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Exploring Poultry Farm Environment for Antibiotic Resistant *Escherichia coli*, *Salmonella* spp., and *Staphylococcus* spp. Having Public Health Significance

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ABSTRACT

Poultry farm could be potential source for antibiotic resistant bacteria. Present study was designed to determine total load of viable bacteria, *Escherichia coli*, *Salmonella* spp. and *Staphylococcus* spp. in different components of poultry farm environments; followed by detection of their antibiogram. A total of 75 samples of six different types (poultry droppings-15, litter-15, poultry feed-15, bird handler's hand wash-10, water-10, and air-10) were collected from five poultry farms. Bacterial total counts were done by spot diffusion method followed by isolation and identification of *E. coli*, *Salmonella* spp., and *Staphylococcus* spp. based on morphology, cultural, staining, and biochemical test. Antimicrobial resistant profiles were determined by disk diffusion method. The mean total bacterial count, *E. coli*, *Salmonella* spp., and *Staphylococcus* spp. count ranged from 3.44±0.65 to 9.22±0.55, 0±0 to 7.12±0.37, 0±0 to 5.84±0.20, and 0±0 to 8.45±.0.15 log CFU/gm or ml, respectively. Of 75 samples, 43 (57.33%), 33 (44%), and 38 (50.67%) samples were positive as *E. coli*, *Salmonella* spp., and *Staphylococcus* spp., respectively. Antibiogram study revealed 42.1% *Staphylococcus* spp. resistance to oxacillin i.e. MRSA in nature. Interestingly, *E. coli* and *Salmonella* showed 48.84% and 54.55% resistance to colistin. In addition, isolated bacteria also showed various degree of resistance against gentamicin, ciprofloxacin, ampicillin, oxytetracycline and chloramphenicol. Antibiotic resistant *E. coli*, *Salmonella* spp., and *Staphylococcus* spp. were detected from poultry farm environments that has the chance to enter into the food chain and poses serious threat to human health.

Introduction

Usage of antibiotics for prevention and control of multifarious bacterial infections has dramatically increased the livestock and poultry production globally in the last few decades. However, the extensive and inappropriate use of antibiotics has resulted in the development of antibiotic resistance (ABR) in bacteria through generating high selection pressure to natural microbial systems (Schwarz *et al.,* 2017). Due to drastic use of antibiotics in poultry farms as therapeutics and growth promoters, ABR has emerged as a burning issue in clinical touchstone and exhibited enormous and multinational public health risk (Boovaragamoorthy *et al.,* 2019; Schwarz *et al.,* 2017). Antimicrobial resistance (AMR), particularly ABR has exposed venturesome treat to public health. It is estimated that around 700,000 human deaths per year can be happened globally due to AMR (Clifford *et al.,* 2018). Nowadays, antibiotic resistant microorganisms are posing inversely cabbalistic and antithetical effects on all the components of one health *i.e.,* animal, human, and environment through circulating extensively in the environmental settings (Aslam *et al.,* 2018; Prestinaci *et al.,* 2015). Indiscrimination and accidental usage of antibiotics in poultry along with lacking of proper knowledge among people facilitate the dissemination of antibiotic-resistant microorganisms in environment surroundings (Li and Webster, 2018). However, poultry is avowed as significant emergence for enhancement of AMR level because of generating sublime selection pressure for ABR in *Escherichia coli*, *Salmonella* spp., and *Staphylococcus* spp. (Thanner *et al.,* 2016). In addition, being gut associated in chicken, these ABR bacteria can act as reservoirs to disseminate from poultry to human, environment, and other animals (Saharan *et al.,* 2020; Thanner *et al.,* 2016). Furthermore, poultry farm environmental settings including feed, litter, water, air, and human hand washing can be contaminated with ABR

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resistant *Escherichia coli*, *Salmonella* spp. and *Staphylococcus* spp. through poultry droppings. These resistant bacteria accumulating into farm environment can also be transmitted directly to human working on farm and leads ominous human health crisis (Chang *et al.,* 2015).

