MICRO-DILUTION ASSAY BASED ANTIBIOGRAM OF *ESCHERICHIA COLI* IN COMMERCIAL CHICKENS IN CHATTOGRAM, BANGLADESH

Nasreen Sultana

Roll No: 0119/05

Registration No.:647

Session: 2019-2020

A thesis submitted in the partial fulfillment of the requirements for the degree of Master of Science in Epidemiology

Department of Medicine and Surgery

Faculty of Veterinary Medicine

Chattogram Veterinary and Animal Sciences University (CVASU)

Chattogram -4225, Bangladesh

JUNE 2022

Authorization

It is my immense pleasure to affirm that I am the sole author of the thesis. I authorize the Chattogram Veterinary and Animal Sciences University (CVASU) to lend this thesis to other institutions or individuals for scholarly research purpose. I further authorize the CVASU to reproduce the thesis by photocopying or by any other means, in total or in part, at the request of other institutions or individuals for scholarly research.

I, the undersigned and author of this work, proclaiming that the electronic copy of this thesis provided to the Central Library, CVASU is an accurate copy of this print thesis submitted within the limits of the technology available.

DR. Nasreen Sultana, DVM

June 2022

MICRO-DILUTION ASSAY BASED ANTIBIOGRAM OF *ESCHERICHIA COLI* IN COMMERCIAL CHICKENS IN CHATTOGRAM, BANGLADESH

Nasreen Sultana
Roll No: 0119/05
Registration No.:647
Session: 2019-2020

This Master's thesis is reviewed thoroughly and found to be satisfactory in all aspects for evaluation

.....
Supervisor
Prof Md. Ahasanul Hoque, PhD
Department of Medicine and Surgery
Faculty of Veterinary Medicine
Chattogram Veterinary and Animal Sciences University, Bangladesh

.....
Chairman of the Examination Committee
Prof Azizunnesa, PhD
Head of the department
Department of Medicine and Surgery
Chattogram Veterinary and Animal Sciences University

Department of Medicine and Surgery

Faculty of Veterinary Medicine

Chattogram Veterinary and Animal Sciences University

Chattogram -4225, Bangladesh

JUNE 2022

Acknowledgements

All gratitude is attributed to Almighty Allah, who has granted me with the ability to successfully complete my thesis for the degree of **Masters of Science** in Epidemiology under **Department of Medicine and Surgery** at Chattogram Veterinary and Animal Science University, Bangladesh.

I would like to acknowledge and give my warmest thanks to my respected supervisor, **Prof Dr Md Ahasanul Hoque**, Department of Medicine and Surgery, CVASU, for his valuable supervision and guidance, which carried me through all stages of writing my thesis.

I'd like to thank the authority and all of the technicians at Poultry Research and Development **(PRTC)** for their initial laboratory support of my research work. I express my deep sense of gratitude to **Roderick Card**, Senior Scientist, Department of Bacteriology; and **Thomas Chisnall**, AMR scientist, Animal and Plant Health Agency (APHA), UK, for their valuable laboratory support in my laboratory work.

It is also my great pleasure to thank **Prof Dr Dirk Pfeiffer** of the City University of Hong Kong and **Guillaume Fournié** of the Royal Veterinary College for supporting me to work on this thesis as part of the **BALZAC** (Behavioral Adaptations in Live Bird Trading and Farming Systems and Zoonosis Control in Bangladesh) spin off project on antimicrobial usage and antimicrobial resistance.

I want to express my thanks and debt to **DR. Nurun Nahar Chisty,** who supported me with data analysis and providing important recommendations, kind suggestions, and very kind instruction for writing my thesis properly. A special thanks to **DR. Mohammed Foysal and DR. Shetu Bhusan Das** for allowing me to use their field and laboratory data in my research.

I gratefully acknowledge **DR. Abdullah Al Sattar** and **DR. Abu Shoeib Muhammad Mohsin** for their suggestions, encouragement, and assistance with my thesis.

I would like to express my heartfelt gratitude **Assoc Prof Tasneem Imam** for her cordial support in my thesis work.

I would like to give thanks to **Prof Dr Azizunnesa**, Head of the Department of Medicine and Surgery and **Prof Dr AMAM Zonaed Siddiki**, Coordinator of Advance Study and Research, CVASU, for their admistrative support in relation to the thesis approval.

Finally, I also express my gratitude to my supportive family, seniors, juniors, and well-wishers.

Table of Contents

List of Tables

List of Abbreviations

Abbreviations	Elaborations
AMR	Antimicrobials Resistance
AMU	Antimicrobials Usage
APEC	Avian Pathogenic *E. coli*
APHA	Animal and Plant Health Agency
AST	Antimicrobial Susceptibility Testing
ATCC	American Type Culture Collection
BAB	Breeders Association of Bangladesh
BA	Blood Agar
BALZAC	Behavioral Adaptations in Live Bird Trading and Farming Systems and Zoonosis Control in Bangladesh
BBI	Bangladesh Business Inspection
BPICC	Bangladesh Poultry Industry Central Council
CDC	Central of Disease Control and Prevention
CFU	Colony Forming Unit
CIAs	Critically Important Antimicrobials
CLSI	Clinical and Laboratory Standard Institute
DLS	District Livestock Services
DVM	Doctor of Veterinary Medicine
E. coli	*Escherichia coli*
EFSA	European Food Safety Authority
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Food and Drug Administrations
GP	Grand Parent
MDR	Multidrug Resistant
MHB	Muller-Hinton Broth
MIC	Minimum Inhibitory Concentration
OHPH	One Health Poultry Hub
P	Probability Value

Abbreviations	Elaborations
PBS	Phosphate Buffer Saline
PS	Parent Stock
ROC	Receiver Operating Characteristics
WHO	World Health Organizations
VIF	Variance Inflation Factor
UK	United Kingdom
US	United States
%	Percentages
≥	Greater than or equal to
≤	Less than or equal to
$	United States Dollar
β	Coefficient
95% CI	95% Confidence Interval

Abstract

Antimicrobial resistance (AMR) is a major health problem, particularly in developing countries like Bangladesh, where there is a paucity of information on the status of AMR at different levels and the associated potential factors. The emergence and spread of multi-resistance to several first-line antimicrobial drugs has made the health management more difficult. The main threat to the poultry industry is AMR and the growth of multidrug resistant bacteria, which endangers food safety. Risk factors associated with the occurrence of AMR in *E. coli* in poultry included the excessive use of antimicrobials, insufficient farm density, poor hygiene standards, and contamination of feeds and eggs, which favors the selection of antimicrobial resistant *E. coli*. There were available studies on the prevalence of AMR in *E. coli* at an individual level (i.e., isolate level) isolated from commercial chicken farms in Bangladesh. But, farm level AMR prevalence and associated risk factors were not identified in any previous study in commercial chicken farms in Bangladesh. Hence, the present study attempted to fill these scientific gaps.

A total of 152 *E. coli* isolates obtained from swab pool samples (cloacal and environmental swabs) of the studied farms (83 broiler and 57 layer farms) through a cross-sectional study conducted in Chattogram between February and July 2019 were evaluated at the UK AMR reference Lab. Broth micro-dilution assay was used to determine the susceptibility of the isolates to a panel of 14 antimicrobials. Farm and farmers' demography and farm bio-security data obtained were used to assess the AMR status with their association at farm level.

Descriptive analysis was performed to calculate the prevalence of AMR in *E. coli* in chickens at farm and individual isolate level. Logistic regression was conducted to determine potential risk factors associated with the occurrence AMR in *E. coli* in chickens at farm level (min/6 antimicrobials per farm vs.7/max antimicrobials per farm) in Chattogram, Bangladesh. Overall farm AMR prevalence was respectively 75% (42.1% in broiler and 32.9% at layer farms. Overall isolate (individual) AMR prevalence was 100% (51.3% from broiler and 48.7% from layer farms. Regardless of production type, multidrug resistant in *E. coli* (3-11 antimicrobials per farm) were identified, as well as farm size was found to have a significant relationship with the

increase level of resistance occurrence. In this study, *E. coli* isolates were more frequently resistant to ciprofloxacin and tetracycline (98.7% in broiler and 97.3% and 100% in layer, respectively), trimethoprim (92.3% and 81.1%), ampicillin (91% and 79.7%) and nalidixic acid (83.3% and 66.2%), whereas colistin (25.6% and 13.5%), cefotaxime (3.9% and 8.1%) and ceftazidime (3.9% and 8.1%) were less frequently resistant antimicrobials.

The findings of high level of AMR prevalence emphasized the development of guidelines for curbing AMR challenge through prudent use of critically important antimicrobials (CIAs) to human, which were revealed as sensitive and stopped the use of highly resistant antimicrobials in commercial chicken. Enhanced veterinarian supervision, strong monitoring systems, farm bio-security management, and increased diagnostic facilities could mitigate the burden of AMR in farms while also reducing public health risk.

Keywords: Chicken farm, Antimicrobial resistance, *E. coli*, Broth micro-dilution method, Risk factors.

Chapter-I: Introduction

Antimicrobial resistance (AMR) is the ability of bacteria to resist exposure to antimicrobials designed to kill them or inhibit their growth (Reygaer et al., 2018; Christaki et al., 2020; Alghoribi et al., 2021).

Although poultry offer many opportunities and benefits to peoples (such as rich and nutritious proteins, employment etc.) (Hassan et al., 2014; Saleque and Ansarey, 2020), there are many challenges in poultry rearing in developing countries including Bangladesh. Controlling infectious diseases such as collibacillosis, salmonellosis, fowl cholera (Rashid et al., 2013; Rahman et al., 2017) and AMR are the main challenges in poultry rearing (Al Amin et al., 2020; Kowalska-Krochmal et al., 2021).

Common bacterial pathogens such as *Salmonella Pullorum/Gallinarum, Pasteurella multocida, Avibacterium paragallinarum, Gallibacterium anatis, Ornitobacterium rhinotracheale, Bordetella avium, Clostridium perfringens, Mycoplasma* in poultry including *Escherichia (E.) coli* become resistance against a range of antimicrobials (Hasan et al., 2011; Nhuang et al., 2017). These pathogens resistance to antimicrobials are acquired in a variety of ways, including indiscriminate use of antimicrobials as a prophylactic (Page et al., 2012), growth promoter (Upadhayay et al., 2014), feed additives (Apata, 2009; Diarra et al., 2014) and therapeutic purposes (Nguyen et al., 2016); violation of antimicrobial withdrawal period (Bushan et al., 2017; Ibrahim et al., 2019). Poor hygiene and a lack of commitment to disease control and prevention have contributed to the spread of AMR strains (Fletcher et al., 2015). Additionally, farm density, hatchery (vertical transmission), fecal contaminated feeds and eggs, contaminated poultry litter, and waste water are risk factors that also contribute to the emergence of resistance pathogens in the poultry sector (Furtula et al., 2013; Bista et al., 2020).

AMR leads to increase morbidity, mortality, disease burden, healthcare expenditure, and reduces livelihoods (Al Amin et al., 2020; WHO, 2021). The direct negative impact of AMR in the animal sector, as well as the poultry industry, is production losses, which ultimately result in reduced food security (Al Amin et al., 2020). Treatment failure induced by AMR pathogens can result in economic losses owing to

high treatment costs, death due to therapeutic failure, and affected birds can also serve as a source of resistant bacteria/genes in poultry sector (Rushton et al., 2014; Nhung et al., 2017). Because of the careless use of antimicrobials for a variety of purposes (David et al., 2002), the frequency of multiple drug resistance in *E. coli* has grown in poultry farming, posing a global public health risk (Osman et al., 2018). Food is also an important factor for the transfer of AMR organisms (Rahman et al., 2017). Diseases caused by AMR pathogens are becoming more difficult to treat. According to the 2019 Centers for Disease Control and Prevention (CDC) AMR Threats Report, AMR bacteria cause more than 2.8 million illnesses and more than 35,000 deaths in the United States (Kadri, 2020). In 2019, due to its impact on human health, the World Health Organization (WHO) included AMR as one of the top ten threats to global health (WHO, 2019).

