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Molecular identification and characterization of *Salmonella* species isolated from poultry value chains of Gazipur and Tangail districts of Bangladesh

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The aim of this study was to identify and characterize *Salmonella* species isolated from poultry value chains of Gazipur and Tangail districts of Bangladesh during the period of October 2015 to May 2016. For this purpose a total of 153 samples (35 chick meconium, 49 cloacal swab, 30 poultry carcass, 14 feed, 16 water, 7 transport swab, 2 floor swab) were collected and were subjected to various cultural, biochemical techniques and polymerase chain reaction (PCR). Furthermore, the isolated *Salmonella* species were characterized by antimicrobial susceptibility testing. Among the samples, 23.53% (n=36) were found to be associated with *Salmonella* species. The *Salmonella* species were identified by observing black centered colonies on XLD agar, positive to MR test and negative to VP and Indole test. All isolates of *Salmonella* species were positive to 16s rRNA gene based PCR (574 bp). Serogrouping of *Salmonella* species were performed by slide agglutination test using commercial *Salmonella* specific polyvalent O (A-I) antisera, *Salmonella* O group B (Factor O: 4, 5, 27) antisera and *Salmonella* O group D (Factor O: 9, 46) antisera. Among the 36 isolates, 30.56% (n=11) belonged to serogroup B and rest of the isolates 69.44% (n=25) to serogroup D. The most prevalent serogroup identified in this study was serogroup D. The isolated *Salmonella* species were subjected to antimicrobial susceptibility testing with the aid of disk diffusion method using 8 antimicrobial agents. All isolates of *Salmonella* species were susceptible to ciprofloxacin, norfloxacin, streptomycin and gentamicin. Out of 36 isolates 100% *Salmonella* species were resistant to erythromycin and tetracycline. The findings of this study revealed the presence of multidrug resistant *Salmonella* species in poultry value chains of Gazipur and Tangail districts of Bangladesh that posseses a serious threat to public and poultry health. To the best of our knowledge, this is the first report on the prevalence, serogrouping and antimicrobial resistance patterns of *Salmonella* species from poultry value chains of selected districts in Bangladesh.

Key words: Isolation, identification, *Salmonella* species, poultry value chains, serogrouping, antibiogram study.

INTRODUCTION

Bangladesh is an agriculture based country. Poultry rearing is considered superior to the others in agricultural sector because of an almost assured and quick return in a relatively short period of time (Saleh et al., 2003).

Poultry industry which has started during 1980_s is an excellent agribusiness (Haque, 2001). Over the last decades surprising development in the poultry sector has been occurred (Rahman, 2003). It has become a vital sector for its employment generation, creating additional income and improving the nutritional level of the country. This sector provides fulltime employment to about 20% and partial employment to about 50% of the rural people (Alam et al., 2003). Development of poultry sector in Bangladesh is being hampered by a number of factors, of which the diseases are considered as the major factor causing 30% mortality of chicken per year (Das et al., 2005). Intestinal bacteria play an important role on health through their effects on gut morphology, nutrition, pathogenesis of intestinal diseases and immune responses (Mead, 2000).

Among the bacterial diseases, salmonellosis has been considered one of the most important infectious disease in both humans and animals (Keusch, 2002). Motile *Salmonellae* (paratyphoid group) infections cause salmonellosis in chickens and have zoonotic significance (Kabir, 2010). Salmonellosis is major problems in the poultry industry in Bangladesh (Haider et al., 2008). *Salmonella* infection is one of the major constraints of poultry farming that hindered its development in Bangladesh (Kamaruddin and Giasuddin, 2003; Das et al., 2005). It causes a variety of acute and chronic diseases of poultry in Bangladesh (Bhattacharjee et al., 1996). Chicks can be infected with *Salmonella* species by vertical transmission through infected parents or by horizontal transmission through hatcheries, sexing in contaminated hatcheries, cloacal infection and transportation of equipment and feed (Opitz et al., 1993). There are >2500 *Salmonella* serovars distributed throughout the world (L Plym and Wierup, 2006).

