Bangl. J. Vet. Med. (2018). 16(1): 115–120 ISSN: 1729-7893 (Print), 2308-0922 (Online)

DETECTION OF MULTI–DRUG RESISTANT *SALMONELLA* FROM MILK AND MEAT IN BANGLADESH

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ABSTRACT

This study was conducted to investigate the prevalence of *Salmonella* spp. in milk, chicken meat and beef and to determine the multi-drug resistance (MDR) profile of *Salmonella* spp. in Mymensingh and Gazipur districts, Bangladesh. A total of 169 samples of milk (n=108), chicken meat (n=51) and beef (n=10) were collected from Bangladesh Agricultural University (BAU) dairy farm, American dairy farm, Gazipur and different small dairy farms of municipal area during July 2016 to June 2017. *Salmonella* spp. were isolated on various selective agar media such as: Salmonella-Shigella (SS) agar, Xylose-Lysine Deoxycholate (XLD) agar, Eosine-Methylene Blue (EMB) agar. Identification of *Salmonella* spp. was done by colony characteristics, Gram staining, biochemical test and Polymerase Chain Reaction (PCR). Multi-drug resistant *Salmonella* spp. was detected by disc diffusion test using 10 commonly used antibiotics. The overall prevalence of *Salmonella* spp. in all food samples was 21.89%. A total of 29 (56.86%) chicken meat, 02 (1.85%) milk, and 06 (60%) beef samples were *Salmonella* spp. positive. Antibiogram study showed that an overall 89.19% of *Salmonella* spp. was found multi-drug resistant. Specifically 100%, 66.67% and 93.10% of the *Salmonella* spp. isolates originated from milk, beef and chicken meat respectively were multi-drug resistant. The result of this study suggests that MDR *Salmonella* spp. is prevalent in the milk and meat which might cause public health hazard if proper hygienic measures are not undertaken at farm and marketing level.

Keywords: *Salmonella*, milk, beef and chicken meat, antibiogram, multidrug resistance, PCR.

INTRODUCTION

A wide range of pathogens play a role in foodborne diseases, most of which are of animal origin and remain as carriers in healthy food animals from which they spread to foods of animal origin and act as most important vehicles of foodborne infections (S´anchez-Vargas *et al.*, 2011). Among the pathogens, *Salmonella* is considered the most prevalent foodborne pathogen worldwide and has long been recognized as an important zoonotic bacterium of economic significance in animals and humans (Carrasco *et al.*, 2012) mainly in the developing countries.

Consumption of raw or unsafe food, cross-contamination, improper food storage, poor personal hygiene practices, inadequate cooling and reheating of food items, and a prolonged time lapse between preparing and consuming food items were mentioned as contributing factors to an outbreak of salmonellosis in humans. The ubiquity of *Salmonella* isolates creates a persistent contamination hazard in all raw foods (Carrasco *et al.*, 2011) and also in animal-origin food products, which are often implicated in sporadic cases and outbreaks of human salmonellosis (Tadesse, 2014).

The prevalence of foodborne *Salmonella* infections has increased dramatically in Bangladesh during the past few years (Al-Salauddin *et al.*, 2015; Islam *et al.*, 2016; Munsi *et al.*, 2016). The prevalence of *Salmonella* in meat and market milk has been reported by several authors and the frequency of detection ranges from 6.79% to 97.6% (Ramya *et al.*, 2012) in chicken meat and 0.17% to 28.6% (Tajbakhsh *et al.*, 2012) in raw milk in India and 21.1% in poultry in Bangladesh (Mahmud *et al.*, 2011).

Antibiotic-resistant *Salmonella* infections in both human and animal are universal concerns, particularly in developing countries where the risk of infection is high because of unhygienic living conditions close contact and sharing of houses between animals and humans (Feasey *et al.*, 2012). Antimicrobial-resistant *Salmonella* spp. have been isolated from different foods of animal origin around the world, which is attributed to the inappropriate use of antimicrobials as therapeutic or prophylactic agents in human and veterinary medicine, as well as the use of growth promoters in animal production (WHO, 2012). However, the sources and transmission routes of *Salmonella* in developing countries are poorly understood due to the lack of coordinated national epidemiological surveillance systems (Aferstein, 2003).

