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Isolation, Molecular Detection and Antibiogram of Multi-drug Resistant Salmonella Typhimurium DT104 from Selected Dairy Farms in Mymensingh, Bangladesh

Shayka Tasnim Pritha, Saifur Rahman, Sadia Afrin Punom, Md. Mizanur Rahman, K. H. M. Nazmul Hussain Nazir, Md. Shafiqul Islam*

Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh
*Corresponding author: shafiq_micro@bau.edu.bd

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Abstract The *Salmonella typhimurium* DT104, an emerging cause of human illness has received an increasing attention due to its multidrug resistant properties. Since it has been isolated from human and other sources including food-producing animals around the world; it has become a worldwide public health concern. Therefore, the present study was designed to isolate, identify and study the antibiogram profile of multidrug resistant *S. typhimurium* DT104 from several dairy farms in Mymensingh district, Bangladesh. A total of 135 fecal samples from diarrhoeic cattle were collected aseptically and subjected for bacterial isolation, molecular detection using PCR followed by antibiogram study. *Salmonella* spp. could be isolated from a total of 39 (28.88%) samples based on cultural and staining methods which were further confirmed by PCR using invA gene specific primers. However, out of 39 *Salmonella* spp., 6 isolates were confirmed as *S. typhimurium* DT104 strain. Results of the antibiotic resistance patterns demonstrated that 100% (39/39) isolates were resistant to erythromycin followed by tetracycline (73.68%), colistin (89.47%), ampicillin (47.36%), gentamicin (21.05%), ciprofloxacin (31.57%), streptomycin (42.10%), enrofloxacin (10.52%) and chlormphenicol (31.57%). Moreover, about 23.07% isolates were resistant to more than 5 antibiotics. However, all the isolates were found to be sensitive to amikacin. These results suggest that antibiotic resistant *S. typhimurium* DT104 strain has been circulating in dairy cattle in Bangladesh which is alarming and may impose threat to livestock and public health due to lack of proper hygienic management. This study will be helpful for the selection of proper antibiotics against salmonellosis in cattle.

Keywords: *antibiogram, cattle, PCR, Salmonella typhimurium DT104*

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1. Introduction

Salmonellosis is a bacterial disease caused by a large group of gram negative, short rod, non-spore forming, non-capsulated, aerobic and facultatively anaerobic bacteria [1]. Under the genus *Salmonella*, the *Salmonella typhimurium* Definitephage Type (DT) 104 causing enteric fever, gastroenteritis and bacteremia recently has been recognized as a potential threat to animal and human health. It was first isolated in the late 1980s. Multidrug-resistant DT104 strains are mostly resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracyclines (ACSSuT resistance type) [2,3,4]. As a consequence, the disease causes substantial economic loss by increasing mortality and poor growth in livestock production especially in calves [3,5]. Apart from these, human health is also at risk due to spreading of the organism in the environment as well as in the food chain system for the zoonotic nature of the organism [4,6].

Salmonella infection in the cattle has become one of the major problems throughout the world since it can be originated from a lot of sources. If an infected animal is imported or introduced to healthy herd the disease can spread from one to another. Cross-infection from other domestic or wild animals may also become source of the disease [7,8,9]. The disease can spread by the infected animal through their faeces, feed, soil, water and other sources in the environment [10,11,12,13,14]. Carrier animals shed the bacteria in their faeces, milk and cross contaminate carcasses at slaughter house [15,16,17,18]. These may cause human *Salmonella* infection which is a serious issue for farm workers, butchers and recognized as professional hazard. The disease can also create food borne infection in human which represents a serious problem in food industry [17,19,20,21].

Now-a-days various antibiotics are being used by the dairy farmers against *Salmonella* infection with or without

prescription. As a result, multiple antibiotics resistant *Salmonella* are increasing at an alarming rate and their negative impact is increasing [7,22,23,24]. The zoonotic multidrug resistant bacteria possess a concern to human health when the drug resistant bacteria transmitted to human via food from animal sources or by environmental factors [24,25,26]. Pathogenic antibiotic-resistant bacteria transmitted to humans via contaminated food of animal origin can compromise the therapeutic value of antibiotics [25,27,28].

In recent times, a significant increase in the occurrence of antimicrobial drug resistance in *Salmonella* strains is of great concern in both developed and developing countries. The exploitation of antimicrobial agents in any environment creates selective pressures that favor the endurance of antibiotic-resistant pathogens. The routine practice of antibiotic utilization to domestic animals as a means of preventing and treating diseases, as well as growth promoter, is an important factor in the emergence of antibiotic-resistant bacteria that are consequently transferred to human [16,29].

