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Detection of multidrug resistant *Salmonella* spp. from healthy and diseased broilers having potential public health significance

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ABSTRACT

Multidrug resistant (MDR) *Salmonella* spp. poses significant global public health concern by causing food-borne infections. This study aimed to detect MDR *Salmonella* spp. from healthy and diseased broiler chickens in the Mymensingh and Jamalpur districts of Bangladesh. Total 70 samples comprising feces (n=20), chicken meat (n=30), and visceral organs i.e. liver, lung, and kidney (n=20) were collected. *Salmonella* were isolated and identified by culture, biochemical tests and PCR. The antibiogram study was performed by the disk diffusion method. By PCR, 30% (21/70; 95% CI: 19.32-40.05%) samples were positive for *Salmonella* spp., of which significantly (p=0.005) higher occurrence were detected in feces (50%; 95% CI: 29.93-70.07%) compared to chicken meat (10%; 95% CI: 3.46-25.62%) and visceral organs (40%; 95% CI: 21.88-61.34%). By antibiogram, all the *Salmonella* isolates were resistant to amoxicillin, and frequently (90.48-19.05%) resistant to tetracycline, ceftazidime, chloramphenicol, colistin, and ciprofloxacin. The significantly higher resistance of chloramphenicol, tetracycline, and ceftazidime were observed in the internal organs of broilers. Interestingly, 80.95% (17/21; 95% CI: 59.99-92.33%) *Salmonella* isolates were MDR in nature. The range of multiple antibiotic resistance (MAR) index of *Salmonella* isolates varied from 0.29 to 0.86. The high occurrence of MDR and MAR *Salmonella* in broilers detected in our present study could reveal a high risk to public health and these organisms could be transmitted to humans through the food supply. We suggest that effective prevention and control measures should be implemented to reduce their potential contamination and to minimize the emergence of antibiotic resistance.

INTRODUCTION

Poultry farming has become a profitable and dependable agricultural business in Bangladesh. In addition, it plays a momentous role in the employment generation and economic growth of Bangladesh [1]. Poultry provides additional income to rural people [2]. Furthermore, poultry delivers about 37% of the total meat supply to the people of Bangladesh and covers more than 12.5% of total daily proteins per capita [3]. But the entry of different infectious diseases e.g. salmonellosis, avian colibacillosis, mycoplasmosis, fowl cholera, avian influenza, Newcastle disease, infectious bronchitis, aspergillosis, and others hinder the further advancement of poultry production [4]. Among them, multidrug resistant (MDR) *Salmonella* spp. are deemed as major botherations in the uplifting of Bangladesh's poultry sector by causing drastic poultry illness and deaths annually [5].

Salmonella spp. is one of the most frequently isolated foodborne pathogens that develops approximately 153

million enteric diseases and 155,000 deaths per year globally [6-8]. In poultry, *Salmonella* spp. is devastating for developing avian salmonellosis, increasing mortality rates, and reducing hatchability and fertility rates [3]. Poultry products especially meat and eggs play a pivotal role in *Salmonella* contamination. Incidences of food-poisoning diseases triggered by these pathogens have been increasing remarkably in the last several years. In human, *Salmonella* spp. cause human salmonellosis. Poultry-originated foods are thought to be the main reasons for human salmonellosis, as poultry especially broilers are important reservoirs of *Salmonella* spp. [9]. In addition, poultry and poultry-originated foods generally act as crucial sources for the sporadic outbreaks of human salmonellosis globally. As *Salmonella* spp. are naturally gut-originated pathogens in poultry, the food supply chain makes an important scope for the transmission of *Salmonella* infections to humans [10].

Antimicrobial resistance (AMR) is considered a worldwide health problem jeopardizing all one-health components [11]. The indiscriminate use of antibiotics triggers selection pressure and develops antibiotic resistance in bacteria [12]. In addition, antibiotics are being used as growth promoters in modern poultry, especially broiler production that also triggers the development of AMR in poultry. Globally, the speculation deaths due to AMR consequences will be more than 300 million per year, if significant steps won't be taken by 2050 [13]. The world critics have warned that the low- and middle-income countries will face the worst impacts of AMR. According to the world health organization, Bangladesh is at high risk of AMR consequences [14].

