PREVALENCE AND ANTIMICROBIAL RESISTANCE OF *SALMONELLA* ISOLATED FROM COMMERCIAL POULTRY FARMS IN BANGLADESH

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Roll No: 0118/03

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JUNE 2020

Authorization

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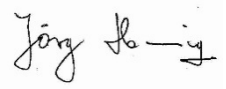

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Abbreviations	Elaborations
AM	Antimicrobials
AMR	Antimicrobial resistance
AST	Antimicrobial susceptibility testing
BA	Blood agar
BALZAC	Behavioural Adaptations in Live Poultry Trading and Farming Systems and Zoonosis Control in Bangladesh
BBS	Bangladesh Bureau of Statistics
BGA	Brilliant green agar
BHI	Brain heart infusion
BLRI	Bangladesh Livestock Research Institute
BPW	Buffered peptone water
CDC	Centers for Disease Control and Prevention
CIAs	Critically important antimicrobials
CLSI	Clinical and Laboratory Standards Institute
CRD	Chronic respiratory disease
DLO	District Livestock Officer
DLS	Department of Livestock Services
DOC	Day old chick
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agriculture Organization
GDP	Gross domestic product
IB	Infectious bronchitis
IBD	Infectious bursal disease
MALDI-TOF	Matrix assisted laser desorption/isonization time of flight
MHA	Mueller hinton agar
MSRV	Modified Semi-solid Rappaport Vassiliadis
ND	Newcastle Disease
OECD	Organization for Economic Co-operation Development
OHPH	One Health Poultry Hub
OIE	Office International des Epizooties (World Organization for Animal Health)
OTC	Oxytetracycline
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
RVO	Netherlands Enterprise Agency

USD	United States dollar
USDA	United States Department of Agriculture
WHO	World Health Organization
WPSA	World's Poultry Science Association
XLD	Xylose lysine deoxycholate
%	Percentages
≥	Greater than or equal to
≤	Less than or equal to
95% CI	95% confidence interval

Abstract

Salmonellosis caused by *Salmonella* spp. belonging to the family Enterobacteriaceae is an important poultry disease and has a great economic impact on the poultry industry as it can result in high mortality and a decrease in productivity. *Salmonella* is also one of the most common zoonotic bacteria that causes food borne illness in humans. Food animals, especially poultry, are an important direct and indirect source for human salmonellosis. The use of antimicrobials benefits producers by controlling pathogens, but contributes to the emergence of antimicrobial resistant bacteria. In addition, consumption of food containing high antibiotic residues can also lead to increased antimicrobial resistance (AMR) in humans. A cross-sectional study was carried out on 140 commercial chicken farms in eight sub-districts of Chattogram, Bangladesh from February to July 2019. This study aimed to assess the farm level *Salmonella* prevalence, describe their association with biosecurity indices and to develop an antibiogram pattern of *Salmonella* spp. on these commercial chicken farms. One pool of cloacal swabs (from 5 birds) and one pool of environmental swabs (5 sites) per farm were collected. Epidemiological data on demographic characteristics and biosecurity practices were obtained through a standardized questionnaire containing closed and open-ended questions, while a physical inspection of the farms was also conducted. *Salmonella* was isolated from cultures on different selective-differential media and further confirmed by Vitek. Antimicrobial susceptibility testing was performed by disc diffusion for 13 antimicrobials of veterinary and/or human health importance. The farm level prevalence of *Salmonella* spp. was 8.4% (95% CI: 3.5-16.6, N=83 broiler farms) and 8.8% (95% CI: 2.9-19.3, N=57 layer farms). The isolation rate of *Salmonella* was significantly higher from environmental than cloacal swabs. The farm prevalence of *Salmonella* spp. based on cloacal swab and environmental swab respectively was 2.4% (95% CI: 0.3-8.4, N=83 broiler farms) and 3.5% (95% CI: 0.4-12.1, N=57 layer farms) and 8.4% (95% CI: 3.5-16.6, N=83 broiler farms) and 8.8% (95% CI: 2.9-19.3, N=57 layer farms). The study identified that broiler farms, which conducted a weekly practice of disinfecting and cleaning the farm surfaces and equipment had a significantly lower level of *Salmonella* prevalence ($p<0.05$).

The farm antimicrobial resistance (AMR) prevalence was 85.7% (95% CI: 42.1-99.6) in broiler farms and 80% (95% CI: 28.4-99.5) in layer farms. The proportion of broiler farms for which isolated *Salmonella* spp. strains were resistant to erythromycin was 100%. Resistance on broiler farms was 86% for amoxicillin, ampicillin, cefalexin, enrofloxacin, ciprofloxacin and pefloxacin respectively and 57% for gentamicin. The resistance profile for *Salmonella* spp. on layer farms showed 100% resistance to amoxicillin, ampicillin, erythromycin and pefloxacin respectively and 60% resistance to cephalexin, doxycycline and enrofloxacin respectively. Some antimicrobials found sensitive for broiler: azithromycin, trimethoprim-sulfonamides combination, neomycin, doxycycline and colistin; for layer: ciprofloxacin, azithromycin, neomycin, trimethoprim-sulfonamides combination, colistin and gentamicin.

This study highlighted high levels of AMR on commercial poultry farms, which requires immediate interventions. Protocols need to be established for judicious use of sensitive antimicrobials in order to improve antimicrobial stewardship. Awareness programs should be developed for the farmers and relevant stakeholders about risk of indiscriminate use of antimicrobials and AMR. Farmers must consult with veterinarians before administration of antimicrobials with performing antimicrobial sensitivity testing (AST). Proper bio-security measures (regular cleaning of farm) should be implemented to improve biosecurity at commercial chicken farms.

Keywords: *Salmonella* Prevalence, Antimicrobial resistance, Poultry farms, Chattogram, Bangladesh

Chapter-I: Introduction

Poultry sub-sector is one of the biggest and important components of livestock which provides quality protein and nutrition to people's meals and confers in economic growth of the country (Raihan and Mahmud, 2008; Hamid et al., 2017). Poultry along with other livestock contributes about 1.43% in national GDP and 13.4% in agricultural GDP and the annual growth rate of poultry is 3.04% (DLS, 2020). Almost 8 million people are directly or indirectly employed with commercial poultry sector, next to the garment industry in this country (USDA, 2019).

The current standing poultry population in Bangladesh is 356.3 million of which 296.6 million is chicken (DLS, 2020). There are around 70 thousand commercial chicken farms in different scales (500-2500 small, 2501-5000 medium and more than 5000 large) which are supported by 16 grandparent farms, 206 small and large-scale breeder farms and 198 registered feed mills (WPSA, 2017; RVO, 2020). Poultry meat contributes approximately 68% of total meat production (76.74 lakh metric tonnes of annual production) of the country which is fulfilled by locally grown backyard chickens as well as commercial chickens (DLS, 2020; RVO, 2020). Total annual egg production of the country is 17.4 billion which fulfill the demand of the country (104 eggs per head per year) (DLS, 2020).

Although the commercial poultry sub-sector in Bangladesh has rapidly been growing over the last two decades, this advancement is facing many challenges of which infectious and non-infectious endemic diseases are important challenges (Hafez and Attia, 2020). The common endemic diseases are colibacillosis, salmonellosis, infectious coryza, fowl cholera, necrotic enteritis, infectious bursal disease, Newcastle disease, avian influenza, infectious bronchitis, avian leucosis and fowl pox (Badruzzaman et al., 2015; Al-Mamun et al., 2019).

Among the aforementioned diseases salmonellosis is one of the most important infectious diseases that makes hindrance to the poultry industry in Bangladesh (Haider et al., 2009; Karim et al., 2017) and poses public health threat because of its zoonotic importance *Salmonella* spp. Salmonellosis caused by *Salmonella* belonging to family Enterobacteriacae which consists of two species with six subspecies and more than 2579

serovars (Grimont and Weill, 2007). Non-motile serotypes *Salmonella enterica* Pullorum and *Salmonella enterica* Gallinarum are highly host adapted to chicken. It's an endemic disease of poultry and causing economic losses through mortality (up to 100%) (Markos and Abdela, 2016) and decrease in productivity (Haider et al., 2009). Motile *Salmonella* of paratyphoid group cause salmonellosis in poultry has public health significance (Dar et al., 2017). In humans, three major infections with gastroenteritis, typhoid fever and paratyphoid fever that may cause serious ailments for young and adults (Li et al., 2014; Wibisono et al., 2020).

Mode of transmission of *Salmonella* occurs vertically and horizontally. The bacteria can spread in hatcher and brooder and transmit horizontally from contaminated eggs (Dos Santos et al., 2019). Horizontal transmission may also occur via direct or indirect contact with infected birds, contaminated food vendors, environment and rodents (Loharikar et al., 2013).

There are not many published epidemiological studies on *Salmonella* in poultry in Bangladesh. Barua et al. (2012 and 2013) reported the farm level *Salmonella* prevalence estimate of 11% in broiler farms and 18% in layer farms in Chattogram. Other studies reported the individual level *Salmonella* prevalence estimate of 1.0% - 71.1% in broiler chickens (Ahmed et al., 2009; Naurin et al., 2012) and 5% - 38.9% in layer chickens in elsewhere in Bangladesh (Naurin et al., 2012; Hossain et al., 2019).

The variable individual level *Salmonella* prevalence was reported in many countries: 2.3% to 8.4% in India (Mir et al., 2010; Kumar et al., 2014), 7.2% in Pakistan (Khan et al., 2019), 12.9% to 92.6% in Thailand (Lampang et al., 2014; Mangmee et al., 2020), 2.5% to 14.9% in Malaysia (Ong et al., 2014; Jajere et al., 2019), 11.2% to 30.3% in China (Zhao et al., 2020; Yu et al., 2021), 5.2% to 14% in Japan (Shahada et al., 2008; Lapuz et al., 2012) and 3.7% to 31.1% in Nigeria (Agada et al., 2014a; Akeem et al., 2017).

The reported farm level prevalence of salmonellosis in different countries: 3.8% in India (Singh et al., 2010), 37.2% in Pakistan (Khan et al., 2019), 55% to 67% in Nepal (Nelson et al., 2020; Sharma et al., 2021), 3.2% in Thailand (Utrarachkij et al., 2012), 20.7% to 96% in Japan (Sasaki et al., 2012; Yamazaki et al., 2016) and 43.6% to 47.9% in Nigeria (Fagbamila et al., 2017; Jibril et al., 2020).

The documented factors that influence the occurrence of *Salmonella* in poultry farms in different parts of the world are poultry reared in caged flocks (Huneau-Salaün et al., 2009), deep liter system (Jibril et al., 2020), closed-house rearing (Sasaki et al., 2012), house with multiple age groups (Mollenhorst et al., 2005; Huneau-Salaün et al., 2009), farm with previous history of *Salmonella* (Cardinale et al., 2004; Agada et al., 2014b), feed sourced from local market (Andino et al., 2014) and careless processing, transportation and distribution of feed (Fagbamila et al., 2017), delivery trucks (feed, eggs and other) enter to the farm premises or parking nearby (Huneau-Salaün et al., 2009; Agada et al., 2014b), keeping sick birds in the same house (Cardinale et al., 2004), supplying untreated drinking water (Djeffal et al., 2018), allowing unnecessary visitors (Cardinale et al., 2004), presence of rodents (Agada et al., 2014b) and presence of other livestock in farm premises (Jibril et al., 2020).