Many Bangladeshi studies reported a high level of AMR prevalence for a wide range of antimicrobials in *E. coli* in commercial chickens at individual isolate level: ampicillin resistance to 70-100% *E. coli* isolates (Al Azad et al., 2019; Sarker et al., 2019; Ievy et al., 2020), trimethoprim resistance to 84% isolates (Al Azad et al., 2019; Sarker et al., 2019;Rahman et al., 2020), tetracycline resistance to 60-90% isolates (Jakaria et al., 2012;Rahman et al., 2017), nalidixic acid resistance to 70% isolates (Bashar et al., 2011; Jakaria et al., 2012) and ciprofloxacin resistance to 82-100% (Akond., 2009; Al Azad et al., 2019; Ievy et al., 2020). However, antibiogram study of *E.coli* by using micro-dilution assay in chicken at both individual isolate and farm levels have rarely been performed in this country.

Multiple international studies documented the following farm factors associated with the occurrence of AMR in *E. coli* in chicken: farm size, farm environments, density of farms, housing conditions, farm hygiene, health status of chickens, commercial feed and biosecurity indices (Nguyen et al., 2015; Mo et al., 2016; Elmi et al., 2021; Mandal et al., 2022). But there are none or limited published works on exploring farm level factors in association with the occurrence of AMR in *E. coli* in broiler and layer farms in Bangladesh.

Although there is an established AMR surveillance program in Bangladesh, funded by Fleming Fund to assess the status of AMR prevalence in *E. coli* in poultry and its dispersion, no complete data is available to assure the long-term implementation of an

AMR management programme (Orubu et al., 2020). Using antimicrobial susceptibility testing (disk diffusion method, minimum inhibitory concentrations: MIC) to treat infectious diseases and select particular treatments against specific pathogens may help decrease antimicrobial usage, ultimately reducing AMR in commercial poultry farms.

Commensal *E. coli* represents a major reservoir for the transmission of AMR to other pathogenic bacteria. *E. coli* has the ability to provide important suggestions on the propagation of AMR (EFSA, 2016). Therefore, this study chose *E. coli* for the present antibiogram study.

With the aforementioned background the present study was therefore conducted with the following specific objectives.

1.1. Objectives

i. To estimate the prevalence of AMR in *E. coli* in commercial chickens at farm and individual isolate level in Chattogram, Bangladesh.
ii. To determine potential risk factors associated with the occurrence AMR in *E. coli* in commercial chickens at farm level in Chattogram, Bangladesh.

1.2. Outcomes

1. Determine the status of AMR in *E. coli* in chickens at farm and individual isolate level which will help take policy decision and intervention.
2. Control of AMR in commercial chicken farms by taking proper interventions against the identified risk factors.
3. The research findings will help revise the existing AMR surveillance in poultry in Bangladesh.

Chapter-II: Review of Literature

The goal of this chapter was to review the previous research findings associated with the Master's thesis "**Micro-dilution assay based antibiogram of *Escherichia Coli (E. coli)* in Commercial Chickens in Chattogram, Bangladesh**" to pin down the scientific gaps and accordingly justify the current investigation. Various published literatures were obtained by searching online sources like PubMed, Hinari, Google Scholar. This chapter is arranged in a series of sections including a review of literatures on i) poultry population, opportunities, benefits and challenges, ii) overview of *E. coli* and its selection for antibiogram investigation, iii) antimicrobial resistance, iv) causes of poultry pathogens becoming resistant to antimicrobials, v) consequence of AMR on poultry and public health and economic consequences, iv) *E. coli* resistant to antimicrobials, vi) prevalence of AMR in *E. coli* in poultry, vi) factors associated with AMR of *E. coli* in poultry, vii) control AMR, viii) comparative epidemiological characteristics of cultural sensitivity test and micro-dilution assay and ix) summary.

2.1. Poultry population, opportunities, benefits and challenges

In Bangladesh, since the beginning of 21st century, the poultry industry has become a significant platform for a quick profit, employment generation, and the production of cheaper animal proteins (Saleque and Ansarey, 2020). Bangladesh's main poultry species are chicken, duck, quail, pigeons, and turkey. Besides, there are four more diverse types of chickens- Broiler, Layer, Sonali, and Local indigenous. In the 2020-2021 production years, Bangladesh had 365.8 million poultry (including 304.1 million chickens) (DLS, 2021). There are currently over 53,000 broiler farms and 18,000 layer farms in different scales in Bangladesh. Sonali chickens account for 28% of the country's chickens. In addition, the facility maintains about 6% of domestic chickens often bred in home gardens in rural areas. Because it grows entirely naturally, the price is also high and demanding compared to other varieties (BBI, 2022).

In Bangladesh, the Department of Livestock Services (DLS) has registered 16 Grand Parent (GP) farms (BPICC, 2020) and 206 parent stock (PS) farms/hatcheries (DLS,

2021). There are 113 poultry companies (BAB, 2020), 96 feed mills (BPICC, 2020) and 30 veterinary pharmaceutical companies (BBI, 2022).

Around 6.0-8.0 million people have been given opportunities to work in the poultry sector in Bangladesh (Ahmed 2019; OHPH, 2020; Saleque and Ansarey, 2020). In 2020-2021, 20574.6 million eggs and 8.44 million metric tons meat were produced against a demand of 17659.2 million and 7.437 million metric tons, respectively. As a result, per capita egg and meat consumption has grown (DLS, 2021).

Despite the fact that poultry farming has increased significantly in Bangladesh in recent years, there are several challenges to progress and economic losses in this promising sector (Rahman et al., 2021). Most poultry farmers are lack of experience in poultry rearing, as well as biosecurity and management system training (Rahman et al., 2020). Lately, poultry farmers have suffered greatly from a lack of security for their farms and investments, since thousands of farms fail each year owing to various disease outbreaks and many due to their inabilities to purchase high-priced chicken components and endure losses from market price drops (Islam et al., 2014).

The advent of several illnesses or diseases, along with an increase in feed and medicine costs, looks to be the major obstacles for this important business in Bangladesh (Mandal and Khan, 2017). To combat the emerging worldwide problem of AMR, which is creating a significant challenge to Bangladeshi poultry producers, increasing production costs along with diagnostic and treatment expenses, as well as economic losses with treatment failure (Hassan et al., 2014).

2.2. Brief overview of *Escherichia coli* and its selection for antibiogram investigation

E. coli, a gram negative, rod shape and facultative anaerobic Coliform bacterium, is commonly found in the intestine of warm blooded animals. Sometimes, they are non-motile or motile by peritrichous flagella (Somaratne et al., 2015).

Commensal *E. coli* represents a major reservoir for the transmission of AMR to other pathogenic bacteria. Besides, AMR *E. coli* has recently arisen as a global concern, with very high levels of resistance to several classes of antimicrobials, and it is considered to be a strong predictor of the selection pressure caused by antimicrobial

use in animals (van den Bogaard et al., 2000 ;Caruso, 2018). *E. coli* has the ability to provide important suggestions on the propagation of AMR (EFSA, 2016). Therefore, this study chose *E. coli* for antibiogram investigation with the specific objectives of estimating the prevalence of AMR in *E. coli* isolated from samples of farm environment and chicken cloacal swabs (at farm and individual isolate level) and associated risk factors (at farm level).

2.3. What is antimicrobial resistance?

There are many ways to define or explain "AMR: A) AMR arises when bacteria acquire the capacity to resist the mechanisms that drugs utilize against them (Reygaer et al., 2018; Christaki et al., 2020); B) AMR has been reported to occur when a drug loses its ability to effectively inhibit bacterial growth; C) AMR is typically caused by antimicrobial destruction or alteration, target alterations (target replacement, target site mutations, target site enzymatic alterations, target site protection, target overproduction, or target bypass and decreased antimicrobials accumulation due to either decreased permeability or increased efflux (Blair et al., 2015; Munita and Arias, 2016).

AMR often develops gradually, making it critical to identify organisms with low levels of resistance that may otherwise serve as the genetic foundation for the development of increasing levels of resistance.

2.4. Causes of poultry pathogens becoming resistant to antimicrobials

Nhuang et al. (2017) published data on AMR in 12 poultry pathogens such as avian pathogenic *E. coli* (APEC), *Salmonella Pullorum / Gallinarum*, *Pasteurella multocida*, Common bacterial pathogens in poultry become resistance against a wide range of antimicrobials by different means, for example indiscriminate usage of antimicrobials as prophylactic (Park et al., 2016), growth promoter (Upadhayay et al., 2014), feed additives at low concentrations for long periods (Apata, 2009; Diarra et al., 2014) and therapeutic purposes (Nguyen et al., 2016; Hassan et al., 2021); violation of antimicrobial withdrawal period (Khatun et al., 2018), lack of an established surveillance and monitoring system of AMR (WHO, 2021); other factors:

farm capacity, and animal husbandry practices (Ibrahim et al., 2019). Poor hygiene and a lack of commitment to disease control and prevention have also contributed to the spread of AMR strains (Fletcher et al., 2015). Furthermore, fecal contaminated feeds and eggs, polluted chicken litter, and waste water are risk factors that also contribute to the emergence of resistant pathogens in the poultry sector (Bista et al., 2020). Resistant bacteria may be transmitted by vertical transmission from parental flocks or contamination in the hatchery environment (Nguyen et al., 2016).

2.5. Consequence of antimicrobial resistance on poultry and public health and economic consequences

The emergence and spread of AMR infections throughout the world poses severe public health and animal health concerns (Roth et al., 2019). AMR against endemic bacterial infections in poultry has been recorded all over the world, including Bangladesh (Marshall et al., 2011). As a result of AMR infections, treatment failure has become increasingly common in both human and animal infectious diseases (Kowalska-Krochmal et al., 2021). Antimicrobial-resistant poultry diseases can result in treatment failure and financial losses, but they can also be a source of resistant bacteria/genes (including zoonotic pathogens) that affect human health (Nhung et al., 2017). Morbidity, mortality, illness burden, and healthcare expense are all increasing as a result of AMR, as are livelihoods (Al Amin et al., 2020). In the United States, AMR bacteria cause over 2.8 million illnesses and over 35,000 deaths (CDC, 2019; Kadri, 2020). It is also predicted that AMR-related diseases death will result in a 2% to 3.5 % drop in global GDP in 2050, totaling between $60 and $100 trillion USD globally (Taylor et al., 2014; Allcock et al., 2017). AMR has a detrimental impact on food security by creating production and economic losses in the livestock and poultry production industries (Mandal and Khan, 2017).

2.6. *E. coli* resistant to antimicrobials

Multidrug resistant *E. coli* in poultry is common in many countries (Hanon et al., 2015; Shecho et al., 2017; Al Azad et al., 2019; Rahman et al., 2020). *E. coli* resistant to the following antimicrobials in poultry reported by many national and international publications: tetracycline, ciprofloxacin, trimethoprim, ampicillin, gentamycin,

nalidixic acid and fluoroquinolones (Elmi et al., 2012; Brower et al., 2017; Al Azad et al., 2019; Sarker et al., 2019; Saha et al., 2020; Dawadi et al., 2021; Mandal et al., 2021; Zou et al., 2021).