E. coli, a zoonotic commensal pathogen, is considered as breakneck organism in worldwide poultry sector leading to sinister economic losses (Kim *et al.,* 2020; Rahman *et al.,* 2020). Notwithstanding most strains of *E. coli* are non-pathogenic, few strains develop gastrointestinal (GI) illness as existing in GI tract as common microbial flora of both human and animals (Tenaillon *et al.,* 2010). In addition, pathogenic strains of *E. coli* can develop urinary tract infections (UTI), abdominal sepsis, meningitis, and septicemia in human leading to zoonotic in nature (Mellata, 2013). *Salmonella* spp. are ubiquitous food-borne pathogens and zoonotic in nature (Abdukhalilova *et al.,* 2016). Poultry can act as natural reservoirs of *Salmonella* spp., transmit to human, and develop Salmonellosis along with septicemia, enteric fever, and gastroenteritis (Varga *et al.,* 2019; Shanta *et al.,* 2017). Several serotypes of *Salmonella* have showed resistance to mostly used antibiotics leading to enhancement of production cost (Nair *et al.,* 2018). *Staphylococcus* spp. is one of the most prevalent human opportunistic pathogens, causing a broad variety of diseases ranging from mild skin and soft-tissue infections to infective endocarditis, osteomyelitis, bacteremia, and necrotizing pneumonia (Al-Talib *et al.,* 2011). Some strains of *Staphylococcus* spp. emerged high level of resistance e.g. methicillin resistant *Staphylococcus aureus* (MRSA) considered as superbug which is posed resistance to almost every obtainable antibiotic used in treatment of Staphylococcal infections (Mamza *et al.,* 2010; Stapleton and Taylor, 2002).

Development of resistance against antibiotics in commensal bacteria is a serious growing problem in modern medicine. The availability of surveillance data on occurrence of AMR bacteria in poultry farming system in Bangladesh, especially in poultry farm environments are crucial to adopt measures to combat AMR related hazards. Present study was therefore carried out using one-health approach to determine total viable bacterial load in poultry farm environment settings followed by detection of *E. coli*, *Salmonella* spp. and *Staphylococcus* spp. and their antibiogram having public health importance.

Materials and Methods

Ethical approval

No ethical approval was required; however verbal permission was taken from the farm owners and farm workers during sample collection.

Study area

Five broiler poultry farms located in Mymensingh district of Bangladesh (24.7539°N, 90.4073°E) (Figure 1) namely Bangladesh Agricultural University (BAU) Poultry Farm, Kewatkhali Poultry Farm, M/S Guru Poultry Farm, S.S. Poultry Farm, and Zakir Poultry Farm selected randomly for the sampling purpose.

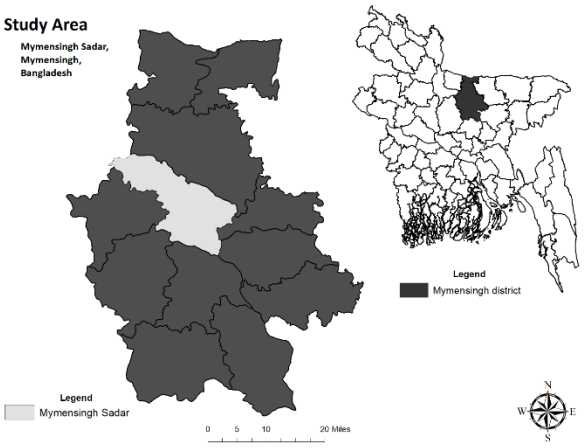

Figure 1. Study area map of Mymensingh Sadar, Mymensingh, Bangladesh. Geographical Information System (GIS) data were collected from DIVA-GIS (http://www.diva-gis.org) and map was created using ArcMap 10.7 software.

Sample collection

A total 75 poultry droppings, litter, feed, bird handler's hand wash, water and air of the broiler shade were collected from five broiler farms for analysis. From each farm 15 samples: three poultry dropping, three poultry litter, three poultry feed, two bird handler's hand wash, two water, and two air samples were collected. All samples except air were collected aseptically using sterile zip-lock bag. Air was sampled using settle pate method as previously described by Mbamalu *et al.* (2015) with few modifications. In brief, instead of nutrient agar, here plate count agar (PCA), eosin methylene blue (EMB) agar, xylose-lysine deoxycholate (XLD) agar and mannitol salt agar (MSA) plates were exposed 1 meter above the ground to different corners of the poultry shades for 10 minutes. Poultry droppings, poultry litter and poultry feed were collected into sterile zip-lock bag using sterile plastic spoons. For bird handler's hand wash using phosphate buffer solution (PBS) was used as described by Sobur *et al.* (2019a). All the samples were transported to the Bacteriology laboratory, Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh maintaining cold chain immediately after collection for bacteriological analysis.

