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...
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.

**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]: 

\[ n = \frac{Z^2pq}{d^2} \]

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, 

\[ n = (1.96)^2\times0.2353\times0.7647/(0.1)^2 = 69.123. \]

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).](image)

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 Deoxyncholate (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’-ATCAGTACCAGTCTTATCTTGTGAT-3’ and R: 5’-TCTGTTTACCGGCATACCAT-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).

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

<table>
<thead>
<tr>
<th>Categories</th>
<th>Sample types</th>
<th>Sample size</th>
<th>Occurrence (%)</th>
<th>95% Cl (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>Feces</td>
<td>20</td>
<td>10 (50)</td>
<td>29.93-70.07</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>Meat</td>
<td>30</td>
<td>3 (10)</td>
<td>3.46-25.62</td>
<td></td>
</tr>
<tr>
<td>Diseased</td>
<td>Visceral organs</td>
<td>20</td>
<td>7 (40)</td>
<td>21.88-61.34</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>70</td>
<td>21 (30)</td>
<td>19.32-40.05</td>
<td></td>
</tr>
</tbody>
</table>

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% Cl: 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

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

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

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

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.

**ACKNOWLEDGMENTS**

The authors are so grateful to farm owners for giving us access to samples during the whole study. The
authors are also very much grateful to Dr. Khalada Zesmin, Upazila Livestock Officer, Kishoreganj, Bangladesh, for her valuable comments and suggestions during the whole study and the preparation of the manuscript.

FUNDING

Authors are very much grateful to the Ministry of Education, Government of Bangladesh for providing fund through a research project (project number: LS2018686) to facilitate the present study.

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.

REFERENCES


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