2.7. Prevalence of antimicrobial resistant in *E. coli* in poultry

The reported prevalence of AMR in *E. coli* in poultry (Both at individual isolate and farm level) based on broth micro-dilution diagnostic assay or cultural sensitivity test are presented in **Tables (2.1 and 2.2).**

Table.2. 1: Prevalence of antimicrobial resistance in *E. coli* in poultry (according to micro-dilution assay)

Country	**Antimicrobial agent:**	**Prevalence: Broiler (at individual level)**	**Prevalence: Layer (at individual isolate level)**	**Reference:**
India	Ampicillin	83.6%		Saharan et al.(2020)
	Azithromycin	91.8%		
	Trimethoprim	100%		
	Tetracycline	58.9%		
	Ciprofloxacin	91.8%		
	Colistin	52.9 to		
Vietnam	Ampicillin	97.8%		Nguyen et al. (2016)
	Ciprofloxacin	73.3%		
	Gentamycin	42.2%		
	Colistin	22.2%		
Nigeria	Ampicillin		80%	Mamza et al.(2010)
	Ciprofloxacin		90%	
	Tetracycline		80%	
	Gentamycin		55%	
China	Ampicillin	100%	83%	Zou et al. (2021),
	Ciprofloxacin	84%	46%	
	Tetracycline	97%	87%	
	Gentamycin	73%	26%	

Country	Antimicrobial agent:	Prevalence: Broiler (at individual level)	Prevalence: Layer (at individual isolate level)	Reference:
	Colistin	73%	4.9%	
Korea	Ampicillin	90.6%		Seo and Lee. (2021)
	Tetracycline	90.6%		
	Ciprofloxacin	100%		
	Gentamycin	13.2%		
Jordan	Ciprofloxacin	66%		Ibrahim et al.(2019)
	Gentamycin	59.4%		
Sudan	Azithromycin	85%		Elomofti et al.(2019)
	Tetracycline	80%		
	Ciprofloxacin	50%		
Germany	Ampicillin	50.1%		Chuppava et al.(2019)
	Tetracycline	8.8%		
Austria	Ampicillin		17.7%	Hess et al.(2022)
	Tetracycline		53.6%	
	Gentamycin		6.2%	
	Nalidixic acid		91.9%	
	Trimethoprim		37.8%	
	Colistin		73.6%	

Table.2. 2: Prevalence of antimicrobial resistant in *E. coli* in poultry mostly at individual level (according to disk diffusion method)

Country	**Antimicrobial agent:**	**Prevalence: Broiler (at individual level)**	**Prevalence: Layer (at individual isolate level)**	**Reference:**
Bangladesh	Ampicillin	70 -100%	25.7%	Hasan et al. (2011), Al Azad et al. (2019) and Sarker et al. (2019)
	Tetracycline	83.7 -92%	90 -100%	Jakaria et al. (2012), Hassan et al. (2014) and Rahman et al. (2017)
	Ciprofloxacin	64 -100%	100%	Kmetova.(2009), Hasan et al. (2011), Hassan et al. (2014) and Al Azad et al. (2019)
	Azithromycin	31.6%		Saha et al. (2020)
	Gentamicin	8.3 -52.4%	51%	Al Azad et al. (2019) and Saha et al. (2020)
	Trimethoprim	50 -94.6%	26.7%	Bashar et al. (2011), Hasan et al. (2011), Rahman et al. (2017), Al Azad et al. (2019) and Sarker et al. (2019)
	Nalidixic acid	91.9%	25.7 -70%	Bashar et al. (2011) and Jakaria et al. (2012)
	Colistin	7.8 -26.5%	63.75%	Hassan et al. (2014), Al Azad et al. (2019), Saha et al. (2020) and Mandal et al. (2021)
India	Ampicillin	29.2 - 84.9%	43.8 -47%	Sahoo et al.(2012), Brower et al. (2017), Bhushan et al. (2017), Muglikar et al. (2019), Kumar and Gupta, (2019) and Khasa and Singh. (2020)

Country	Antimicrobial agent:	Prevalence: Broiler (at individual level)	Prevalence: Layer (at individual isolate level)	Reference:
	Tetracycline	74.3 - 84%	42.8 -47%	Sahoo et al. (2012), Samanta et al. (2014), Hussain et al. (2017), Kumar and Gupta, (2019) and Khasa and Singh. (2020)
	Ciprofloxacin	33.3 -78.3%	39.4%	Bhushan et al. (2017), Hussain et al. (2017), Sharma et al. (2017), Kumar and Gupta. (2019) and Kumar and Kumar. (2020)
	Azithromycin	31.6 -85.7%		Sharma et al. (2017)
	Gentamicin	25 - 51.8%	65.2%	Joshi et al. (2012), Samanta et al.(2014), Bhushan et al.(2017), Hussain et al. (2017), Kumar and Gupta. (2019) and Khasa and Singh. (2020)
	Trimethoprim	55.6 - 57.1%		Sharma et al. (2017), Kumar and Gupta. (2019) and Kumar and Kumar. (2020)
	Nalidixic acid	58.3%	86.7%	Samanta et al. (2014) and Kumar and Kumar. (2020)
	Colistin	85.7%		Sharma et al. (2017)
China	Ampicillin	100%	83.0%	Li et al. (2015), Kamboh et al. (2018) and Xu et al. (2019)
	Tetracycline	97.4%	89.3%	Wang et al. (2010), Kamboh et al. (2018) and Xu et al. (2019)
	Ciprofloxacin	83%	45.8 %	Wang et al. (2010), Li et al. (2015) and Kamboh et al. (2018)

Country	Antimicrobial agent:	Prevalence: Broiler (at individual level)	Prevalence: Layer (at individual isolate level)	Reference:
	Gentamicin	26.2 - 73.1%	26.1%	Li et al. (2015) and Xu et al. (2019)
	Trimethoprim	100%	80%	Xu et al. (2019)
	Nalidixic acid	96.2%	77.6%	Xu et al. (2019)
Vietnam	Ampicillin	100% (farm level) 78.9- 86%	80%	Nguyen et al. (2015), Nhung et al. (2015) and Bui et al. (2018)
	Tetracycline	100% (farm level) 83.4 - 84.7%	85%	Nguyen et al. (2015), Nhung et al. (2015) and Bui et al. (2018)
	Ciprofloxacin	91.8% (farm level) 24.9 - 32.5%	33%	Nguyen et al. (2015), Nhung et al. (2015) and Bui et al. (2018)
	Trimethoprim	100% (farm level) 52.1 - 69.7%	70%	Nguyen et al. (2015), Nhung et al. (2015) and Bui et al. (2018)
	Gentamicin	96.6% (farm level) 19.9%	20%	Nguyen et al. (2015) and Bui et al. (2018)
	Nalidixic Acid	100% (farm level) 80%	78%	Nguyen et al. (2015) and Bui et al. (2018)

Country	Antimicrobial agent:	Prevalence: Broiler (at individual level)	Prevalence: Layer (at individual isolate level)	Reference:
	Colistin	22.2%		Nguyen et al. (2015)
Malaysia	Ampicillin	90% at farm level 51.9 -87.5%	72% (farm level)	Kamaruzzaman et al. (2020), Mariappan et al. (2021), Elmi et al. (2021) and Ibrahim et al. (2021)
	Ciprofloxacin	19.4%		Kamaruzzaman et al. (2020) and Elmi et al.(2021)
	Tetracyclin	91%	71% (farm level)	Mariappan et al. (2021)
	Nalidixic acid	52.2%		Kamaruzzaman et al. (2020) and Elmi et al. (2021)
	Gentamicin	20.2 -23.3%		Kamaruzzaman et al. (2020), Elmi et al. (2021) and Ibrahim et al. (2021)
	Trimethoprim	74.2 -83.3%		Kamaruzzaman et al. (2020), Elmi et al. (2021) and Ibrahim et al. (2021)

Regardless of farm and individual level, types of diagnostic assays and countries the AMR prevalence in *E. coli* in broiler was reported to occur 70 to100% for ampicillin followed by 50 to 100% for trimethoprim, 74 to 100% for tetracycline, 78 to 100% for nalidixic acid, 26 to 73% for gentamicin and 22.2 to 73.1 % for colistin. High AMR prevalence in layer farms was also found to be 25 to 83% for ampicillin, 27 to 80% for trimethoprim, 40 to 100% for tetracycline, 25 to 87% for nalidixic acid, 20 to 51% for gentamicin, and 5 to 64% for colistin at farm and individual levels in different countries **(Tables 2.1 and 2.2).** Although individual level AMR prevalence in *E. coli* in poultry was reported to be common in Bangladesh and other countries, farm level AMR prevalence in *E. coli* in poultry was however rarely documented in Bangladesh **(Tables 2.1 and 2.2)** which is the potential gap of AMR studies in scientific literature.

2.8. Factors associated with the occurrence of antimicrobial resistance in *E. coli* in poultry

Several factors were previously identified with the occurrence of AMR in *E. coli* in commercial poultry farms which are presented in Table 2.3.

Table.2.3: Significant factors ($p \leq 0.05$) associated with the occurrence of antimicrobial resistance in *E. coli* in commercial poultry farm

Country	Factors at farm level	Categories	Broiler: Odds ratio	Layer: Odds ratio	References
Norway	Status of previous flock in same house	Yes	12.7		Mo et al.(2016)
	Allow transport and personnel enter into the farm	Occasional	9.3		
	Always disinfect the	No	0.1		

Country	Factors at farm level	Categories	Broiler: Odds ratio	Layer: Odds ratio	References
	floor between the production cycles				
Malaysia	Water source	Pump water	2.0		Elmi et al. (2021)
		Surface water	1.6		
	Farm size	Small scale	2.5		
	Source of sample	Sewage samples	7.4		
Belgium	Clean hygienic condition of the treatment reservoir	Yes	5.2		Persoons et al.(2011)
	Acidification of drinking water	No	3.5		
	>3 feed changes/cycle	Yes	8.3		
	Litter material	Straw	5.1		
Vietnam	Farm size	Small	6.4		Nguyen et al.(2015)
	Change of shoes/boots	Yes	2.4		
	Use of commercial feed	Yes	2.5		
	Day old chick from other sources	Yes	4.9		
	Density	Yes	1.3		

Country	Factors at farm level	Categories	Broiler: Odds ratio	Layer: Odds ratio	References
	Experience	Yes	1.0		
Jordan	Water Source	Artesian wells	18.1		Ibrahim et al. (2019)
	Get prescription before antibiotics	No	13.4		
	Distance in relation to other farms	Very close	23.8		
South Africa	Used antimicrobial agents for treatment	Yes		4.6	Adesiyun et al. (2020)
	Used antimicrobial agents as growth promoters	Yes		1.5	
	Used antimicrobial agents for prophylaxis	Yes		1.4	
Switzerland	Flock size	2000 to 4000		0.2	Harisberger et al.,2011
		More than 4000		0.2	
	Egg boxes	Reused		4.4	
		Less than 3 peoples		2.4	

Country	Factors at farm level	Categories	Broiler: Odds ratio	Layer: Odds ratio	References
	Other poultry production	Yes		0.2	
Bangladesh	Season	Winter	8.4		Mandal et al. (2022)
	Specific shoes for staffs	No	8.6		
	Follow veterinarian prescription	No	18.5		

Table 2.3 highlights the general overview of AMR in Bangladesh and other countries by determining risk factors for the prevalence of AMR in *E. coli* in poultry at farm level (Broiler and Layer). Most of the risk factor studies conducted on broiler farms. However, there is a single publication on determining broiler farm level risk factors associated with AMR in Bangladesh that justify the current investigation to identify potential risk factors associated with the development of AMR at the farm level.

2.9. Control the development of antimicrobials resistance

AMR can be reduced by continuous AMR surveillance and the implementation of concrete interventions based on data identified as risk factors for AMR reported in previous investigations (Acharya et al., 2019; WHO, 2021; Mandal et al., 2022; Saleem et al., 2022). Improvements to the farm management system, increased farmer knowledge about antimicrobial use, and alternative usage of growth boosters such as prebiotics and probiotic etc, can assist to reduce AMR risk in poultry farms around the world (Barroga et al., 2020; Moffo et al., 2020; Hassan, 2021). Implementing diagnostics facilities to treat infectious diseases and select specific treatments against specific pathogens using antimicrobial susceptibility tests (Disc diffusion method, Minimum inhibitory concentrations (MIC) etc.) may assist to decrease antimicrobial use and AMR in commercial poultry farms (Caruso et al., 2018; Barroga et al., 2020).