Sample processing

Solid or semisolid samples (poultry droppings, litter, feed) and liquid samples (water, bird handler's hand washings) were mixed with 0.1% peptone water for preparing dilution to count total bacterial load. Briefly, solid or semisolid and liquid samples were weighed as 10g and 10 ml, respectively and followed by adding and mixing well into separate sterile beaker containing 90 ml 0.1% peptone water to have initial dilution. In epilogue, ten-fold serial dilutions were prepared to enumerate total bacterial count. On the other hand, collected samples except agar plates exposed to air were transferred into sterile test tubes containing 5 ml nutrient broth followed by incubated aerobically at 37°C overnight for bacterial growth. The agar plates exposed to air were directly kept under incubator for culture.

Total viable bacteria, E. coli, Salmonella and Staphylococcus count

The total viable counts were made using plate-serial dilution spotting (SP-SDS) as described by Thomas *et al.* (2015), In brief, initially ten-fold dilutions (10^{-1}-10^{-6}) of each sample were prepared in Eppendorf tubes (1.5 ml) containing 0.1% peptone water. Earlier, PCA, EMB, XLD, and MS agar plates were divided and marked separately to count total bacterial load (Total Viable Count, TVC), *E. coli* (Total Coliform Count, TCC), *Salmonella* spp. (Total *Salmonella* count, TS_1C), and *Staphylococcus* spp. (Total *Staphylococcus* Count, TS_2C), respectively. After that, 3 drops of diluted broth each containing 10 µl broth were inoculated into each divided parts of selected agar plates separately; followed by incubation at 37°C for 24 hours for development of single colonies. The observation of colonies with any types, metallic sheen, black center, and yellow color on PCA, EMB, XLD, and MS agar media, respectively were identified as growth of any bacteria, *E. coli*, and *Salmonella* spp., and *Staphylococcus* spp., respectively. Finally, CFU was calculated based on average count of 3-30 colonies per 10 µl from particular dilution were recorded as colony forming unit (cfu)/gm or ml samples.

Isolation and identification of bacteria

Isolation and Identification of *E. coli*, *Salmonella* spp., and *Staphylococcus* spp. from collected samples were based on cultural characteristics on selective media followed by staining characteristics under Gram's staining and biochemical test. Initially, the broth culture with bacterial growth were streaked on EMB, XLD, and MS agar media; followed by incubation aerobically for overnight at 37°C. Growth of metallic sheen colonies on EMB agar, black center colonies on SS agar and golden yellow colonies on MS agar were considered as *E. coli*, *Salmonella* spp., and *Staphylococcus* spp. respectively. Those colonies were then subjected to morphological study by Gram staining and biochemical tests namely sugar fermentation test, methyl red test Voges-Proskauer test, indole test, coagulase test, catalase test (Sobur *et al.,* 2019a; Sobur *et al.,* 2019b; Zaman *et al.,* 2020; Ievy *et al.* 2020).

Antimicrobial susceptibility test

Isolated bacteria subjected to antimicrobial susceptibility test by disk diffusion method as described by Bauer *et al.* (1966). Seven commonly used antibiotics namely- colistin (10µg), chloramphenicol (30µg), ciprofloxacin (5µg), ampicillin (2µg), gentamycin (10µg), oxacillin (1µg), and oxytetracycline (30µg) were used for antimicrobial susceptibility test. These tests were conducted on Mueller Hinton agar media (Himedia, India) with purely growth of bacteria having a concentration of bacterial growth equal to 0.5 McFarland standard (HiMedia, India). Finally, zone of growth inhibitions was computed as sensitive, intermediate, and resistant based on standards provided by Clinical and Laboratory Standards Institute (CLSI, 2016).

