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Original Article

Epidemiology and antimicrobial resistance profiles of *Salmonella* in chickens, sewage, and workers of broiler farms in selected areas of Bangladesh

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Abstract

Introduction: A cross-sectional study was conducted to determine the prevalence and AMR pattern of *Salmonella* isolated from broiler chickens, farm sewage, and farm workers. This study also aimed at identifying the risk factors for *Salmonella* infection in chickens.

Methodology: Cloacal swabs (n = 50) from broiler chickens, farm sewage (n = 50), and hand washed water of farm workers (n = 50) were collected along with data on farm management and antimicrobial usage from 50 broiler farms of Mymensingh and Gazipur districts. All samples were analysed for the presence of *Salmonella* using selective media and PCR assay. Antimicrobial susceptibility test was done for ten antimicrobials by disk diffusion test. Risk factor analyses were carried out by multivariable logistic regression using SPSS.

Results: The overall *Salmonella* prevalence was 66% (99/150). *Salmonella* prevalence were 82% and 72% in cloacal swabs and farm sewage samples, respectively. From hand washed water, 44% of the samples were positive for *Salmonella*. *Salmonella* infection in broiler chickens was significantly associated with farming experience (≤ 5 years) and age of birds (≥ 11 days). Similar pattern of antimicrobial resistance was observed in *Salmonella* isolated from three types of samples, and high resistances were observed to colistin, doxycycline, ciprofloxacin and ceftazidime. Moreover, isolates from all the three sources showed high percentage of multidrug-resistance (80.6% to 97.6%).

Conclusions: The findings of this study reveal that antimicrobial-resistant *Salmonella* are prevailing at animal-human-environment interface, which needs an integrated approach to limit further spread of AMR.

Key words: Antimicrobial resistance; *Salmonella*; Risk factors; broiler chickens; Bangladesh.

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Introduction

Foodborne diseases are a growing public health concern in recent times, and their importance for health is progressively recognized [1]. In 2015 the World Health Organization (WHO) estimated that each year over 600 million people suffer from 31 foodborne diseases and 420,000 deaths occur globally [2]. Most illnesses occur due to the consumption of contaminated food and water [1]. A large variety of zoonotic pathogens are responsible for the occurrence of those diseases. Among those pathogens, *Salmonella* is a significant contributor to foodborne illness in both developing and developed countries [3]. It has been estimated that around 94 million people are infected and 155,000 people died each year with non-typhoidal *Salmonella* [1]. Food animals, especially poultry, act as an important reservoir of *Salmonella*, which serves as a potential source of human infection [4]. A lot of epidemiological studies have identified the involvement of poultry foodstuffs with *Salmonella* burden in humans [4,5]. In previous decades, the development of antimicrobial resistance (AMR) in various organisms posing an emerging threat to public health. Widespread resistance of *Salmonella* in poultry samples is reported to antimicrobial drugs which is thought to be mainly due to the extensive use of antimicrobials on poultry farms [6]. The presence of AMR *Salmonella* in poultry raises the risk of human infection as those organisms can be transmitted to humans not only through the food supply but also via water or direct contact with birds. During the past decades, poultry production in Bangladesh has expanded and the sector accounts for 14% of the total value of livestock output and is growing rapidly [7]. Poultry meat alone contributes a considerable 37% of the total meat production here [8]. Beyond this development, increasing tendencies of poultry farmers were seen to use broad-spectrum antimicrobials not only for therapeutic purposes but also for growth promotion at sub-therapeutic doses, use of unauthorized antimicrobials and thus contributing to the development of antimicrobial resistance [9]. Numerous factors can influence bacterial resistance on farms, such as flock health status, farm management practices, and the environment [10]. Therefore, to minimize the overuse of antimicrobial drugs, information regarding the antimicrobial usage, particularly in poultry production system is necessary to know. It is also needed to pay more attention to the peoples who engaged in the poultry farms to reduce or eliminate the risk of transmission of AMR *Salmonella* and other pathogenic microbes. One option to improve the situation and to reduce the load of *Salmonella* at the farm is by identifying, and reducing, possible risk factors. Some studies report different risk factors (smallest flock size, summer season, poor biosecurity practices), variable prevalence of *Salmonella* (11% - 71.1%) and high percentage of AMR of *Salmonella* isolated from cloacal swabs of broiler chickens [11-16]. However information on *Salmonella* prevalence, along with the AMR pattern of *Salmonella* in one health setting, (poultry farm-workers-farm sewage) is not well documented in Mymensingh and Gazipur districts of Bangladesh. Although to elucidate the possibility of transmission of antimicrobial resistant *Salmonella* between humans, broiler chickens and environment, it is necessary to know the current status of AMR distribution of *Salmonella* at these sources.

Therefore, this study was conducted in broiler farms in two selected districts (Mymensingh and Gazipur) of Bangladesh with the aim to estimate the prevalence of *Salmonella* in broiler chickens, sewage and farm workers as well as to identify the risk factors for *Salmonella* infection in the farms. Furthermore, the antimicrobial resistance pattern of isolated *Salmonella* was investigated.

Methodology

Study area, design and population

The study was carried out in Mymensingh and Gazipur districts of Mymensingh and Dhaka division, respectively. These districts were selected due to the high density of broiler farms. It was a cross-sectional study and was conducted on 50 broiler farms between December 2018 and April 2019. Five upazilas (sub-unit of district, i.e. third tier of administrative units) from each district and five broiler farms in each upazila were randomly selected.

Sample collection

A total of 500 cloacal swab samples were collected from 50 broiler farms (ten birds from each farm). In addition, farm sewage (n = 50) and hand washed water of farm workers (n = 50) were collected from the corresponding farm. However, cloacal swabs collected from ten birds in each farm were pooled for bacteriological analysis. Thus, a total of 150 samples were analysed for the presence of *Salmonella.*

Swabs were collected from the cloacae of broiler chickens using sterile swab sticks moistened with 1 ml Buffered Peptone Water (BPW) (Himedia, India). Farm sewage samples were taken aseptically into a sterile falcon tube from the farm surroundings environment. Hand washed water samples were collected from the respective farm workers. The hands of the workers were washed directly in 100 ml of sterile distilled water and then taken into a sterile falcon tube and sealed. Hand washed water samples were taken at the same time point after the broiler chickens were sampled. Samples were then transported to the laboratory within 2 hours of collection in a cool box with ice for bacteriological culture and isolation.