The detection of MDR *Salmonella* spp. from broilers was previously recorded in Bangladesh [5, 15, 16]. However, it needs regular surveillance to determine the actual prevalence of *Salmonella* in broilers in Bangladesh. Therefore, the present study was carried out to detect MDR *Salmonella* in both healthy (feces, and meat) and diseased (visceral organs) broiler samples.

MATERIALS AND METHODS

Sample size calculation

The sample size of our present study was calculated following by the prevalence of *Salmonella* spp. (23.53%) isolated from broilers in Bangladesh [16]. The formula we followed for the sample size calculation was described previously [17]: $n = Z^2pq/d^2$, where, n = desired sample size, Z = the standard normal deviation (1.96 at 95% confidence level), p = prevalence (23.53% or 0.2353), q = 1-p = 1-0.2353 = 0.7647, d = precision at 10% (d = 0.1). So, $n = (1.96)^2 \times 0.2353 \times 0.7647 / (0.1)^2 = 69.123$. Therefore, we collected 70 samples from broiler chickens.

Sampling site and sampling

This study was performed from June 2018 to November 2019 in Mymensingh (24.7539° N, 90.4073° E) and Jamalpur (24.9250° N, 89.9463° E) districts of Bangladesh. The study areas are showed in Figure 1.

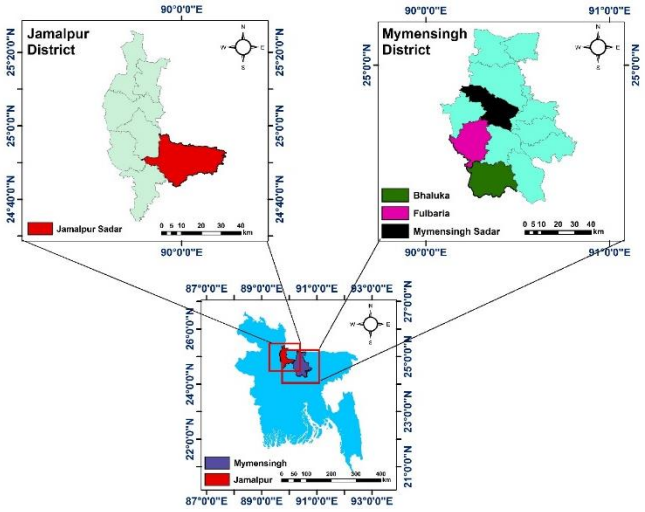

Figure 1. Study area map produced by ArcMap (version 10.7) software (ESRI, Redlands, CA, USA).

A total of 70 broiler samples comprising feces (n=20), chicken meat i.e. thigh, breast, and wings (n=30) from healthy birds, and visceral organs i.e. liver, lungs, and kidneys (n=20) from diseased birds were collected aseptically. Sterile cotton buds were used to collect freshly dropped fecal samples. Meat samples were collected by processing broilers from different markets. By post-mortem examination, visceral organs were collected from each bird that had lesions of avian salmonellosis. 5 gm of each samples was collected aseptically. Immediately after collection, samples were taken into sterile zip-lock bags with particular tag

numbers and transferred to the laboratory maintaining a cool chain. After bringing to the laboratory, samples were seeded to sterile test tubes containing 5 ml sterile nutrient broth and incubated overnight at 37°C. All the experimental procedures and protocols used in this study were approved by the animal welfare and experimentation ethics committee of Bangladesh agricultural university (No. AWEEC/BAU/2019(28)).

Isolation of *Salmonella* spp.

Isolation of *Salmonella* spp. was performed by culture on Xylose Lysine Deoxycholate (XLD) agar (HiMedia, India) plates. Overnight enriched samples were streaked on XLD agar plates and incubated aerobically for 18-24 hours at 37°C to get pure colonies. Black-centered colonies on XLD agar plates were suspected as the growth of *Salmonella* spp. Gram's staining and biochemical tests (urease test, sugar fermentation test, methyl red test, Voges-Proskauer test) were performed for further confirmation [18].