Results

Total viable bacteria, E. coli, Salmonella and Staphylococcus count

Among the samples analyzed, poultry litter was found to carry maximum TVC and TS_2C, whereas maximum TCC was recorded in poultry droppings. Both farm water and farm air manifested minimum TS_1C. The overall bacterial load of different samples are represented in Table 1. In farm-wise, maximum TVC and TCC were detected in BAU Poultry Farm and Zakir Poultry Farm, respectively, whereas maximum TS_1C, and TS_2C were detected in S.S. Poultry Farm. The S.S. Poultry Farm showed minimum TVC and TCC, in contrary, M/S Guru & S.S. Poultry Farm revealed minimum TS_1C, and TS_2C (Table 1 and 2).

Bacterial isolation and identification

Among the 75 samples, 43 (57.33%), 33 (44%), and 38 (50.67%) were found to be positive for *E. coli*, *Salmonella* spp., and *Staphylococcus* spp., respectively. The highest prevalence of *E. coli* (80%, 12/15) and *Salmonella* spp. (66.67%, 10/15) were detected in poultry droppings, whereas highest occurrence of *Staphylococcus* spp. (100%, 10/10) were detected in farm air samples. Conversely, farm air samples showed lowest occurrence of *E. coli* (30%, 30/10), whereas poultry feed samples exhibited lowest *Salmonella* spp. (20%, 3/15) and *Staphylococcus* spp. (20%, 3/15). The overall occurrence of isolated bacteria from different samples of selected poultry farms are represented in Figure 2.

Antimicrobial susceptibility test

Antimicrobial susceptibility test revealed that all the *E. coli* and *Salmonella* spp. were found resistance to oxacillin, whereas *Staphylococcus* spp. exhibited highest resistance against ampicillin (71.05%). Additionally, several antimicrobial agents revealed frequently resistant to all type of isolates e.g. ampicillin, colistin, gentamicin, oxytetracycline to *E. coli*; ampicillin, colistin, gentamicin, chloramphenicol, ciprofloxacin, oxy-tetracycline to *Salmonella* spp.; and oxy-tetracycline, chloramphenicol, ciprofloxacin to *Staphylococcus* spp. Interestingly, 42.10% *Staphylococcus* spp. were also found resistant to oxacillin, i.e., phenotypically MRSA in nature. The overall antibiotic resistance profiles of the isolated bacteria are presented in figure 3.

Table 1. Bacterial load of different samples of poultry farm

Sample	TVC (Mean log CFU±SD/gm or ml)		TCC (Mean log CFU±SD/gm or ml)		TS_1C (Mean log CFU±SD/gm or ml)		TS_2C (Mean log CFU±SD/gm or ml)	
	Max	Min	Max	Min	Max	Min	Max	Min
Poultry droppings	8.93± 0.52	6.96 ± 0.21	7.12 ± 0.37	5.49 ± 0.42	5.81 ± 0.04	3.39 ±. 0.35	7.66±.0.24	6.17 ±.0.16
Poultry litter	9.22± 0.55	7.53 ± 0.72	7.00 ± 0.08	5.57 ± 0.97	5.84 ± 0.20	4.62 ±. 0.05	8.45±.0.15	5.32 ±.0.33
Poultry feed	6.79± 0.04	5.79 ± 0.07	5.49 ± 0.64	4.89 ± 0.61	3.84 ± 0.62	3.17 ±. 0.15	2.01 ± 0.08	0 ± 0
Hand washings	6.93± 0.54	5.67 ± 0.80	5.83 ± 0.57	4.54 ± 0.70	4.79 ± 0.49	3.28 ±. 0.24	6.52 ± 0.10	5.01 ± 0.25
Farm water	4.54 ± 0.71	3.44 ± 0.55	3.47 ± 0.10	2.00 ± 0.18	2.78 ± 0.24	0 ± 0	3.36 ±0.05	0 ± 0
Farm air	7.54 ± 0.24	5.63 ± 0.19	4.15 ± 0.30	0 ± 0	3.01 ± 0.10	0 ± 0	1.93 ±0.14	0 ± 0

(TVC= Total viable Count, TCC=Total coliform count, TS_1C= Total *Salmonella* count, TS_2C= Total *Staphylococcus* count, CFU= Colony forming unit, SD= Standard deviation, Max= Maximum, Min= Minimum).