Assessment of human traffic control system

Human traffic control system, one of the components of biosecurity measures to limit the human entry into the farm, was categorized as good, moderate, and poor. Scoring was done based on the presence of foot-bath, a fence around the farm, gate, and restriction in the entry. The presence of each component was scored as one and the highest possible score was 4. Human traffic control system was considered as good when the score was 4, 3 score as moderate, and < 3 score as poor.

Scoring on biosecurity practices

Biosecurity practices of the farm was evaluated on 11 items including human traffic control system indicators, specific clothes, and shoes for staff, disposal of litter, management of sick and dead birds, presence of other poultry houses within 500 meters and using of the farm area. For scoring 1 point was given for “appropriate practice” and 0 for “inappropriate” practice in case of each item. Each farm was scored out of highest possible score which was 11. The scores of all the 25 farms in each district were summed up and then the average biosecurity score was calculated for each district.

Data collection

Demographic data of the farms along with data on antimicrobial usage (Supplementary Table 1) were collected using a pre-tested questionnaire on mobile data collection app, KoboCollect (https://www.kobotoolbox.org/) through face to face interview of the farmers and geo-location of the farms was also recorded. Voluntary and informed written consents were obtained from the farmers before the collection of data.

Bacteriological culture, isolation, and confirmation

Culture for *Salmonella* was performed as per the standard procedures [17]. Briefly, each sample was transferred to 5 ml Nutrient broth (NB) (Himedia, India) after pre-enrichment in 1 ml BPW and incubated aerobically at 37 °C for 24 h. Subsequently, a loopful of the overnight NB culture was streaked onto Xylose Lysine Deoxycholates (XLD) agar (Himedia, India) and incubated at 37 °C for 24 h. At least two black colonies with red periphery typical for *Salmonella* were sub-cultured and identified by observing under microscope after Gram's staining. Each identified colony with typical morphology was assessed by catalase test and biochemically identified by inoculating onto triple sugar iron (TSI) agar (Himedia, India), methyl red-Voges Proskauer (MR-VP) broth (Himedia, India), peptone water (Himedia, India) for indole test and incubated at 37 °C for 18-24 h and interpreted according to the guidelines of International Organization for Standardization (ISO) [17]. The boiling method was used for the extraction of DNA and the DNA was quantified using nanodrop spectrometer (NanoDrop One, Thermo Fisher Scientific, USA). DNA concentration of more than 100 ng/ µl was maintained to use it for PCR assay. The biochemically positive *Salmonella* isolates were then subjected to PCR for further confirmation with gene-specific primers targetting the Internal Transcribed Spacer (ITS) region of 16S-23S rRNA as described by Chiu *et al*. [18]. The oligonucleotide primer sequences were ITS Forward (5'-TGC GGC TGG ATC ACC TCC TT-3') and ITS Reverse (5'-TAT AGC CCC ATC GTG TAG TCA GAA C-3') with fragment size 312 bp.

Antimicrobial susceptibility testing

The antimicrobial susceptibility testing of *Salmonella* isolates was performed against ten antimicrobials belonged to six classes by Kirby-Bauer disk diffusion technique except colistin. Briefly, isolates were grown on NB at 37 °C until reaching a 0.5 McFarland turbidity standard. Then 150 µl test suspension was spread evenly on Mueller-Hinton (MH) agar (Himedia, India) plates and then antimicrobial disks, which includes levofloxacin (LEV, 5 µg), ciprofloxacin (CIP, 5 µg), ceftazidime (CAZ, 30 µg), ceftriaxone (CRO, 30 µg), cefotaxime (CTX, 30 µg), amoxicillin-clavulanic acid (AMC, 30 µg), doxycycline (DO, 30 µg), imipenem (IPM, 10 µg), and meropenem (MEM, 10 µg) (Biomaxima, Poland) were dispensed and incubated at 37ºC for 18-24 h. For colistin the minimum inhibitory concentrations (MIC) were determined by broth microdilution method. The interpretation of the results was based on the guidelines of CLSI (Supplementary Table 2) [19]. Isolates showing resistance to at least 1 antimicrobial agent in ≥ 3 antimicrobial classes were classified as multidrug-resistant (MDR) [20].

Statistical analysis

An initial descriptive analysis was done to calculate the frequencies and percentages. Chi-square test (Z-test for proportions) and Fisher's exact test (if one or more expected cell frequencies were less than 5) were performed to find out any significant differences in proportions (prevalence, phenotypic resistance percentages etc.) among different types of samples. Initially, univariable logistic regression was done to find out the relationship between the *Salmonella* infection in broiler chickens and each of the 11 independent variables. The variables with p-values <0.3 in univariable analysis were included in the multivariable logistic regression. Finally, in the multivariable logistic regression model the associations between eight candidate variables and *Salmonella* infection were analyzed. Non-significant terms ($p > 0.05$) were eliminated from the model using backward selection procedure and the correlations between the variables were evaluated. The final model was assessed for the goodness-of-fit using the Hosmer-Lemeshow test. In the final model the variables with a p-value of ≤ 0.05 were considered as risk factors for *Salmonella* infection in broiler chickens. The SPSS version 22.0 (IBM, Somers, NY) was used for the statistical analysis.

Results

Description of farm demographics, management and biosecurity practices

In Gazipur district, 48% of the farmers had more than 5 years of farming experience, which was comparatively higher than in Mymensingh district (24%) (Table 1). The average biosecurity score of the farms was relatively higher in Gazipur (6.44) than Mymensingh (5.32) district. Eighty four percent and 92% farms in Gazipur and Mymensingh districts, respectively had flock size of ≤ 2,000 birds. Thirty five farms were found to sell the litter as manure. Practice of burial of dead birds was observed in 88% and 96% of the farms in Gazipur and Mymensingh district,

Table 1. Demographic information of 50 broiler chicken farms in Mymensingh and Gazipur districts.