Several studies were carried out about the detection of *Salmonella* species in poultry in Bangladesh (Islam et al., 2016; Parvej et al., 2016; Al-salauddin et al., 2015; Jahan et al., 2013). Al-salauddin et al. (2015) recently conducted a study to isolate and identify the *Salmonella* species present in broiler meat. From 18 dressed broiler meat samples 31.66% *Salmonella* species were isolated. 55% *Salmonella* species were also isolated from 80 cloacal swab samples by Islam *et al*. (2016). 45% (n=27) bacterial isolates out of 60 samples were identified as *Salmonella* species from dressing water and environmental swabs by Jahan et al. (2013). This indicated a high rate of *Salmonella* contamination in poultry and live bird markets of Bangladesh, therefore salmonellosis status of a farm needs to be determined for its proper control and management (Ahmed et al., 2009). But no work has been done yet in Bangladesh to identify the *Salmonella* species from different phases of poultry value chains (hatchery → farm → transport → live bird markets) at a time. Therefore, the present study was designed to isolate and identify *Salmonella* species as well as serogrouping the isolated *Salmonella* species.

MATERIALS AND METHODS

Sample collection

A total of 153 samples (Table 1) were collected from poultry value chains of three different upazilas of Gazipur and Tangail districts of Bangladesh. During the collection of samples precautionary measures were taken to avoid contamination and ice boxes were used to maintain cool chain. Then the collected samples were brought to the Bacteriology Laboratory of the Department of Microbiology and Hygiene, BAU, Mymensingh for isolation, identification, serogrouping and antimicrobial susceptibility testing of *Salmonella* species.

Cultural characterization and isolation of *Salmonella* species

The samples were then cultured under aerobic conditions at 37ºC for 24 h using XLD agar. The growing colonies of *Salmonella* species were characterized morphologically using Gram's stain according to the method described by Merchant and Packer (1967) and motility test with hanging drop slide (Cowan, 1974).

Identification of *Salmonella* species by biochemical tests

Biochemical characterizations of the *Salmonella* isolates were performed with Sugar fermentation test, Methyl Red test (MR) and Voges-Proskauer test (V-P) (Cheesbrough, 1985).

Preparation of DNA templates

DNA template was prepared by boiling method described by Queipo-Ortuno et al. (2007). 250 µl distilled water was taken into Eppendorf tube and a pure *Salmonella* colony was picked up and mixed with the distilled water. The tubes then transferred to boiling water and boiled for 10 min then immediately transferred to ice for cold shock about 10 min and then centrifuged at 10,000 rpm for 10 min. Supernatant were collected and used as DNA template during PCR.

16S rRNA gene based PCR for identification of the genus *Salmonella*

The PCR analyses, reaction was carried out in a final volume of 20 µl containing 10 µl of master mixture (Promega, USA), 1 µl of each primer (forward and reverse/16s rRNA), 3 µl of DNA and 5 µl of deionized water. The amplification was carried out as follows:

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Table 1. Summary of collected samples from poultry value chains of Gazipur and Tangail districts of Bangladesh.

Type of samples	No. of collected samples	
	Gazipur district	Tangail district
Chick meconium	25	10
Cloacal swab	35	14
Whole Carcass	22	08
Feed	10	04
Water	12	04
Transport swab	05	02
Floor swab	02	-
Total	**111**	**42**

Initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 50°C for 30 s and extension at 72°C for 30 s. The final extension was conducted at 72°C for 5 min. After amplification, the samples were stored at 4°C. PCR products were analyzed by 1.5% agarose (Invitrogen, USA) gel electrophoresis and the bands were visualized with UV light after staining with ethidium bromide (0.5 µg/ml) for 10 min in a dark place. Bands were visualized and images were captured on a UV transilluminator (Biometra, Germany).

Serogrouping of *Salmonella* by O-antigen test

Salmonella agglutinating antiserum poly "O" and poly "A-I" (S & E Reagents Lab, Bangkok, Thailand) was used to perform the serotyping of the isolated Salmonella species. One drop of normal saline (0.85% NaCl) was added as control on a glass slide by the use of a wire. A loop full of culture from the Nutrient agar (NA) plate was transferred onto the glass slide and mixed with the drop of saline. Agglutination within 30 seconds indicated that it's rough strains. The strains can't be used for serotyping. Serotyping was continued with antisera if no agglutination was recorded. One drop of *Salmonella* agglutinating antisera (Poly A-I) was added on each test area on the slide. A loop full of culture from NA plate was added to each spot of antiserum. Mixed carefully the culture with the O-serum. The glass slide was rocked gently for one minute. Agglutination with the antisera indicated that the strain has an O-antigen. It was a screen procedure. Then tested with O group B and O group D. Some strain agglutinated with O group B (O: 4, 5, 27) and some strain agglutinated with O group D (O: 9, 46).