Table 2. Overall result on bacterial load in different farms

Bacterial load	Max / Min	(Mean log CFU±SD/gm or ml)	Sample	Source of sample
TVC	Maximum	9.22 ± 0.55	Poultry litter	BAU Poultry Farm
	Minimum	3.44 ± 0.55	Water	S.S. Poultry Farm
TCC	Maximum	7.12 ± 0.37	Poultry droppings	Zakir Poultry Farm
	Minimum	0 ± 0	Air	S.S. Poultry Farm
TS_1C	Maximum	5.84 ± 0.20	Poultry litter	S.S. Poultry Farm
	Minimum	0 ± 0	Water & Air	M/S Guru & S.S. Poultry Farm
TS_2C	Maximum	8.45±.0.15	Poultry litter	S.S. Poultry Farm
	Minimum	0 ± 0	Poultry feed, Water & Air	BAU & M/S Guru Poultry Farm

(TVC= Total viable Count, TCC=Total coliform count, TS_1C= Total *Salmonella* count, TS_2C= Total *Staphylococcus* count, CFU= Colony forming unit, SD= Standard deviation, Max= Maximum, Min= Minimum)

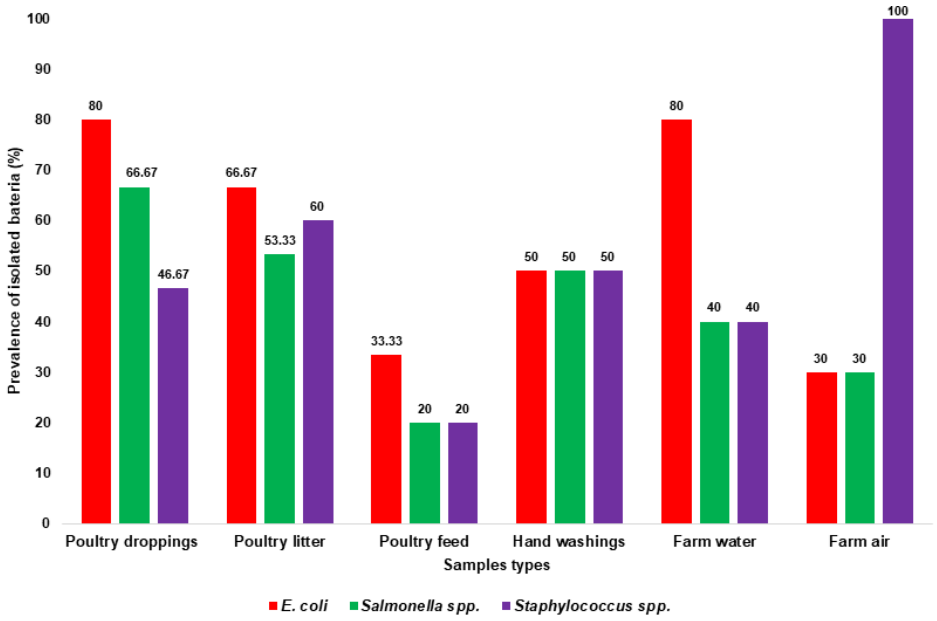

Figure 2. Occurrence of *E. coli, Salmonella* and *Staphylococcus* in the poultry farm samples