Variables	Districts	
	Mymensingh, No. of farms (%) (n = 25)	**Gazipur, No. of farms (%) (n = 25)**
Farming experience (years)		
≤ 5	19 (76)	13 (52)
> 5	6 (24)	12 (48)
Flock size (no. of birds)		
≤ 2000	23 (92)	21 (84)
> 2000	2 (8)	4 (16)
Other poultry house within 500m	15 (60)	1 9(76)
Using of farm premises		
Only for poultry farming	20 (80)	19 (76)
Integrated with other farming	5 (20)	6 (24)
Human traffic control system*		
Poor (< 3)	18 (72)	12 (48)
Moderate (3)	7 (28)	13 (52)
Specific clothes for staff	0	6 (24)
Specific shoes for staff	6 (24)	16 (64)
Disposal of litter		
Compost	3 (12)	2 (8)
Sell as manure	15 (60)	20 (80)
Throw into nearby pit	9 (36)	9 (36)
To biogas plant	0	3 (12)
Management of dead bird		
Burial	24 (96)	22 (88)
Thrown away	3 (12)	7 (28)
To garbage bin	0	3 (12)
Cleaning of feeder		
Daily	8 (32)	6 (24)
Alternate day	16 (64)	13 (52)
Once a week	1 (4)	0
Twice a week	0	6 (24)
Cleaning of waterer		
Daily	19 (76)	21 (84)
Alternate day	5 (20)	2 (8)
Once a week	1 (4)	0
Twice a week	0	2 (8)
Wastewater management		
Compost pit	0	1 (4)
Pond	16 (64)	7 (28)
Drainage system	9 (36)	17 (68)
Use of antibiotics	23 (92)	23 (92)
Purpose of antibiotics use		
Therapeutic	15 (65.2)	8 (34.8)
Preventive & Therapeutic	6 (26)	6 (26)
Therapeutic & Growth promoter	1 (4.34)	2 (8.7)
Preventive, Therapeutic & Growth promoter	1 (4.34)	7 (30.4)
By whom suggestion antibiotics were used		
Company representatives	7 (28)	12 (34.3)
Experienced farmer	0	2 (5.7)
Feed dealer	9 (36)	19 (54.3)
Self	4 (16)	0
Veterinarian	5 (20)	2 (5.7)

N: No. of farms; * Total score: 4; [Foot-bath- 1, Fence around the farm- 1, Gate- 1 and Entry restricted-1].

respectively. In response to a question asked 'whether they use antimicrobials on their farm', 92% farmers from both districts replied that they use antimicrobials, and the proportions of farmers use antimicrobials only for therapeutic purpose were 65.2% and 34.8% in Mymensingh and Gazipur districts, respectively. We found that in 11 farms antimicrobials were used as growth promoter also. In 28 farms antimicrobials were used according to the suggestion of the feed dealers and 19 farms followed the advise of company representatives regarding antimicrobial use (Table 1). Ciprofloxacin, amoxicillin, colistin, and oxytetracycline were found to be used more frequently on the farms (Figure 1).

Prevalence and distribution of Salmonella isolates

Among 150 samples, *Salmonella* was recovered from 99 samples. All the isolates were further confirmed by PCR as they generated 312 bp fragment size on amplification (Figure 2), and thus the overall prevalence was 66% (Table 2). *Salmonella* prevalence was recorded 82% and 72% in cloacal swab and farm sewage samples respectively which differed significantly ($p < 0.05$) to the prevalence of *Salmonella* recovered from hand washed water (44%). Between two districts, the prevalence of *Salmonella* in cloacal swab samples were almost similar (80% and 84% in Mymensingh and Gazipur, respectively). However, in case of other two types of samples higher prevalence of *Salmonella* were observed in Mymensingh than Gazipur district (Table 2). However, no significant difference was observed in prevalence of *Salmonella* in each type of sample between Mymensingh and Gazipur districts.

Risk factors for Salmonella infection in broiler chickens

Irrespective of district all the 50 farms were included for the risk factors analysis for *Salmonella* infection in broiler chickens. The results of univariable analysis showed that farming experience of the farmers and flock size were significantly ($p < 0.05$) associated with *Salmonella* infection in broiler chickens (Table 3). However, the final multivariable logistic model identified two potential risk factors associated with the occurrence of *Salmonella* (Table 4), which were the farming experience of the poultry farmers (≤ 5 years) and age of the birds (≤ 11 days).

Figure 1. List of antimicrobials used in the broiler farms.

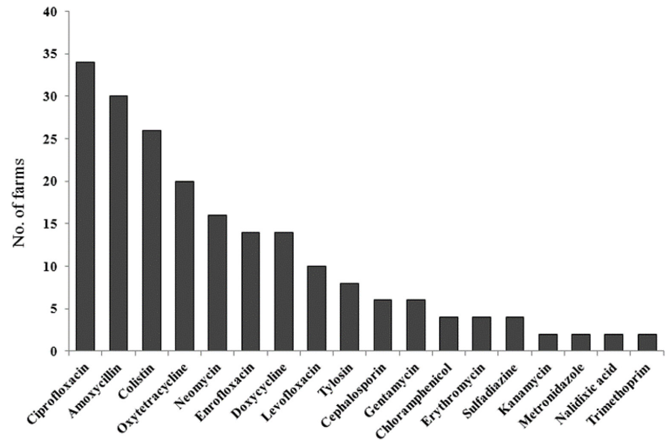
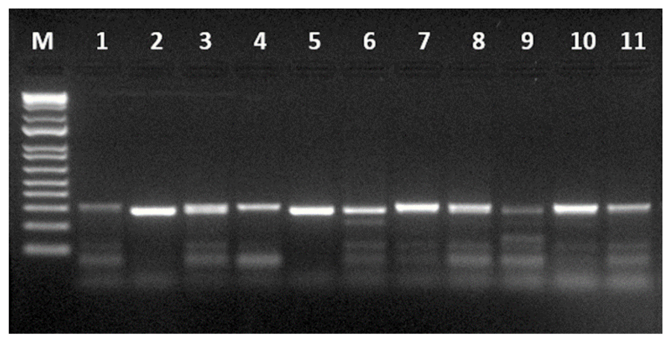


Figure 2. Gel electrophoresis of PCR amplicon of ITS sequence of *Salmonella* spp.



Lane M: 100kb DNA ladder, lane 1-10: Sample isolates, lane 11: Positive control of *Salmonella* spp.