DNA extraction and PCR confirmation of *Salmonella* spp.

Isolated *Salmonella* spp. were finally confirmed by polymerase chain reaction (PCR) targeting the *invA* gene (F: 5'-ATCAGTACCAGTCGTCTTATCTTGAT-3' and R: 5'-TCTGTTTACCGGGCATACCAT-3') with 211 amplicon size [19]. For PCR, bacterial DNA was extracted by boiling and freeze-thawing method as previously described [20]. Briefly, initially 1 ml of overnight enriched culture was centrifuged at 5,000 rotation per minute (rpm) for 5 minutes and the supernatant was discarded. Subsequently, a similar process was performed after mixing 1 ml of phosphate buffer solution (PBS). After discarding supernatant, the pellet was suspended to 200 µL PBS; followed by boiling and cooling of the suspension for 10 minutes in each step. Finally, the suspension was again centrifuged for 10 minutes at 10,000 rpm and the supernatant was collected as genomic DNA. The collected genomic DNA was then stored at -20°C for further use.

A final volume of 20 µL consisting of 10 µL of the master mix (2X) (Promega, Madison, WI, USA), 4 µL of nuclease-free water, 1 µL of each primer, and 4 µL of genomic DNA (50 ng/ µL) was used to carry out the PCR amplification. The thermo-cycle conditions were as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 2 min, extension at 72°C for 45 s, and final extension was conducted at 72°C for 45 s.

After amplification, PCR products were analyzed by 1.5% agarose (Invitrogen, USA) gel electrophoresis, stained with ethidium bromide (0.5 µg/ml) for 10 min in a dark place, and finally, the expected amplicon sizes were audited and captured under ultra-violet trans-illuminator (Biometra, Germany). A 100 bp DNA ladder (Promega, Madison, WI, USA) was used to check the targeted amplicon size.

Antibiotic susceptibility test

The antibiotic susceptibility test (AST) was done by the disk diffusion method [21]. Seven commonly used antibiotics under seven classes were employed: penicillins (amoxicillin- 30 µg), fluoroquinolones (ciprofloxacin- 5 µg), amphenicols (chloramphenicol- 30 µg), polypeptides (colistin- 10 µg), aminoglycosides (gentamicin- 10 µg), tetracyclines (tetracycline- 30 µg), and cephalosporins (ceftazidime- 30 µg). The AST was done by spreading freshly *Salmonella* growth culture having an equal concentration of 0.5 McFarland solution on Mueller-Hinton agar (HiMedia, India) plates. The guidelines of the clinical and laboratory standard institute [22] were followed to interpret the results. Any isolates showing resistance against three or more classes of antibiotics were deemed as MDR [23]. Furthermore, the multiple antibiotic resistance (MAR) index was evaluated by the following formula: MAR= a/b, where ''a" denotes the number of antibiotics which were resistant to a particular isolate, and ''b" denotes the total number of antibiotics tested [24].

Statistical analysis

Data obtained from this study were incorporated in Microsoft Excel-2010 (Los Angeles, CA, USA), and exported to the GraphPad Prism 8.4.2 (GraphPad Software, Inc.) and the Statistical Package for the Social Sciences (SPSS) software (IBM SPSS- version 25.0, USA) for statistical analysis. By SPSS, a Pearson chi-square test for goodness-of-fit was performed to observe the possible variations in the occurrence of *Salmonella* spp. and the resistance profiles of different antibiotics among different collected samples. Statistically significant *p*-value was less than 0.05. Furthermore, GraphPad Prism following the Wilson/Brown Hybrid method as previously described [25] was used to calculate the binomial 95% confidence intervals.

RESULTS

Occurrence of *Salmonella* isolates

Out of 70 samples, 46 (65.71%, 95% confidence interval: 54.04-75.75%) samples were positive for *Salmonella* spp. based on their colony characteristics and biochemical tests. Of these 46 isolates, 30% (21/70) samples were PCR positive for *Salmonella* spp. targeting *invA* gene; among which healthy broiler sample- feces (50%, 10/20) exhibited significantly higher occurrence of *Salmonella* spp., compared to internal organs (40; 7/20) from diseased broilers, and meat (10%, 3/30) samples from healthy broilers (Table 1).