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There are reports of high prevalence of resistance in *Salmonella* isolates from Bangladesh (Hasan *et al.*, 2014). Studies conducted in different parts of our country have demonstrated the presence of *Salmonella* in human beings (Nesa *et al.*, 2011) and in different food animals and food products (Al-Salauddin *et al.*, 2015; Rahman *et al.*, 2004). However, there is scant information on the prevalence of foodborne *Salmonella* spp. and MDR in different food items such as chicken meat in Mymensingh (Al-Salauddin *et al.*, 2015) and in Gazipur (Rahman *et al*., 2004). But no report on the prevalence of *Salmonella* spp. in beef from Mymensingh district as per literature review. Therefore, this study reports the presence of *Salmonella* including its antimicrobial-resistant profiles in foods of animal origin like chicken meat, beef and milk in Mymensingh and Gazipur districts.

MATERIALS AND METHODS

Study area and collection of samples

The samples were collected randomly from farms and local markets situated in Mymensingh and Gazipur district of Bangladesh. A total of 169 samples comprised of poultry meat (n=51), beef (n=10) and milk (n=108) samples were tested from July 2016 to December 2017. Aseptically collected meat samples were placed in sterile plastic bags and transported to the Department of Medicine using an icebox. Milk samples were collected from Bangladesh Agricultural University (BAU) dairy farm, American dairy farm, Gazipur, and several small dairy farms. Aseptically 8-10 ml of milk was collected in test tube directly from teat of lactating cow and local market and transported to the Department of Medicine using an icebox. Five to ten grams of chicken (breast and cloacal muscle) meat and beef were collected aseptically in zipper bag from local municipal market and transported to the laboratory using ice box.

Sample preparation

Ten to twenty grams of meat were mixed with 10 ml of peptone (0.1%) water then homogenized suspension was prepared using sterilized pestle and mortar.

Isolation and identification of *Salmonella*

The homogenized meat suspension (4 to 5 ml) was inoculated into nutrient broths (5 ml/test tube), nutrient agar, SS agar, XLD agar and EMB agar). The inoculated media were incubated at 37^0C for 24±2 hours. Culture positive samples were subcultured several times to be pure culture. Gram staining and biochemical tests and sugar fermentation test were performed (Cheesbrough, 1985).

Polymerase chain reaction (PCR)

The DNA of *Salmonella* spp. was extracted by heat lysis method with slight modification (Momtaz *et al.*, 2012). The PCR assays were performed to detect *Salmonella* spp. using primer pairs. The nucleotide sequence (5'-3') for the *Salmonella* common primer used for this study were: R- ATG TTG TCC TGC CCC TGG TAA GAG and F- ACT GGC GTT ATC CCT TTC TCT TGG with an amplicon size of 496 bp (Al-Salauddin *et al.*, 2015). Briefly, for the detection of 16SrRNA gene PCR reaction mixture were 25 µl consisting of 5.5 µl RNAse free water, 12.5 µl PCR master mixture (Thermo Scientific, EU), 5 µl DNA template, 1 µl (20 pmol) primer. The PCR amplification was done by Initial denaturation at 95^0C for 5 minutes followed by 30 cycle of denaturation at 95^0 C for 30 seconds, annealing at 62^0C for 1 minute and extension at 72^0C for 1 minute. The final extension was at 72^0C for10 minutes. PCR amplify products were subjected to gel (1.5% agarose, Takara, Japan) electrophoresis with ethidium bromide fluorescence (100 v for 30 minutes) and visualized in gel documentation system via UV transilluminator (302 nm).

Antimicrobial susceptibilty test

Antimicrobial susceptibility of *Salmonella* spp., was performed by the disc diffusion according to the guidelines of the CLS1 (2012). Ten commercially available antibiotics (Oxoid, UK) e.g., oxytetracycline (30µg), ciprofloxacin (5µg), gentamicin (10µg), erythromycin, (15µg), azithromycin (15µg), sulphonamide-trimethoprim (25µg), neomycin (10µg), amoxicillin (10µg), doxycycline (10µg) and amikacin (30µg) were used for antibiogram study.