Prevalence of antimicrobial-resistant Salmonella

Irrespective of sample types, the results of the antimicrobial susceptibility test revealed that the highest resistance was observed against colistin (88.9%) and doxycycline (84.8%) followed by ciprofloxacin (78.8%) and ceftazidime (64.6%) (Table 5), while the lowest resistance was observed against levofloxacin (15.2%).

Table 2. Prevalence of *Salmonella* spp. in three different types of samples of Mymensingh and Gazipur districts.

Sample type	No. (%) of *Salmonella* isolates		OR	95% CI	p-value	Prevalence of *Salmonella* spp.
	Mymensingh (n = 25)	Gazipur (n = 25)				
Cloacal swab	20 (80)	21 (84)	1.31	0.3-5.6	1.00	82[a]
Farm sewage	20 (80)	16 (64)	2.25	0.63-8.06	0.208	72[a]
Hand washed water	14 (56)	8 (32)	2.7	0.85-8.57	0.087	44[b]
Total	54 (72)	45 (60)				66

N: No. of farms; OR: Odds ratio; CI: Confidence interval; [a,b] Values with different superscripts within the same column differ significantly ($p < 0.05$).

Table 3. Univariable analysis of the risk factors associated with *Salmonella* infection in broiler chickens[#].

Variables	Prevalence No. (%)	OR[b]	95% CI[c]	P-value
Experience of farming (years)[a]				
≤ 5 (n = 32)	29 (90.6)	4.83	1.03-22.55	0.045
> 5 (n = 18)	12 (66.7)	Ref.	-	-
Season[a]				
Winter (Dec. - Feb.) (n=30)	23 (76.7)	Ref.	-	-
Pre-monsoon (March) (n=20)	18 (90)	2.74	0.51-14.8	0.242
Using of farm premises				
Only for poultry farming (n=39)	31 (79.5)	Ref.	-	-
Integrated with other farming (n = 11)	10 (90.9)	2.58	0.28-23.23	0.398
Distance of natural water body (meters)				
≤ 10 (n = 23)	20 (87)	1.9	0.42-8.66	0.405
> 10 (n = 27)	21 (77.8)	Ref.	-	-
Age of birds (days)[a]				
1-10 (n = 7)	4 (57.1)	Ref.	-	-
11-20 (n = 23)	20 (87)	5	0.73-34.3	0.102
21-35 (n = 20)	17 (85)	4.25	0.61-29.5	0.143
Flock size[a]				
≤ 2000 (n = 44)	38 (86.4)	6.3	1.02-38.9	0.047
> 2000 (n = 6)	3 (50)	Ref.	-	-
Human traffic control system*[a]				
Poor (< 3) (n = 30)	26 (86.7)	2.17	0.5-9.3	0.299
Moderate (3) (n = 20)	15 (75)	Ref.	-	-
Specific shoes for staff				
Yes (n = 22)	19 (86.4)	Ref.	-	-
No (n = 28)	22 (78.6)	0.58	0.12-2.63	0.48
Litter condition[a]				
Wet (n = 13)	12 (92.3)	3.31	0.37-29.4	0.283
Dry (n = 37)	29 (78.4)	Ref.	-	-
Litter turning[a]				
Alternate day (n = 18)	16 (88.9)	Ref.	-	-
Once a week (n = 22)	16 (72.7)	0.33	0.05-1.9	0.217
Twice a week (n = 10)	9 (90)	1.13	0.09-14.2	0.927
Involvement of farmer with other livestock farm[a]				
Yes (n = 22)	20 (90.9)	3.33	0.61-18	0.162
No (n = 28)	21 (75)	Ref.	-	-

N: No. of farms; [#] Irrespective of district all the 50 farms were included for the risk factors analysis; [*] Total score=4; [Foot-bath- 1, Fence around the farm- 1, Gate-1 and Entry restricted-1]; [a] Variables included in the multivariable logistic model; [b] Odds ratio; [c] Confidence interval.

Table 4. Final multivariable logistic regression model for risk factors for *Salmonella* infection in broiler chickens.

Variables	OR[a]	95% CI[b]	p-value
Experience of farming (years)			
≤ 5 (n = 32)	14.17	1.75-114.7	0.013
> 5 (n = 18)	Ref.	-	-
Age of birds (days)			
1-10 (n = 7)	Ref.	-	-
11-20 (n = 23)	18.55	1.18-292.2	0.038
21-35 (n = 20)	15.71	1.11-222.67	0.042

N: No. of farms; [a] Odds ratio; [b] Confidence interval.

Of note, resistance to carbapenems namely imipenem and meropenem were 36.4% and 18.2% respectively, though they are not used in poultry practices in Bangladesh. None of the isolates were resistant to ceftriaxone. Sample types (cloacal swabs, farm sewage and hand washed water) when took into account, the similar high resistance pattern of *Salmonella* isolates was observed against colistin, doxycycline, ciprofloxacin, and ceftazidime (Table 5).

Multidrug resistance of Salmonella

About 90% of *Salmonella* isolates exhibited MDR, of which 97.6% isolates were from cloacal swabs, 90.9% from hand washed water, and 80.6% from farm sewage samples (Table 6). Significant ($p < 0.05$) difference was observed between the prevalence of MDR *Salmonella* isolated from cloacal swab and farm sewage. Between the districts, higher percentage of MDR *Salmonella* was observed in Mymensingh (94.4%) than Gazipur (84.4%) district. Around 68% of the isolates showed resistance to 4 to 6 antimicrobials

Table 5. Resistance profile of *Salmonella* isolates to ten antimicrobials.