Antimicrobial resistance (AMR) is a global animal and public health threat (Nhung et al., 2017; Holubar, 2020). AMR is frequently occurred against endemic bacterial pathogens in poultry in the world including Bangladesh. Multidrug resistant *Salmonella* has become a major public health concern around the world (Marshall and Levy, 2011). Pattern of AMR is more severe against *Salmonella* isolates obtained from poultry according to the past non-systematic and non-epidemiology studies in Bangladesh: 42.7% to 100% amoxicillin (Hassan et al., 2014; Mridha et al., 2020), 71.4% to 99% ampicillin (Mahmud et al., 2011; Hossain et al., 2019), 31% to 65% cephalexin (Akond et al., 2012; Sultana et al., 2014), 50% to 52% doxycycline (Hassan et al., 2014; Sultana et al., 2014), 80% to 100% tetracycline (Akond et al., 2012; Mridha et al., 2020), 25% to 47.3% azithromycin (Sultana et al., 2014; Mridha et al., 2020), 87.5% enrofloxacin (Hassan et al., 2014), 7.1% to 40% ciprofloxacin (Mahmud et al., 2011; Hossain et al., 2019), 82% erythromycin (Akond et al., 2012; Mridha et al., 2020), 9% to 46% gentamicin (Mahmud et al., 2011; Mridha et al., 2020).

Published factors associated with the occurrence of AMR in poultry in Bangladesh and elsewhere in the world were: high magnitude of antimicrobial use in poultry production practices provokes selection pressure on bacteria to become resistant (Van Boeckel et al., 2015), antimicrobial agent given to the poultry as prophylaxis, growth promoter or

treatment purposes and practice of using multiple drugs with broad-spectrum antibiotics can cause AMR (Akond et al., 2012; Ferdous et al., 2019; Sarker et al., 2020), metaphylactic use of antimicrobial is also responsible for AMR (Serwecińska, 2020), employment of essential antibiotics (Agyare et al., 2018; Ferdous et al., 2019), unscrupulous use of antibiotics in poultry feed during poultry production (Mridha et al., 2020) and knowledge gap about withdrawal period (Sarker et al., 2020) may increase the possibility of spreading microbial resistance in environment.

With the aforementioned background the present epidemiological study was designed with the following specific objectives.

1. Estimate the farm level prevalence of *Salmonella* in broiler and layer chickens in Chattogram, Bangladesh.
2. Determine the association between the farm level *Salmonella* prevalence and the bio-security indices in Chattogram, Bangladesh.
3. Describe the antibiotic resistance pattern of *Salmonella* isolates obtained from broiler and layer chicken farms in Chattogram, Bangladesh.

1.1. Outcomes

1 Estimated farm level *Salmonella* prevalence in broiler and layer chickens in Chattogram, Bangladesh.

2 Identification of the farm management level factors associated with the farm level *Salmonella* prevalence in Chattogram, Bangladesh.

3 Establishment of farm level antibiograms of *Salmonella* isolates in Chattogram, Bangladesh.

Chapter-II: Review of Literature

The overall goal of this chapter was to review past research findings related to the Master's project "***Prevalence and antimicrobial resistance of Salmonella isolated from commercial poultry farms in Bangladesh***" to identify the gaps and justify the present research. Various published literatures were obtained by searching online sources like PubMed, Google Scholar and Web of Science. This chapter is arranged in a series of sections including a review of literatures on Bangladesh poultry production, farming challenges, *Salmonella* and *Salmonella* prevalence, associated risk factors, *Salmonella* diagnosis, treatment, prevention and control and antibiotic resistance pattern and its public health consequences.

2.1. Poultry production

Bangladesh is pre-eminently an agricultural country with dense human population. The poultry sub-sector plays an important role in bringing agricultural growth up. This fast growing sub-sector has proved to be an attractive economic activity, accounting for 14 percent of the total value of livestock outputs. It is also considered more beneficial than any other agricultural sub-sector for quick profit, income generation and cheaper animal protein production (Islam et al., 2016). Poultry meat contributes 37% of total meat production of livestock origin in Bangladesh (WPSA, 2017). It helps in improving livelihood including poverty reduction with food and nutritional security in rustic community of Bangladesh. Moreover, poultry meat and eggs are well accepted by all religions, social, economic and demographic groups (Simon, 2009). It also becomes diversified, produces healthier further processed food and growing as multi-dimensional and stable industry as the needs of consumers, society and government (Rahman et al., 2017).

The demand for poultry meat and eggs are mostly fulfilled by locally grown backyard poultry (chicken, duck, goose) as well as from commercial chicken in different scales: small (flock size: 500-2500), medium (2501-5000) and large-scale (>5000) poultry enterprises (Personal communication: BALZAC project, 2018)

Although the poultry sector has remarkably intensified over the last two decades in Bangladesh, per capita animal protein consumption from poultry is still low (6.3 kg in Bangladesh vs. 2.4 kg in India, 6.6 kg in Pakistan, 48.7 kg in Malaysia, 7.8 kg in Indonesia, 7.8 kg in Thailand, 14 kg in China, 16.2 kg in Viet Nam, 17.7 kg in Japan and 18.7 kg in Korea) (Kawsar et al., 2013; WPSA, 2017; OECD, 2020). However, due to high-income generation and population growth with urbanization, demand for poultry meat and eggs has been increased (Islam and Jabbar, 2010; Hamid et al., 2017).

In Bangladesh, there were a total of 356.3 million poultry (296.6 million chickens) in the 2019-2020 production years (DLS, 2020). There are 65-70 thousand commercial chicken farms which are supported by 16 grandparent farms, 206 small and large-scale breeder farms and 198 registered feed mills producing 5.3-5.4 million metric tonnes industrial feeds (WPSA, 2017). Commonly available commercial chicken strains in Bangladesh are Cobb 500, Ross 308, Habbard, Indian River meat, Tiger Sasso and Arber acre (broiler) and Hyline Brown/White, ISA Brown, Novogen Brown/White, Shaver 579, Hi-Sex Brown/White, and Bovine White (layer). The poultry sub-sector is not only providing a key source of protein, but also creating a great employment opportunities for almost 8 millions of people in this country (USDA, 2019).

2.2. Challenges of poultry farming

There are many challenges in poultry farming in Bangladesh. These include lack of policy and policy implementation, insufficient veterinary services, lack of skilled manpower, poor disease surveillance and data management systems along with poor laboratory support, poor strategies of disease prevention and control measures, feed dealer dependency, unsatisfactory market facilities etc (Rahman et al., 2004; Kawsar et al., 2013; Msoffe et al., 2016; Masud et al., 2020).

Like many countries disease is the top most challenge in poultry rearing in Bangladesh. Reported common infectious diseases in Bangladesh are colibacillosis, salmonellosis, infectious coryza, fowl cholera, necrotic enteritis, infectious bursal disease, Newcastle disease, avian influenza, infectious bronchitis, avian leucosis and fowl pox (Roy et al.,

2012; Al-Mamun et al., 2019). As the current MS research is focused on *Salmonella*, the literatures below have been given on this.

2.2.1. *Salmonella* and its transmission

Salmonellosis caused by *Salmonella* spp. belonging to the family of Enterobacteriaceae is an endemic disease in poultry in Asia as well as in Bangladesh which is characterized by anorexia, diarrhea, dehydration, weakness and high mortality, drop in egg production, and reduce fertility and hatchability (Shivaprasad, 2000; Kabir, 2010; Jahan et al., 2013; Cosby et al., 2015). *Salmonella* spp. are classified as non-motile serotypes *Salmonella enterica* Pullorum and *Salmonella enterica* Gallinarum and many motile paratyphoid *Salmonella*. *Salmonella enterica* Pullorum and *Salmonella enterica* Gallinarum are highly host-adapted to chicken. Paratyphoid infections can be caused by any one of the many non-host-adapted *Salmonella*. *S. enterica* Typhimurium, *S. enterica* Enteritidis, *S. enterica* Kentucky, and *S. enterica* Heidelberg are among the most common *Salmonella* infections in poultry across the world (Shah et al., 2017). *S. enterica* Typhimurium, *S. enterica* Enteritidis are more pathogenic than others. The motile *Salmonella*, Paratyphoid group, is of public health significance and disseminate via contamination and mishandling of poultry products (Dar et al., 2017).

According to World Health Organization, non-typhoidal salmonellosis due to (*S. enterica* Typhimurium, *S. enterica* Enteritidis, *S. enterica* serovar Newport, *S. enterica* serovar Heidelberg) causes 1.3 billion cases of acute gastroenteritis or diarrhea and 3 million deaths annually (WHO, 2018; Wibisono et al., 2020).

The mode of *Salmonella* transmission can be both vertical and horizontal (Foley et al., 2008; Hedican et al., 2010; Wensley and Coole, 2013; Gieraltowski et al., 2016). Vertical transmission can occur from parent to the infants. *Salmonella* infection caused by *Salmonella enterica* Enteritidis has a particular predilection for poultry reproductive system. Transovarian infection can occur if the mother has systemic infection that results in ovary infection. *Salmonella* migrates from cloaca into reproductive organs. They get lodged in the ovary and passed in to the eggs. *Salmonella* can also spread to the hatcher and brooder horizontally (Dos Santos et al., 2019; Wibisono et al., 2020).

Horizontal transmission can occur direct or indirect contact with infected birds, contaminated food vendors or environment and infected rodents (Hedican et al., 2010; Loharikar et al., 2013). *Salmonella* may transmit among the birds in a flock through fecal shedding (Agyare et al., 2018). Transmission may take place by aerogens/fomites, polluted drinking water, polluted feeds, dirty cages and faeces of infected birds (Zamora-Sanabria and Alvarado, 2017).

Some serotypes of *Salmonella* are transmitted from infected breeder to young birds through contamination of outer shell surface (Gantois et al., 2009). The farms are the primary source of *Salmonella* but downstream processing steps may also amplify *Salmonella* contamination. Sanitary management in slaughterhouses and poultry carcasses may become contaminated during processing steps (Lee et al., 2019). Transmission between farms may occur due to poor biosecurity (Koutsoumanis et al., 2019).

2.2.2. *Salmonella* prevalence

The variable individual level prevalence of Salmonellosis was reported in poultry across the world: 2.3% to 8.4% in India (Mir et al., 2010; Kumar et al., 2014), 7.2% to 34% in Pakistan (Asif et al., 2017; Khan et al., 2019), 12.9% to 92.6% in Thailand (Lampang et al., 2014; Mangmee et al., 2020), 2.5% to 14.9% in Malaysia (Ong et al., 2014; Jajere et al., 2019), 11.2% to 30.3% in China (Zhao et al., 2020; Yu et al., 2021), 5.2% to 14% in Japan (Shahada et al., 2008; Lapuz et al., 2012) and 3.7% to 31.1% in Nigeria (Agada et al., 2014a; Akeem et al., 2017). The reported farm level prevalence of salmonellosis across the world is as follows: 3.8% in India (Singh et al., 2010), 55% to 67% in Nepal (Nelson et al., 2020; Sharma et al., 2021), 3.2% in Thailand (Utrarachkij et al., 2012), 20.7% to 96% in Japan (Sasaki et al., 2012; Yamazaki et al., 2016), 43.6% to 47.9% in Nigeria (Fagbamila et al., 2017; Jibril et al., 2020) and 50% in Canada (Arsenault et al., 2007).

Investigation of *Salmonella* prevalence in poultry in Bangladesh is limited. However, some published *Salmonella* prevalence in poultry in this country are 21.1% in layer chicken in Savar, Dhaka (Mahmud et al., 2011), 71.1% in broiler chicken and 38.9% in layer chicken in Mymensingh (Naurin et al., 2012) and 23.3% non-motile *Salmonella* in broiler or layer

chicken in Dhaka metropolitan city (Akond et al., 2012) at individual level. The reported farm level motile *Salmonella* prevalence are 11% in broiler farm and 18% in layer farm in Chattogram (Barua et al., 2012 and 2013). The cited Bangladesh studies were either small-scale in nature or not-properly followed epidemiological study designs.