2.10. Diagnostic tests

Two basic methods of antimicrobial susceptibility testing are available to laboratories: i) Disc diffusion method and ii) broth micro-dilution assay.

2.10.1. Disk diffusion method

The disk diffusion method (also known as the Kirby-Bauer method) works on the principle that antimicrobial molecules create a dynamically changing gradient of antimicrobial concentrations by diffusing out from a disk into the agar while the organism being tested begins to divide and grows toward critical mass (Kuper et al., 2009). A wide zone of inhibition refers to a high level of sensitivity in this test. The bacteria are more susceptible to the drug in the disk when the zone is wider. Although the extent of the zone of inhibition has an inverse correlation with the MIC, it should not be utilized to calculate a MIC value (Mahon et al., 2011).

2.10.2. Micro-dilution assay

Broth micro-dilution method is considered as quantitative because they can measure the MIC. The MIC is defined as the lowest concentration of an antibiotic that inhibits visible growth of a microorganism. This method is considered as the reference method for susceptibility testing because of their high levels of reproducibility (Kuper et al., 2009).

Table.2. 4 : Comparison between the tests

Points	**Disk diffusion method**	**Micro-dilution assay**
Sensitivity	92.6% for Methicillin-resistant *Staphylococcus* (Farahani et al., 2013)	98.9% for Methicillin-resistant *Staphylococcus* (Farahani et al., 2013)
Specificity	93.4% for Methicillin-resistant *Staphylococcus* (Farahani et al.,2013)	100% for Methicillin-resistant *Staphylococcus* (Farahani et al.,2013)
Cost per test	It is the least costly of all susceptibility methods.	It is more costly than disc diffusion method. The cost

Points	Disk diffusion method	Micro-dilution assay
	AtReller et al. (2009), mention that approximate cost per test is $2.50 to $ 5.00.	of the pre-prepared panels range from approximately $10 to $22 (Reller et al., 2009).
Interpretations	The zone diameters of each drug are interpreted using criteria published by the Clinical and Laboratory Standards Institute or those included in the US Food and Drug Administration (FDA)-approved product insert for the discs. The disk diffusion test results are "qualitative," in the sense that they provide a susceptibility category (i.e., susceptible, intermediate, or resistant) rather than a MIC (Kuper et al., 2009).	According to the European EUCAST (European Committee on Antimicrobial Susceptibility Testing) and the American CLSI (Clinical and Laboratory Standards Institute), the determined MIC value must be compared with MIC clinical breakpoints to determine whether the strain is susceptible or resistant to the antibiotic (Kowalska-Krochmal et al., 2021).
Advantages	The advantages of the disk approach include test simplicity (no special equipment required), availability of categorical data for simple interpretation, and flexibility in disk selection for testing (Reller et al., 2009).	The advantages of the micro-dilution technique include the creation of MICs, the reproducibility and convenience of having pre-prepared panels, and the savings in reagents and space that result from the test's reduction. If an automated panel reader is used, it can also help with the generation of

Points	Disk diffusion method	Micro-dilution assay
		computerized reports (Reller et al., 2009).
Disadvantages	The disadvantages of the disk test include the lack of mechanization or automation of the test, which may lead in antimicrobial misclassification. This technique is also incapable of testing fastidious and slow-growing bacteria accurately (Reller et al., 2009).	The main disadvantage of the micro-dilution method is the restricted number of drugs available in standard commercial panels (Reller et al., 2009).

The above-mentioned data **(Table 2.4)** clearly shows that the sensitivity and specificity of the micro-dilution assay are higher than the disc diffusion method, implying that there is less risk of misclassification of antimicrobials as sensitive or resistant. We therefore considered micro-dilution assay for this antibiogram study to evaluate antibiogram of *E. coli* in poultry in Chattogram, Bangladesh.

2.11. Summary

The review highlights the gap between AMR prevalence and its associated risk factors at farm level in commercial poultry farms in Bangladeshi studies. Very few studies are found on AMR prevalence and associated risk factors in layer farms in Bangladesh. The study aimed to estimate the prevalence of AMR in *E. coli* in commercial chickens (both broiler and layer) at farm and individual isolate level in Bangladesh. This study also conducted to find out the associated risk factors with the occurrence of AMR prevalence in poultry at farm level. By estimating the specific resistance pattern using the micro-dilution method and identifying the risk factors, proper management measures on the farm may be introduced to tackle the AMR issue, which is a serious health issue in both animals and humans.

Chapter-III: Materials and Methods

3.1. *E. coli* Isolates used for the study

A total of 162 *E. coli* isolates were sent to the UK AMR reference laboratory for conducting broth micro-dilution assay to evaluate AMR in *E coli* to a 14 panel of antimicrobials. Before conducting the broth micro-dilution assay, all the isolates were re-cultured by the standard bacteriological culture protocol (Arshad et al., 2012) of which 152 were re-isolated.

Samples (5 cloacal swabs in a pool from 5 randomly selected birds per farm and 5 environmental swab samples from a shed floor in another pool per farm) for *E. coli* testing and epidemiological data (farmer and farm demography, farm bio-security and antimicrobial usage data obtained through a cross-sectional study on 140 commercial chicken farms (83 broiler and 57 layer farms) in 8 upazillas of Chattogram district, Bangladesh (Bhusan, 2021, MS Thesis). The detailed description of the cross-sectional study is given as **Appendix II.**

3.2. Broth micro-dilution assay

The broth micro dilution test was used to determine the minimum inhibitory concentration (MIC) in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2020). After vortexing 3–5 colonies of *E. coli* from blood agar with phosphate buffer saline (PBS), the turbidity was adjusted to the 0.5 MacFarland turbidity standard. With the use of a multi-channel pipette, the 5×10^4 CFU/well inoculums were utilized in a Muller-Hinton broth to inoculate the 96 wells Thermofisher sensititre EUVSEC plates, following the standard micro-broth dilution method (Gail and John, 1995; Miles and Amyes, 1996). This EUVSEC plates containing a dilution series (ranging from 0.12 µg/ml to 128 µg/ml) of each of the following antimicrobials: ampicillin, azithromycin, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, colistin, gentamicin, meropenem, nalidixic acid, sulfamethoxazole, tetracycline, tigecycline and trimethoprim. *Escherichia coli* ATCC 25922 was used for quality control purpose .The sensititre plate was sealed and incubated for 16-20 hours at 34-36º C in a non-CO_2 incubator. The lowest

concentrations of antimicrobials that inhibited a color change (Kowalska-Krochmal et al., 2021) or prevented growth in the wells of the micro-dilution trays after incubation were used to calculate the MICs of the test solutions. Based on the interpretation guidelines by CLSI guideline (2020) for dilution susceptibility testing, the results were categorized as susceptible or resistant (CLSI, 2010).

Table.3. 1: Threshold level of minimum inhibitory concentration of different antimicrobial agents (CLSI, 2020); MIC: Minimum Inhibitory Concentration

Serial No	**Test agent**	**MIC Breakpoints (microgram/ml)** **Resistance**
1.	Ampicillin	≥32
2.	Azithromycin	≥32
3.	Cefotaxime	≥4
4.	Ceftazidime	≥16
5.	Chloramphenicol	≥8
6.	Ciprofloxacin	≥4
7.	Colistin	≥4
8.	Gentamicin	≥16
9.	Meropenem	NA
10.	Nalidixic acid	≥32
11.	Sulfamethoxazole	NA
12.	Tetracycline	≥16
13.	Tigecycline	≥0.5
14.	Trimethoprim	≥16

3.3. Statistical evaluation

3.3.1. Data entry and cleaning

Data from the field and the lab were imported into Microsoft Excel 2016. Data cleaning, coding, scoring and integrity were checked for validation and consistency, and then exported to STATA IC-16 (StataCrop, 4905, Lakeway Drive, College Station, Texas 77845, USA) for epidemiological analysis.

3.3.2. Descriptive analysis

All the farms were scored for a list of total 17 biosecurity and hygienic practices. For the ideal practice, a score of "1" was provided and zero was provided to the risky practice. Then all the scores were summed to calculate the total **"biosecurity score"** of an individual farm. Thus, each farm was provided a biosecurity score between 0 and 17.

Descriptive analysis was performed on the data of AMR in *E. coli* at farm and individual level. If *E. coli* isolate determined in any of sample type (cloacal swab pool or environmental swab pool) from a farm was resistant to any of the tested antimicrobials, then the farm was tested as AMR positive farm. Accordingly, the overall farm AMR prevalence was computed by counting number of AMR positive farms divided by the total number of tested farms. Then mean and median of **"number of antimicrobials used"** and **"biosecurity score"** were presented to the specific number of resistant antimicrobials per farm in a tabular format **(Tables 3.1 and 3.2)**. An *E. coli* isolate resistant to any of the tested antimicrobials was considered as AMR isolate. Accordingly, the overall isolate AMR was calculated by using number of AMR isolates divided by total number of isolates tested. The prevalence of AMR in *E. coli* at individual isolate level was calculated by using the number of the specific resistant antimicrobial divided by the total number of *E. coli* isolates tested **(Table 4.3).** The results were expressed as frequency number, percentage and 95% confidence interval (CI).

3.3.3. Risk factor analysis

3.3.3.1. Univariate analysis to assess the status of antimicrobials resistance (min/6 antimicrobials vs. 7/max antimicrobials per farm) associated with potential farm level factors

Univariate logistic regression was performed to assess the association between farm level AMR status and each of the following factors: biosecurity score, number of antimicrobials used, farm size, farmer's experience and farmers education (No education and some level of education).

3.3.3.2. Multivariate analysis to assess the status of antimicrobial resistance (min/6 antimicrobials vs. 7/max antimicrobials per farm) associated with potential farm level factors

Factors determined as significant at $p \leq 0.2$ from univariate logistic regression were forwarded to multivariable logistic regression. The independent factors were checked for multi-collinearity before performing the multivariable logistic regression. The factors were considered to be non-collinear if the variance inflation factor was <10. Backward stepwise logistic regression analysis was applied to fit the model. At first a full model was run and only variables with $p \leq 0.05$ in the likelihood ratio test were retained.

Biologically plausible interactions among the main factors were also tested by using an interaction term between each two factors and retained in the final stage if not significant ($p > 0.05$) in the likelihood ratio test. Confounding was checked by removing one by one variable in the stepwise backward procedure. A variable was considered as a confounder if after removing it, the regression coefficients of the remaining variables showed a relative change (≥15%) (Dohoo et al., 2003). The sensitivity of the final model was then assessed for goodness-of-fit using the Hosmer–Lemeshow test described by Dohoo et al. (2003) while the post estimation of predictive ability was determined using the receiver operating characteristics (ROC) curve (Dohoo et al. 2003). The outputs were presented for each adjusted predictor variable as coefficient (β), p value, and 95% CI.

Chapter-IV: Results

4.1. Farm prevalence of antimicrobial resistance in *E. coli* in chickens along with the level of usage of antimicrobials and bio-security status in commercial chicken farms of Chattogram.

Overall farm AMR prevalence was 75% (42.1% at broiler and 32,9% at layer farm level; N=140 farm).

