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Multidrug-Resistant *Salmonella* spp. Isolated from Apparently Healthy Pigeons in a Live Bird Market in Chattogram, Bangladesh

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ABSTRACT

Multidrug-resistant *Salmonella* could pose a severe public health threat. The current study aimed to investigate the prevalence of antibiotic resistance and some antibiotic-resistant genes in *Salmonella* spp. isolated from pigeons in a live bird market, Chattogram, Bangladesh. A total of 100 cloacal swab samples were collected aseptically from apparently healthy pigeons in the live bird market, namely Riazuddin Bazar in Chattogram city, Bangladesh. Different bacteriological and biochemical tests were used for the isolation and identification of *Salmonella* spp. The susceptibility test of *Salmonella* isolates to different antibiotics was performed by the disk diffusion method. PCR assay using specific primers was used for antibiotic resistance genes detection. The results indicated that the prevalence of *Salmonella* spp. was 29% in sampled birds. The highest antibiotic resistance rate was found to be ampicillin (93.1%), followed by both sulfamethoxazole-trimethoprim and tetracycline (86.2%). In contrast, 65.5% of isolates were found sensitive to ciprofloxacin, followed by colistin (62.1%), kanamycin (55.2%), and gentamicin (48.3%). 96.6% of *Salmonella* isolates were classified as multidrug-resistant and harbored *bla*TEM, *tet*A, *sul*1, and *sul*2 genes. In conclusion, pigeons as carriers of antibiotic-resistant *Salmonella* spp. may pose a health risk to other birds and humans.

Keywords: Antibiogram, Antibiotic resistance genes, Pigeons, Prevalence, *Salmonella*

INTRODUCTION

Food animals have been recognized as a reservoir of resistant bacteria and a source of foodborne infections for humans (Szmolka and Nagy, 2013). Food chain cycle act as a vehicle to transmit antibiotic-resistant infectious agents from farm animals to humans (Molbak et al., 2002).

Pigeons (*Columbia livia*) have an important role in dispersing the bacterial agents to free-range poultry and have been considered a fecal contaminator of drinking water sources and rural harvests (Lillehaug et al., 2005). These birds are in contact with humans at home, farms, and live bird markets (LBM), and are responsible for the transmission of several diseases through their droppings (Weber, 1979). Several pathogenic microbes such as *E. coli*, *Salmonella* spp., *Cryptococcus* spp., and *Chlamydia* spp. are carried by pigeons (Tanaka et al., 2005).

LBMs are the most significant terminal hub of the poultry business in Asian countries, where individuals purchase live or freshly butchered poultry (Sarker et al., 2019a). Pigeons in LBMs are originated from various sources and territories and stocked in confined spaces at high densities. Moreover, at LBMs, customers come in close and direct interaction with live or processed poultry. Therefore, unhygienic conditions in LBMs may provoke the dissemination of infectious agents from pigeon to pigeon and pigeon to human. In the farms and LBMs, the apparently healthy pigeons are one of the sources of human salmonellosis (Hosain et al., 2012). Therefore, LBMs have an important role in the transmission of *Salmonella* in human food chain. To date, very little work on prevalence of antibiotic-resistant *Salmonella* in pigeons has been conducted in Bangladesh. Therefore, the present study aimed to determine the prevalence of antibiotic resistance and some resistance genes in *Salmonella* isolated from pigeons in LBM in Chattogram, Bangladesh.

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MATERIALS AND METHODS

Ethical approval

The study protocol was approved by the Institutional Animal Ethics Committee, Chattogram Veterinary and Animal Sciences University, Bangladesh.

Samples collection

A total of 100 cloacal swab samples were aseptically collected from 100 apparently healthy pigeons in a LBM in Chattogram, Bangladesh during the period from February to April 2018. Using a simple random technique, 10 samples were collected from each shop. The swab samples were transferred to Falcon tubes containing 5 ml of buffered peptone water (BPW) (Oxoid, UK) and immediately transported to the laboratory in an icebox.