Antimicrobial susceptibility testing

All *Salmonella* species were tested against eight commonly used antibiotics (HiMedia, India) by the method of disk diffusion as described by Bauer et al. (1966). The zones of growth inhibition were compared with the zone size interpretative standards as described by Clinical and Laboratory Standard Institute (2011). *E. coli* ATCC 25922 was used as a quality control organism in this study. At least two separate experiments were performed for confirmation of all susceptibility data.

RESULTS AND DISCUSSION

A total of 153 samples (35 chick meconium, 49 cloacal swab, 30 poultry carcass, 14 feed, 16 water, 7 transport swab, 2 floor swab) were collected from poultry value chains of three different upazilas of Gazipur and Tangail districts of Bangladesh. Out of 153 samples, 147 samples were collected from hatcheries, farms and transports. Out of 147 samples, 30 (20.4%) samples have shown positive for *Salmonella* species (Table 2). A total of 6 samples were collected from 2 live bird markets of Gazipur district. All 6 (100%) samples were positive for *Salmonella* species in collected broiler meat, floor swab and water samples of Sreepur upazilla in Gazipur district as presented in Table 2.

For the cultural examination of *Salmonella* species several selective media such as XLD and SS agar were used which were also used by a number of researchers (Hyeon et al., 2011; Muktaruzzaman et al., 2010). In this study, *Salmonella* species were produced translucent, black smooth, small round colonies on SS agar and pink color colonies with black centre on XLD agar (Table 3). These findings were similar to the findings of other authors (Muktaruzzaman et al., 2010; Sujatha et al., 2003; Khan et al., 2005).

In Gram's staining, the morphology of the isolated *Salmonella* species exhibited Gram negative small rod arranged in single or paired (Table 3) which was supported by several researchers (Freeman, 1979; Buxton and Fraser, 1977; Merchant and Packer, 1967). Biochemical tests were performed for the identification of *Salmonella* species. In carbohydrate fermentation test, the isolates that fermented glucose, maltose and produced acid and gas but did not ferment lactose those indicated positive for *Salmonellae* as was stated by Buxton and Fraser (1977). All the isolates were found positive for MR test and negative to Indole test and V-P test (Table 4) which was supported by Cheesbrough (1985). Motility test was elementary basis for the detection of motile and non-motile *Salmonella* species. In motility test, 26 isolates were non motile and 10 isolates were motile (Table 3). That means the non-motile 26 isolates were either *Salmonella enterica* serotype *gallinarum or pullorum* and other isolates were motile species of *Salmonella* which was supported by Grimont et al. (2000).

In this study molecular identification was done by PCR, in which 16s rRNA gene was amplified for the detection of isolated *Salmonella* species and the results are shown in Figure 1. All conditions and results found in the PCR were similar by the findings of the several researchers (Ziemer and Steadham, 2003; Lin and Tsen, 1996).

Salmonella species were isolated from 30 apparently healthy broiler samples and positive isolates were 36.67% (n=11). In this study the incidence of *Salmonella* species in whole carcass was closely similar with the results reported by several researchers (Hossain et al., 2015; Ahmed et al., 2009; Zhao et al., 2001).

There were presence of 8.16% (n=4) *Salmonella*

Table 2. Summary of isolated *Salmonella* species from poultry value chains.

Placement (no. of farms)	No. of collected samples										*Salmonella* species No. of isolates (%)									
	Hatchery	Farm					Live bird market				Hatchery	Farm					Live bird market			
	CM	CS	F	W	WC	T	LWC	W	FS	Total	CM	CS	F	W	WC	T	LWC	W	FS	Isolates
Gazipur sadar, Gazipur (3)	15	21	6	6	12	3	1	1	1	66	2(13.3)	5(23.8)	1(16.6)	0(0)	4(33.3)	1(33.3)	1(100)	1(100)	1(100)	16(24.2)
Sreepur, Gazipur (2)	10	14	4	4	8	2	1	1	1	45	1(10)	2(14.2)	1(25)	0(0)	2(25)	0(0)	1(100)	1(100)	1(100)	9(20)
Total (Gazipur) (5)	25	35	10	10	20	5	2	2	2	111	3(12)	7(20)	2(20)	0(0)	6(30)	1(20)	2(100)	2(100)	2(100)	25(22.5)
Tangail sadar, Tangail (2)	10	14	4	4	8	2	-	-	-	42	1(10)	3(21.4)	2(25)	1(25)	3(37.5)	1(50)	-	-	-	11(26.19)
Gross total (7)	35	49	14	14	28	7	2	2	2	153	4(11.4)	10(20.4)	4(28.6)	1(7.1)	9(32.1)	2(28.6)	2(100)	2(100)	2(100)	36(23.53)

CM=Chick meconium, CS =Cloacal swabs, F=Feed, W=Water, WC=Whole carcass (broiler meat), T=Transport swabs, LWC=Live whole carcass and FS=Floor swabs.