2.2.3. Factors in association with *Salmonella*

The following reported factors increase the occurrence of *Salmonella* in poultry farms in Bangladesh (based on a few studies) and other parts of the world: Poultry reared in caged flocks (Huneau-Salaün et al., 2009), deep liter system (Mollenhorst et al., 2005; Jibril et al., 2020), closed-house rearing (Sasaki et al., 2012), house with multiple age groups (Mollenhorst et al., 2005; Huneau-Salaün et al., 2009), farm with previous history of *Salmonella* (Cardinale et al., 2004; Huneau-Salaün et al., 2009; Agada et al., 2014b), feed sourced from local market (Andino et al., 2014) and careless processing, transportation and distribution of feed (Fagbamila et al., 2017), delivery trucks (feed, eggs and other) enter to the farm premises or parking nearby (Huneau-Salaün et al., 2009; Agada et al., 2014b), keeping sick birds in the same house (Cardinale et al., 2004), supplying untreated drinking water (Agada et al., 2014b; Djeffal et al., 2018), allowing unnecessary visitors (Cardinale et al., 2004), presence of rodents (Agada et al., 2014b) and presence of other livestock in farm premises (Jibril et al., 2020).

Documented individual level factors in association with *Salmonella* are as follows: layer chickens are commonly affected than broiler chickens (Mouttotou et al., 2017). *Salmonella* infection in day-old chick increase the risk of *Salmonella* infection in later stage (Cardinale et al., 2004).

Factors, as follow, have previously been identified as protective factors for *Salmonella* in poultry and poultry farms: vaccination flock against *Salmonella* (Davies and Breslin, 2003; Agada et al., 2014b), cleaning and disinfection of fixed equipment and decontamination of surface by using detergents (Cardinale et al., 2004; Donado-Godoy et al., 2012; Ferdous et al., 2019; Jibril et al., 2020), boot disinfection, hand washing practice before entering the farm and using foot bath (Agada et al., 2014b), disposal of dead birds and poultry waste by

composting on the farm in a container (Huneau-Salaün et al., 2009; Donado-Godoy et al., 2012; Jibril et al., 2020) and presence of fence around the farm (Jibril et al., 2020).

2.3. Antibiotic use and abuse

Antibiotics are widely used in commercial poultry for different purposes in countries like Bangladesh: i) therapeutic, ii) prophylactic and iii) growth promotion. In developing countries farmers can easily use antibiotics without the prescription of registered veterinarians and can purchase antibiotics over counter (Mutua et al., 2020; Phares et al., 2020). Besides registered veterinarian, non-veterinary staff and farmers themselves apply antibiotics based on tentative diagnosis (Boamah et al., 2016; Ferdous et al., 2019; Sarker et al., 2020). Commonly used antibiotics in poultry in Bangladesh and neighboring countries are **access group**: amoxicillin, ampicillin, cephalexin, doxycycline, gentamicin, sulfamethoxazole-trimethoprim and tetracycline; **watch group**: azithromycin, ciprofloxacin, erythromycin, levofloxacin, lincomycin, neomycin, norfloxacin, oxytetracycline and pefloxacin and **reserve group**: colistin, fosfomycin and polymyxin B (McGettigan et al., 2017; WHO, 2017; Ferdous et al., 2019).

Reported prevalence of antibiotics usage in poultry in different countries are as follows: amoxicillin 33%, doxycycline 51%, ciprofloxacin 37.0%, neomycin 39%, oxytetracycline 11%, enrofloxacin 20%, sulfamethoxazole-trimethoprim 30% and colistin 57% in Bangladesh (Islam et al., 2016; Ferdous et al., 2019; Imam et al., 2020); oxytetracycline 13%, doxycycline 100%, neomycin 63%, enrofloxacin 100%, colistin 100% and tylosin 100% in Pakistan (Mohsin et al., 2019); amoxicillin 76%, erythromycin 25%, norfloxacin 48%, oxytetracycline 39%, tetracycline 11% and sulfamethoxazole-trimethoprim 11% in China (Xu et al., 2020); amoxicillin 15%, doxycycline 28%, azithromycin 33%, erythromycin 28%, neomycin 67% and oxytetracycline 48% in Nigeria (Awogbemi et al., 2018); doxycycline 2%, ciprofloxacin 5%, enrofloxacin 9%, sulfadimidine 18%, sulfamethoxypyridazine 3%, tylosin 5% and oxytetracycline 49% in Tanzania (Azabo et al., 2020), doxycycline 47%, ciprofloxacin 57%, norfloxacin 57%, enrofloxacin 57%, sulfamethoxazole 54%, tetracycline 47%, oxytetracycline 47% and colistin 3% in

Cameroon (Kamini et al., 2016). These results are also given a tabular form below for better understanding.

Table 2.1: Reported prevalence of antibiotic usage in poultry in different countries

Antibiotics	Bangladesh	Pakistan	China	Tanzania	Cameroon	Nigeria
Amoxicillin	33% (Imam et al., 2020)	---	76% (Xu et al., 2020)	---	---	15% (Awogbemi et al., 2018)
Doxycycline	51% (Imam et al., 2020)	100% (Mohsin et al., 2019)	---	2% (Azabo et al., 2020)	47% (Kamini et al., 2016)	28% (Awogbemi et al., 2018)
Oxytetracycline	11% (Ferdous et al., 2019)	13% (Mohsin et al., 2019)	39% (Xu et al., 2020)	49% (Azabo et al., 2020)	47% (Kamini et al., 2016)	48% (Awogbemi et al., 2018)
Ciprofloxacin	37% (Imam et al., 2020)	---	---	5% (Azabo et al., 2020)	57% (Kamini et al., 2016)	---
Azithromycin	---	---	---	---	---	33% (Awogbemi et al., 2018)
Erythromycin	---	---	25% (Xu et al., 2020)	---	---	28% (Awogbemi et al., 2018)
Enrofloxacin	20% (Islam et al., 2016)	100% (Mohsin et al., 2019)	---	9% (Azabo et al., 2020)	57% (Kamini et al., 2016)	---
Norfloxacin	---	---	48% (Xu et al., 2020)	---	57% (Kamini et al., 2016)	---
Sulfamethoxazole-trimethoprim	30% (Imam et al., 2020)	---	11% (Xu et al., 2020)	---	---	---
Gentamicin	---	---	---	---	---	5% (Awogbemi et al., 2018)
Neomycin	39% (Imam et al., 2020)	63% (Mohsin et al., 2019)	---	---	---	67% (Awogbemi et al., 2018)
Colistin	57% (Imam et al., 2020)	100% (Mohsin et al., 2019)	---	---	3% (Kamini et al., 2016)	---
Tylosin	---	100% (Mohsin et al., 2019)	---	5% (Azabo et al., 2020)	---	---

So, the indiscriminate use of antibiotics may lead to develop resistance against pathogens as well as commensal organisms. Also, overuse of antibiotics attributes to develop antibiotic resistant genes in bacteria is occurred. In broiler production system, farmers use antibiotics heavily in the farm as prophylactics to keep the birds safe and with dealer recommendation to administer antibiotics from Day 1 until selling of mature birds (Masud et al., 2020). Antibiotic usage data in poultry farms are deficient due to lack of surveillance system and negligence of poultry producers, feed producers and pharmaceutical companies not keeping data of antimicrobial consumption or sales (Van Boeckel et al., 2015).

2.4. Antibiotic resistance

Several Bangladesh and international studies on AMR against *Salmonella* in poultry have been reviewed and found the following findings: In Bangladesh 42.7% to 100% resistance develop against amoxicillin (Hassan et al., 2014; Mridha et al., 2020). 71.4% to 99% ampicillin (Mahmud et al., 2011; Hossain et al., 2019), 31% to 65% cephalexin (Akond et al., 2012; Sultana et al., 2014), 50% to 52% doxycycline (Hassan et al., 2014; Sultana et al., 2014), 80% to 100% tetracycline (Akond et al., 2012; Mridha et al., 2020), 25% to 47.3% azithromycin (Sultana et al., 2014; Mridha et al., 2020), 87.5% enrofloxacin (Hassan et al., 2014), 7.1% to 40% ciprofloxacin (Mahmud et al., 2011; Hossain et al., 2019), 82% erythromycin (Akond et al., 2012; Mridha et al., 2020) and 9% to 46% gentamicin (Mahmud et al., 2011; Mridha et al., 2020) develop resistance against *Salmonella*.

In India 50% amoxicillin (Singh et al., 2010), 12.1% to 95.7% ampicillin (Harsha et al., 2011; Sharma et al., 2019), 70% to 100% doxycycline (Singh et al., 2010; Waghamare et al., 2018), 23.1% to 100% tetracycline (Singh et al., 2013; Sharma et al., 2019), 21.4% azithromycin (Waghamare et al., 2018), 6.1% to 82.9% ciprofloxacin (Harsha et al., 2011; Sharma et al., 2019), 83.3% to 100% erythromycin (Waghamare et al., 2018; Sharma et al., 2019), 14.3% enrofloxacin (Waghamare et al., 2018), 26.2% norfloxacin (Waghamare et al., 2018), 50% levofloxacin (Sharma et al., 2019), 88% neomycin (Waghamare et al., 2018) and 4.8% colistin (Waghamare et al., 2018) become resistant to *Salmonella*.

In Pakistan found 80% amoxicillin (Khan et al., 2019), 66.6% to 88.4% ampicillin (Shah and Korejo, 2012; Wajid et al., 2019), 64.5% to 89.7% tetracycline (Shah and Korejo, 2012; Khan et al., 2019), 28.6% azithromycin (Asif et al., 2017), 42.9% to 50% ciprofloxacin (Asif et al., 2017; Wajid et al., 2019), 40.6% erythromycin (Shah and Korejo, 2012), 58.7% neomycin (Shah and Korejo, 2012), 31.4% gentamicin (Wajid et al., 2019) and 94.4% pefloxacin (Wajid et al., 2019) resistance of *Salmonella* in poultry. The above cited results on AMR are also given a tabular form below for better understanding.

Table 2.2: Reports on AMR pattern of *Salmonella* in different countries

Antibiotics	Bangladesh	India	Pakistan
Amoxicillin	**42.7- 100%** (Hassan et al., 2014; Mridha et al., 2020)	**50%** (Singh et al., 2010)	**80%** (Khan et al., 2019)
Ampicillin	**71.4-99%** (Mahmud et al., 2011; Hossain et al., 2019)	**12.1-95.7%** (Harsha et al., 2011; Sharma et al., 2019)	**66.6-88.4%** (Shah and Korejo, 2012; Wajid et al., 2019)
Cephalexin	**31-65%** (Akond et al., 2012; Sultana et al., 2014)	---	---
Doxycycline	**50-52%** (Hassan et al., 2014; Sultana et al., 2014)	**70-100%** (Singh et al., 2010; Waghamare et al., 2018)	---
Tetracycline	**80-100%** (Akond et al., 2012; Mridha et al., 2020)	**23.1-100%** (Singh et al., 2013; Sharma et al., 2019)	**64.5-89.7%** (Shah and Korejo, 2012; Khan et al., 2019)
Azithromycin	**25-47.3%** (Sultana et al., 2014; Mridha et al., 2020)	**21.4%** (Waghamare et al., 2018)	**28.6%** (Asif et al., 2017)
Enrofloxacin	**87.5%** (Hassan et al., 2014)	**14.3%** (Waghamare et al., 2018)	---
Ciprofloxacin	**7.1-40%** (Mahmud et al., 2011; Hossain et al., 2019)	**6.1-82.9%** (Harsha et al., 2011; Sharma et al., 2019)	42.9-50% (Asif et al., 2017; Wajid et al., 2019)
Pefloxacin	---	---	**94.4%** (Wajid et al., 2019)
Norfloxacin	---	**26.2%** (Waghamare et al., 2018)	---
Levofloxacin	---	**50%** (Sharma et al., 2019)	---
Erythromycin	**82%** (Akond et al., 2012; Mridha et al., 2020)	**83.3-100%** (Waghamare et al., 2018; Sharma et al., 2019)	**40.6%** (Shah and Korejo, 2012)
Gentamicin	**9-46%** (Mahmud et al., 2011; Mridha et al., 2020)	---	**31.4%** (Wajid et al., 2019)
Neomycin	---	**88%** (Waghamare et al., 2018)	**58.7%** (Shah and Korejo, 2012)
Colistin	---	**4.8%** (Waghamare et al., 2018)	---

These above mentioned data on antimicrobial resistance reflect the overall situation in poultry and poultry products (egg and meat) of Bangladesh and neighboring countries. But the farm level AMR prevalence study from cloacal and environmental sample was not commonly reported. In the current study, we therefore tried to focus on farm level antibiogram pattern for human important antibiotics.

