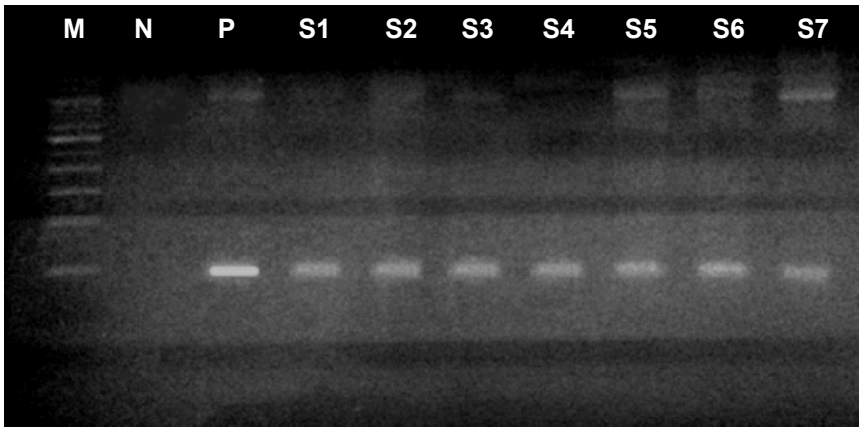


**Fig. 1**. Results of PCR under UV illuminator (bands corresponding to the 100 bp marker line indicate *Salmonella* positive samples); M = marker, N = negative control, P = positive control, S1–S7 = representative samples, bp = base pair.

**Table 3.** Overall susceptibility and resistance patterns of *Salmonella* isolates to selected antibiotics as defined by the Clinical and Laboratory Standards Institute (CLSI, 2009)

| Antimicrobial class | Antimicrobial drug | Abbreviation | Susceptible | Resistant* |
|---|---|---|---|---|
| Aminoglycosides | Gentamicin | GEN | 100.00% | – |
| Aminoglycosides | Neomycin | NM | 100.00% | – |
| Aminoglycosides | Streptomycin | STR | 37.04% | 62.96% |
| β-lactams | Amoxicillin | AMX | – | 100.00% |
| Macrolides | Erythromycin | ERY | – | 100.00% |
| Quinolones | Ciprofloxacin | CIP | 100.00% | – |
| Folate pathway inhibitors | Cortimoxazole | CTX | 18.52% | 81.48% |
| Tetracyclines | Tetracycline | TET | 44.44% | 55.56% |

**Salmonella* isolates showing intermediate resistance to some antibiotic was considered as resistant as per CLSI recommendations.

that prevalence of *Salmonella* in cattle was 8.7% (Kim-Yong Hwan *et al.,* 2000) and 12% (Rasha *et al.,* 2014) which was almost similar to present findings. These results disagree with others who isolated *Salmonella* from cattle at higher rates reaching 43.8 % and 25.3% (Sato *et al.,* 2001; Murinda *et al.*, 2002).

A total of 17 (8.5%) cattle faecal samples were PCR positive for *Salmonella* which is higher than data of Aida *et al.* (2012) who reported only 3.2% PCR positive cases. Gallegos-Robles *et al*. (2009) isolated and detected *Salmonella* from cattle faeces through microbiological and PCR methods, estimating a prevalence of 55% e.g. much higher than that in the present study. The presence of *invA* gene in all strains and clinical isolates of *Salmonella* has been reported (Dahshan *et al*., 2010). The detection of *Salmonella* in clinical samples by PCR was more rapid than conventional culture methods (Stone *et al*., 1994) which take 4–7 days for isolation of *Salmonella* and are more laborious and require more personnel (Van der Zee *et al*., 2000). Molecular testing is more accurate than conventional microbiological techniques (Jungkind, 2001).

The antibiotic disk diffusion test showed that some isolates were resistant to streptomycin (62.96%), amoxicillin (100%), erythromycin (100%), cortimoxazole (81.48%) and tetracycline (55.56%). On the other hand, highest number of *Salmonella* isolates was sensitive to ciprofloxacin (100%), gentamicin (100%) and neomycin (100%). Isolation of antibiotic-resistant *Salmonella* from food and water is of great importance for public health. A high percentage of *Salmonella* isolates from healthy and sick animals were resistant to two or more antimicrobial agents (Esaki *et al.,* 2004). These organisms spread through faeces of apparently healthy animals and are disemined in the environment. There are reports of the release of *S.* Typhimurium in a pig plant (Tanaka *et al.,* 2014). The animals were apparently healthy, the release was very high for several days of post inoculation. Therefore even normal faeces could be a source for herd infection.

It has been reported that multiresistant *S.* Enteritidis (resistant to two or more antimicrobial agents) can reach up to 51.6% with different patterns (de Oliveira *et al.,* 2005). In general, the most commonly seen resistance patterns were to sulfisoxazole, streptomycin and tetracycline while ciprofloxacin resistance was the least common. In Japan, *S.* Typhimurium isolated from various types of animals showed that 20% of the isolates were resistant to ampicillin and 24% to tetracyclines (Esaki *et al.,* 2004). In 2008, 31 *Salmonella* strains were isolated in cattle belonging to 12 different serovars, and the transduction of microbial resistance from *S.* Heidelberg to *S.* Typhimurium was demonstrated (Zhang & LeJeune, 2008). Later on, a 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 *Salmonella* strains isolated, mainly from pigs, showed multidrug resistance, oxytetracycline being the agent showing the highest resistance (69.1%) (Junod *et al.,* 2013).

## CONCLUSION

The control and monitoring of *Salmonella* from various animal sources is of great importance for public health. *Salmonella* are usually dispersed in the environment and animals are asymptomatic carriers of

the disease. The prevention of *Salmonella* is not easy and depends on good animal husbandry and veterinary practices. The rapid and proper diagnosis of animal disease can prevent damages inflicted on livestock industry.

The presence of organisms in faeces is a potential risk of public health contaminating the environment as well as animal food. Therefore control of infections and establishment of strategic antibiotic therapy should be a priority.

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