Isolation and identification of *Salmonella*

Samples were incubated overnight in BPW at 37 °C for enrichment. For selective enrichment, 100 µl of the pre-enriched sample was transferred to Rappaport-Vassiliadis (RV) medium (Oxoid, UK), incubated at 41.5 °C for 24 hours. A loopful of positive enrichment in RV was streaked onto Salmonella-Shigella (SS) agar (Oxoid, UK) and xylose lysine deoxycholate (XLD) agar (Oxoid, UK), incubated at 37°C for 24 hours. At least two single typical *Salmonella* colonies were randomly picked up and subjected to biochemical tests (triple sugar iron [TSI], indole, urease, oxidase, and catalase tests) (Begum et al., 2018). Positive *Salmonella* isolates were preserved into brain heart infusion (BHI) broth (Oxoid, UK) with 15% glycerol at -80 °C.

Extraction of chromosomal DNA

For the PCR, total DNA was extracted from the isolated bacterial agents using the boiling method (Sánchez et al., 2010). In brief, 2-3 pure cultured colonies were mixed with 200 µl of deionized water into 1.5 ml sterile Eppendorf tube, followed by boiling for 15 min. After boiling, it was kept on ice immediately for 10 min, centrifuged for 2 min at 15000 rpm. Finally, the collected supernatant was used as a DNA template for PCR.

Molecular detection of *Salmonella*

Genotypic confirmation of *Salmonella* was done by targeting the *sdi*A gene as previously described by Halatsi et al. (2006). The sequence of the specific primer pair for the *sdi*A gene is presented in Table 1. PCR amplification was accomplished with a 25 µl reaction mixture containing deionized water (10.5 µl), GoTaq master mix (Promega, USA) (12.5 µl), forward and reverse primers (0.5 µl each), and DNA template (1 µl). The thermal profile consisted of an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 50 °C for 1 min, elongation at 72 °C for 1 min, and final extension at 72 °C for 10 min. PCR products were then electrophoresed by running in 1.5% agarose gel stained with ethidium bromide (Sigma-Aldrich, USA).

Table 1. Oligonucleotide primers used in the study

Target genes	Primers sequence (5´-3´)	Amplicon size (base pair)	References
*bla*TEM	F: TACGATACGGGAGGGCTTAC R: TTCCTGTTTTTGCTCACCCA	716	Belaaouaj et al. (1994)
*tet*A	F: GCTACATCCTGCTTGCCTTC R: CATAGATCGCCGTGAAGAGG	210	Karczmarczyk et al. (2011)
*sul*1	F: CGGCGTGGGCTACCTGAACG R: GCCGATCGCGTGAAGTTCCG	433	Sunde (2005)
*sul*2	F: CGGCATCGTCAACATAACCT R: TGTGCGGATGAAGTCAGCTC	721	Lanz et al. (2003)
*sdi*A (*Salmonella*)	F: AATATCGCTTCGTACCAC R: GTAGGTAAACGAGGAGCAG	274	Halatsi et al. (2006)

F: forward, R: reverse

Antibiotic susceptibility test

To assess the antibiotic susceptibility of *Salmonella* isolates, the disk diffusion method was performed on Mueller-Hinton agar (Oxoid, UK) plates as described by CLSI (2012). The isolates were tested against 10 commonly used antibiotics using antibiotic disks including ampicillin (10 µg), ceftriaxone (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), kanamycin (30 µg), tetracycline (30 µg), sulfamethoxazole-trimethoprim (25 µg), colistin sulfate (10 µg), chloramphenicol (30 µg) and nalidixic acid (30 µg) (Oxoid, UK). The sensitivity results were interpreted according to CLSI (2012). Multidrug-resistant (MDR) was defined as isolate being resistant to at least three antimicrobial agents from different classes (Tenover, 2006).