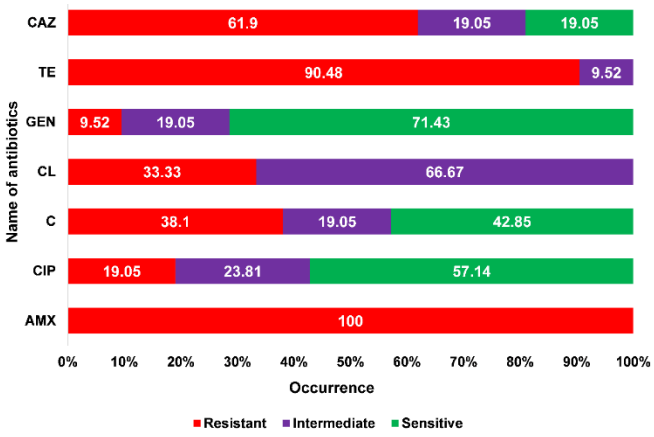

Figure 2. Antibiogram profiles of *Salmonella* isolated from broiler samples. Here, AMX= Amoxicillin, CIP= Ciprofloxacin, C= Chloramphenicol, CL= Colistin, GEN= Gentamicin, TE= Tetracycline, CAZ= Ceftazidime.

Table 1. Occurrence of *Salmonella* spp. from different broiler samples

Categories	Sample types	Sample size	Occurrence (%)	95% CI (%)	*p*-value
Healthy	Feces	20	10 (50)	29.93-70.07	0.005
	Meat	30	3 (10)	3.46-25.62	
Diseased	Visceral organs	20	7 (40)	21.88-61.34	
	Total	70	21 (30)	19.32-40.05	

Here, CI= Confidence interval, *A p-value less than 0.05 was deemed as statistically significant.

Antibiogram profiles of isolates *Salmonella* spp.

From the antibiotic susceptibility test, all the *Salmonella* isolates were resistant to amoxicillin; frequently resistant to tetracycline (90.48%), ceftazidime (61.90%), chloramphenicol (38.10%), and colistin (33.33%). On contrary, gentamicin showed higher sensitivity to *Salmonella* isolates (Figure 2). *Salmonella* from visceral organs (diseased broiler samples) revealed peak resistance against most of the used antibiotics, where a statistically significant correlation was found for chloramphenicol, tetracycline, and ceftazidime (Table 2).

Occurrence of MDR patterns and MAR index of *Salmonella* isolates

Out of 21 *Salmonella* isolates, 17 (80.95%; 95% CI: 59.99-92.33%) were MDR in nature. Overall, nine resistance patterns were audited, among them, the highest 23.53% (4/17; 95% CI: 9.56-47.26%) *Salmonella* isolates showed the resistance pattern no. 9 (AMX-TE-CAZ). One isolate showed resistance against six classes of antibiotics (six antibiotics) (pattern no. 1). The antibiotic resistance profile of each *Salmonella* isolate was found to vary with MAR indices ranging from 0.29 to 0.86. All the *Salmonella* isolates were resistant against at least two antibiotics representing two classes (Table 3).

DISCUSSION

Avian Salmonellosis is a major threat to both the poultry industry (causing serious economic losses) and human health (showing zoonotic significance). In addition, infections developed by MDR *Salmonella* spp. are difficult to control. Broiler meat, eggs, fecal materials, and visceral organs have been recorded as cardinal sources of *Salmonella* contamination [26]. Here, we reported the detection of MDR *Salmonella* from broiler chickens which show serious public health significance.