Antibiotic name	Cloacal swab (n = 41)			Farm sewage (n = 36)			Hand washed water (n = 22)			Total (n = 99)		
	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)
Levofloxacin	4 (9.8)	11 (26.8)	26 (63.4)	6 (16.7)	6 (16.7)	24 (66.7)	5 (22.7)	6 (27.3)	11 (50)	15 (15.2)	23 (23.2)	61 (61.6)
Ciprofloxacin	35 (85.4)	6 (14.6)	0	26 (72.2)	10 (27.8)	0	17 (77.3)	5 (22.7)	0	78 (78.8)	21 (21.2)	0
Ceftazidime	25 (61)	4 (9.8)	12 (29.3)	24 (66.7)	3 (8.3)	9 (25)	15 (68.2)	2 (9.1)	5 (22.7)	64 (64.6)	9 (9.1)	26 (26.3)
Ceftriaxone	0	2 (4.9)	39 (95.1)	0	2 (5.6)	34 (94.4)	0	2 (9.1)	20 (90.9)	0	6 (6.1)	93 (93.9)
Cefotaxime	11 (26.8)	10 (24.4)	20 (48.8)	10 (27.8)	15 (41.7)	11 (30.6)	8 (36.4)	6 (27.3)	8 (36.4)	29 (29.3)	31 (31.3)	39 (39.4)
Amoxyclav	15 (36.6)	14 (34.1)	12 (29.3)	9 (25)	9 (25)	18 (50)	4 (18.2)	6 (27.3)	12 (54.5)	28 (28.3)	29 (29.3)	42 (42.4)
Colistin	41 (100)	0	0	30 (83.3)	0	6 (16.7)	17 (77.3)	0	5 (22.7)	88 (88.9)	0	11 (11.1)
Doxycycline	37 (90.2)	1 (2.4)	3 (7.3)	30 (83.3)	2 (5.6)	4 (11.1)	17 (77.3)	3 (13.6)	2 (9.1)	84 (84.8)	6 (6.1)	9 (9.1)
Imipenem	14 (34.1)	13 (31.7)	14 (34.1)	13 (36.1)	11 (30.6)	12 (33.3)	9 (40.9)	9 (40.9)	4 (18.2)	36 (36.4)	33 (33.3)	30 (30.3)
Meropenem	6 (14.6)	5 (12.2)	30 (73.2)	7 (19.4)	7 (19.4)	22 (61.1)	5 (22.7)	5 (22.7)	12 (54.5)	18 (18.2)	17 (17.2)	64 (64.6)

N: No. of isolates; R: Resistant; I: Intermediate; S: Susceptible.

Table 6. Multidrug resistance (MDR)* observed among different types of *Salmonella* isolates.

Sample type	No. (%) of MDR *Salmonella* isolates		Prevalence of MDR *Salmonella*
	Mymensingh	Gazipur	
Cloacal swab (n = 41)	20 (100)	20 (95.2)	97.6^{a}
Farm sewage (n = 36)	17 (85)	12 (75)	80.6^{b}
Hand washed water (n = 22)	14 (100)	6 (75)	90.9^{ab}
Total (n = 99)	51 (94.4)	38 (84.4)	89.9

N: No. of isolates; * MDR when isolates are non-susceptible to at least 1 antimicrobial agent in ≥ 3 antimicrobial categories; ^{a,b} Values with different superscripts within the same column differ significantly ($p < 0.05$).

Table 7. The distribution of phenotypic resistance of *Salmonella* isolates to number of antimicrobials and antimicrobial classes.

No. of antibiotics	Cloacal swab No. (%) of isolates (n = 41)	Farm sewage No. (%) of isolates (n = 36)	Hand washed water No. (%) of isolates (n=22)	Total No. (%) of isolates (n = 99)
2	$1 (2.4)^{a}$	$6 (16.7)^{b}$	0	7 (7.1)
3	6 (14.6)	5 (13.9)	7 (31.8)	18 (18.2)
4	11 (26.8)	8 (22.2)	7 (31.8)	26 (26.3)
5	16 (39)	9 (25)	4 (18.2)	29 (29.3)
6	6 (14.6)	5 (13.9)	1 (4.5)	12 (12.1)
7	0	$3 (8.3)^{ab}$	$2 (9.1)^{b}$	5 (5.1)
8	1 (2.4)	0	1 (4.5)	2 (2)
No. of antibiotic classes				
2	$1 (2.4)^{a}$	$7 (19.4)^{b}$	$2 (9.1)^{ab}$	10 (10.1)
3	6 (14.6)	7 (19.4)	7 (31.8)	20 (20.2)
4	21 (51.2)	12 (33.3)	9 (40.9)	42 (42.4)
5	12 (29.3)	9 (25)	4 (18.2)	25 (25.3)
6	1 (2.4)	1 (2.8)	0	2 (2)

[*Penicillin β-lactamase inhibitors*: Amoxyclav; *Tetracyclines*: Doxycycline; *Quinolones*: Levofloxacin, Ciprofloxacin; *Cephalosporins*: Ceftazidime, Ceftriaxone, Cefotaxime; *Polymyxins*: Colistin; *Carbapenems*: Imipenem, Meropenem]; N = No. of isolates; ^{a,b} Values with different superscripts within the same row differ significantly ($p < 0.05$).

while 25% isolates were resistant to 2 to 3 and 7% isolate to 7 to 8 antimicrobials (Table 7). A high percentage of isolates (~80%) from cloacal swabs and hand washed water showed resistance to 3 to 5 antimicrobials while it was 61.1% in case of farm sewage.

Antimicrobial class-wise resistance analysis revealed that a total of 88% isolates were resistant to 3 to 5 classes of antimicrobials (Table 7). Sample type-wise analysis revealed that more than 90% isolates from cloacal swabs and hand washed water showed resistance to 3 to 5 classes of antimicrobials while it was 78% in case of farm sewage. Two isolates (one from cloacal swab, one from farm sewage) were resistant to all the six classes of antimicrobials.

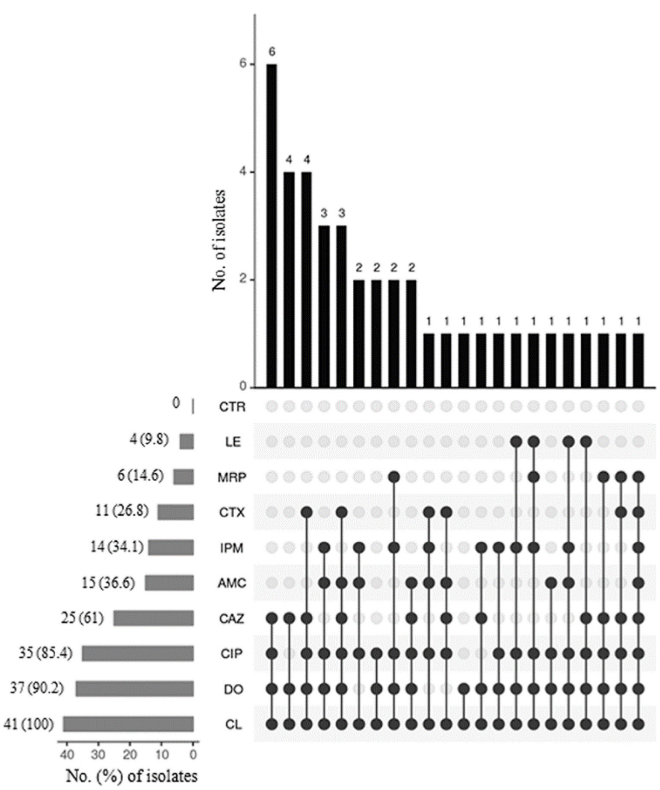
Resistance phenotypes pattern of Salmonella