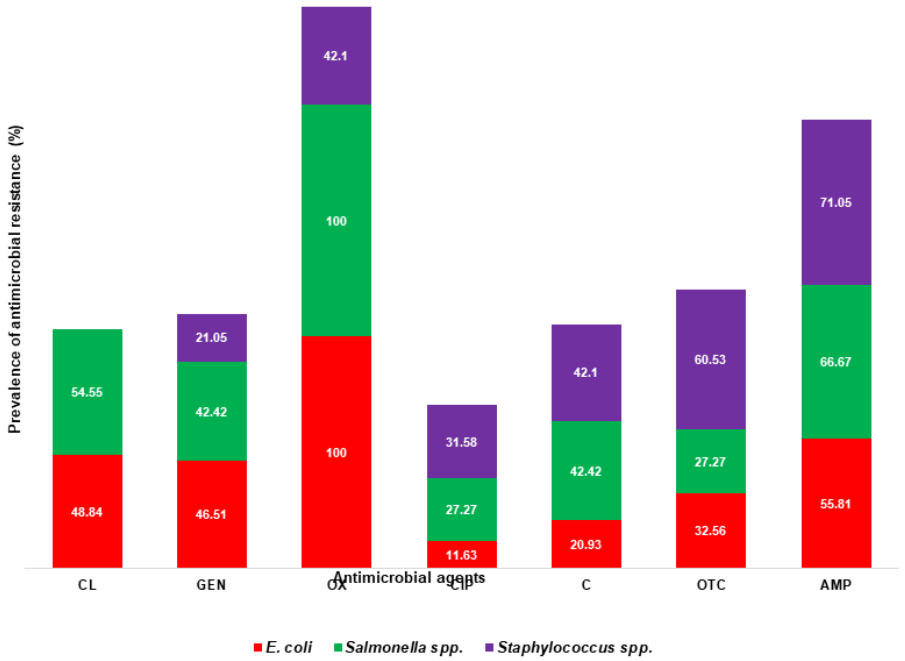

Figure 3. Overall antibiotic profiles of isolated bacteria in the poultry farm samples. CL= Colistin, GEN= Gentamicin, OX= Oxacillin, CIP= Ciprofloxacin, C= Chloramphenicol, OTC= Oxytetracycline, AMP= Ampicillin.

Discussion

Antibiotic resistance is a serious global health issue affecting all the components of one health. The indiscriminate administration of antimicrobial agents for therapeutic purpose and as growth promoting agent to maintain increase growth and production in poultry industries has resulted in the emergence of antibiotic resistance in many of the avian bacterial pathogens. Here we investigated the load of selective bacterial population in poultry and various components of poultry farm environments as well as their antibiotic resistance pattern.

Present study revealed the widespread occurrence of bacteria in poultry environments. The maximum TVC was detected in poultry litter followed by in droppings, farm air, handlers' hand washings, poultry feed, and farm water. Previously, Nasrin *et al.* (2007) reported higher bacterial count in fecal materials; (103.5±3.62 $\times 10^5$ CFU/gm) and poultry litter (37.0±1.79 $\times 10^5$ CFU/gm); followed by poultry feed (6.5±1.87 $\times 10^5$ CFU/gm) and drinking water (31.33±1.12 $\times 10^5$ CFU/ml). Presence of increased level of bacterial load in poultry litter and poultry droppings is not unexpected. Poultry gut which is full of varieties of microbes are released from poultry through droppings (fecal materials), contaminate and accumulate into the litter (Pan and Yu, 2014; Borda-Molina *et al.,* 2018; Diaz *et al.,* 2019). Similarly, load of total *E. coli*, *Salmonella* spp. and *Staphylococcus* spp. counts were also found higher in poultry dropping and litter compared to other samples tested.

Poultry feed could be potential source for pathogens. In this study analyzed 33.33% feed were found to be contaminated with *E. coli*, 20% with *Salmonella* spp., and 20% with *Staphylococcus* spp. as reported by others (Rahman *et al.,* 1999; Alam *et al.* 2020). These contaminations of poultry feed may be due to haphazard employment of technologies during processing, storage, transportation of poultry feed or may be originated from nitrogenous wastes (Uwaezuoke and Ogbulie, 2008). In addition, the exhibition of pathogens in feed from our present study suggests that feed can be a potential source of *E. coli, Salmonella* spp., and *Staphylococcus* spp. in poultry. Presence of bacteria in water samples recorded from our present study exhibited that contaminated water can be common source for multiple bacteria in poultry farm. *Salmonella* spp. in drinking water in poultry farms has earlier been reported from Gazipur and Tangail district of Bangladesh (Al-Mamun *et al.,* (2017)). Occurrence of *Salmonella* spp. in the farm water samples may be linked with fecal contamination of the water at any point of the water supply, storage and distribution system into the farm that need further investigation. Detection of bacteria in the poultry handlers' hand washings suggest need for the better hygiene and sanitation. Like previous study, we also detected *E. coli* in air samples in the poultry farm (Duan *et al.,* 2008). Pathogens found as bioaerosol form may

develop allergies, difficulties in immune, nervous, and respiratory functions (Konieczny *et al.,* 2016). Detection of *E. coli*, *Salmonella* spp., and *Staphylococcus* spp. in air samples of poultry farms revealed high risks for the poultry farm workers.