2.5. Public health significance of antibiotic resistance

Antibiotic resistant bacteria from food animals may become pathogenic to human (Hinton, 1988). Every new antibiotic become resistant as well as other classes to patients vulnerable to infections and are not possible to treat with available antibiotics (Kouyos et al., 2011). It may cause complication to human health with untreatable and prolonged infection. As a consequence healthcare cost becomes higher (Manyi-Loh et al., 2018).

2.6. Summary of the review

The review indicates information gaps about assessing farm *Salmonella* prevalence in commercial chicken in Bangladesh and associated factors. The review points to inconsistent AMR prevalence study against *Salmonella* spp. for human important antibiotics. Moreover, the aforementioned cited Bangladeshi studies were not epidemiologically well designed. Therefore, the study aimed to appraise farm *Salmonella* prevalence, associated risk factors and antibiogram pattern of *Salmonella* in Chattogram, Bangladesh.

Chapter-III: Materials and methods

3.1. Study area description

Chattogram, is an ancient district of Bangladesh, located in south-eastern part of the country (between 21°54' and 22°59' N and 91°17' and 92°13' E). It is bounded on the north by Tripura State of India, on the east by Khagrachhari, Rangamati and Bandarban districts, on the south by Cox's Bazar district and on the west by the Bay of Bengal, Feni and Noakhali districts. It has a total area of 5282.92 sq. km. with the total population of 7,616,352. The population density is 1,442 per sq km (BBS, 2013). There are great diversities in ethnic groups of Muslim, Hindu, Buddhist, Christian and many other tribes. The literacy rate of the district is 58.9% (BBS, 2013). This district consists of 15 upazilas and 3 metro thanas.

In Bangladesh, there were a total of 356.3 million poultry (including 296.6 million chickens) in the 2019-2020 production years (DLS, 2020). There are 65-70 thousand commercial chicken farms in various scales which are supported by 16 grandparent farms, 206 small and large-scale breeder farms and 198 registered feed mills producing 5.3-5.4 million metric ton industrial feeds (WPSA, 2017). Chattogram has 18 million poultry population, regardless of production types, which contribute to 5.1% (n~356 million, (OHPH, 2020) of total poultry population in Bangladesh (DLS, 2020). There are 4882 broiler farms, 559 layer farms, 295 Sonali farms and 20 breeder farms in Chattogram (Personal communication: Dr. Md. Reajul Huq, DLO, Chattogram, 2020).

3.2. Study period and design

A cross-sectional study was carried out between February and July 2019.

3.3. Population

3.3.1. Reference population

All commercial broiler and layer poultry farms under Chattogram district were considered as the reference population of the study.

3.3.2. Source population

To cover maximum geographical area of Chattogram district, Gupta et al. (2020) selected eight upazilas according to some criteria such as presence of water bodies, forests, hills and distance from Chattogram city. Poultry farms belonging to these upazilas of Chattogram district were chosen as the source population for the present study. They included Anowara, Chandanaish, Fatickchari, Lohagara, Potiya, Rangunia, Raozan and Sitakunda.

3.3.3. Epidemiology unit and sampling frame

A farm consisting of at least 500 birds was considered as the smallest epidemiological unit of the study. Accordingly there were a total 1748 commercial poultry farms (1493 broiler and 255 layer farms) and distribution of the farms in the sampling frame by upazillas (See Table 3.1). The sampling frame was developed by Gupta et al. (2020) through consultation with the relevant stakeholders or offices: Chattogram Livestock services, government and private poultry practitioners, feed and chick dealers and pharmaceuticals representatives. Then Gupta et al. (2020) selected farms by using simple random sampling.

Table 3.1: Total number of poultry farms in sampling frame in studied upazilas

Upazilla	**Broiler farm**		**Layer farm**	
	No of farms	Size: Min-Max	No of farms	Size: Min-Max
Anowara	187	500-4000	24	500-5000
Chandanaish	169	500-5500	25	1000-6500
Fatickchari	221	500-4800	33	500-5500
Lohagara	172	500-3500	36	1000-13000
Potiya	215	500-5000	28	500-5000
Rangunia	208	500-3000	52	500-7000

Raozan	156	500-3500	27	500-6000
Sitakunda	165	500-7000	30	500-8000
Total	1493	500-7000	255	500-13000

3.4. Sample size calculation

The main objective of this study was to identify prevalence of AMR of *Salmonella* spp. Sample size was calculated according to the main objective. A total of 139 farms were required for the current study assuming the expected AMR prevalence of 90% (if a farm having 50% of commonly used antibiotics being resistant against indicator organism *Salmonella* spp, then this farm was classified as an AMR farm), ±10 precision, 95% CI and a design effect of 4 (Formula: N = Design effect * $p(1-p)/E^2$) (OpenEpi, 2013).

3.5. Sampling technique

A proportionate probability of random sampling technique was applied to enroll the required number of farms (N= 83 broiler farms and N= 57 layer farms). Some farms were excluded as they were not operating or had no birds during field visit and neighboring farms were included as replacement.

If a farm had one shed, data and sample were then collected from that shed. If a farm had more than 1 shed and same kind of antimicrobials used in all sheds, data and sample were taken from the shed with oldest chickens. If a farm had more than 1 shed and multiple antimicrobials used in different sheds, data and sample were taken from the shed with highest number of antimicrobials used.

Table 3.2: Farm distribution according to production type in studied upazilas

Upazila	**Broiler farm**		**Layer farm**	
	No of farms	Size: Min-Max	No of farms	Size: Min-Max
Anowara	13	700-2500	2	1150-4000
Chandanaish	11	700-5000	4	1000-4400
Fatickchari	10	1000-3175	8	1200-4945
Lohagara	10	650-2000	9	1400-11044
Potiya	11	850-4100	9	500-4500
Rangunia	13	500-3000	9	500-6500
Raozan	8	850-2000	6	1000-5192
Sitakunda	7	500-5000	10	500-8000
Total	83	500-5000	57	500-11044

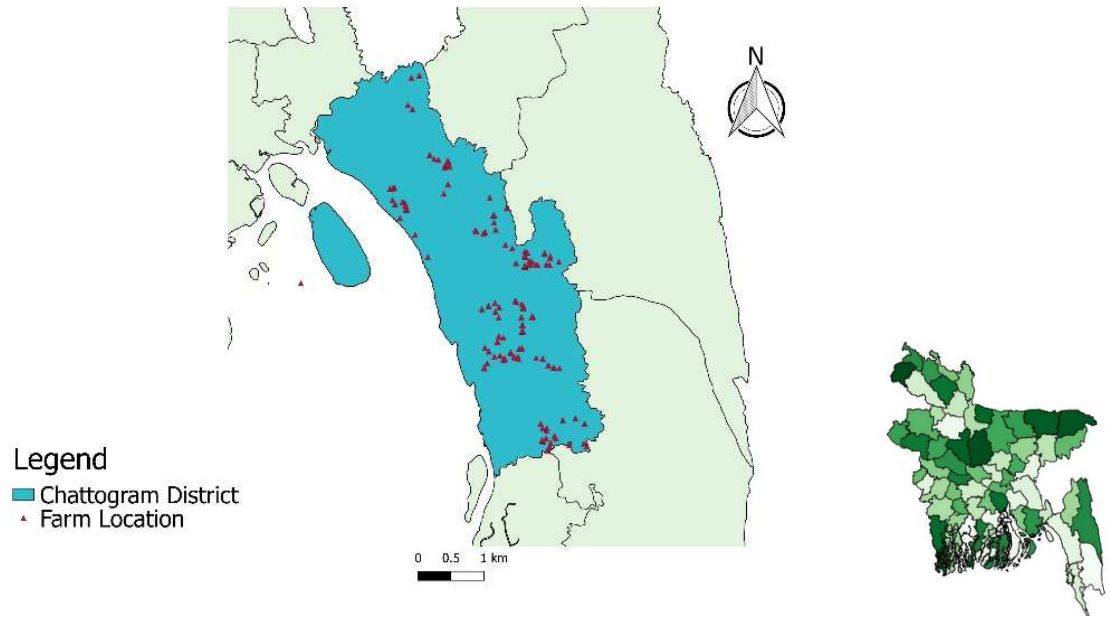

Figure 3.1. Location of selected poultry farms in Chattogram district

3.6. Data collection

3.6.1. Questionnaire development, validation and interviews

A questionnaire was drafted as per targeted objectives. Before drafting a thorough literature review and some peer-consultation was performed to identify the areas to develop questionnaire. The drafted questionnaire was thoroughly peer-reviewed to locate gaps and re-structured accordingly. Then the questionnaire was piloted on five broiler and five layer farms to check the consistency and time requirement for the questionnaire administration. Afterwards, the questionnaire was modified according to the findings of the pilot study.

The questionnaire composed of the following information: i) poultry farm related information including farm location, type of the production system, number of sheds, population of birds, ii) farmer's demography like name, address, gender, educational status and iii) husbandry practices like farm hygiene, biosecurity, water bath facility, cleaning and disinfection, isolating sick birds, cleaning egg trays, disposal of dead birds, manure

and farm wastage. Closed ended, open ended and mixed types of questions were incorporated in the questionnaire. The full questionnaire is given as **Appendix-I**.

A team of 3 members made the field trips during the study period and covered 4-5 farms each day. Among the team members, one conducted the interview, one collected the biological samples and other took the photographs of the antimicrobials used in the farms and close inspection. Before visiting the field the team communicated with the local veterinarian and then communicated with the farmers to set date of interview for data collection and biological sampling. Verbal consent was obtained from each participant farmer before administering the questionnaire and sample collection. All the farmers had incentivized with a soap and a liquid hand-wash.

3.7. Sample collection, transportation, preservation and storage

Cloacal and environmental swab samples were collected from each selected poultry farms. For single-housed farms samples were collected from a single flock. For the farms containing more than one house, samples were collected from older or oldest flock. Cloacal samples were collected from randomly selected 5 birds and pooled in a 5 ml sterile falcon tube containing Stuart transport medium (Neogen, Lansing MI). Environmental swab samples were collected from middle and 4 corners of each selected farm and then pooled in a 15 ml sterile falcon tube containing buffered peptone water (BPW) (Neogen, Lansing MI) with unique identity number. All tubes were then kept in an insulate box containing ice packs and transferred to the laboratory within 4-6 hours. The samples were kept in -20°C until further analysis.