Diversity of resistant phenotypes of *Salmonella* among the isolates presented in Figure 3-5. Twenty two resistance patterns were found among the 41 *Salmonella* isolates recovered from broiler chickens. The most common resistance pattern was ceftazidime-ciprofloxacin-doxycycline-colistin (6), followed by ceftazidime-doxycycline-colistin (4) and cefotaxime-ceftazidime-ciprofloxacin-doxycycline-colistin (4). In case of farm sewage, 25 resistance patterns were observed and among them doxycycline-colistin, ceftazidime-ciprofloxacin-doxycycline-colistin and amoxyclav-ceftazidime-ciprofloxacin-doxycycline-colistin were frequently observed. Sixteen phenotypic resistance patterns were identified from hand washed water isolates where the most common pattern was imipenem-ciprofloxacin-doxycycline-colistin (4) followed by ceftazidime-doxycycline-colistin (3) and ceftazidime-ciprofloxacin-doxycycline-colistin (2).

Discussion

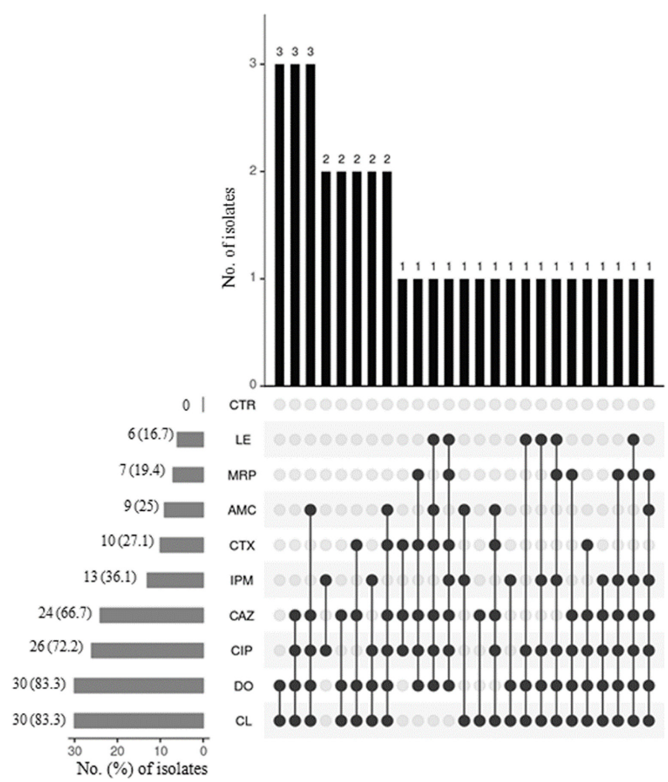
The results of the study revealed an overall 66% prevalence of *Salmonella* in cloacal swabs, farm sewage, and hand washed water samples. About 80% of the cloacal swab samples were found positive for *Salmonella*. This was in agreement with the previous reports of Naurin *et al.* who found 71.1% prevalence of *Salmonella* in cloacal swabs from broiler chickens of Bangladesh [14]. However, variable prevalence of *Salmonella* (18% to 70%) in different types of poultry

Figure 3. Phenotypic resistance pattern of *Salmonella* spp. isolated from broiler chickens (n = 41).



CTR: ceftriaxone, LE: levofloxacin, MRP: meropenem, CTX: cefotaxime, IPM: imipenem, AMC: amoxyclav, CAZ: ceftazidime, CIP: ciprofloxacin, DO: doxycycline, CL: colistin.

Figure 4. Resistance phenotypes pattern of *Salmonella* spp. isolated from farm sewage (n = 36).



CTR: ceftriaxone, LE: levofloxacin, MRP: meropenem, CTX: cefotaxime, IPM: imipenem, AMC: amoxyclav, CAZ: ceftazidime, CIP: ciprofloxacin, DO: doxycycline, CL: colistin.

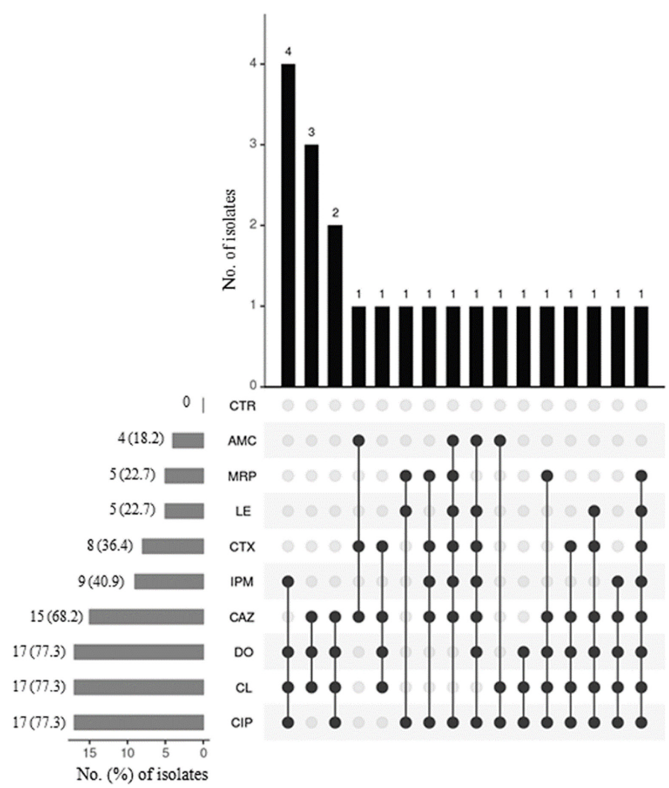
samples was reported by earlier researchers [13,21]. Relatively lower percentage of *Salmonella* was obtained from hand washed water of farmers than cloacal swabs which was in accordance with the earlier report of Akond *et al.* who reported 26.3% and 6% prevalence of *Salmonella* in cloacal swab and hand washed water samples respectively [11]. In our study, there was a possibility of pre-contaminations of farm worker's hands from other sources. However, samples from farm workers were collected just after working on the farm. Moreover, sterile distilled water was used for washing the hand and collected into the sterile tubes. The present study revealed lower prevalence of *Salmonella* in hand washed water and sewage samples of Gazipur than Mymensingh district. Farmers having more experience in poultry farming and presence of drainage system for wastewater management in many farms of Gazipur that were found in our study might be a possible explanation.