RESULTS

Salmonella spp. were isolated and identified based on culture, staining and biochemical characteristics. *Salmonella* spp., produced turbid growth on nutrient broth (Figure 1) and smooth white to grayish white colony on nutrient agar with peculiar fetid odor, pink colonies on EMB agar and black centered colonies on SS agar (Figure 2) and XLD agar (Figure 3). On Gram staining *Salmonella* spp., were found Gram negative, small rod and arranged as single or paired. Among five basic sugars only dextrose, maltose and mannitol were fermented with the production of acid and gas but lactose and sucrose were not fermented by most of the isolates.

Salmonella spp. were detected from different food sample the overall prevalence of *Salmonella* spp. in all food samples was 21.89%. A total of 29 (56.86%) chicken meat, 02 (1.85%) milk, and 06 (60%) beef samples were *Salmonella* spp. positive (Table 1)

Salmonella spp. was reconfirmed by PCR. The amplicon size of PCR product was 496 bp (16SrRNA gene). One beef and one chicken meat samples have shown *Salmonella* spp. positive (Figure 5).

The result of antibiotic sensitivity test is shown in Table 2. *Salmonella* spp. isolated from chicken meat was resistant to erythromycin (100%), doxycycline (79.31%), sulphonamide-trimethoprim (75.86%), azithromycin (72.41%) and oxytetracycline (66.67%) and sensitive to ciprofloxacin (83.33%) and gentamicin (86.21%). *Salmonella* spp. isolated from beef samples was resistant to erythromycin (83.33%), azithromycin (83.33%), oxytetracycline (66.67%) and doxycycline (66.67%); and sensitive to gentamicin (100%), neomycin (100%) ciprofloxacin (83.33%), amikacin (83.33%). *Salmonella* spp. isolated from milk samples were 100% sensitive to gentamicin, neomycin and ciprofloxacin, and 100% resistant to erythromycin, doxycycline and amoxycilin. Overall 89.19% isolates of *Salmonella* spp. were found multi-drug resistant (Table 3, Figure 4). About 100%, 66.67% and 93.10% of the *Salmonella* spp. isolates originated from milk, beef and chicken meat respectively were multi-drug resistant (resistant to three or more antibiotics).

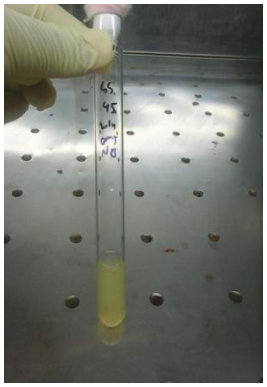

Figure 1. Growth of *Salmonella* spp. in nutrient broth

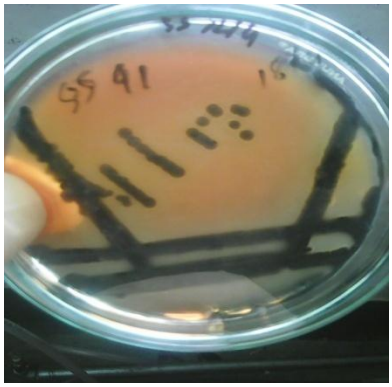

Figure.2. Formation of black centered colony of *Salmonella* spp. on SS agar.

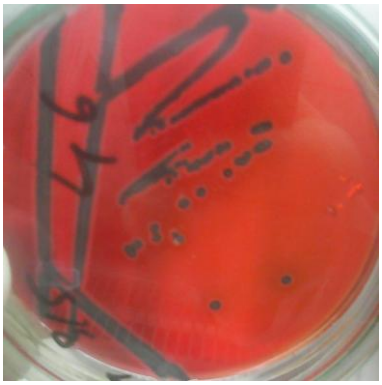

Figure 3. Formation of Black centered single colony of *Salmonella spp.* on XLD agar

Figure.4. Antibacterial susceptibility test of *Salmonella* sp on Muller Hinton Agar (recovered from XLD agar).Resistant to Oxytetracycline (OT), Azythromycin (AZM), Doxycycline (DO), and Susceptible to Amikacin (AK), Ciprofloxacin (CIP).