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Detection of antibiotic resistance genes

All of the phenotypically resistant *Salmonella* isolates were subjected to PCR to detect the ampicillin resistance gene (*bla*TEM), tetracycline resistance gene (*tet*A), and sulfonamide resistance gene (*sul*1 and *sul*2) according to the previously published study (Sarker et al., 2019b). The sequence of primers used for target gene amplification is presented in Table 1.

Statistical analysis

Data were entered into an Excel spreadsheet (Microsoft Corporation, USA). Descriptive statistics were used to analyze the data by an online epidemiological calculator (Sergeant, ESG, 2019).

RESULTS

Prevalence and characteristic of *Salmonella*

The prevalence of *Salmonella* in the collected samples was 29% (29/100) (95% CI: 21.01-38.54). Distinctive *Salmonella* colonies on XLD agar were pink color with black centered, and on SS agar produced small, smooth, round, and black centered colonies. *Salmonella* isolates were positive to TSI and negative to indole, urease, oxidase, and catalase test.

Antibiotic resistance patterns

Antibiogram study of *Salmonella* showed that the isolates were highly resistant to ampicillin (93.1%, 27/29), tetracycline (86.2%, 25/29) and sulfamethoxazole-trimethoprim (86.2%, 25/29), followed by nalidixic acid (72.4%, 21/29), chloramphenicol (51.7%, 15/29) and ceftriaxone (48.28%, 14/29). To the contrary, the highest susceptibility rate was found against ciprofloxacin (65.5%, 19/29), followed by colistin (62.1%, 18/29) and kanamycin (55.2%, 16/29) (Figure 1). Of the 29 *Salmonella* isolates, 28 (96.6%) disclosed the MDR patterns (Table 2).

Table 2. Antibiotic resistance patterns and distribution of targeted resistant genes among 29 *Salmonella* isolates recovered from cloacal swab samples of pigeons in a live bird market in Chattogram, Bangladesh

Phenotypic pattern of antibiotic resistance	MDR isolate	Genotypic pattern of antibiotic resistance			
		*bla*TEM	*tet*A	*sul*1	*sul*2
AMP-SXT	-	+	-	+	+
AMP-TE-SXT	+	+	+	-	-
AMP-TE-CT-C	+	-	-	-	-
AMP-TE-SXT-NA	+	+	+	+	+
AMP-SXT-C-K	+	+	-	-	-
AMP-SXT-CIP-NA	+	+	-	-	+
AMP-SXT-CN-C-NA	+	-	-	+	-
AMP-CRO-TE-SXT-NA	+	+	+	-	-
AMP-TE-SXT-CT-C	+	+	+	-	+
CRO-TE-SXT-CN-NA	+	-	+	+	-
AMP-TE-SXT-CN-NA	+	+	-	-	+
AMP-TE-SXT-CN-CT-CIP	+	+	+	+	-
AMP-TE-SXT-C-NA-K	+	-	-	-	+
TE-SXT-CN-C-CIP-K	+	-	+	-	-
AMP-CRO-TE-SXT-CT-NA	+	+	-	+	+
AMP-TE-CN-CT-C-K	+	+	+	-	-
AMP-CRO-TE-SXT-C-NA	+	+	+	-	+
AMP-CRO-TE-SXT-CIP-NA	+	-	-	+	+
AMP-TE-SXT-CN-C-NA	+	+	+	-	-
AMP-CRO-TE-SXT-NA-K	+	+	-	-	+
AMP-CRO-TE-SXT-CN-C-NA	+	-	+	+	+
AMP-CRO-TE-SXT-CIP-NA-K	+	+	-	-	-
AMP-CRO-TE-SXT-C-NA-K	+	+	+	+	-
AMP-TE-SXT-CN-CT-CIP-NA	+	+	+	-	+
AMP-CRO-TE-SXT-CT-CIP-NA	+	-	+	+	-
AMP-CRO-TE-CN-CT-C-NA-K	+	+	-	-	-
AMP-CRO-TE-CT-C-CIP-NA-K	+	+	+	-	-
AMP-CRO-TE-SXT-C-CIP-NA-K	+	+	-	+	+
AMP-CRO-TE-SXT-CN-C-CIP-NA-K	+	+	+	+	+
Total	29	21	16	12	14