The *invA* gene of *Salmonella* usually comprises specific DNA sequences which proves the *invA* as a compatible gene to detect *Salmonella* genotypically [27]. In addition, the *invA* gene is available in almost all *Salmonella* serovars. This gene encodes a protein (inner membrane) that assists *Salmonella* to invade their epithelial cells [3]. In this study, the overall occurrence of *Salmonella* spp. targeting *invA* gene in broiler samples was 30% (21/70) which is lined with the previous study conducted in Bangladesh [15]. Conversely, both higher [5] and lower [16] prevalence rate of *Salmonella* spp. from broilers than our study were also recorded previously in Bangladesh. Globally, variable findings as 7.9% [26] and 0.75% [28] were recorded previously. This observed variations in the occurrence of *Salmonella* spp. might have linkage with the variations of the

Table 2. Resistance profiles of *Salmonella* isolated from broiler samples

Categories	Sample types (n)	Antibiotic Resistance Pattern (%)													
		CIP (%)	*p*-value	C (%)	*p*-value	GEN (%)	*p*-value	TE (%)	*p*-value	AMX (%)	*p*-value	CL (%)	*p*-value	CAZ (%)	*p*-value
Healthy	Feces (10)	1 (10)	0.286	2 (20)	0.02	1 (10)	0.244	10 (100)	0.001	10 (100)	NC	2 (20)	0.068	4 (40)	0.018
	Meat (3)	0 (0)		0 (0)		1 (33.33)		1 (33.33)		3 (100)		0 (0)		1 (33.33)	
Diseased	Visceral organs (8)	3 (37.5)		6 (75)		0		8 (100)		8 (100)		5 (62.5)		8 (100)	
	Total (21)	4 (19.05)		8 (38.10)		2 (9.52)		19 (90.48)		21 (100)		7 (33.33)		13 (61.90)	

Here, AMX= Amoxicillin, CIP= Ciprofloxacin, C= Chloramphenicol, CL= Colistin, GEN= Gentamicin, TE= Tetracycline, CAZ= Ceftazidime. *A *p*-value less than 0.05 was deemed as statistically significant.

Table 3. Occurrence of multidrug resistance and multiple antibiotic resistance index of *Salmonella* isolated from broiler samples

Pattern No.	Antibiotic resistance patterns	No. of Antibiotics (classes)	No. of isolates	Overall MDR isolates (%)	MAR Index
1	AMX, C, CL, GEN, TE, CAZ	6 (6)	1	17/21 (80.95)	0.86
2	AMX, CIP, C, TE, CAZ	5 (5)	1		0.71
3	AMX, C, CL, TE, CAZ	5 (5)	2		
4	AMX, C, TE, CAZ	4 (4)	2		0.57
5	AMX, CIP, TE, CAZ	4 (4)	1		
6	AMX, CIP, C, TE	4 (4)	2		
7	AMX, CL, TE, CAZ	4 (4)	1		
8	AMX, CL, TE	3 (3)	3		0.43
9	AMX, TE, CAZ	3 (3)	4		
10	AMX, GEN	2 (2)	1	-	0.29
11	AMX, TE	2 (2)	2	-	
12	AMX, CAZ	2 (2)	1	-	

Here, AMX= Amoxicillin, CIP= Ciprofloxacin, C= Chloramphenicol, CL= Colistin, GEN= Gentamicin, TE= Tetracycline, CAZ= Ceftazidime, MDR= Multidrug resistant, MAR= Multiple antibiotic resistance.

management systems of farms (biosecurity, hygiene, sanitary, etc.), sample size, types of samples, geographical and seasonal distributions, and method related factors. The occurrence of *Salmonella* in broilers suggests that the farms' and poultry processing environments might contain poor-hygienic protocols. Furthermore, the presence of virulence gene *invA* in *Salmonella* isolates denotes their pathogenicity which can develop foodborne pathogens after introducing into food.