3.8. Laboratory evaluation

3.8.1. Sample preparation

Salmonella was isolated from both sample types (cloacal and environmental samples) by the standard microbiological methods according to ISO 6579 Amendment 1: Annex D. Initial enrichment of each sample was in buffered peptone water (BPW) (Neogen, Lansing MI) in a ratio of 1:10 and incubated between 34°C and 38°C for 18 hours.

3.8.2. Bacteriological test

From pre-enrichment cultured broth, 100 µl of the overnight culture (divided into 3 separate drops) on to novobiocin (Oxoid®, Oxoid Ltd, Basingstoke, United Kingdom) supplemented Modified Semisolid Rappaport Vassiliadis (MSRV) agar (Oxoid®, Oxoid Ltd, Basingstoke, United Kingdom) and incubated for 24 hours at 41.5 °C. Production of any gray white, turbid zone from center of inoculation on the MSRV agar plates was suspected for *Salmonella* and streaked on to brilliant green (BG) agar (Oxoid®, Oxoid Ltd, Basingstoke, United Kingdom) and xylose lysine deoxycolate (XLD) agar (Neogen, Lansing MI) by using an inoculating loop which was dipped into the periphery of the opaque zone. Plates were incubated overnight at 37°C. After incubation the plates were examined for the presence of typical colonies of *Salmonella*. In case of BGA, light pink colony against a rose pink background and red colonies with black centers on XLD agar were observed. Any suspected *Salmonella* colonies on BG or XLD agar were verified and confirmed by biochemical tests including TSI agar slant reaction of typical *Salmonella* (yellow/acidic butt, pink/alkaline slant while middle of the tube appeared as black due to H_2S production), indole reaction and citrate utilization test.

Suspected colonies were transferred to 5% blood agar (BA) (Blood agar base, Oxoid®, Oxoid Ltd, Basingstoke, United Kingdom). After overnight incubation at 37°C these were grown in brain heart infusion (BHI) broth (Neogen, Lansing MI). All the positive isolates were stored at -80°C using 50% glycerol. All samples were shipped later to the Bangladesh Livestock Research Institute (BLRI) for Vitek confirmation. The detailed bacteriological test protocols are presented in **Appendix-II**.

3.8.3. Culture sensitivity test

Cultural sensitivity test of *Salmonella* through disk diffusion method was conducted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2018a) using Kirby-Bauer disc diffusion assay. *Salmonella* colonies from BA were mixed with the phosphate buffer saline (PBS) by vortexing and the turbidity was adjusted to the 0.5 MacFarland turbidity standard. Then the broth was streaked on Mueller Hinton (MH)

agar (Difco Laboratories, Sparks, MD, USA) plate. Antibiotic discs were placed aseptically on the surface of the inoculated plates with the help of a multidisc dispenser. A 12-cartridge dispenser was used to dispense antibiotic discs (Oxoid®, Oxoid Ltd, Basingstoke, United Kingdom) of amoxicillin (10 µg), ampicillin (10 µg), cephalexin (30 µg), doxycycline (30 µg), erythromycin (15 µg), enrofloxacin (5 µg), gentamicin (10 µg), neomycin (10 µg), ciprofloxacin (5 µg), azithromycin (15 µg), colistin (10 µg), pefloxacin (5 µg), sulfonamide and trimethoprim (25 µg). These antibiotics were selected based on common antibiotics used in the farms. The plates were then inverted and incubated at 37°C for 16 to 18 hours. After incubation the plates were examined and the diameters of the zones of complete inhibition were observed through automated inhibition zone reader (Scan® 4000) and interpreted. The breakpoints for the interpretation of resistance and susceptibility were those recommended by the CLSI guideline (CLSI, 2018b) and EUCAST guideline (EUCAST, 2018). All breakpoints were not available in one guideline, thereby both guidelines were followed. Zones of inhibition were classified as susceptible, intermediate and resistant categories based on the CLSI guideline. The detailed cultural sensitivity test procedure is presented in **Appendix-III**.

3.9. Case definition

A farm was considered as *Salmonella* positive if only either of the pooled sample type (cloacal or environmental swabs) were tested positive.

If equal or more than 50% of commonly used antibiotics become resistant against a *Salmonella* isolate in a farm, then this farm was categorized as an AMR farm.

3.10. Statistical evaluation

3.10.1. Data entry and cleaning

The unit of observation was the farm. Field and laboratory data were entered in Microsoft excel 2016. Data cleaning, coding and integrity were checked for validation and consistency, and then exported to STATA IC-13 (StataCrop, 4905, Lakeway Drive, College Station, Texas 77845, USA) for epidemiological analysis.

3.10.2. Descriptive analysis

At first, the farm prevalence of *Salmonella* spp was calculated by the number of *Salmonella* positive farms divided by the total number of farms tested. The prevalence of *Salmonella* was then distributed by production types and sample types.

The prevalence of AMR was calculated by the number of antibiotic resistant farms (when 50% or more antibiotics tested were resistant in a farm) divided by the total number of *Salmonella* positive farms and the results were then distributed by production type. Antimicrobial specific AMR prevalence was calculated by the total number of resistant *Salmonella* isolates to each individual antimicrobial divided by the total number of resistant and sensitive *Salmonella* isolates to each individual antimicrobial.

The results were expressed as frequency numbers, percentage and 95% confidence interval.

3.10.3. Univariate analysis

Fisher's exact test was conducted to assess the association between the occurrence of *Salmonella* at farm level (Broiler/Layer) (Yes or No) and each of different farm biosecurity indices. The following bio-security indices were tested: isolation of sick birds (Yes/No/Partial), washing facilities like hand-washing (Yes/No/Partial), changing clothes or shoes before entering to the farm for employees and visitors (Yes/No/Partial/NA), vehicles decontamination before entering and leaving farm (Yes/No/Partial/NA), using foot bath (Yes/No/Partial), source of drinking water (Deep well/Shallow well/Pond), cleaning and disinfection of the farm (Yes/No/Partial), washing egg-tray (Yes/No/Partial/NA), employee having training on farm biosecurity (Yes/No/NA), rearing other animals in farm premises and employee living in farm premises (Yes/No/NA). The cut value of $p \leq 0.05$ was used as level of significance.

Chapter-IV: Results

4.1. Description of farm and farmer demography

Demography of farms and farmers has been presented in **Table 4.1.** Of 140 surveyed farms, 59.3% were broiler and 40.7% were layer farms. Small-scale farms (500-2500 birds per farm) dominated (90% broiler farms and 49% layer farms) over other scales. Most of the farms had a single shed (96% broiler and 83% layer). Only one farmer was female. Education of most of the farmers was secondary to graduation level (76% broiler farmers and 91% layer farmers). Most of the farmers experienced in poultry rearing in the study area.

Table 4.1: Demographic characteristics of commercial chicken farms and farmers in Chattogram, Bangladesh

Characteristics	**Category**	**Broiler farmers (N=83)** **n (%)**	**Layer farmers (N=57)** **n (%)**
Flock size	500-2500	75 (90.4)	28 (49.1)
	2501-5000	8 (9.6)	20 (35.1)
	>5000	0 (0.0)	9 (15.8)
No. of sheds	1	80 (96.4)	47 (82.5)
	>1	3 (3.6)	10 (17.5)
Gender	Male	82 (98.8)	57 (100)
	Female	1 (1.2)	0 (0.0)
Educational status	No education or primary	20 (24.1)	5 (8.8)
	Secondary or graduation	63 (75.9)	52 (91.2)
Experience of poultry farming	0-5 years	29 (34.9)	9 (15.8)
	6-10 years	13 (15.7)	14 (24.6)
	>10 years	41 (49.4)	34 (59.6)

4.2. Farm level prevalence of *Salmonella* infection

The farm *Salmonella* spp. prevalence was 8.4% (95% CI: 3.5-16.6, N=83 broiler farms) and 8.8% (95% CI: 2.9-19.3, N=57 layer farms). The farm *Salmonella* spp. prevalence based on cloacal swab and environmental swab respectively was 2.4% (95% CI: 0.3-8.4, N=83 broiler farms) and 3.5% (95% CI: 0.4-12.1, N=57 layer farms) and 8.4% (95% CI: 3.5-16.6, N=83 broiler farms) and 8.8% (95% CI: 2.9-19.3, N=57 layer farms) (**Table 4.2**).

Table 4.2: Prevalence of *Salmonella* infection in commercial poultry at farm level in Chattogram, Bangladesh

	Broiler farm (N=83)		**Layer farm (N=57)**	
Type of samples	**% (n)**	**95% CI**	**% (n)**	**95% CI**
Cloacal swab	2.4 (2)	0.3-8.4	3.5 (2)	0.4-12.1
Environmental swab	8.4 (7)	3.5-16.6	7.0 (4)	1.9-17.0
Either cloacal or environmental swab	8.4 (7)	3.5-16.6	8.8 (5)	2.9-19.3

N: Number of farms; CI: Confidence Interval

4.3. Association between farm *Salmonella* prevalence and each of bio-security indices

None of the factors was significantly associated with the occurrence of *Salmonella* infection at layer farms in Chattogram (**Table 4.3**). Only "Weekly disinfecting and cleaning the farm surfaces and equipment" was in association significantly with the occurrence of *Salmonella* infection at broiler farms in Chattogram (**Table 4.3**).

Table 4.3: Association between farm *Salmonella* prevalence and each of bio-security indices

Variables		Broiler farm (N=83)			Layer farm (N=57)		
	Category	% (+)	-	*p*	% (+)	-	*P*
Isolation of sick birds in separate shed	No	7.8 (4)	47	0.77	11.1 (1)	8	1.00
	Yes	10.3 (3)	26		9.1 (4)	40	
	Partial	0	3		0	4	
Washing facility before entering to farm	No	14.3 (4)	24	0.288	13.0 (3)	20	0.446
	Yes	5.6 (3)	51		6.1 (2)	31	
	Partial	0	1		0	1	
Hand washing before entering in to farm	No	16.1 (5)	26	0.177	14.3 (4)	24	0.511
	Yes	5.4 (2)	35		3.9 (1)	25	
	Partial	0	15		0	3	
Changing clothes/shoes before entering in to farm (Employees)	No	9.6 (7)	66	1.000	7.3 (3)	38	0.637
	Yes	0	7		13.3 (2)	13	
	Partial	0	1		0	1	
	NA	0	2				
Changing clothes/shoes before entering in to farm (Visitors)	No	8.9 (7)	72	1.000	11.4 (5)	39	1.00
	Yes	0	2		0	7	
	Partial	0	1		0	1	
	NA	0	1		0	5	
Checking and decontamination of vehicles before entering in to farm	No	8.9 (4)	41	0.594	17.7 (3)	14	0.158
	Yes	20.0 (1)	4		13.3 (2)	13	
	Partial	0	2		0	3	
	NA	6.5 (2)	29		0	22	
Decontamination of vehicles before leaving farm	No	10.6 (5)	42	0.807	15.8 (3)	16	0.153
	Yes	0	4		18.2 (2)	9	

	Partial	0	1		0	5	
	NA	6.5 (2)	29		0	22	
Functioning foot bath facility	No	8.6 (7)	74	1.000	11.1 (5)	40	0.609
	Yes	0	2		0	11	
	Partial				0	1	
Source of drinking water	Deep well	10.0 (4)	36	0.734	8.8 (3)	31	1.00
	Shallow well	7.1 (3)	39		8.7 (2)	21	
	Pond	0	1				
Disinfecting and cleaning the farm surfaces and equipment weekly	No	26.7 (4)	11	0.03	0	2	1.000
	Yes	5.5 (3)	52		10.2 (5)	44	
	Partial	0	13		0	6	
Washing egg tray being brought back from market	No	---			20.0 (2)	8	0.081
	Yes	---			4.7 (2)	41	
	Partial	---			50.0 (1)	1	
	NA	---			0	2	
Employees having training on biosecurity measures (at least once)	No	8.2 (5)	56	0.212	9.4 (5)	48	1.000
	Yes	50.0 (1)	1		0	2	
	Others	5.0 (1)	19		0	2	
Employee living within farm premises	No	5.0 (1)	19	1.000	9.1 (1)	10	1.00
	Yes	9.8 (4)	37		9.1 (4)	40	
	NA	9.1 (2)	20		0	2	
Presence of other birds/animals in the farm	No	9.5 (2)	19	0.887	11.1 (4)	32	1.00
	Yes	10.0 (2)	18		0	8	
	NA	7.1 (3)	39		7.7 (1)	12	

****Fisher's exact test*

4.4. Antibiogram pattern of *Salmonella* isolates

The farm AMR prevalence for Salmonella isolates was estimated to be 85.7% (95% CI: 42.1-99.6, N=7) in broiler farms and 80% (95% CI: 28.4-99.5, N=5) in layer farms. AMR pattern to each antimicrobial in the study is given as follows.