AMP: ampicillin, SXT: sulfamethoxazole-trimethoprim, TE: tetracycline, CT: colistin sulfate, C: chloramphenicol, NA: nalidixic acid, K: kanamycin, CIP: ciprofloxacin, CN: gentamicin, CRO: ceftriaxone, MDR: multidrug-resistant, *bla*TEM: ampicillin resistance gene, *tet*A: tetracycline resistance gene, *sul*1 and *sul*2: sulfa drug resistance genes.

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Antibiotic resistance genes

Out of 27 ampicillin-resistant isolates, 77.8% (21/27) isolates carried ampicillin resistance gene *bla*TEM. The prevalence of *tet*A in *Salmonella* isolates that were phenotypically resistant to tetracycline was 64% (16/25). Among isolates that were phenotypically resistant to sulfamethoxazole-trimethoprim, the prevalence of *sul*1, and *sul*2 genes were 48% (12/25), and 56% (14/25), respectively. The distribution of resistance genes along with resistance patterns of *Salmonella* isolates are presented in Table 2.

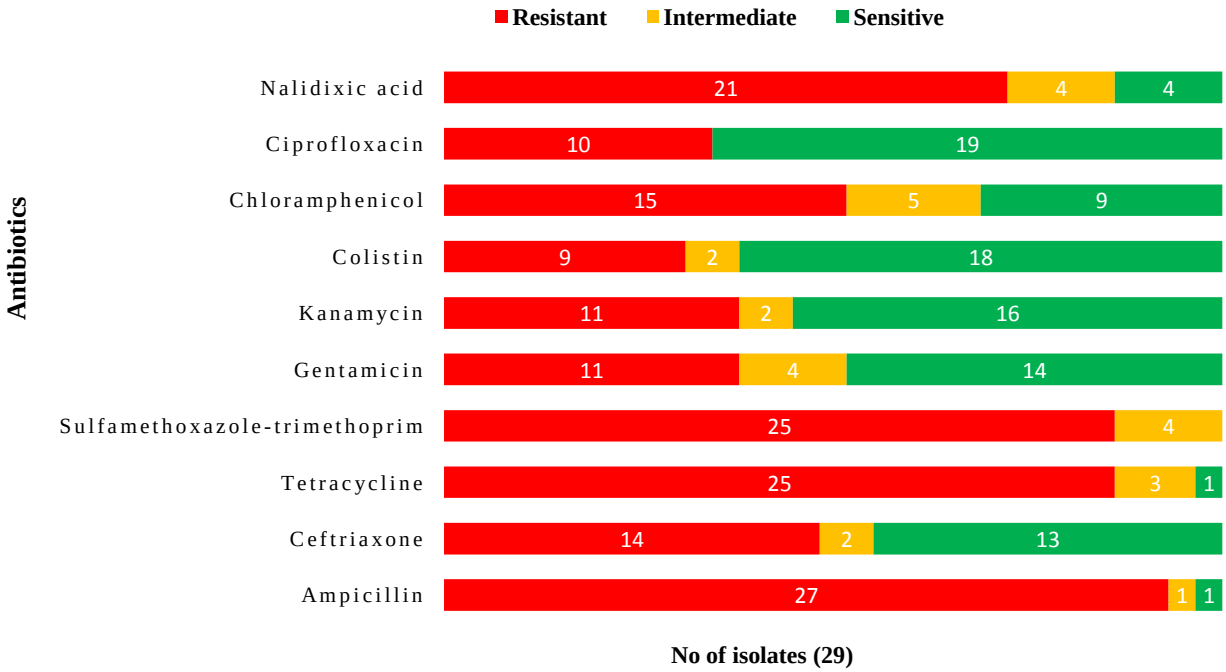

Figure 1. Antibiogram profile of *Salmonella* isolates recovered from cloacal swab samples of pigeons in a live bird market in Chattogram, Bangladesh