In Bangladesh many research works have already been performed on the isolation and characterization of *Salmonella* species from cattle, poultry and other hosts [8,18,19,29,30]. Recently antibiotic resistant *Salmonella* species has also been identified from various sources by different reserachers in Bangladesh [8,19,23,29,30]. Although the multidrug resistant *S. typhimurium* DT104 strain has become a major health concern throughout the world, to the best of our knowledge there is no published report in Bangladesh yet. Therefore, the present study was undertaken with a view to isolate, identify and study the antibiogram profile of multidrug resistant *S. typhimurium* DT104 from different dairy farms in Mymensingh district of Bangladesh.

2. Materials and Methods

2.1. Sample Collection

A total number of 135 samples including 103 faeces samples, 13 soil samples, 9 water samples, 10 feed samples were collected aseptically from various dairy farms in Mymensingh district during the period from July 2018 to February 2019 and then transported to the bacteriology laboratory in the dept. of Microbiology and Hygiene, Bangladesh Agricultural University (BAU) maintaining cool chain.

2.2. Isaolation and Identification of Salmonella spp.

The collected samples were incubated at 37°C for 24 hours at nutrient broth (Difco, England). Then the enriched samples were cultured in XLD agar (HI media, India) and SS agar (HI media, India) by streak plate method and incubate for 24 hours at 37°C for the isolation of *Salmonella* spp. The suspected *Salmonella* were identified by Gram staining method to determine their staining characteristics, morphology and arrangements. Then the cultural and staining positive samples were identified by biochemical tests such as sugar fermentation test (Dextrose, Sucrose, Lactose, Maltose and Mannitol), Indole and MR-VP tests. The isolated *Salmonella* were further confirmed as *Salmonella typhimurium* by PCR.

2.3. Genomic DNA Extraction and Polymerase Chain reaction (PCR)

The genomic DNA was extracted by boiling method [31]. Briefly, 1 mL of overnight incubated broth culture was centrifuged for 5 minutes at 10000 rpm. Then the supernatant was discarded and the pellet was mixed in 200 µl of sterile distilled water. The mixture was then boiled for 10 minutes and immediately placed on ice for cold shock followed by centrifugation at 10000 rpm for 10 minutes at 4°C. The supernatant was collected and used as template DNA.

PCR was performed with genus specific *invA* primers (Fw: 5'-ATCAGTACCAGTCGTCTTATCTTGAT-3' and Rv: 5'-TCTGTTTACCGGGCATACCAT-3') [32]. Thermal cycler conditions for *invA* primer were set as denaturation at 94°C for 5 minutes, 29 cycles of 94°C for 30 seconds, 52°C for 2 minutes, 72°C for 45 seconds and final extension cycle at 72°C for 5 minutes. Another PCR was performed with DT104 species specific primers (Fw: 5'-GTCAGCAGTGTATGGAGCGA-3' and Rv: 5'-AGTAGCGCCAGGACTCGTTA-3') with some modifications [3]. The genes were amplified by denaturation at 95°C for 5 minutes, 30 cycles of 95°C for 1 minute, 57°C for 1 minute, 72°C for 1 minute and final extension cycle at 72°C for 10 minutes.

2.4. Antimicrobial Susceptibility Test

Antimicrobial susceptibility of the isolated organisms was performed against 10 commonly used antibiotics by Kirby-Bauer disc diffusion method [33] on Muller Hinton agar (HI media, India) according to CLSI [34]. The standardized antibiotic discs were amplicillin (25 µg/disc), chloramphenicol (30 µg/disc), streptomycin (10 µg/disc), tetracycline (10 µg/disc), ciprofloxacin (5 µg/disc), erythromycin (15 µg/disc), gentamicin (10 µg/disc), enrofloxacin (5 µg/disc), colistin (10 µg/disc) and amikacin (30 µg/disc).