E. coli resistant to antimicrobials per farm were evident at 7-11 antimicrobials in 59.3%, 4-6 in 37.3% and 1-3 in 3.4% of broiler farms. Almost identical pattern of *E. coli* resistant to antimicrobials per farm was found in layer farms: 7-12 antimicrobials in 45.7%, 4-6 in 45.7% and 2-3 in 8.7% of layer farms. Neither mean antimicrobial usage nor mean farm bio-security score had apparent influence on farm AMR prevalence in the studied farms **(Tables 4.1 and 4.2).**

Table.4. 1: Farm prevalence of antimicrobial resistance in *E. coli* in chickens along with the level of antimicrobial usage and bio-security standards in broiler farms, Chattogram (N=83 farms, n= number of resistant farms)

Number of antimicrobial resistance in *E. coli* per farm	**Prevalence, % (n)**	**Antimicrobial usage per farm**		**Farm biosecurity score**	
		Mean	Median	Mean	Median
0	29 (24)	4	4	6	6
1	1.2 (1)	4		7	
3	1.2 (1)	4		8	
4	4.8 (4)	2	3	8	8
5	9.6 (8)	3	3	6	7
6	12 (10)	3	2	8	7
7	20.5 (17)	3	3	11	7
8	10.8 (9)	4	3	7	7
9	8.4 (7)	3	4	6	6
11	2.4 (2)	4	4	6	6

Table.4. 2: Farm prevalence of antimicrobial resistance in *E. coli* in chickens along with the level of antimicrobial usage and bio-security standards in layer farms, Chattogram (N=57 farms, n= number of resistant farms)

Number of antimicrobial resistance in *E.coli* per farm	**AMR prevalence, %(n)**	**Antimicrobial usage per farm**		**Farm biosecurity score**	
		Mean	median	Mean	Median
0	19 (11)	3	2	9	9
2	1.7 (1)	4		12	
3	5.3 (3)	3	4	9	9
4	10.5 (6)	3	3	9	11
5	17.5 (10)	2	3	8	9
6	8.7 (5)	3	2	10	9
7	10.5 (6)	2	3	12	10
8	17.5 (10)	3	3	11	11
9	1.7 (1)	8		11	
10	3.5 (2)	4	4	10	10
11	1.7 (1)	1		11	
12	1.7 (1)	3		8	

4.2. Individual (isolate level) prevalence of antimicrobial resistance in *E. coli* in chickens of commercial chicken farms of Chattogram

Overall AMR prevalence at isolate level was 100% in both production types (51.3% in broiler chickens and 48.7% in layer chickens).

In broiler farms, *E coli* isolates were more frequently resistance to ciprofloxacin and tetracycline (98.7%), trimethoprim (92.3%), ampicillin (91%), nalidixic acid (83.3%), chloramphenicol (53.9%) and azithromycin (41%). Whereas, in layer farms, *E. coli* isolates were more commonly resistant to ciprofloxacin (100%), tetracycline (97.3%), trimethoprim (81.1 %), ampicillin (79.7%), nalidixic acid (66.2%), chloramphenicol (40.5 %) and azithromycin (32.4%) (**Table.4.3**).

Table.4. 3: Individual prevalence of antimicrobial resistance in *E. coli* in chickens of commercial chicken farms of Chattogram (152 *E. coli* isolates from 140 farms; n=number of antimicrobial resistance in isolates where multiple numbers of antimicrobials in each isolate were considered in counting; CI=Confidence Interval)

	Broiler (78 *E. coli* isolates)		**Layer (74 *E. coli* isolates)**	
Test agent	**AMR prevalence % (n)**	**95% CI**	**AMR prevalence % (n)**	**95% (CI)**
Ampicillin	91 (71)	84.5/97.5	79.7 (59)	70.4/ 89.1
Azithromycin	41 (32)	29.9/52.2	32.4 (24)	21.5/43.4
Cefotaxime	3.9 (3)	0.5/8.2	8.1 (6)	0.2/14.4
Ceftazidime	3.9 (3)	0.5/8.2	8.1 (6)	0.2/14.4
Chloramphenicol	53.9 (42)	42.5/65.1	40.5 (30)	29.1/51.9
Ciprofloxacin	98.7 (77)	96.2/101.3	100 (74)	1
Colistin	25.6 (20)	15.7/35.5	13.5 (10)	5.5/21.5
Gentamicin	43.6 (34)	32.3/54.8	21.6 (16)	12.02/31.2
Meropenem				
Nalidixic acid	83.3 (65)	74.9/91.8	66.2 (49)	55.2/77.2
Sulfamethoxazole	-			-
Tetracycline	98.7 (77)	96.2/101.3	97.3 (72)	93.5/101.1
Tigecycline	1.3 (1)	1.2/3.8	1.4 (1)	1.3/4.04
Trimethoprim	92.3 (72)	86.3/98.4	81.1(60)	71.9/90.2

4.3: Farm prevalence of antimicrobial resistance in *E. coli* (min/6 vs. 7/max antimicrobials per farm) and associated risk factors in Chattogram

4.3.1. Univariate analysis

Five factors were used for univariable logistic regression in both broiler and layer farms to assess their individual associations with the farm AMR in *E. coli* (min/6 antimicrobials vs.7/max per farm).

In case of broiler farms, only number of antimicrobials used was determined as a influencing factor for farm AMR in *E. coli* (p=01). Therefore, multivariate analysis was not performed for this data set.

In case of layer farms, two factors were significantly associated with farm AMR in *E. coli*: (i) number of antimicrobials used (p=0.1) and ii) farm size (p=0.03). Hence, these two variables were used for multivariate analysis.

Table.4 4: Univariate risk factor analysis for farm antimicrobial resistance in *E. coli* in Chattogram (β-Coefficient; CI-Confidence Interval)

Factor	**Broiler farm**			**Layer farm**		
	β	**95% CI**	**P**	**β**	**95% CI**	**P**
Number of antimicrobials used	1.4	0.9/2.1	0.11	1.4	0.9/2.0	0.1
Farm size	1.0	1.0/1.0	0.52	1.0	1.0/1.0	0.03
Farmer's education	1.0	0.7/1.7	0.90	1.0	0.6/1.8	0.94
Biosecurity score	0.9	0.6/1.2	0.39	1.1	0.9/1.5	0.3
Farmer experience	0.7	0.5/1.2	0.23	1.0	14.0	0.95

4.3.2. Multivariate analysis

Neither confounding nor interaction was detected in the model. No significant multi-collinearity was found among the independent factors. The models were well fitted with the p value of 0.44 for goodness of fit test and value of 0.74 for area under ROC.

The farm size in layer was significantly associated with farm AMR in *E. coli* (β=1.36; 95% CI: 0.88/ 2.10; p=0.03).

Table.4. 5: Multivariate risk factor analysis for farm antimicrobial resistance in *E. coli* in Chattogram (β-Coefficient; CI-Confidence Interval)

Factor	**Broiler farms**			**Layer farms**		
	β	**95% CI**	**P**	**β**	**95% CI**	**P**
Number of antibiotics used	1.4	0.9/2.1	0.11	1.4	0.9/2.1	0.16
Farm size	-	-	-	1.00	0.9/1.0	0.03

Chapter-V: Discussion

Poultry provide many opportunities and benefits to peoples (for example rich and nutritious proteins, employment etc.) (Hassan et al., 2014). However, there are many challenges in poultry rearing in developing countries including Bangladesh of which controlling endemic and epidemic infectious diseases and antimicrobial resistance (AMR) are the main obstacles (Kowalska-Krochmal et al., 2021). AMR occurs due to number of reasons: indiscriminate usage of antimicrobials as prophylactic (Page et al., 2012), growth promoter (Upadhayay et al., 2014), feed additives (Apata, 2009; Diarra et al., 2014) and therapeutic purposes (Nguyen et al., 2016); violation of antimicrobial withdrawal period (Khatun et al., 2018), no established surveillance and monitoring system of AMR (WHO, 2020). Other factors that are involved with AMR are farm capacity, and animal husbandry practices (Bushan et al., 2017; Ibrahim et al., 2019; Hedman et al., 2020). In the present study we considered *E. coli*, a commensal bacterium which is capable of spreading antimicrobial resistance to other pathogenic bacteria.

The present study estimated the prevalence of AMR in *E. coli* isolated from cloacal swabs of chickens and farm environment (at farm and individual isolate level) and associated risk factors (farm level). Significant findings of the study, their implications, limitations, conclusions, recommendations and future directions have thoroughly been discussed under various headings as follows.

4.1. Farm antimicrobial resistance prevalence and multi-antimicrobial resistance in *E. coli* at farm level and associated factors

The overall farm AMR prevalence was high. Many preceding studies reported high AMR prevalence at the farm level, 90% to 100% (broiler) and 71% to 72% (layer) in Vietnam and Malaysia (Kamaruzzaman et al., 2020; Mariappan et al., 2021).

E. coli resistance to multi-antimicrobials (3-11 per farm), regardless of the production types, was found in the present study. Here, increase farm size was significantly associated with the increase occurrence of AMR in *E. coli* at layer farms. These findings correspond to many international studies (Brower et al., 2017; Adesiyun et al., 2020) where Brower et al. reported that large flocks in small, enclosed areas, a

lack of sufficient sanitary measures, and the uncontrolled use of broad-spectrum antimicrobials all contribute to the emergence of MDR at the farm level. Adesiyun et al. (2020) discovered various risk factors in large scale layer farms, including the use of antimicrobial agents, pest infestations (insects, rats, etc.), faced rodent difficulties, encountered feral bird problems, and employed antimicrobial agents as growth boosters. Although the present study was not able to determine many factors other than "Farm size", earlier studies identified many other factors associated with the farm level (both broiler and layer) occurrence of AMR such as water source, waste water, poultry litter, farm density, faecal contamination of feeds and eggs and commercial feed (Furtula et al., 2013; Pruden et al., 2013; Ibrahim et al., 2019). Ibrahim et al. reported, in addition, air linked with high farm density is associated with the occurrence of MDR in *E. coli* at farm. Egg shell and fecal contaminated feed were also identified as risk factors with the occurrence of MDR in *E.coli* at layer farm level (Dawadi et al., 2021). Antimicrobial use as growth promoter in feed for layer chickens was determined as a risk factor associated with the occurrence of MDR at farm level (Imam et al., 2020). Nguyen et al. (2016) found the increase number of antimicrobial use is associated with the increase occurrence of MDR in *E. coli* at farm level (both layer and broiler).

Though literatures on poultry farm level MDR and factors in association are not available in Bangladesh, the frequent and extensive use of antimicrobials, combined with poor biosecurity and hygienic practices in small-medium scale poultry production in this country are commonly occurred (Alam et al., 2019; Ferdous et al., 2019; Parvin et al., 2020) which may contribute to developing MDR at farm level (Parvin et al., 2020). Though we discovered no apparent association between total antimicrobial usage on farms and AMR, a similar finding was reported in another Vietnam investigation (Nguyen et al., 2016).

4.2. Antimicrobial resistance in *E. coli* in chicken at individual (isolate level)

Overall isolate AMR prevalence was 100% (51.3% from broiler and 48.7% from layer farms) which are supported by previous studies (Elmofti et al., 2019; Ibrahim et al., 2019).

Our analysis discovered that *E. coli* resistance to a wide range of antimicrobials commonly used in broiler farms is quite frequent (such as 98.3%). *E. coli* isolates resistance to tetracycline and ciprofloxacin each, 91.5% isolates resistance to trimethoprim and ampicillin, 84.8% isolates resistance to nalidixic acid and 62.7% isolates resistance to chloramphenicol). The high resistance of these antimicrobials also found in *E coli* isolates obtained from layer farms in this study. In both production systems, *E. coli* isolates showed a higher resistance to ciprofloxacin, tetracycline, trimethoprim, ampicillin, and nalidixic acid.

The aforementioned findings of the present study are very much consistent with the findings of several international publications. The AMR of *E. coli* in poultry was documented as follows: ampicillin in 97.8% *E. coli* isolates (Nguyen et al., 2016), trimethoprim 95.5% (Ibrahim et al., 2019), tetracycline 80-90% (Elmofti et al., 2019; Ibrahim et al., 2019), nalidixic acid 91.2% (Hess et al., 2022) and ciprofloxacin 73.3% (Nguyen et al., 2016). Unfortunately there are not available Bangladesh publications on antibiogram of *E. coil* in poultry assessed by broth micro-dilution assay. However, literatures of antibiogram of *E. coil* in poultry in Bangladesh evaluated by disk diffusion method supports our findings (Jakaria et al., 2012; Rahman et al., 2017, Al Azad et al., 2019). These cited studies reported the antibiogram pattern of *E. coli* in poultry as follows: ampicillin resistance 70-100% (Al Azad et al., 2019; Sarker et al., 2019; Ievy et al., 2020), trimethoprim 84% (Al Azad et al., 2019; Sarker et al., 2019; Rahman et al., 2020), tetracycline resistance 60-90% (Jakaria et al., 2012; Rahman et al., 2017), nalidixic acid 70% (Bashar et al., 2011; Jakaria et al., 2012) and ciprofloxacin 82-100% (Akond., 2009; Al Azad et al., 2019; Ievy et al., 2020). These findings are in agreement with the findings of the present study.