Table 3. Results of cultural, morphological and motility characteristics of isolated *Salmonella* species.

Colony morphology		Staining characteristics	Motility
Xylose-Lysine Deoxycholate agar	Salmonella-Shigella agar	Pink short rod shaped, Gram negative bacteria arranged in single or paired.	+ve or -ve
Black centered colony.	Translucent, black smooth round colonies.		

"-Ve"=negative, "+Ve" = positive.

Table 4. Biochemical reaction patterns of *Salmonella* species.

Bacteria	Sugar fermentation properties					MR	VP	Indole
	Dextrose	Maltose	Sucrose	Lactose	Mannitol			
Salmonella species	AG	AG	-ve	-ve	AG	+ve	-ve	-ve

MR= Methyl red, VP = Voges-Proskauer reaction. , AG = Acid and Gas, "-Ve"=negative, "+Ve" = positive.

species in collected samples of chick meconium (49) from hatchery and this findings was very close to the findings of several researchers (Nasrin et al., 2012). These findings also indicate that vertical transmission of *Salmonella* species occurred to the chick that was similar to the findings reported by several researchers (Cason et al., 1994; Bailey et al., 1994).

In this study, 28.57% (n=7) of *Salmonella* species were positive in transport swabs samples (n=7). 28.57% (n=4) of *Salmonella* species were positive in feed samples (n=14). The prevalence of *Salmonella* species in feed sample was closely similar to the findings of Islam et al. (2014). There was also 18.75% (n=3) *Salmonella* species were present in water samples (n=16). The results of this study were closely related with the results of several authors (Saha et al., 2012; Samanta et al., 2014).

About 49 cloacal swab samples were collected for the research work. Among 49 cloacal swab

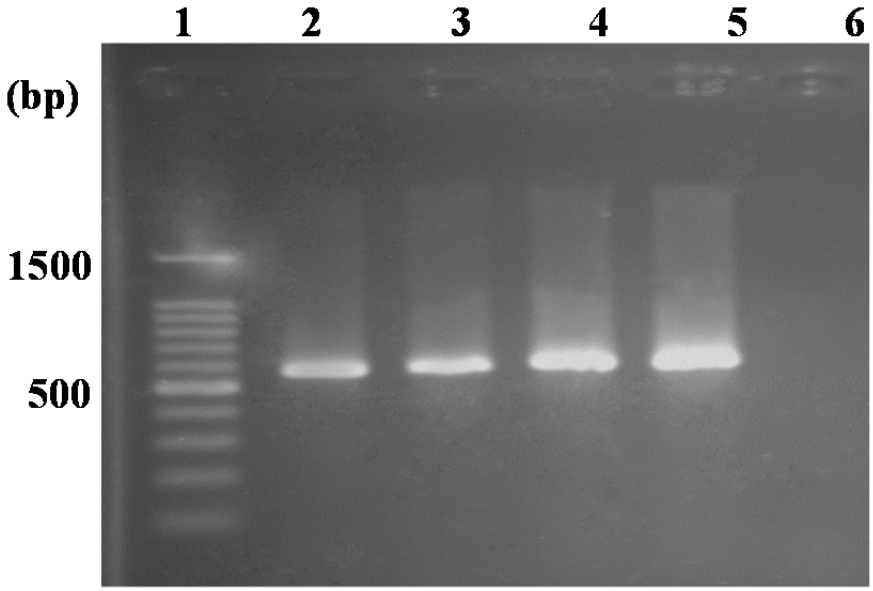

Figure 1. Detection of *Salmonella* species by 16s rRNA gene based PCR. Lane 1: 100 bp DNA ladder (Promega, USA); Lane 2, 3, 4, 5: DNA of *Salmonella;* Lane 6: Negative control.

Table 5. Serogrouping of *Salmonella* species.