In this study, a multivariable logistic regression model identified farming experience (≤ 5 years) and age of birds (≥ 11 days) as factors significantly associated with the occurrence of *Salmonella* in broiler chickens. This might be due to the lack of knowledge and awareness of the farmers on *Salmonella* infection in the poultry flocks. Agada *et al*. stated that farmers who had less knowledge of salmonellosis were at higher risk of *Salmonella* infection in their poultry farms [22]. Birds older than 10 days were seen to be at higher risk for *Salmonella* infection, is in contrast with the result of Ansari-Lari *et al.* who found no association between *Salmonella* infection and age of broilers [23]. *Salmonella* contaminated chickens become intestinal carriers and shedding microorganism through their faeces that increase over time [24]. This might be the reason behind the high prevalence of *Salmonella* in cloacal samples of older birds. In addition, several studies found similar results demonstrating higher *Salmonella* prevalence in older poultry flocks as *Salmonella* can spread more within the poultry houses during the growing period [24, 25].

High resistance percentage was observed against particular antimicrobials, notably colistin, doxycycline, ciprofloxacin, and ceftazidime. Since a long period, colistin (last resort of drug for human therapy) was used indiscriminately in the poultry farms in Bangladesh [26]. Recently, manufacture, sale and distribution of colistin and its formulations for poultry production has been banned by Bangladesh government for tackling colistin resistance. However, pack size of 1 L and above is still permitted to use in breeder flocks in Bangladesh. In our study, in total nearly 90% *Salmonella* isolates were resistant to this drug. It is much higher compared to the findings of Hassan *et al*. who reported 50% of the *Salmonella* isolates of chickens were resistant to colistin [27]. Overuse of the drug with an intention to achieve a better results may be attributed to this high resistance of colistin. Resistance to doxycycline was found 84.8%, which was in accordance with the previous study conducted by Rahman *et al.* and Parvej *et al.* who reported 79.3% doxycycline resistance in chicken meat and 81.8% in cloacal swab isolates of Bangladesh [21,28].

Among the quinolones, resistance to ciprofloxacin showed 78.8% which is similar to the report of Hassan *et al.* who reported 87.5% ciprofloxacin resistance in chicken isolates of Bangladesh [27]. Although, few years ago, the resistance to ciprofloxacin in Bangladesh was less than 20%, which indicated a growing degree of resistance to ciprofloxacin in the poultry population in this country [11]. This could be due to the fact that the increasing tendency of farmers to use antimicrobials for prophylaxis and growth promotion [9]. This has threatened the efficacy of quinolones, the preferred antibiotics for the treatment of *Salmonella*-associated

Figure 5. Phenotypic resistance pattern of *Salmonella* spp. isolated from hand washed water (n = 22).



CTR: ceftriaxone, LE: levofloxacin, MRP: meropenem, CTX: cefotaxime, IPM: imipenem, AMC: amoxyclav, CAZ: ceftazidime, CIP: ciprofloxacin, DO: doxycycline, CL: colistin.

diseases in humans. However, considerable susceptibility (61.6%) was explored to levofloxacin which was slightly lower than the recent report of Parvin, *et al.* [29]. Among third-generation cephalosporins, none of the isolates were resistant to ceftriaxone. Higher susceptibility to ceftriaxone was in agreement with the previous findings of Islam *et al.* who reported more than 90% sensitivity of *Salmonella* isolated from cloacal swab to ceftriaxone in Bangladesh [30]. However, 64.6% and 29.3% resistance of *Salmonella* were seen against ceftazidime and cefotaxime, respectively, which is discordant with the findings of Mahmud *et al.* and Sultana *et al.* who reported lower resistance to ceftazidime (37.5%) and cefotaxime (19%), respectively [13, 31]. Although third generation cephalosporins are not commonly used in poultry production in Bangladesh, it is quite unclear why higher susceptibility was found to ceftriaxone but not to ceftazidime and cefotaxime. However, the difference in resistance percentages to antimicrobials of cephalosporin group was reported earlier [32]. The frequent cross-resistance between antimicrobial classes including cephalosporin and the ability of antimicrobial resistance genes to alter the microbial community due to continuous antimicrobial usage might be the reason behind this [33,34].

Carbapenem antibiotics are considered as the drug of choice to treat many multidrug-resistant infections and used as a last line of defense in severe bacterial infections in humans [35]. However, in the present study, about one-third (36.4%) of the isolates were resistant to imipenem and 18.2% to meropenem though these antibiotics are not used in poultry practice, even in large animal practice in Bangladesh. Very little information was found regarding the carbapenem resistance of *Salmonella* isolated from poultry farm and farm environment in Bangladesh. However, our findings stated a higher percentage of carbapenem resistance of *Salmonella* compared to in India and Japan where less than 8% resistance was reported in poultry [36,37]. This type of antimicrobial resistance is acquired mainly by mutational events or gene acquisition via horizontal gene transfer [38]. Moreover, cross-resistance in other antimicrobial classes due to the result of long term and intensive use of broad-spectrum antimicrobial agents alone or in combinations in the agricultural sector might be the reason behind the emergence and spread of carbapenem resistance [39].