The concerning level of antimicrobial resistance in the cited and the current studies above could have occurred due to numerous driving factors. Some are given as follows: i) the unregulated use of antimicrobials and dietary supplements may be the

major source of AMR (Dawadi et al., 2021); ii) AMR is caused in both production systems by ambiguous labeling of feed items about their antimicrobial levels and these types of antimicrobial compounds are also supplied through drinking water (Elmofti et al., 2019) to improve production performance; iii) Besides AMU, husbandry practices, poor washing and disinfection and feed varieties may all lead to AMR (Callens et al., 2015; Nguyen et al., 2015); Resistant bacteria may be transmitted by vertical transmission from parental flocks or contamination in the hatchery environment (Nguyen et al., 2016).

E coli isolates resistant to cefotaxime and ceftazidime were identified as less frequent in both production systems in the present study which were found as highly resistant in Austria (Hess et al., 2022). This is a promising finding of our study because these antibiotics are critically important to human medicine (Scott et al., 2019; WHO, 2019; Lhermie et al., 2020; Zou et al., 2021). We must therefore take appropriate measures to preserve these drugs as sensitive, as these antimicrobials are also essential for the management of poultry infections. It is therefore, before administering any antibiotics, bacteriology confirmation is required, along with coordinated action for control and prevention of rising antimicrobial usage in poultry with the guidance of a veterinarian (Manyi-Loh et al., 2018; Acharya et al., 2019).

In the current study, *E coli* isolates resistant to colistin was also found less frequent (17-29%) in both production types. Similar results were also revealed in the research conducted in Vietnam (Nguyen et al., 2016). It's especially excellent news for human medicine, as the WHO has put colistin in its Essential Medicines List Access, Watch, and Reserve (AWaRE) classification (WHO, 2010). Colistin usage in feed is prohibited in developing countries like as India, Nepal, and China (Acharya et al., 2019; Walia et al., 2019; Dewadi et al., 2021). Colistin in small pack is also banned in Bangladesh (Al Amin et al., 2020; Islam, 2021). Antibiotics are also prohibited in feed in Bangladesh under the Animal Feed Act (Ministry of Fisheries and Livestock, Fish Feed and Animal Feed Act, 2010). Furthermore, the use of colistin at random in animals prohibited in Bangladesh (Dawadi et al., 2021; Hassan et al., 2021). Limit the use of antimicrobials as growth promoters and implement a strong awareness program to prevent the use of antimicrobials at sub-therapeutic levels, which can contribute to remain colistin sensitive.

4.3. Limitations of the study

4.3.1. Information bias or recall bias

Information bias could have occurred as the interviewers were dependent on the respondents. However, before starting the primary field research, the questionnaire was carefully piloted, and field investigators (veterinarians) were properly trained to avoid collecting inaccurate data.

4.3.2. Sample size

The sample size was very small as *E. coli* positive farms were added only in this research. This smaller sample size has possibly contributed to the few significant risk factors identified in this study. The broiler type and the layer type farms could have been merged to increase the sample size, but the farm settings and management system of the two types of farms are quite different and so merging them could be subject to confounding.

4.3.3. Study area coverage

This cross-sectional study was conducted in eight upazillas of the Chattogram district. So, the findings of this research are unable to represent the overall AMR pattern and its associated risk factors in poultry across Bangladesh.

4.3.4. Misclassification (resistant/ sensitive)

As we used a diagnostic test of micro-dilution assay having high sensitivity (98.9%) and specificity (100%) (Farahani et al., 2013) at UK AMR reference laboratory, the misclassification of diagnosis was very unlikely to occur in this study.

Chapter-VI: Conclusion, Recommendations and Future direction

6.1. Conclusion

- Overall AMR prevalence was high at farm and isolate level.
- Multi-antimicrobial resistance in *E. coli* in poultry was evidenced.
- The level of *E. coli* resistance to antimicrobials significantly increased with the increase of flock size.
- *E. coli* isolates showed a higher resistance pattern to ciprofloxacin, tetracycline, trimethoprim, ampicillin, nalidixic acid, chloramphenicol, azithromycin and gentamicin in both production systems. However, cephalosporine and colistin remain sensitive in both farm types.

6.2. **R**ecommendations

- Resistant antimicrobials found in the study should be stopped immediately, while sensitive antimicrobials should be used prudently.
- Colistin and ciprofloxacin have recently been banned for usage in veterinary field. Colistin, cefotaxime and ceftazidime were shown to be sensitive in this study. To keep colistin, cefotaxime and ceftazidime sensitive, a strong "National Act Plan" should be developed. Farm bio-security management (including farm hygiene) and judicious usage of antimicrobials based on proper diagnosis of the disease could reduce the burden of AMR in farms having larger flocks.
- Judicious use of antimicrobials; consultation with veterinarians; diagnosis of disease for specific treatment use; bird vaccination; use of prebiotics and postbiotics; proper farm density and floor hygiene; reducing bird stress; maintaining ideal temperature, and so on.
- A proper protocol of antimicrobial use should be prepared with the help of a registered veterinarian and follow the selection of antimicrobials with proper susceptibility testing.

- Routine monitoring of AMR, increased diagnostic facilities and facilities for antimicrobial susceptibility tests such as disc diffusion method.

6.3. Future directions

6.3.1. This study was only performed in Chattogram district. So, there is question of representativeness of the study findings across Bangladesh. Hence, any future study should be focused on covering wider geographical areas.

6.3.2. Further molecular level investigation for AMR gene determination and changes in sequences should be targeted.

References

Acharya KP, and Wilson RT. 2019. Antimicrobial resistance in Nepal. Frontiers in Medicine, 6:105.

Adesiyun AA, Nkuna C, Mokgoatlheng-Mamogobo M, Malepe K, Simanda L. 2020. Food safety risk posed to consumers of table eggs from layer farms in Gauteng Province, South Africa: Prevalence of *Salmonella* species and *Escherichia coli*, antimicrobial residues, and antimicrobial resistant bacteria. Journal of Food Safety, 40(3):e12783.

Al Amin M, Hoque MN, Siddiki AZ, Saha S, Kamal MM. 2020.Antimicrobial resistance situation in animal health of Bangladesh. Veterinary World, 13(12):2713.

Alam MU, Rahman M, Islam MA, Asaduzzaman M, Sarker S, Rousham E, Unicomb, L. 2019. Human exposure to antimicrobial resistance from poultry production: assessing hygiene and waste-disposal practices in Bangladesh. International Journal of Hygiene and Environmental Health, 222(8):1068-1076.

Al Azad MAR, Rahman MM, Amin R, Begum MIA, Fries R, Husna A, Khairalla AS, Badruzzaman ATM, El Zowalaty ME, Lampang KN, Ashour HM. 2019. Susceptibility and multidrug resistance patterns of *Escherichia coli* isolated from cloacal swabs of live broiler chickens in Bangladesh. Pathogens, 8(3):118.

Alghoribi MF, Alqurashi M, Okdah L, Alalwan B, Al Hebaishi YS, Almalki A, Alzayer MA, Alswaji AA, Doumith M, Barry M. 2021. Successful treatment of infective endocarditis due to pandrug-resistant *Klebsiella pneumoniae* with ceftazidime-avibactam and aztreonam. Scientific Reports, 11(1):1-9.

Allcock S, Young EH, Holmes M, Gurdasani D, Dougan G, Sandhu MS, Solomon L, and Török ME. 2017. Antimicrobial resistance in human populations: challenges and opportunities. Global Health, Epidemiology and Genomics, 2:10-12.

Apata D. 2009. Antibiotic resistance in poultry. International Journal of Poultry Science. 8(4): 404-408.

Arshad HM, Mohiuddin OA, Azmi MB. 2012. Comparative in vitro antibacterial analysis of different brands of cefixime against clinical isolates of *Staphylococcus aureus* and *Escherichia coli*. Journal of Applied Pharmaceutical Science, 2(1):109-113.

Baddour LM, Wilson WR, Bayer AS, Fowler Jr VG, Bolger AF, Levison ME, Ferrieri P, Gerber MA, Tani LY, Gewitz MH, Tong DC. 2005. Infective endocarditis: diagnosis, antimicrobial therapy, and management of complications: a statement for healthcare professionals from the committee on rheumatic fever, endocarditis, and kawasaki disease, council on cardiovascular disease in the young, and the councils on clinical cardiology, stroke, and cardiovascular surgery and anesthesia, American heart association: endorsed by the infectious diseases society of America. Circulation, 111(23):e394-e434.

Barroga TRM, Morales RG, Benigno CC, Castro SJM, Caniban MM, Cabullo MFB, Agunos A, de Balogh K, Dorado-Garcia A. 2020. Antimicrobials used in backyard and commercial poultry and swine farms in the Philippines: a qualitative pilot study. Frontiers in Veterinary Science, 7:329.

Bashar T, Rahman M, Rabbi FA, Noor R, Rahman MM. 2011. Enterotoxin profiling and antibiogram of *Escherichia coli* isolated from poultry feces in Dhaka district of Bangladesh. Stamford Journal of Microbiology.1(1): 51-57.

BBI (Bangladesh Business Inspection). 2020. Report of the self-sufficiency in protein: poultry industry in Bangladesh. [cited:2022,Feb 1] Available online: https://businessinspection.com.bd/poultry-industry-in-bangladesh/.

Bhushan C, Khurana A, Sinha R, Nagaraju M. 2017. Antibiotic resistance in poultry environment: Spread of resistance from poultry farm to agricultural field. Centre for Science and Environment, New Delhi. [cited: 2022, Feb 27] Available online: https://cdn.cseindia.org/userfiles/report-antibiotic-resistance-poultry-environment.pdf.

Bista S, ThapaShrestha U, Dhungel B, Koirala P, Gompo TR, Shrestha N, Adhikari N, Joshi DR, Banjara MR, Adhikari B, Rijal KR. 2020. Detection of plasmid-mediated colistin resistant mcr-1 gene in *Escherichia coli* isolated from infected chicken livers in Nepal. Animals, 10(11):2060.

Blair J, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJ. 2015. Molecular mechanisms of antibiotic resistance. Nature Reviews Microbiology, 13(1):.42-51.

BPICC (Bangladesh Poultry Industry Central Council). 2020. Reports on poultry industry. [cited: 2022,Feb 27] Available online:http://www.bpicc-poultry.com/pages.php?pageID=basic-info&mID=2.

Brower CH, Mandal S, Hayer S, Sran M, Zehra A, Patel SJ, Kaur R, Chatterjee L, Mishra S, Das BR, Singh P. 2017. The prevalence of extended-spectrum beta-lactamase-producing multidrug-resistant *Escherichia coli* in poultry chickens and variation according to farming practices in Punjab, India. Environmental Health Perspectives, 125(7):077015.

Bui TKN, Bui TMH, Ueda S, Le DT, Yamamoto Y, Hirai I. 2018. Potential transmission opportunity of CTX-M-producing *Escherichia coli* on a large-scale chicken farm in Vietnam. Journal of Global Antimicrobial Resistance, 13:1-6.

Bushan, 2021. MS thesis on epidemiology of farm *Escherichia Coli* infection and antibiogram of *Escherichia Coli* in commercial chickens in Chattogram, Bangladesh. [cited: 2022, Feb 27] Available online: https://cvasu.ac.bd/office/advanced-studies-and- research.