Isolates	No. (%) of *Salmonella* species		
Salmonella species (n=36)	Poly A-I	Group B (O:4,5,27)	Group D (O:9,46)
	36 (100%)	11 (30.56%)	25 (69.44%)

samples 20.41% (n=10) samples were positive for *Salmonella* species. In between two districts higher percentage was observed 21.4% (n=3) in Tangail district of Bangladesh. The results of this study were in close relation with the results of several researchers (Islam et al., 2016; Parvej et al., 2016; Sarker et al., 2012). Cloacal swabs have been used to provide evidence of persistent intestinal colonization by *Salmonella* in individual birds reported by Gast et al. (1997). Among the 153 collected samples from two different districts, the total *Salmonella* species were isolated as 23.53% (n=36). The isolated *Salmonella* species in Gazipur district was 22.52% (n=25) and in Tangail district was 26.19% (n=11). Results of this study were closely related with the results of several researchers (Al-Ferdous et al., 2013; Kabir, 2010).

From the collected 153 samples, 36 *Salmonella* species were isolated. Among the 36 isolates, 30.56% (n=11) belonged to serogroup B and 69.44% (n=25) isolates belonged to serogroup D as presented in Table 5. The most prevalent serogroup identified in this study was serogroup D. The results of serogrouping correlated with the results of motility test where 26 isolates were non motile and 10 isolates were motile. These findings were in agreement with the result reported by several researchers (Mahmud et al., 2011; Arroyo and Arroyo, 1995).

Antimicrobial susceptibility analysis is presented in Tables 6 and 7. In this study it was revealed that *Salmonella* species were sensitive to ciprofloxacin, gentamicin and norfloxacin. This result was supported by a number of researchers (Jahan et al., 2013; Al-Ferdous et al., 2013; Khan et al., 2005). Out of the 36 *Salmonella* isolates 100% (n=36) were resistant to tetracycline which was similar to the report of (Lu et al., 2011). 13 (36.11%) were resistant to 2 agents E-TE. 6 (16.67%) were resistant to 3 agents E- AMX-TE. 9 (25%) were also resistant to 3 agents E-AZM-TE. Another 8 (22.22%) were resistant to 4 agents AMX-AZM-E-TE. Similar studies were also observed by several researchers (Al-Ferdous et al., 2013; Jahan et al., 2013; De et al., 2012; Hyeon et al., 2011; Khan et al., 2005). Resistant profile of *Salmonella* species were recorded some multi-drug resistant *Salmonella* species, which was similar to the result of some researchers (Al-Ferdous et al., 2013;

Table 6. Antimicrobial susceptibility pattern of *Salmonella* species by disk diffusion method.

Antimicrobial agents	No. (%) of *Salmonella* species		
	S	I	R
Amoxicillin	17 (47.22%)	5 (13.89%)	14 (38.89%)
Azithromycin	7 (19.44%)	12 (33.33%)	17 (47.22%)
Ciprofloxacin	36 (100%)	0 (0%)	0 (0%)
Erythromycin	0 (0.0%)	0 (0%)	36 (100%)
Gentamicin	36 (100%)	0 (0%)	0 (0.0%)
Norfloxacin	36 (100%)	0 (0%)	0 (0.0%)
Streptomycin	36 (100%)	0 (0%)	0 (0%)
Tetracycline	0 (0.0%)	0 (0%)	36 (100%)

S= Susceptible; I= Intermediate; R= Resistance

Table 7. Antimicrobial resistance profiles of *Salmonella* species.

Isolates	Resistance profiles	No. of isolates (%)
Salmonella species (n=36)	No resistance demonstrated	–
	Resistant to 2 agent (E-TE)	13 (36.11%)
	Resistant to 3 agents (E- AMX-TE)	6 (16.67%)
	Resistant to 3 agents (E-AZM-TE)	9 (25%)
	Resistant to 4 agents (AMX-AZM-E--TE)	8 (22.22%)
	Total resistant isolates	**36 (100%)**

AMX=Amoxicillin, AZM=Azithromycin, E=Erythromycin, GEN=Gentamicin, ClP=Ciprofloxacin, NOR=Norfloxacin, TE=Tetracycline, S=Streptomycin.

Jahan et al., 2013).

Conclusions

The findings of this study revealed the presence of multidrug resistant *Salmonella* species in poultry value chains of Gazipur and Tangail districts of Bangladesh that poses a serious threat to public and poultry health. Nevertheless, more studies are needed to clearly understand the genomic diversity in *Salmonella* species as well as molecular mechanisms for the development of antimicrobial resistance.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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