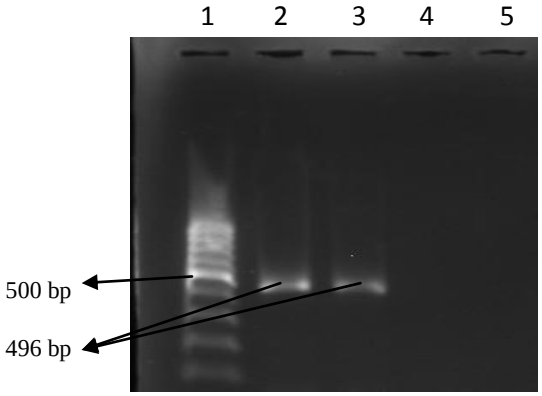

Figure 5. Amplification of PCR product; Lane 1:100 bp ladder, Lane 2 & 3: Specific for *Salmonella* spp. **(**Size-496bp for 16SrRNA gene). Lane 4 & 5 negative**.**

Table 1. Prevalence of *Salmonella* spp. in food samples

Sample	Sample tested	Positive	Prevalence (%)	95% CI
Milk	108	02	1.85	0.32-7.19
Chicken meat	51	29	56.86	42.32-70.37
Beef	10	06	60.00	27.37-86-31
Total	169	37	21.89	16.06-29.03

Table 2. Antibiogram study of *Salmonella* spp. isolates from food samples

Antibiotics	Chicken meat (n=29)		Milk (n=02)		Beef (n=6)	
	R=n (%)	S=n (%)	R=n (%)	S=n (%)	R=n (%)	S=n (%)
Ciprofloxacin	5 (17.24)	24 (83.33)	0	2(100)	01(16.67)	5(83.33)
Gentamicin	04(13.79)	25(86.21)	0	2(100)	0	6(100)
Erythromycin	29(100)	0	02 (100)	0	05(83.33)	1(16.67)
Azithromycin	21(72.41)	8(27.59)	01(50)	1(50)	05(83.33)	1(16.67)
Sulphonamide and Trimethoprim	22(75.86)	7(24.14)	01(50)	1(50)	02(33.33)	4(66.67)
Neomycin	05(17.24)	24(82.76)	0	2(100)	0	6(100)
Amoxicillin	13(44.83)	16(55.17)	02(100)	0	02(33.33)	4(66.67)
Doxycycline	23(79.31)	6(20.69)	02(100)	0	04(66.67)	2(33.33)
Amikacin	10(34.48)	19(65.52)	-	-	01(16.67)	5(83.33)
Oxytetracycline	04(66.67)	02(33.33)	-	-	04(66.67)	02(33.33)

R= Resistant *Salmonella*; S= Sensitive *Salmonella*

Table 3. Detection of multi-drug resistant *Salmonella* spp. in food samples

Name of samples	No. of *Salmonella* isolated	No. of MDR *Salmonella*	Prevalence of MDR *Salmonella* (%)
Milk	02	02	100
Chicken meat	29	27	93.10
Beef	06	04	66.67
Total	37	33	89.19

DISCUSSION

The overall prevalence of *Salmonella* spp. in all food samples was 21.89% and 56.86% of chicken meat were positive for *Salmonella* spp. which is in agreement with Parbati *et al.* (2017) who isolated 53.33 % *Salmonella* spp. from poultry and poultry environment; and with the Islam MJ (2016) who isolated 55% salmonella spp. from healthy poultry and their cloacal swab. But our result differs with the findings of other authors where the range of reported prevalence varied from 18% to 31.66% (Barua *et al.*, 2012; Al-Salauddin *et al.*, 2015). Sharma and Das (2016) isolated 43% *Salmonella* in chicken carcass at Assam, India. Dallal *et al.* (2009) reported 21.6% and 15.7% *Salmonella* spp. in unpackaged and packaged chicken meat; 29.4% and 33.3% in packaged and unpackaged beef in retail store shop respectively. These variations might be due to source of sample collection, hygienic condition of the farm and personal hygiene of the processors and handlers.