In the current study, a significantly higher occurrence of *Salmonella* spp. was observed in fecal samples (50%) of healthy broilers in relation to visceral organs (40%) of diseased broilers, and meat samples (10%) of healthy broilers. Previously several studies reported the presence of *Salmonella* spp. in broiler meat [29], fecal materials [5], and visceral organs [15]. The significantly higher occurrence of *Salmonella* in fecal materials is not unusual, as *Salmonella* are naturally found in the gastrointestinal tract of avian species [30]. These *Salmonella* contaminations can be introduced into the production system from broilers via feces, contaminated water or feed, and others. In addition, the presence of *Salmonella* in feces samples indicates that broiler droppings can shed *Salmonella* to other birds of the flocks. The presence of *Salmonella* spp. in meat samples denotes that *Salmonella* spp. have the potential to be transmitted to humans via the food supply chain. Furthermore, consumption of undercooked poultry and poultry products contaminated by *Salmonella* has also the potential in the transmission of *Salmonella* to humans [28].

Antimicrobial resistance is an emerging problem in the world and has the most significant public health challenge of this century globally [31]. Poultry and poultry products are huge sources of antibiotic reservoirs [32]. In our present study, all the *Salmonella* isolates were resistant to amoxicillin, and frequently resistant to tetracycline, ceftazidime, chloramphenicol, and colistin. Visceral organs exhibited a higher occurrence of antibiotic resistance compared to other selected samples (in the most antibiotics used). In addition, *Salmonella* resistance to tetracycline, ceftazidime, and chloramphenicol was significantly higher in visceral organs of diseased broilers. Interestingly, *Salmonella* isolates showed resistance to ceftazidime (61.90%) and colistin (33.33%) which is alarming for both human and animal health-care facilities. Ceftazidime is a 3rd generation cephalosporin antibiotic which usually used to treat severe bacterial infections in humans [33]. In addition, colistin is a reserved group of antibiotics which generally used only in severe infections developed by MDR Gram-negative bacteria [34]. However, MIC and molecular assays should be employed before drawing any conclusions.

Infections caused by MDR and MAR bacteria are serious global health concern as it is expensive for treatment and it may cause fatal consequences. MDR *Salmonella* has emerged as a cardinal human health issue throughout the world. The alarming situation was that 80.95% of *Salmonella* isolates were MDR in nature. Previously, Alam et al. [5] detected 100% MDR *Salmonella* spp. from broilers in Bangladesh. In addition, MAR indices of isolated *Salmonella* from our study were ranged from 0.29 to 0.86. More than 0.29 of MAR index denotes that antibiotics were frequently used in the sources from where *Salmonella* were isolated showing high-risk sources for MDR and MAR bacteria. The development of MDR and MAR in *Salmonella* may be the results of selective pressure triggered by the misuse and overuse of antibiotics in broilers [5]. These MDR and MAR *Salmonella* show severe public health significance by transmitting to humans through the food supply chain. In addition, these MDR and MAR bacteria can also spread in the environments and transfer their resistance genes to other bacteria horizontally.

CONCLUSION

High occurrence of MDR *Salmonella* spp. detected in our present study reveals a potential human and animal health risk. There is potential in the transmission of *Salmonella* spp. from broilers to one-health components through the food chain, and ultimately to contaminate them. Future studies including the detection of virulence and antibiotic resistance genes of *Salmonella* spp. from healthy and diseased broilers may clarify the actual dynamics of their transmission and dissemination to one-health components. Effective control strategies and sustained implementation of comprehensive risk reduction practices including strict biosecurity throughout the production continuum are required to minimize the emergence of MDR and MAR zoonotic *Salmonella* pathogens.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHOR'S CONTRIBUTIONS

Conceptualization, M.F.R.K. and M.T.R.; Sample collection, M.T. and M.S.I.; Methodology, M.T. and M.S.I.; Software, M.S.I.; Validation, M.F.R.K. and M.T.R.; Formal analysis, M.S.I., M.A.S. and M.T.R.; Investigation, M.T., M.S.I., S.I. and M.N.; Data curation, M.S.I. and M.T.; Writing-original draft preparation, M.S.I. and M.T.; Writing- review and editing, M.S.I., M.A.S., M.F.R.K., F.M.B. and M.T.R.; Visualization, M.S.I., and M.T.R.; Supervision, M.F.R.K. and M.T.R.; Fund acquisition, M.F.R.K. and M.T.R.; Critical revisions and writing, M.F.R.K. and M.T.R. All authors have read and agreed to the published version of the manuscript.

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