In present study, *E. coli* were isolated most frequently (57%) from the samples analyzed, followed by *Staphylococcus* spp. (50.67%) and *Salmonella* spp. (44%). As ubiquitous in nature, detection of these bacteria in poultry farm environments was not surprising, since these are mostly found as part of the poultry gut microbiota (Pan and Yu, 2014). Previously several studies also isolated *E. coli*, *Salmonella* spp., and *Staphylococcus* spp. from different samples of poultry farm environments (Nasrin *et al.,* 2007; Skora *et al.,* 2016; Chat *et al.,* 2019). Occurrence of *E. coli*, *Salmonella* spp., and *Staphylococcus* spp. in the poultry environments may be due to the improper management, unhygienic condition and inadequate biosecurity measures of poultry farms.

Among the antibiotic resistance bacteria MRSA had been receiving public health attention for over a decade because of their zoonotic potential (Zaman *et al.,* 2020). In this study about 42.10% *Staphylococcus* spp. were also found resistant to oxacillin. Previously, Ali *et al.* (2017) detected MRSA from different poultry farm of Bangladesh. However, the presence of MRSA in poultry farm has to be considered as serious health issues because of the potentiality of these MRSA to transmit to personnel working in the farm. Isolated *E. coli* and *Salmonella* spp. also showed various degree of resistance to antibiotic including ampicillin, gentamicin chloramphenicol and colistin. Occurrence of antibiotic resistance level recorded in current study is not surprising in context of Bangladesh. In Bangladesh, antibiotics are being used extensively and inappropriately to treat infectious diseases in poultry, animals, and human (personal communication). Amer *et al.* (2018) detected *E. coli* from broiler farms in Egypt which were resistant against oxytetracycline (85%), ampicllin (80%), chloramphenicol (65%), gentamicin (55%) and oxacillin (30%). Another study (Zhao *et al.,* 2016) showed that *Salmonella* spp. isolated from free ranged chicken in china were found to be resistant against ampicillin (57.9%), gentamicin (23.7%), chloramphenicol (13.2%) and ciprofloxacin (13.2%). In addition, recently Mridha *et al.* (2020) recorded that 80%, 12.73%, and 9.09% *Salmonella* spp. isolates were resistant against tetracycline, ciprofloxacin, and gentamicin, respectively in Dhaka, Gazipur, and Tangail districts of Bangladesh. Furthermore, Roy *et al.* (2017) detected resistant *Salmonella* spp. and *Staphylococcus* spp. isolates from poultry feed in Bangladesh.

As per WHO AWaRe (access, watch, reserve) classification, colistin is considered as a reserve group of antibiotics. This is one of the last-resort antimicrobials used for the treatment of multidrug-resistant Gram-negative bacteria. However, many reports are now available describing resistance to colistin (Sobur *et al.,* 2019c; Yin *et al.,* 2017; Zhang *et al.,* 2018a). Here we found about 48.84% isolated *E. coli* and 54.55% *Salmonella* resistant to colistin. Farmers often use colistin in the production of food animals including in poultry to enhance growth. Antibiotic itself, acts as a selective pressure to induce resistance (Peterson and Kaur, 2018). Poultry and livestock also act as major reservoir and transmitter of colistin resistance (Hoelzer *et al.,* 2017). Previously, few reports recorded in the development of colistin resistance bacteria from poultry and poultry environments (Sobur *et al.,* 2019c; Zhang *et al.,* 2018a; Zhang *et al.,* 2018b; Shang *et al.,* 2018). In addition, a study conducted in Vietnam showed that the development of colistin resistance bacteria is associated with extensive and blind use of colistin in poultry and livestock industry (Nguyen *et al.,* 2016). The presence of antibiotic resistant along with colistin resistant bacteria in poultry farm environments exposes hazardous health significance in poultry and working personnel in farms. We suggest monitoring of poultry farm on the use of colistin and other antibiotics so that development of resistance could be kept at minimum level.

Conclusion

Detection of antibiotic resistance *E. coli*, *Salmonella* spp., and *Staphylococcus* spp. in poultry farm environments is of public health concern. From poultry farm and farm environments, they can transmit to human causing health problems. In addition, they can also enter into the food chain. Further detail molecular epidemiological studies are required to suggest better farm management to reduce the AMR related hazards in poultry farm.

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Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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