Callens B, Faes C, Maes D, Catry B, Boyen F, Francoys D, de Jong E, Haesebrouck F and Dewulf J. 2015. Presence of antimicrobial resistance and antimicrobial use in sows are risk factors for antimicrobial resistance in their offspring. Microbial Drug Resistance, 21(1):50-58.

Caruso G. 2018. Antibiotic resistance in *Escherichia coli* from farm livestock and related analytical methods: A review Journal of Association of Official Agricultural Chemists International, 101(4):916-922.

CDC (Centers for Disease Control and Prevention). 2019. Antibiotic resistance threats in the United States, 2019. Atlanta, GA: Centers for disease control and prevention; 2019. [cited: 2022 April 6] Available from: https://www.cdc.gov/drugresistance/pdf/threatsreport/2019-ar-threats-report-508.pdf .

Christaki E, Marcou M, Tofarides A. 2020. Antimicrobial resistance in bacteria: mechanisms, evolution, and persistence. Journal of Molecular Evolution, 88(1):26-40.

Chuppava B, Keller B, Abd El-Wahab A, Sürie C, Visscher C. 2019. Resistance reservoirs and multi-drug resistance of commensal *Escherichia coli* from excreta and manure isolated in broiler houses with different flooring designs. Frontiers in Microbiology, 10:2633.

CLSI (Clinical and Laboratory Standard Institute). 2020. Performance standards for antimicrobial susceptibility testing. 30th ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute; 2020. [cited: 2022 Jan 1]. Available from: https://www.nih.org.pk/wp-content/uploads/2021/02/CLSI-2020.pdf.

Dawadi P, Bista S, Bista S. 2021. Prevalence of colistin-resistant *Escherichia coli* from poultry in South Asian developing countries. Veterinary Medicine International. 2021. [cited: 2022 Jan 1]. Available from: https://www.hindawi.com/journals/vmi/2021/6398838/.

Diarra MS, Malouin F. 2014.Antibiotics in Canadian poultry productions and anticipated alternatives. Frontiers in Microbiology. 5:282.

DLS (Department of Livestock Services). 2021. Livestock economy at a glance. [cited : 2022 Apr 24]. Available from: http://www.dls.gov.bd/site/page/22b1143b-9323-44f8-bfd8-647087828c9b/Livestock-Economy.

EFSA (European Food Safety Authority). 2016. Summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2016 [cited: 2022 Feb 27]. Available from: https://www.efsa.europa.eu/en/efsajournal/pub/5182.

Elmi SA, Simons D, Elton L, Haider N, Abdel Hamid MM, Shuaib YA, Khan MA, Othman I, Kock R, Osman AY. 2021. Identification of risk factors associated with resistant *Escherichia coli* isolates from poultry farms in the east coast of peninsular Malaysia: A cross sectional study. Antibiotics.10(2): 117.

Elmofti HA, Almofti Y, Abuelhassan NN, Omer NN. 2019. Identification and antibiotic resistance patterns of *Escherichia coli* isolated from broilers farms in Bahri locality/Sudan. Acta Scientific Nutritional Health. 3:197-203.

Farahani A, Mohajeri P, Gholamine B, Rezaei M, Abbasi H. 2013. Comparison of different phenotypic and genotypic methods for the detection of methicillin-resistant *Staphylococcus aureus.* North American Journal of Medical Sciences, 5(11):637.

Ferdous J, Sachi S, Al Noman Z, Hussani SAK, Sarker YA, Sikder MH. 2019. Assessing farmers' perspective on antibiotic usage and management practices in small-scale layer farms of Mymensingh district, Bangladesh. Veterinary World, 12(9):1441.

Fletcher S. 2015. Understanding the contribution of environmental factors in the spread of antimicrobial resistance. Environmental Health and Preventive Medicine, 20(4):243-252.

Furtula V, Jackson CR, Farrell EG, Barrett JB, Hiott LM, Chambers PA. 2013. Antimicrobial resistance in *Enterococcus* spp. isolated from environmental samples in an area of intensive poultry production. International Journal of Environmental Research and Public Health, 10(3):1020-1036.

Hanon JB, Jaspers S, Butaye P, Wattiau P, Méroc E, Aerts M, Imberechts H, Vermeersch K, Van der Stede Y. 2015. A trend analysis of antimicrobial resistance in commensal *Escherichia coli* from several livestock species in Belgium (2011–2014). Preventive Veterinary Medicine, 122(4):443-452.

Harisberger M, Gobeli S, Hoop R, Dewulf J, Perreten V, Regula G. 2011. Antimicrobial resistance in Swiss laying hens, prevalence and risk factors. Zoonoses and Public Health, 58(6):377-387.

Hasan B, Faruque R, Drobni M, Waldenström J, Sadique A, Ahmed KU, Islam Z, Parvez MH, Olsen B, Alam M. 2011. High prevalence of antibiotic resistance in pathogenic *Escherichia coli* from large-and small-scale poultry farms in Bangladesh. Avian Diseases, 55(4):689-692.

Hassan MM, Amin KB, Ahaduzzaman M, Alam M, Faruk MS, Uddin I. 2014. Antimicrobial resistance pattern against *E.coli* and *Salmonella* in layer poultry. Research Journal for Veterinary Practitioners, 2(2):30-35.

Hassan MM, Kalam MA, Alim MA, Shano S, Nayem MRK, Badsha MR, Al Mamun, MA, Hoque A, Tanzin AZ, Nath C, Khanom H 2021. Knowledge, attitude, and practices on antimicrobial use and antimicrobial resistance among commercial poultry farmers in Bangladesh. Antibiotics, 10(7):784.

Hedman HD, Vasco KA, Zhang L. 2020. A review of antimicrobial resistance in poultry farming within low-resource settings. Animals, 10(8):1264.

Hess C, Troxler S, Jandreski-Cvetkovic D, Zloch A, Hess M. 2022. *Escherichia coli* isolated from organic laying hens reveal a high level of antimicrobial resistance despite no antimicrobial treatments. Antibiotics, 11(4):467.

Hussain A, Shaik S, Ranjan A, Nandanwar N, Tiwari SK, Majid M, Baddam R, Qureshi IA, Semmler T, Wieler LH, Islam MA. 2017. Risk of transmission of antimicrobial resistant *Escherichia coli* from commercial broiler and free-range retail chicken in India. Frontiers in Microbiology, 8:2120.

Ibrahim RA, Cryer TL, Lafi SQ, Basha EA, Good L, Tarazi YH. 2019. Identification of *Escherichia coli* from broiler chickens in Jordan, their antimicrobial resistance, gene characterization and the associated risk factors. BMC Veterinary Research, 15(1):1-16.

Ibrahim S, Wei Hoong L, Lai Siong Y, Mustapha Z, CW Zalati CW, Aklilu E, Mohamad M, Kamaruzzaman NF. 2021. Prevalence of antimicrobial resistance (AMR) *Salmonella* spp. and *Escherichia coli* isolated from broilers in the East Coast of Peninsular Malaysia. Antibiotics, 10(5):579.

Ievy S, Islam M, Sobur M, Talukder M, Rahman M, Khan MFR. 2020. Molecular detection of avian pathogenic *Escherichia coli* (APEC) for the first time in layer farms in Bangladesh and their antibiotic resistance patterns. Microorganisms, 8(7): 1021.

Imam T, Gibson JS, Foysal M, Das SB, Gupta SD, Fournié G, Hoque MA, Henning J. 2020. A cross-sectional study of antimicrobial usage on commercial broiler and layer chicken farms in Bangladesh. Frontiers in Veterinary Science, 7:576113.

Islam MM. 2021. Bacterial resistance to antibiotics: access, excess, and awareness in Bangladesh. Expert Review of Anti-infective Therapy, 19(8):973-981.

Jakaria ATM, Islam MA, Khatun MM. 2012. Prevalence, characteristics and antibiogram profiles of *Escherichia coli* isolated from apparently healthy chickens in Mymensingh, Bangladesh. Microbes and Health, 1(1):27-29.

Jorgensen JH, Turnidge JD. 2015. Susceptibility test methods: dilution and disk diffusion methods. Manual of Clinical Microbiology:1253-1273.[cited: 2022 Apr24]. Availablefrom:https://onlinelibrary.wiley.com/doi/epdf/10.1128/9781555817381.ch71.

Joshi S, Singh R, Singh SP. 2012. Antibiotic resistance profile of *Escherichia coli* isolates from colibacillosis in and around Pantnagar, India. Veterinary World, 5(7): 405.

Kadri SS. 2020. Key takeaways from the US CDC's 2019 antibiotic resistance threats report for frontline providers. Critical Care Medicine.[cited: 2022 Apr 22]. Available from:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7176261/.

Kamaruzzaman NF, Saeed S. 2020. Prevalence of antimicrobial resistance (AMR) *Salmonella* spp. and *Escherichia coli* isolated from broilers in east coast Malaysia.[cited: 2022 Apr 22]. Available from: https://www.preprints.org/manuscript/202012.0679/v1.

Kamboh AA, Shoaib M, Abro SH, Khan MA, Malhi KK,Yu S. 2018. Antimicrobial resistance in enterobacteriaceae isolated from liver of commercial broilers and backyard chickens. Journal of Applied Poultry Research, 27(4):627-634.

Khairu Islam M, Forhad Uddin M, Mahmud Alam M. 2014. Challenges and prospects of poultry industry in Bangladesh. European Journal of Business and Management, 6:116-127.

Khasa V, Singh P. 2020. Isolation and antibacterial susceptibility of *Escherichia coli* from poultry. Journal of Pharmacognosy and Phytochemistry, 9(1):1182-1184.

Khatun R, Howlader AJ, Ahmed S, Islam N, Alam K, Haider S, Mahmud MS, Hasan MA. 2018. Validation of the declared withdrawal periods of antibiotics. Universal Journal of Public Health, 6(1):14-22.

Kowalska-Krochmal B, Dudek-Wicher R. 2021. The minimum inhibitory concentration of antibiotics: Methods, interpretation, clinical relevance. Pathogens, 10(2):165.

Kumar S, Gupta R. 2019. Risk factors for antimicrobial resistance in *Escherichia coli* isolates from poultry in Haryana. Indian Journal of Animal Research, 53(7):918-925.

Kumar A, Kumar M. 2020. Antimicrobial spectrum of *Escherichia coli* isolated from poultry faecal samples in and around Ranchi. International Journal of Current Microbiology and Applied Sciences, 9(9): 2921-2925.

Kuper KM, Boles DM, Mohr JF, Wanger A. 2009. Antimicrobial susceptibility testing: a primer for clinicians. Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy, 29(11):1326-1343.

Lhermie G, La Ragione RM, Weese JS, Olsen JE, Christensen JP, Guardabassi L. 2020. Indications for the use of highest priority critically important antimicrobials in the veterinary sector. Journal of Antimicrobial Chemotherapy, 75(7):1671-1680.

Li Y, Chen L, Wu X, Huo S. 2015. Molecular characterization of multidrug-resistant avian pathogenic *Escherichia coli* isolated from septicemic broilers. Poultry Science, 94(4):601-611.

Mahmud, R. 2020. "COVID-19 and the future for Bangladesh's poultry sector." One Health Poultry Hub.[cited: 2022 Jun 8]. Available from:https://www.onehealthpoultry.org/blog-posts/covid-19-and-the-future-for-bangladeshs-poultry-sector/.

Mahon CR, Lehman DC, Manuselis G. 2018. Textbook of diagnostic microbiology-e-book. Elsevier Health Sciences,Sixth edition, p:1045.

Mamza SA, Egwu GO, Mshelia GD. 2010. Beta-lactamase *Escherichia coli* and *Staphylococcus aureus* isolated from chickens in Nigeria. Veterinaria Italiana, 46(2):155-165.