We observed only 1.85% *Salmonella* spp. in milk samples. However, Munsi *et al.* (2015) reported 35.71 % milk samples to be positive for *Salmonella typhi* in vendor milk and 0 % in brand milk. We have collected milk aseptically from teat directly and the prevalence of *Salmonella* spp. in our samples was very low because of no intermediate contamination. The high prevalence of *Salmonella* spp. in vendor milk was probably due to adding contaminated water or unhygienic handling, using contaminated utensils, etc. (Antunes *et al.*, 2003). Among 37 culture positive *Salmonella* spp., only two samples were confirmed by PCR. Similar findings was also reported by Yadav *et al.* (2017) where one out of 18 culture positive samples was found to be InvA gene positive (*Salmonella*

typhimurium), and 5 samples were turned out as SefA gene positive (*Salmonella enteridis*). Smilarly, Parimal *et al.* (2002) also confirmed 92 isolates as PCR positive out of 569 *Salmonella* positive samples. *Salmonella* spp. isolated from chicken meat were resistant to Erythromycin (100%), Doxycycline (79.31%), sulphonamide-trimethoprim (75.86%), azithromycin (72.41%) and oxytetracycline (66.67%) and sensitive to ciprofloxacin (83.33%) and gentamicin (86.21%). Similar findings had also been reported by other authors (Rahman *et al.*, 2004; Al-salauddin *et al*., 2015; Obi and Ike, 2015; Parbati *et al*., 2017; Parvez *et al.*, 2016). *Salmonella* spp. isolated from beef sample were resistant to erythromycin (83.33%), azithromycin (83.33%), oxytetracycline (66.67%) and doxycycline (66.67%); and sensitive to gentamicin (100%), neomycin (100%) ciprofloxacin (83.33%), amikacin (83.33%). Similarly Dallal *et al.* (2009) also reported *Salmonella* spp. from beef sample as 100 % resistant to erythromycin and tetracycline; 60 % resistant to sulphamethoxazole, and all isolates were susceptible to ciprofloxacin and gentamicin. Azithromycin is normally used in humans, pet animals and poultry .However, *Salmonella* isolated form foods of animal origin were found to be resistant to azithromycin. This indicates interspecies transmission of resistance gene which might pose serious public health threats.

Salmonella spp. isolated from milk samples were 100% sensitive to gentamicin, neomycin and ciprofloxacin, and 100% resistant to erythromycin, doxycycline and amoxicilin. Other authors also found *Salmonella* isolates of raw and fermented milk origin as 100% sensitive to ciprofloxacin and gentamicin and resistant to erythromycin (85.7%) and amoxicillin (78.6%) (Tamba *et al.*, 2016; Munsi *et al.*, 2015).

Multi- drug resistant *Salmonella* isolates were found in different food samples. Although we did not check the pathogenicity of the isolates, the gene responsible for multi-drug resistance may transfer to consumer via food and results in serious public health hazard. An overall 89.19% isolates of *Salmonella* spp. were found to be multi-drug resistant. About 100%, 66.67% and 93.10% of the *Salmonella* spp. isolates originated from milk, beef and chicken meat respectively were multi-drug resistant. This MDR result is higher than that of Al-Salauddin *et al.*, (2015) who reported 16.67% isolates of *Salmonella* originated from broiler meat as multidrug resistant (MDR); and also higher with the findings of Dallal *et al.* (2009) who reported 23.5% of the *Salmonella* strains as MDR to two or more antibiotic families. These findings showed that MDR in *Salmonella* spp., prone to increase with the time due to indiscriminate use of antibiotics in dairy and poultry industry, pet animal practice and in human practice in Bangladesh. Rationale use of this drug may prevent development of resistant isolates of *Salmonella* spp., in future.

CONCLUSION

The presence of *Salmonella* spp. in food indicates contamination with polluted water; the improper handling, processing and storage and poor personal hygiene and sanitation. *Salmonella* isolates of food animal and poultry origin found to be resistant to drugs commonly used in humans like azithromycin. This indicates interspecies transmission of resistance gene which might pose serious public health threats. The antibiogram profile of the isolates will help choosing the most effective antibiotics to treat the diseases conditions caused by *Salmonella* spp. in cattle and poultry in Bangladesh.

ACKNOWLEDGEMENTS

The author would like to acknowledge the Ministry of Education, Bangladesh for financial support (Project no.267/2015.MoE) for funding the research.

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