DISCUSSION

In this study, the prevalence of *Salmonella* in pigeons was lower than the previous reports in Bangladesh (Hosain et al., 2012; Saifullah et al., 2016), which reported the prevalence rates of 40.28% and 37.5% in pigeons, respectively. In Copenhagen, pooled fecal samples of pigeons showed a prevalence rate of 22.8% for *Salmonella* (Pasmans et al., 2004), while in Iran, prevalence of *Salmonella* in cloacal samples isolated from pigeons was 15.6% (Akbarmehr, 2010).

In this study, *Salmonella* isolates were highly resistant to ampicillin, followed by tetracycline and sulfamethoxazole-trimethoprim (86.2%) and nalidixic acid (72.4%). A comparable result was obtained by Saifullah et al. (2016), who stated a high rate of resistance to ampicillin (88.2%), while a high sensitivity rate to nalidixic acid (76.5%) in *Salmonella* spp from apparently healthy pigeons. Hosain et al. (2012) reported that 80% of *Salmonella* isolated from pigeons were resistant to ampicillin, followed by tetracycline (60%) and sulfamethoxazole (20%) from Bangladesh. The highest resistance rate of these antibiotics may be due to the long-term use in veterinary practice. Ampicillin, tetracycline, and sulfonamide are regularly prescribed antibiotics in poultry treatment in Bangladesh (Saifullah et al., 2016). Moreover, cross resistance to similar classes of antibiotics is also responsible for the high resistance rates.

In food animals, ciprofloxacin is one of the broadly used antibiotics that is regularly prescribed for poultry practice in Bangladesh (Azad et al., 2019). The resistance to ciprofloxacin is a worldwide issue since it could complicate clinical therapy both in humans and livestock. Resistance to colistin (31%), one of the significant findings of our study which is worrisome. The last resort drug, colistin is being expansively prescribed in veterinary practice, however because of nephrotoxicity and neurotoxicity, its use is restricted in human practice (Hassan et al., 2015). The findings of the present study disclosed that 65.5%, 55.2%, and 48.3% of *Salmonella* isolates were sensitive to ciprofloxacin, kanamycin, and gentamicin, respectively while Hosain et al., (2012) reported 60% sensitivity rate to kanamycin and gentamicin. The present study indicated that 96.6% of *Salmonella* isolates were MDR. A number of previous researches reported MDR *Salmonella* in Bangladesh (Khan et al., 2005; Rahman et al., 2011). A high incidence of MDR strains may be occurred due to the aimless use of antibiotic agents.

In the present study, the antibiotic resistance genes among *Salmonella* isolates were detected. The presence of *bla*TEM, *tet*A, and *sul*2 genes in *Salmonella* isolates were reported by Adelowo et al. (2014) and Messaili et al. (2019)

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that had similar frequencies with our findings. The frequency of antibiotic resistance genes in *Salmonella* is quite variable in poultry, which may be due to differences in antibiotic use patterns in different regions.

CONCLUSION

The prevalence of MDR *Salmonella* in pigeons in live bird markets is a public health concern, therefore, it is recommended to maintain strict hygienic measures, proper cage, and litter management to diminish the load and spread of MDR *Salmonella* and ensure customers health and safety.

DECLARATIONS

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Competing interests

The authors declare that they have no conflict of interest.

Authors' contribution

Zamila Bueaza Bupasha, Abdul Ahad and Md Samun Sarker designed the study plan. Zamila Bueaza Bupasha, Md Bayzid and Md Samun Sarker performed the laboratory experiments. Sharna Karmakar and Rahima Akter analyzed the data. Ruhena Begum and Zamila Bueaza Bupasha drafted the manuscript. Abdul Ahad and Md Samun Sarker revised the manuscript. All authors read and approved the final manuscript for publication.

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