3. Results

A total of 135 samples (103 faeces samples, 13 soil samples, 9 water samples, 10 feed samples) from 3 different farms and random households were subjected to isolation and identification of *Salmonella* spp. The translucent, smooth, small round colonies with black centre were produced by *Salmonella* spp. on SS agar and XLD agar [35]. Gram's staining of the suspected organism revealed as pink colored short rod shaped bacteria arranged in single or paired. The isolates were methyl red positive but indole and VP negative [35]. All the isolates of *Salmonella* spp. fermented dextrose, maltose and mannitol with acid and gas production but did not ferment sucrose and lactose. Among the samples, 39 (28.89%) were found to be *Salmonella* positive based on the above mentioned cultural and biochemical properties (Table 1). The isolates were further confirmed as *Salmonella* spp. by

amplification of *invA* gene (211 bp) (Figure 1). For the detection of *S. typhimurium* DT104 serover, species specific DT104 primers were used and 6 (15.38%) isolates were detected as positive by amplification at 162 bp (Figure 2, Table 1).

Table 1. Results of isolated and indentified Salmonella spp. and S. typhimurium DT104 from the collected samples

Name of the farm	No. of samples	No.of Salmonella positive samples	No. of *Salmonella typhimurium* DT104 positive samples
Research Animal Farm, Dept. of Surgery and Obstetrics, BAU	8	1	-
BAU Dairy Farm	17	10	-
Trishal Military Farm	94	23	6
Household sample	16	5	-
Total	135	39(28.89%)	6 (15.38%)

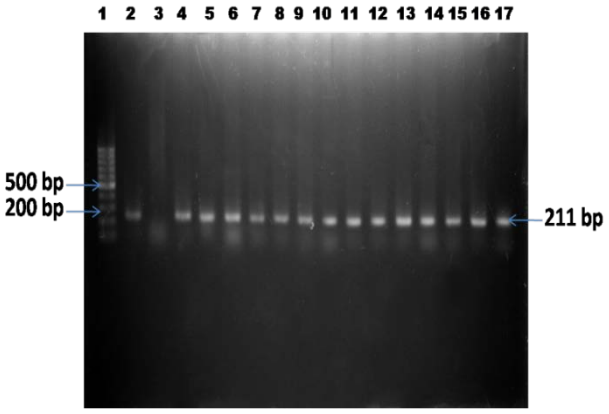

Figure 1. Electrophoresis results of PCR products of *Salmonella* isolates showing specific bands on 1.5% Agarose gel using *InvA* primers. Lane 1:1000bp Ladder, Lane 2: Positive control, Lane 3: Negative control, Lane 4-17: Positive samples

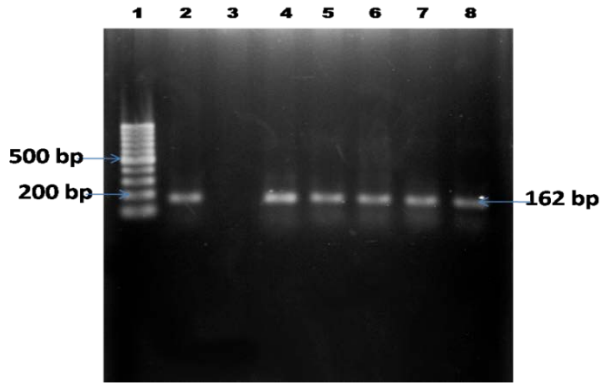

Figure 2. Electrophoresis results of PCR products of *Salmonella typhimurium* DT104 showing specific bands on 1.5% Agarose gel using DT104 specific primers. Lane 1:100bp DNA Ladder, Lane 2: Positive control, Lane 3: Negative control, Lane 4-8: Positive samples

Antibiotic sensitivity test with 10 commercially available antibiotic discs revealed that *Salmonella* isolates of dairy farm were 100% resistant to erythromycin followed by tetracycline (73.68%), colistin (89.47%), ampicillin (47.36%), gentamicin (21.05%), ciprofloxacin (31.57%), streptomycin (42.10%), enrofloxacin (10.52%) and chlormphenicol (31.57%). About 23.07% (n=9) isolates were resistant to more than 5 antibiotics. All the isolates were 100% sensitive to amikacin. The results of the antibiotic sensitivity tests are shown in Figure 3 & Figure 4.