Looking at the MDR pattern of the isolates we observed that around 98% isolates from cloacal swabs were MDR, and a relatively lower percentage (80.6%) of MDR *Salmonella* was found in farm sewage. This was supported by Alam *et al.* who reported that isolates from cloacal swab exhibit wider spectrum of resistance than isolates from other sources [40]. Relatively higher percentage of MDR was observed in the present study compared to the observation of Hui and Cui *et al.* who reported MDR percentage ranged from 48-65% in chickens [41,42]. Our results on MDR *Salmonella* in farm sewage converse with the report of Thakur *et al.* who reported a higher percentage of MDR in farm environment than fecal samples [43]. The routine use of antimicrobials at sub-therapeutic level for growth-promotion purposes and disease prevention which was also found in our study, could be responsible for the emergence of MDR *Salmonella* [44]. Similar phenotypic resistance pattern of the *Salmonella* isolates recovered from cloacal swabs of broiler chickens, hand washed water of farm workers and farm sewage led us to assume preliminarily that there was possible transmission of *Salmonella* between broiler chickens and farm workers or vice-versa, and finally to the environment. A previous report revealed that higher proportions of multi-drug resistant organisms were observed among *Salmonella* isolates from farm workers with livestock contact than among isolates from workers with no livestock contact [45]. The transmission of *Salmonella* occurs among production animals, wild birds, environment, and humans that lived amongst or in close proximity to them [46,47]. If we could do pulsed-field gel electrophoresis and random amplified polymorphic DNA analysis then we could infer the possible transmission of *Salmonella* between the sources. However, as we found a high resistance of *Salmonella* against a number of antimicrobials, it is very likely to transmit those resistant *Salmonella* from broiler chickens to humans or vice-versa.

Conclusions

This study estimates the high prevalence of *Salmonella* in the broiler farm settings (broiler chickens, farm sewage and farm workers) in Mymensingh district of Bangladesh with low biosecurity score. The high resistance to a number of antimicrobials and considerably high percentage of MDR *Salmonella* in broiler farms pose a threat to the public health. The indiscriminate use of antimicrobials is considered as the major factor behind this high resistance. The availability of antimicrobials and lack of guidance in using antimicrobials could be the contributing factor to the irrational use of antimicrobials in poultry farms. Finally, we may conclude that farmer's awareness and experience translating into good practices regarding various aspects of farm biosecurity in poultry rearing can reduce the load of *Salmonella* infection on the broiler farms as well as limit the transmission of resistance to humans and environment.

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Conflict of interests: No conflict of interests is declared.

Annex – Supplementary Items

Supplementary Table 1. Questionnaire used for collection of data in broiler chicken farms.

Serial No.	Questions	Responses
1.	Farm ID	
2.	Date	
3.	GPS data	Latitude: Longitude:
4.	Name of the farm/ farm owner	
5.	Address of the farm	Village: Upazila: District: Division: Dhaka/Mymensingh
6.	Mobile no. of the owner/ respective person	
7.	Farm registration	Yes/ No
8.	Duration of farming (months)	
9.	Season	Winter/ Pre-monsoon
10.	Number of birds	
11.	Farm premise is used for	Only for poultry farm/ Integrated with fish farming/ Integrated with agricultural farming/ Integrated with other animal farming
12.	Human traffic control system	Presence of foot bath: Yes/ No Fence around the farm: Yes/ No Presence of gate: Yes/ No Restriction on entry: Yes/ No
13.	Sheds accessible to rodents	Yes/ No
14.	Sheds accessible to wild birds	Yes/ No
15.	Other poultry house within 500 meters	Yes/ No
16.	Source of chicks (Hatchery)	
17.	Age of birds (days)	
18.	Flock vaccinated	Yes/ No
19.	Litter disposal	Compost/ Sell as manure/ Throw into nearby pit/ To biogas plant
20.	Management of sick birds	Kept in the farm/ Separated
21.	Management of dead birds	Burial/ Thrown away/ To garbage bin
22.	Control of rodents (regular inspection, baits)	Yes/ No
23.	Drinking water origin	Public/ Private/ Natural source
24.	Treatment of water	Yes/ No
25.	Type of drinker/ waterer	Nipple drinker/ Bell drinker/ Linear tray/ Others
26.	Cleaning of waterer	Daily/ Alternate day/ Twice a week/ Once a week
27.	Poultry feeder	Automatic/ Manual
28.	Cleaning of feeder	Daily/ Alternate day/ Twice a week/ Once a week
29.	Specific clothes for staff	Yes/ No
30.	Specific shoes for staff	Yes/ No
31.	Cleanliness of clothes and shoes	Yes/ No
32.	Litter condition	Wet/ Dry
33.	Litter turning	Alternate day/ Twice a week/ Once a week
34.	Wastewater management	Drainage/ Pond/ Compost pit
35.	Distance of natural water body (meters)	
36.	Disease outbreak in the last one year	
37.	Use of antibiotics	Yes/ No
38.	If yes, purpose	Preventive/ Therapeutic/ Growth promoter
39.	By whom suggestion	Veterinarian/ Company representative/ Feed dealer/ Experienced farmer/ Self
40.	Name of the antibiotics	
41.	Cleaning and disinfection of floor between batches	Yes/ No

Supplementary Table 2. Breakpoints of the antimicrobial disks for *Salmonella* spp.

Antimicrobial agent	Disk content	Interpretive categories and zone diameter breakpoints, nearest whole mm		
		Susceptible	Intermediate	Resistant
Penicillin β-lactamase inhibitors:				
Amoxyclav	30 µg	≥ 18	14-17	≤ 13
Tetracyclines:				
Doxycycline	30 µg	≥ 14	11-13	≤ 10
Quinolones:				
Levofloxacin	5 µg	≥ 17	14-16	≤ 13
Ciprofloxacin	5 µg	≥ 31	21-30	≤ 20
Cephalosporins:				
Ceftazidime	30 µg	≥ 21	18-20	≤ 17
Ceftriaxone	30 µg	≥ 23	20-22	≤ 19
Cefotaxime	30 µg	≥ 26	23-25	≤ 22
Polymyxins:				
Colistin[#]	10 µg	≤ 2 µg/ml (MIC)	-	> 2 µg/ml (MIC)
Carbapenems:				
Imipenem	10 µg	≥ 23	20-22	≤ 19
Meropenem	10 µg	≥ 23	20-22	≤ 19

[#] For colistin the minimum inhibitory concentrations (MIC) were determined by broth microdilution method.