Salmonella spp. isolates obtained from broiler farms were 100% resistant to erythromycin followed by each of amoxicillin, ampicillin, cefalexin, enrofloxacin, ciprofloxacin and pefloxacin (85.7%) and gentamicin (57.1%) (**Table 4.3**). However, resistance level of the following antimicrobials was low: azithromycin and trimethoprim-sulfonamides combination (42.9%), neomycin (28.6%), doxycycline and colistin (14.3%) (**Table 4.3**).

Salmonella spp. isolates obtained from layer farms were 100% resistant to each of amoxicillin, ampicillin, erythromycin and pefloxacin followed by cephalexin, doxycycline and enrofloxacin (60% each). Resistance rates were comparatively lower in ciprofloxacin, azithromycin, neomycin, trimethoprim-sulfonamides combination (40% each) and colistin (20%). No resistance was found to gentamicin (**Table 4.4**).

Table 4.4: Antibiogram pattern of *Salmonella* spp. isolates obtained from the broiler farms in Chattogram, Bangladesh

Antibiotic	**Farm 1 (E)**			**Farm 2 (C)**			**Farm 2 (E)**			**Farm 3 (C)**			**Farm 3 (E)**			**Farm 4 (E)**			**Farm 5 (E)**			**Farm 6 (E)**			**Farm 7 (E)**			**N (% resistance)**
	R	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	
Amoxicillin (A)	-	+	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	6 (85.7)
Ampicillin (A)	-	-	+	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	6 (85.7)
Erythromycin (W)	+	-	-	+	-	-	+	-	-	+	-	-	-	+	-	+	-	-	+	-	-	+	-	-	+	-	-	7 (100)
Enrofloxacin (A)	-	-	+	+	-	-	+	-	-	+	-	-	-	-	+	+	-	-	+	-	-	+	-	-	+	-	-	6 (85.7)
Doxycycline (A)	-	-	+	+	-	-	+	-	-	-	+	-	-	+	-	-	+	-	-	+	-	-	-	+	-	+	-	1 (14.3)
Gentamicin (A)	-	-	+	-	+	-	+	-	-	+	-	-	-	-	+	+	-	-	+	-	-	-	-	+	-	-	+	4 (57.1)
SXT (A)	-	-	+	-	-	+	-	-	+	+	-	-	-	-	+	+	-	-	+	-	-	-	-	+	-	-	+	3 (42.9)
Ciprofloxacin (W)	-	+	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	6 (85.7)
Neomycin (W)	-	-	+	-	-	+	-	-	+	-	-	+	-	+	-	+	-	-	+	-	-	-	+	-	-	+	-	2 (28.6)
Azithromycin (W)	-	-	+	-	-	+	+	-	-	-	+	-	-	-	+	-	-	+	+	-	-	-	+	-	+	-	-	3 (42.9)
Cefalexin (A)	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	-	-	+	6 (85.7)
Pefloxacin (W)	-	-	+	+	-	-	+	-	-	+	-	-	-	-	+	+	-	-	+	-	-	+	-	-	+	-	-	6 (85.7)
Colistin (R)	-	-	+	-	-	+	+	-	-	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	1 (14.3)
Types of AM usages	CT, CN, Flor			SXT, DO, AMX,Tylo, Sulfaclozine Na						CT, AMX						CT, AMX, CIP			AMX, CT, N, DO, OTC, CIP, E, SXT			EX, CT			CIP, EX, E, Tylo, CT, N,DO, SXT, OTC			

***R**=Resistant, **I**=Intermediate, **S**=Sensitive*

***AMX**: Amoxicillin; **CIP**: Ciprofloxacin; **CN**: Gentamicin; **DO**: Doxycycline; **EX**: Enrofloxacin; **E**: Erythromycin; **N**: Neomycin; **CT**: Colistin; **OTC**: Oxytetracycline; **SXT**: Sulfamethoxazole and trimethoprim; **Flor**: Florfenicol;**Tylo**: Tylosin*

***A**: Access; **W**: Watch; **R**: Reserve; **E**: Environmental swab sample; **C**: Cloacal swab sample*

Table 4.5: Antibiogram pattern of *Salmonella* spp. isolates obtained from the layer farms in Chattogram, Bangladesh

Antibiotic	**Farm 1 (E)**			**Farm 2 (E)**			**Farm 3 (E)**			**Farm 4 (C)**			**Farm 4 (E)**			**Farm 5 (C)**			**N (% of resistance)**
	R	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	
Amoxicillin (A)	+	-	-	+	-	-	+	-	-	+	-	-	-	+	-	+	-	-	5 (100)
Ampicillin (A)	+	-	-	+	-	-	+	-	-	+	-	-	-	-	+	+	-	-	5 (100)
Erythromycin (W)	+	-	-	+	-	-	+	-	-	-	+	-	+	-	-	+	-	-	5 (100)
Enrofloxacin (W)	+	-	-	-	-	+	+	-	-	-	-	+	-	-	+	+	-	-	3 (60.0)
Doxycycline (A)	+	-	-	+	-	-	+	-	-	-	+	-	-	+	-	-	+	-	3 (60.0)
Gentamicin (A)	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	0 (0.0)
SXT (A)	-	-	+	-	-	+	+	-	-	-	-	+	-	-	+	+	-	-	2 (40.0)
Ciprofloxacin (W)	-	+	-	-	+	-	+	-	-	+	-	-	-	-	+	-	+	-	2 (40.0)
Neomycin (W)	-	-	+	-	+	-	+	-	-	+	-	-	-	-	+	-	+	-	2 (40.0)
Azithromycin (W)	+	-	-	+	-	-	+	-	-	-	-	+	-	-	+	-	+	-	2 (40.0)
Cephalexin (A)	+	-	-	-	-	+	-	-	+	+	-	-	+	-	-	+	-	-	3 (60.0)
Pefloxacin (W)	+	-	-	+	-	-	+	-	-	-	+	-	+	-	-	+	-	-	5 (100.0)
Colistin (R)	-	-	+	-	-	+	+	-	-	-	-	+	-	-	+	-	-	+	1 (20.0)
Types of AM usages	CIP, SXT, CTC, Tylo, AMX, CT, OTC, Tiamulin			Levofloxacin, AMX, Til, EX			AMX, E, SXT, EX									AMX, CT Tiamulin, CTC, DO			

***R**=Resistant, **I**=Intermediate, **S**=Sensitive;*

***AMX**: Amoxicillin; **CIP**: Ciprofloxacin; **CN**: Gentamicin; **DO**: Doxycycline; **EX**: Enrofloxacin; **E**: Erythromycin; **N**: Neomycin; **CT**: Colistin; **OTC**: Oxytetracycline; **SXT**: Sulfamethoxazole and trimethoprim; **Flor**: Florfenicol; **Tylo**: Tylosin; **Til**: Tilmicosin*

***A**: Access; **W**: Watch; **R**: Reserve; **E**: Environmental swab sample; **C**: Cloacal swab sample*

Chapter-V: Discussion

The prevalence study of *Salmonella* in commercial poultry farms and associated farm bio-security indices with antibiogram pattern have rarely been performed in Bangladesh. To fill these scientific gaps the present study attempted to estimate farm level *Salmonella* prevalence and associated farm bio-security indices along with antibiotic sensitivity pattern. In this chapter, significant findings of the study, their implications, limitations, conclusions, recommendations and future directions have thoroughly been discussed under various headings as follows.

5.1. Prevalence of *Salmonella* in commercial poultry farms

The overall farm *Salmonella* spp. prevalence was low in the current study. Variable farm *Salmonella* prevalence was reported by many earlier national and international studies: 11% (broiler) and 18% (layer) in Chattogram, Bangladesh (Barua et al., 2012 and 2013),1.0% to 71.1% (broiler) and 5% to 46.2% (layer) in Dhaka, Gazipur, Mymensingh, Dinajpur and Naogaon districts of Bangladesh (Ahmed et al., 2009; Rahman et al., 2011; Naurin et al., 2012; Hossain et al., 2019), 11% in Malaysia (Ong et al., 2014), 15.3% in Korea (Ha et al., 2018), 20.7% in Japan (Sasaki et al., 2012), 11% in Nigeria (Jibril et al., 2020), 8.6% in France (Le Bouquin et al., 2010), 25.6% in USA (Dailey et al., 2017) and 5% in Brazil (Giombelli and Gloria, 2014). This variability of farm *Salmonella* prevalence might have occurred due to different factors such as sample size, sample types, culture and culture media, isolation methods, seasonal influence and local environmental conditions (Arkali and Çetinkaya, 2020) and biosecurity, hygiene, and sanitation of the farms (Alam et al., 2020) which have been discussed below in details.

In the present study *Salmonella* isolation rate was significantly higher from environmental (8.4% in broiler and 7% in layer) than cloacal swabs (2.4% in broiler and 3.5% in layer) which are very much consistent with numerous earlier studies (García et al., 2011; Adesiyun et al., 2014; Abdi et al., 2017; Djeffal et al., 2018). Reported reasons for environmental samples to be better samples in isolating *Salmonella* were unhygienic

farming condition, improperly cleaned and disinfected poultry house, overcrowding and absence of biosecurity measures (Frederick and Huda, 2011; Ong et al., 2014) and direct and indirect fecal contamination poultry environment (Carrique-Mas and Davies,2008).

5.2. Association between the presence of *Salmonella* in poultry farm and bio-security indices

Only weekly practice of disinfecting and cleaning the farm surfaces and equipment was significantly associated with lower *Salmonella* prevalence on broiler farms in Chattogram. This finding is in close agreement with multiple earlier studies (Namata et al., 2009; Donado-Godoy et al., 2012). The organic and inanimate objects present on the surface of poultry farms may favour the growth of micro-organisms. Cleaning helps remove such debris from the surface and accelerate disinfecting procedures (Cardinale et al., 2004). Other bio-security indices tested here were not found as significant factors for the presence of *Salmonella* in poultry farms in this study which may be due to small sample size with low *Salmonella* prevalence. However, many global studies identified the following bio-security indices that were strongly associated with the prevalence *Salmonella* in commercial poultry (broiler/layer): keeping sick and healthy birds' together without using any dedicated isolation space (Cardinale et al., 2004), no clothes or shoes changing facilities for employees or visitors before entering into farms (Sasaki et al., 2012), no functional footbath at entrance of poultry farm (Agada et al., 2014b), disinfection of vehicles before entering and leaving farms occurred infrequently (Sasaki et al., 2012) and presence of other birds/animals in the farm (Jibril et al., 2020) and supplying untreated drinking water (Sasaki et al., 2012; Agada et al., 2014b; Djeffal et al., 2018).