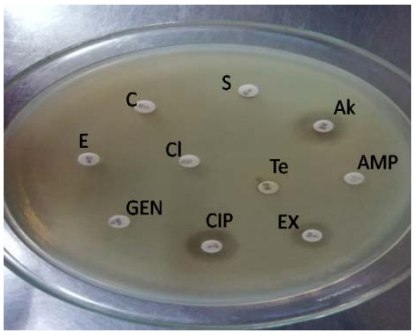

Figure 3. Antibiogram profile of *Salmonella typhimurium* DT104 in dairy farm samples

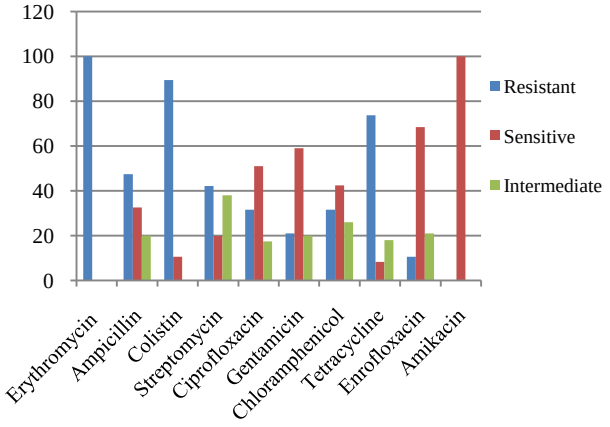

Figure 4. Summary of the antibiogram profile of *Salmonella* spp. against 10 antibiotics

4. Discussion

Disease from multidrug resistant *Salmonella* is a major problem now a days in dairy farm industry. *S. typhimurium* DT104 is one of the potential public health threats in Bangladesh because of its multi-drug resistance characteristics. It transmits horizontally between herds through personal contact, contaminated equipments and slurry, and a DT104 infected herd might pose a risk for epidemiological related herds, or herds located within the same area [36]. There is also a clear association between infections of farm animals, or foods of animal origin, and human infection, showing that DT104 readily infects people in contact with infected animals or their products [37,38,39]. But till now to the best of our knowledge there is no report of isolation and molecular identification of *S. typhimurium* DT104 in Bangladesh. Therefore, isolation, identification and antibiogram study of *Salmonella typhimurium* DT104 was carried out from various dairy farms in Mymensingh district, Bangladesh.

The present study demonstrated that, among 135 dairy farm environmental samples 39 (28.88%) were positive for *Salmonella* spp. and among them 6 (15.38%) isolates were confirmed by PCR to be *S. typhimurium* DT104 positive from cow diarrhoeal samples. The findings of the present study suggest that, *S. typhimurium* DT104 could easily infect nearby dairy farms by spreading to

environment through water, equipment and workers. It can also cause zoonosis to the farm workers as they handle the infected animals. Human could be affected by consuming undercooked meat and un-treated milk of the infected animals. The results of the present study indicate that high level of multidrug resistant *Salmonella* are present in environment which is a great threat for human specially farm workers as well as the consumers.

In the present study, all the *Salmonella* spp. isolates were found to be sensitive to amikacin. By this finding we can recommend the veterinary practitioners to treat *Salmonella* infection with amikacin. Recently, antibiotic resistant *Salmonella* was isolated and characterized from dairy farms and its environment in Bangladesh [30]. *Salmonella* isolates were resistant to erythromycin (88.89%) and tetracycline (75.73%) which was similar to our study. About 23.07% (n=9) isolates were resistant to more than 5 antibiotics which was similar to the recent findings [16,24,27,30]. Colistin is an antibiotic that is being used increasingly as a 'last-line' of defence to treat infections caused by MDR Gram-negative bacteria, when essentially no other options are available [40]. It is the matter of concern that, we observed high level of resistance (about 89.47%) toward colistin. No resistance of *Salmonella* spp. isolated from pig slaughtered house was found in Spain in 2007 [41] which is opposite to our findings. However, colistin resistant *Salmonella* were found in human and poultry in England and Wales in 2016 [42]. Those findings indicate that colistin resistant *Salmonella* is increasing day by day. With the view to our present findings, we can assume that the situation of colistin resistance has been arising due to continuous use of antibiotics without proper guidance from registered veterinary practitioners. The farmers don't maintain proper dose and accurate antibiotics which leads to emergence of drug resistant pathogen in the environment. The accurate dose of antibiotic to animal should be carefully controlled and proper hygienic management in the farm should be ensured otherwise it will become a great global problem because of wide host range of *Salmonella* spp. and its serious zoonotic significance [43].

5. Conclusion

The findings of this study suggest that antibiotic resistant *S. typhimurium* DT104 strain has been circulating in dairy cattle in Bangladesh which is alarming and may impose threat to livestock and public health due to lack of proper hygienic management. This study will be helpful for field veterinarians to select the proper antibiotics against salmonellosis in cattle.

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Conflict of Interests

The authors have declared that no conflict of interests exists.

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