Use of hand sanitizer before entering into farms (Namata et al., 2009), washing egg trays immediately after being brought back from markets significantly reduced the farm *Salmonella* prevalence. However, comprehensive farm bio-security measures only can significantly reduce the level of common infectious diseases in farms in the study areas (Trampel et al., 2014).

5.3. Antibiogram pattern of *Salmonella*

Regardless of the production type the farm AMR prevalence (Definition of AMR positive farm: 50% or more antimicrobials used in a farm were resistant to *Salmonella* isolate) are so high (~85%) which indicate indiscriminate use of antimicrobials in the studied farms. This farm level AMR prevalence is novel finding. This could also be due to long term use of antimicrobials in poultry farms (Eguale, 2018). It admits the colonization of resistant *Salmonella* in poultry considerably and contamination of poultry products and finally get access to human through food chain (Lu et al., 2011).

In broiler farms, the current study determined resistance to multiple antibiotics against *Salmonella* isolates (100% resistant to erythromycin, 86% to amoxicillin, ampicillin, cefalexin, enrofloxacin, ciprofloxacin and pefloxacin each and 57% to gentamicin). Similar multidrug resistant pattern was observed in *Salmonella* isolates obtained from layer farms (100% resistant to each of amoxicillin, ampicillin, erythromycin and pefloxacin and 60% resistant to each of cephalexin, doxycycline and enrofloxacin). These findings were supported by multiple studies in Bangladesh (Mahmud et al., 2011; Jahan et al., 2013; Hassan et al., 2014; Parvej et al., 2016; Hossain et al., 2019; Alam et al., 2020) and neighboring countries where poultry production systems are similar (Yildirim et al., 2011; Agada et al., 2014a; Thung et al., 2016). The high rates of resistance found in the current study can be described by the spread of antimicrobial agent given to the poultry as prophylaxis, growth promoter or treatment purposes and practice of using multiple drugs with broad-spectrum antibiotics (Rahman et al., 2018; Ferdous et al., 2019). Our findings therefore suggest serious poultry health and public health threat.

AMR in poultry pathogens results in treatment failure, leading to economic losses as well as burden of untreated poultry diseases but importantly act as a source of resistant bacteria to human (Nhung et al., 2017).

Common infections caused by resistant bacteria become unfortunate with limited treatment options and higher mortality rate also occurs in such infections (Paphitou, 2013). In human, significant economic losses occur as a consequence of AMR by increasing medication costs due to treatment failure with prolong hospitalization (Friedman et al., 2016; Jajere, 2019). Alarming issue is that if effective measures are not taken immediately, by 2050

death of 10 million people and economic losses equivalent to 100 trillion USD will occur and 11% fall in animal production (O'Neill, 2016; World Bank, 2017).

Different types of antimicrobials are used in poultry production around the world but a large number of them are considered as critically important for human medicine (Landoni and Albarellos, 2015; WHO, 2019). Worldwide estimation shows that more than 60% of all antibiotics produced are used in animal production as therapeutic or non-therapeutic purposes (Van Boeckel et al., 2015; Agyare et al., 2019). The classes that are closely related with human medicine are β-lactams (penicillins and cephalosporins); sulphonamides with or without trimethoprim; tetracyclines; macrolides, lincosamides and streptogramins; and quinolones (including fluoroquinolones) (Phillips et al., 2004; Nhung et al., 2017; Jajere et al., 2019).

Colistin is a last resort antibiotic for the treatment of multidrug-resistant Gram-negative bacterial infection. Extensive use of colistin in poultry for therapeutic purposes and hence human consumption through the food chain has been documented in low and middle-income countries (Kumar et al., 2020). Several countries have banned colistin in animal use (Maron et al., 2013; Walsh and Wu, 2016).

Resistance can be declined when antibiotic use is decreased and discontinued. Resistant strains are replaced by susceptible strains when the selection pressure is removed (Schrag and Perrot, 1996; Phillips et al., 2004). Therefore, the antibiotics that become already resistant should stop applying in the field for a certain time, nationally or globally.

Our study also identified some sensitive antimicrobials (In broiler: azithromycin and trimethoprim-sulfonamides combination, neomycin, doxycycline and colistin; in layer: ciprofloxacin, azithromycin, neomycin, trimethoprim-sulfonamides combination colistin and gentamicin). Many earlier studies are aligned with our findings (Mahmud et al., 2011; Agada et al., 2014a; Im et al., 2015; Cui et al., 2016; Mridha et al., 2020). These results are promising but these sensitive drugs should be used judiciously in poultry farms to keep them effective for long time. Antibiotic should be used when necessary and then, appropriately. Some effective measures like avoidance of unnecessary use of antibiotics, avoid using Critically Important Antimicrobials (CIAs) for humans, give emphasize on relevant vaccinations, effective biosecurity measures and isolation of sick birds as well as

keeping record should be implemented (Read and Woods, 2014; Magnusson et al., 2019). AMR surveillance are necessary to monitor antimicrobial uses in poultry farms (Hedman et al., 2020). World Organization for Animal Health (OIE) claimed that significant number of national veterinary services do not meet the optimal requirements (Forman et al., 2012).Quality veterinary services are essential for mitigating misunderstanding about antimicrobial use in animal and bacterial resistance. One Health Approach is necessary to decrease the burden of AMR (Yang et al., 2019).

5.4. Limitation of the study

As the main objective of the present study was to estimate AMR prevalence, sample size was therefore calculated based on statistical assumptions centering farm AMR prevalence against indicator organism. Hence estimation of farm *Salmonella* prevalence by using the current sample size was biased. Therefore, the farm *Salmonella* prevalence status and the associated factors in the present study should be interpreted cautiously.

Some level of information bias (recall bias in particular) might have occurred because of interviewees' responses mostly based on their memories. There were a few farms that had registered books to maintain farm database. However, before starting the main survey the questionnaire was properly piloted and field investigators were properly trained to prevent from recording incorrect information.

Although we isolated *Salmonella* with ISO 6579 Amendment 1 bacterial cultural protocol Eriksson and Aspan (2007) estimated an excellent sensitivity (98%) and specificity (100%) and performed cultural sensitivity test by using the protocol described by Mensah et al. (2019) having excellent sensitivity (94%) and specificity (93%), there could be lab technician error. However, our lab technician was experience in *Salmonella* isolation.

As farm *Salmonella* prevalence was poor with statistically biased and small sample size we were not able to conduct multivariate logistic regression analysis to determine significant risk factors associated with the occurrence of *Salmonella* at farm level.

Chapter-VI: Conclusion, Recommendations and Future direction

6.1. Conclusion

In this study, the overall farm *Salmonella* prevalence was low. *Salmonella* isolation rate was significantly higher from environmental than cloacal swabs. Weekly practice of cleaning and disinfecting farm surface and equipment significantly reduce the *Salmonella* prevalence in broiler farms.

Salmonella was confirmed resistant against erythromycin amoxicillin, ampicillin, cephalexin, enrofloxacin, ciprofloxacin, pefloxacin and gentamicin in broiler and layer farms. However, azithromycin, trimethoprim-sulfonamides combination, neomycin, doxycycline and colistin remain sensitive in both types of farms.

6.2. Recommendations

Resistant antimicrobials identified in the study should be stopped immediately and identified sensitive antimicrobials should be used judiciously. It is recommended to introduce antimicrobial use protocol for poultry farms in the study areas. Selection of antibiotics for treatment should be justified based on antimicrobial susceptibility testing results of disc diffusion. Although colistin remained sensitive, this reserve group of antibiotic for human must be stopped using in poultry. These findings should be discussed with the farmers participated in the study along with local veterinarians and feed and drug dealers to make aware about risk of indiscriminant use of antimicrobials and AMR.

Farmers must seek veterinary advice from registered veterinarian before applying antibiotics. The respective authorities should take necessary steps for proper monitoring of withdrawal periods and improvement of antimicrobial stewardship.

Several simple implementations like weekly practice of disinfecting and cleaning farm surfaces and equipment, isolating sick birds from others, using foot-bath, changing clothes and shoes before entering to the farm, proper disposal of dead birds and farm wastage can be approached to increase biosecurity at commercial chicken farms. Proper management of vehicles, air, feed and water supply should be well monitored.

6.3. Future direction

- Study of antibiogram pattern as well as determination of minimum inhibitory concentration (MIC) and AMR genes should be carried out in future.
- An AMR epidemiological study should also be conducted to determine the association between farm level factors and the occurrence of AMR at farm level.
- A farm *Salmonella* prevalence study and factors in association with a proper sample size calculated based on statistical assumptions should be conducted in future.
- Further study should be conducted to identify *Salmonella* isolates with PCR confirmation, serotyping and whole genome sequencing (WGS).

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Appendix-I

Assessment of antimicrobial usage on commercial poultry farms and, attitudes and behaviours of antimicrobial usage by commercial poultry farmers and attitudes and behaviours of antimicrobial sales and distribution by traders of antimicrobials in Bangladesh

<u>Demographic/Socioeconomic characteristics of the interviewee</u>

(Tick the boxes and fill in the blanks)

Date of interview:	_____ (day)	_____(month)	_____(Year)
Farm ID			
Name of the interviewee:			
What is your farm type?	0= Meat type (Broiler)		1= Egg type (Layer)
Status of the interviewee on farm:	0=Owner 1=Manager 2=Worker 3=Owner's spouse 4=Owner's son 5=Owner's daughter 6=Other _____		

Poultry Information

How many chickens do you have in the farm today?			
What is your current production system?	0=All-in-All out	1=Continuous	2=Both
How many sheds you have in your farm?			
Do you use antimicrobial/antibiotics/medicine/ vitamins/minerals in your farm?	0=No	1=Yes	
If yes, do you use different amount of antimicrobial/medicine/vitamins/antibiotics in different sheds?	0=No	1=Yes	
1. If yes, in which shed is the highest amount of antimicrobial/medicine/vitamins or antibiotics used? THIS IS THE SHED TO BE SAMPLED (if we get ans here then ques 21 will not appear)	0=Shed 1 3= Shed 4	1= Shed 2 4= Shed 5	2=Shed 3 5=Shed 6 6=Other shed (specify)_____
If the same amount of antimicrobial/medicine/vitamins are used accross, do you have birds of different age on your farm?	0=No	1=Yes	
2. If yes, in which shed are the oldest birds? THIS IS THE SHED TO BE SAMPLED	0=Shed 1 3= Shed 4	1= Shed 2 4= Shed 5	2=Shed 3 5=Shed 6 6=Other shed

If no (all birds are of the same age) then THE SHED TO BE SAMPLED will be selected randomly.			(specify)___ _
How many chickens you have in the shed today from which faecal sample is taken?			
What is the age of the poultry in the shed from which faecal sample is collected?	_____ (day)	_____ (month)	_____ (Year)
What are the ages of the poultry from other sheds?			
If, all in all out, then collect the age for one batch (as all the chickens are of same age, so all sheds will be of same ages)			
If, continuous then collect age for different batches			
1st Shed of same age	_____ __ (day)	_____ (month)	_____ (Year)
2nd Shed of same age	_____ __ (day)	_____ (month)	_____ (Year)
3rd Shed of same age	_____ __ (day)	_____ (month)	_____ (Year)
4th Shed of same age	_____ __ (day)	_____ (month)	_____ (Year)
5th Shed of same age	_____ __ (day)	_____ (month)	_____ (Year)
6th Shed of same age	_____ __ (day)	_____ (month)	_____ (Year)
Others_____			

Farm bio-security and hygiene related information

(Answers will be observed/asked by the interviewer)

Is the farm surrounded by a protective fence?	0=No	1=Yes	
3. In addition to the people involved in rearing poultry (listed in ques 23),who has access to your farm?	0=Feed suppliers	1=Other farm owners	2=Other farm workers
	3=Relatives	4=Egg traders	5=Poultry traders
	6=Poultry vaccinator	7=Government Veterinarians	8=Private Veterinarians
	9=Feed delivery person	10=Owner/worker from another farm	11=Others - _____
Does anyone who are involved in poultry keeping go to other commercial poultry farms?	0=No	1=Yes	
	0=daily	1=consequtive days	2=once in a week

If yes in question 63, then how frequently does he/they visit in the last month?	3=once in a fortnight	4=once in a month	5=others _____

(Answers will be observed/asked by the interviewer)

(Tick appropriate answers)	**Yes**	**Partial**	**No**
1. Do you isolate the sick birds in a separate shed?			
2. What do you do with dead birds?			
3. What do you do with your manure?			
4. Does washing facility exist for the visitors/employees before entering farm/shed/premises?			
5. Do the visitors/employees use washing facility before entering farm/shed?			
6. Do the employees change clothes and shoes before entering the farm/shed?			
7. Do the visitors change clothes and shoes before entering the farm/shed?			
8. Are the vehicles checked and decontaminated before entering farm?			
9. Are the vehicles decontaminated when leaving the farm?			

10. Do you have footbaths available & used, and disinfectant water changed within 6 hours?			
11. What types of water you allow for drinking or cooling at the farm?			
12. Do you weekly disinfect and clean the farm surfaces and equipments?			
13. Are egg trays washed when bringing back from market?			
14. Are farm employees given training on biosecurity measures?			
15. How long do you keep the shed empty between two consecutive batches?			
16. Are farm workers live within the farm premises?			
16.1. If yes, do they rear their own poultry birds within the farm premises?			

Other demographic and Farm information

Mobile number of the interviewee:	
Address of the farm:	
Name of the poultry farm:	
Village:	
Ward:	
Union	
Upazilla/Thana:	
Latitude:	
Longitude:	

Experience of the interviewee in poutry farming:	0=< 6 months	1= (6-12) months 2= (1-5) years	3= (6-10) years 4=>10 years
Age (in years)			
Gender:	0=Male	1=Female	
Education:	0=No education	1=Up to Primary	2=Up to Secondary
	3=Up to higher secondary	4=Graduate	5=Post graduate
	6=Dakhil	7=Fazil	
Marital status:	0=Single	1=Married	2=Divorced
	3=Widow	4=Others _____	
Religion:	0=Muslim	1=Hindu	2=Christian
	3=Buddhist		
Which is the source provides the largest income to your household?	0= Poultry rearing	1=Livestock rearing	2=Fishing
	3= Daily worker	4= Grocery	5= Non-Government Organization
	6= Family business	7= Agriculture	8= Government organization
			9=Others_____
Monthly Net Income (in BDT)			
What type of breed/strain you have in the farm currently? (THIS QUES will come if	0=Novogen Brown 1= White Hyline Brown 2= White Shaver 579 3= ISA Brown	4= Hi-Sex Brown 5=White Bovine White 6= Others_____	

interviewer tick egg type)			
What type of breed/strain you have in the farm currently? (THIS QUES will come if interviewer tick meat type)	1=Cobb 500 2=Ross 308 3= Indian River Meat 4= Tiger Sasso, 5=Habbard and Arber acre		

Appendix-II

Pre-enrichment in Buffered peptone water

The swab sample stored at -20 °C was thawed at room temperature and inoculated into Buffered peptone water (Neogen, Lansing MI) at a ratio of 1:10 and then incubated between 34°C and 38°C for 18 h. After incubation, they were then separately incubate in Modified Semi-solid Rappaport-Vassiliadis (MSRV) media.

Modified Semi-solid Rappaport Vassiliadis Agar inoculation

Sample properly grown in buffered peptone water were further inoculated into MSRV (Oxoid®, Oxoid Ltd, Basingstoke, United Kingdom) and incubated at 41.5 °C for 24 h. Grey-white, turbid opaque growth in MSRV was suspected as *Salmonella* spp. (Mir et al., 2015). Positive samples in MSRV were then inoculated in Xylose lysine deoxycholate (XLD) agar for differentiating the organisms.

Xylose Lysine Deoxycholate (XLD) inoculation

Sample properly grown in MSRV were inoculated into xylose lysine deoxycholate (Neogen, Lansing MI) agar and incubated at 37 °C for 24 h. The positive growth in XLD indicates the presence of *Salmonella* spp.

Biochemical tests

Further confirmation of *Salmonella* spp. is supported by some specific biochemical tests

Citrate utilization test

Suspected samples were inoculated in Simmons citrate (Neogen, Lansing MI) agar that ferment citrate and change of color from greenish to royal blue. No color change indicates negative result.

Triple sugar iron test

Same samples were also inoculated in triple sugar iron (Neogen, Lansing MI) agar. Development of red color in slant, yellow in butt, gas production or not and H_2S production indicates positive for *Salmonella* spp.

Maintenance of pure culture and stock

For isolation of pure culture the bacteria were grown in selective media: XLD agar were again sub-cultured in blood agar (Oxoid®, Oxoid Ltd, Basingstoke, United Kingdom). After confirmation of pure culture by observation of colonies in BA, colonies were reinoculated in brain heart infusion (Neogen, Lansing MI) broth and incubated at 37 °C for 24 h for bacterial multiplication as per manufacture instruction. 50% glycerol solution was prepared by diluting 100% glycerol with phosphate buffered saline. Then 700 µl overnight cultures were transferred in sterilized cryovial with 300 µl of 50% glycerol and stored at -80°C as stock for longer time preservation. Entire procedure of bacteriological culture has been attached as sketch below.

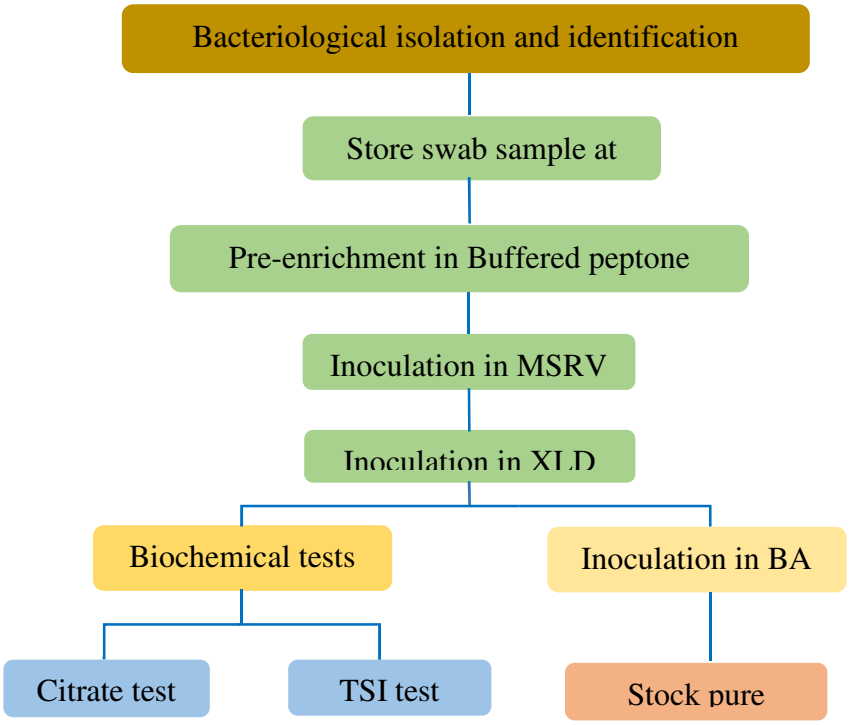

Figure 1. Flow chart of bacteriological isolation and identification

Appendix-III

Mueller-Hinton agar plate preparation

Mueller-Hinton (Oxoid®, Oxoid Ltd, Basingstoke, United Kingdom) agar plates were prepared according to manufacture instruction. MHA plates were stored at 4 °C in sealed packages. These plates were removed from refrigerator at least 15 minutes before use. If excess moisture on agar surface, the plates were then placed in a laminar flow hood at room temperature to remove access liquid till dry

Preparation of inoculum

Subculture of *Salmonella* spp. was prepared the previous day. Using a sterile inoculating loop, four or five isolated colonies from subculture were touched and suspended in 2 ml sterile saline. After vortexing the saline tube, turbidity of the suspension was adjusted with 0.5 McFarland standard to achieve an equivalent turbidity.

Mueller-Hinton agar plate inoculation

A sterile cotton swab was dipped into the 0.5 McFarland adjusted suspension and rotated against the side of the tube with firm pressure to remove excess fluid. MHA plate was inoculated by streaking the swab three times over the entire plate for an even distribution of inoculum and the rim of the agar. Leaving the lid ajar, allowed the plate to sit at room temperature at least 3 to 5 minutes.

Placement of discs to inoculated agar plates

Antimicrobial-impregnated disks were placed on the agar surface by using a multidisc dispenser. Each disk was pressed with sterilized forceps to ensure complete contact with agar surface. Then the plates were inverted and placed in an incubator to set 35°C ± 2°C.

Measuring zones and interpreting results

All plates were examined after 16 to 18 hours incubation. Plates were placed in automated inhibition zone reader (Scan® 4000) and recorded the zone diameter.

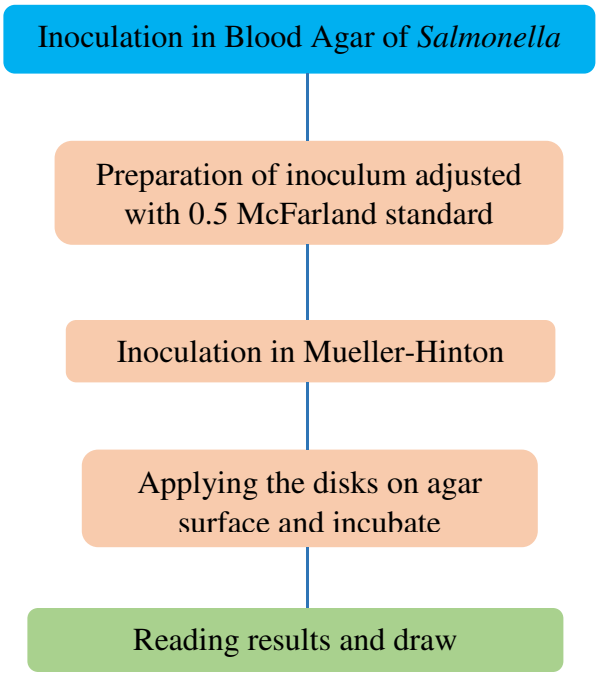

Figure 1. Disk diffusion AST process

Biography

Mohammad Foysal passed the Secondary School Certificate Examination, SSC, in 2008 obtaining GPA 5.00 (A+) and then Higher Secondary Certificate Examination, HSC, in 2010 obtaining GPA 5.00 (A+). Mr. Foysal obtained his Doctor of Veterinary Medicine Degree in 2016 from Chattogram Veterinary and Animal Sciences University, CVASU, Bangladesh. Now, he is a candidate for the degree of MS in Epidemiology under the Department of Medicine and Surgery, Faculty of Veterinary Medicine, CVASU. He has immense interest working in epidemiology and anthropology of AMR.