Mandal MS, Khan ALFR. 2017. Poultry industry in Bangladesh: Which way to sustainable development. In Proceedings of the 10th International Poultry Show and Seminar. WPSA-BB. [cited:2022 May 23]. Available from:http://wpsa-bb.com/wp-content/uploads/2018/07/Keeynote-paper-10th-Show-17.pdf.

Mandal AK, Talukder S, Hasan MM, Tasmim ST, Parvin MS, Ali MY, Islam MT. 2022. Epidemiology and antimicrobial resistance of *Escherichia coli* in broiler chickens, farmworkers, and farm sewage in Bangladesh. Veterinary Medicine and Science, 8(1):187-199.

Mariappan V. Vellasamy KM, Mohamad NA, Subramaniam S, Vadivelu J. 2021. One health approaches contribute towards antimicrobial resistance: Malaysian Perspective. Frontiers in Microbiology,12:718774.

Marshall BM, Levy SB. 2011. Food animals and antimicrobials: impacts on human health. Clinical Microbiology Reviews, 24(4):718-733.

Mo SS, Kristoffersen AB, Sunde M, Nødtvedt A, Norström M. 2016. Risk factors for occurrence of cephalosporin-resistant *Escherichia coli* in Norwegian broiler flocks. Preventive Veterinary Medicine, 130:112-118.

Moffo F, Mouiche MMM, Kochivi FL, Dongmo JB, Djomgang HK, Tombe P, Mbah, CK, Mapiefou NP, Mingoas JPK, Awah-Ndukum J. 2020. Knowledge, attitudes, practices and risk perception of rural poultry farmers in Cameroon to antimicrobial use and resistance. Preventive Veterinary Medicine, 182:105087.

Muglikar DM, Kalyani IH, Desai D, Patel JM, Patel DR, Makwana P, Solanki JB. 2019. Serotyping and antimicrobial susceptibility pattern of avian pathogenic *Escherichia coli*. International Journal of Current Microbiology and Applied Sciences, 8(12): 505-511.

Munita JM, Arias CA. 2016. Mechanisms of antibiotic resistance. Microbiology Spectrum, 4(2):4-2.

Nguyen VT, Carrique-Mas JJ, Ngo TH, Ho HM, Ha TT, Campbell JI, Nguyen TN, Hoang NN, Pham VM, Wagenaar JA, Hardon A. 2015. Prevalence and risk factors for carriage of antimicrobial-resistant *Escherichia coli* on household and small-scale chicken farms in the Mekong Delta of Vietnam. Journal of Antimicrobial Chemotherapy, 70(7):2144-2152.

Nguyen NT, Nguyen HM, Nguyen CV, Nguyen TV, Nguyen MT, Thai HQ, Ho MH, Thwaites G, Ngo HT, Baker S, Carrique-Mas J. 2016. Use of colistin and other critical antimicrobials on pig and chicken farms in southern Vietnam and its association with resistance in commensal *Escherichia coli* bacteria. Applied and Environmental Microbiology, 82(13):3727-3735.

Nhung NT, Cuong NV, Campbell J, Hoa NT, Bryant JE, Truc VNT, Kiet BT, Jombart T, Trung NV, Hien VB, Thwaites G. 2015. High levels of antimicrobial resistance among *Escherichia coli* isolates from livestock farms and synanthropic rats and shrews in the Mekong Delta of Vietnam. Applied and Environmental Microbiology, 81(3):812-820.

Nhung NT, Chansiripornchai N, Carrique-Mas JJ. 2017. Antimicrobial resistance in bacterial poultry pathogens: a review. Frontiers in Veterinary Science, 4:126.

Orubu ESF, Zaman MH, Rahman MT, Wirtz VJ. 2020. Veterinary antimicrobial resistance containment in Bangladesh: Evaluating the national action plan and scoping the evidence on implementation. Journal of Global Antimicrobial Resistance, 21:105-115.

Osman KM, Kappell AD, Elhadidy M, ElMougy F, El-Ghany WAA, Orabi A, Mubarak AS, Dawoud TM, Hemeg HA, Moussa IM, Hessain AM. 2018. Poultry hatcheries as potential reservoirs for antimicrobial-resistant *Escherichia coli*: A risk to public health and food safety. Scientific Reports, 8(1):1-14.

Park YH, Hamidon F, Rajangan C, Soh KP, Gan CY, Lim TS, Abdullah WNW, Liong MT. 2016. Application of probiotics for the production of safe and high-quality poultry meat. Korean Journal for Food Science of Animal Resources, 36(5):567.

Parvin R, Nooruzzaman M, Kabiraj CK, Begum JA, Chowdhury EH, Islam MR, and Harder T. 2020. Controlling avian influenza virus in Bangladesh: Challenges and recommendations. Viruses, 12(7):751.

Pruden A, Larsson DJ, Amézquita A, Collignon P, Brandt KK, Graham DW, Lazorchak JM, Suzuki S, Silley P, Snape JR, and Topp E. 2013. Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment. Environmental Health Perspectives, 121(8):878-885.

Rahman MA, Rahman AKMA, Islam MA, Alam MM. 2017. Antimicrobial resistance of *Escherichia coli* isolated from milk, beef and chicken meat in Bangladesh. Bangladesh Journal of Veterinary Medicine, 15(2):141-146.

Rahman M, Husna A, Elshabrawy HA, Alam J, Runa NY, Badruzzaman ATM, Banu NA, Al Mamun M, Paul B, Das S, Khairalla AS. 2020. Isolation and molecular characterization of multidrug-resistant *Escherichia coli* from chicken meat. Scientific Reports, 10(1):1-11.

Rahman M, Chowdhury EH, Parvin R. 2021. Small-scale poultry production in Bangladesh: Challenges and impact of COVID-19 on sustainability. German Journal of Veterinary Research, 1(1):19-27.

Rashid MH, Xue C, Islam MR, Islam MT, Cao Y. 2013. A longitudinal study on the incidence of mortality of infectious diseases of commercial layer birds in Bangladesh. Preventive Veterinary Medicine, 109(3-4):354-358.

Reller LB, Weinstein M, Jorgensen JH, Ferraro MJ. 2009. Antimicrobial susceptibility testing: a review of general principles and contemporary practices. Clinical Infectious Diseases, 49(11):1749-1755.

Reygaert WC. 2018. An overview of the antimicrobial resistance mechanisms of bacteria. AIMS Microbiology, 4(3):482.

Rushton J, Ferreira JP, Stärk KD. 2014. Antimicrobial resistance: the use of antimicrobials in the livestock sector.[cited: 2022 May 8]. Available from:https://www.oecd-ilibrary.org/agriculture-and-food/antimicrobial-resistance_5jxvl3dwk3f0-en.

Saha O, Hoque MN, Islam OK, Rahaman M, Sultana M, Hossain MA. 2020. Multidrug-resistant avian pathogenic *Escherichia coli* strains and association of their virulence genes in Bangladesh. Microorganisms, 8(8): 1135.

Saharan VV, Verma P, Singh AP. 2020. *Escherichia coli, Salmonella* spp., and *Staphylococcus aureus* susceptibility to antimicrobials of human and veterinary importance in poultry sector of India. Journal of Food Safety, 40(1):12742.

Sahoo TK, Sahoo L, Sarangi LN, Panda SK, Panda HK. 2012. Prevalence, isolation, characterisation and antibiogram study of pathogenic *Escherichia coli* from different poultry farms of Odisha. Journal of Advanced Veterinary Research, 2(3): 169-172.

Saleem Z, Godman B, Azhar F, Kalungia AC, Fadare J, Opanga S, Markovic-Pekovic V, Hoxha I, Saeed A, Al-Gethamy M, Haseeb A. 2022. Progress on the national action plan of Pakistan on antimicrobial resistance (AMR): A narrative review and the implications. Expert Review of Anti-infective Therapy, 20(1):71-93.

Saleque MA, Ansarey FH. 2020. Poultry industry in Bangladesh: Challenges and solutions. [cited: 2022May 25]. Available from:https://www.daily-sun.com/printversion/details/502289/Poultry-Industry:-Challenges-and-Solutions.

Samanta I, Joardar SN, Das PK, Das P, Sar TK, Dutta TK, Bandyopadhyay S, Batabyal S, Isore DP. 2014. Virulence repertoire, characterization, and antibiotic resistance pattern analysis of *Escherichia coli* isolated from backyard layers and their environment in India. Avian Diseases.58(1): 39-45.

Sarker MS, Mannan MS, Ali MY, Bayzid M, Ahad A, Bupasha ZB. 2019. Antibiotic resistance of *Escherichia coli* isolated from broilers sold at live bird markets in Chattogram, Bangladesh. Journal of Advanced Veterinary and Animal Research, 6(3):272.

Seo KW, Lee YJ. 2021. Molecular characterization of fluoroquinolone-resistant *Escherichia coli* from broiler breeder farms. Poultry Science, 100(8):101250.

Sharma S, Galav V, Agrawal M, Faridi F, Kumar B. 2017. Multi-drug resistance pattern of bacterial flora obtained from necropsy samples of poultry. Journal of Animal Health and Production, 5:165-171.

Somaratne N, Hallas G. 2015. Review of risk status of groundwater supply wells by tracing the source of coliform contamination. Water, 7(7):3878-3905.

Taylor J, Hafner M, Yerushalmi E, Smith R, Bellasio J, Vardavas R, Bienkowska-Gibbs T, Rubin J. 2014. Estimating the economic costs of antimicrobial resistance: model and results.[cited:2022 Jun 5]. Available from:http://resp.llas.ac.cn/C666/handle/2XK7JSWQ/3811.

Upadhayay UPPDD, Vishwa PCV. 2014. Growth promoters and novel feed additives improving poultry production and health, bioactive principles and beneficial applications: the trends and advances-a review. International Journal of Pharmacology, 10(3):129-159.

Walia K, Sharma M, Vijay S, Shome BR. 2019. Understanding policy dilemmas around antibiotic use in food animals and offering potential solutions. The Indian Journal of Medical Research, 149(2):107.

Wang XM, Liao XP, Zhang WJ, Jiang HX, Sun J, Zhang MJ, He XF, Lao DX, Liu YH. 2010. Prevalence of serogroups, virulence genotypes, antimicrobial resistance, and phylogenetic background of avian pathogenic *Escherichia coli* in south of China. Foodborne Pathogens and Disease, 7(9):1099-1106.

WHO (World Health Organization). 2019. "World Health Organization ten threats to global health in 2019."[cited: 2022 Jan 19]. Available from: https://www.globalcitizen.org/en/content/top-health-threats-2019/.

WHO (World Health Organization) 2019."Critically important antimicrobials for human medicine." (2019).[cited: 2022 Mar 20]. Available from: https://www.who.int/publications/i/item/9789241515528.

WHO (World Health Organization). 2019. WHO releases the 2010 AWaRE classification antibiotics. 2019. [cited: 2022 june 8]. Available from: https://www.who.int/news/item/01-10-2019-who-releases-the-2019-aware-classification-antibiotics.

WHO (World Health Organization). 2021. "Global antimicrobial resistance and use surveillance system (GLASS) report: 2021". [cited: 2022 Apr 24]. Available from:https://apps.who.int/iris/bitstream/handle/10665/341666/9789240027336-eng.pdf.

WHO (World Health Organization). 2021. WHO strategic priorities on antimicrobial resistance: preserving antimicrobials for today and tomorrow.[cited: 2022 Apr 24].
Availablefrom:https://apps.who.int/iris/bitstream/handle/10665/351719/9789240041387-eng.pdf.

Xu X, Sun Q, Zhao L. 2019. Virulence factors and antibiotic resistance of avian pathogenic in eastern China. Journal of Veterinary Research, 63(3):317-320.

Zou M, Ma PP, Liu WS, Liang X, Li XY, Li YZ, Liu BT. 2021. Prevalence and antibiotic resistance characteristics of extra-intestinal pathogenic *Escherichia coli* among healthy chickens from farms and live poultry markets in China. Animals, 11(4):1112.

Appendix-I

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

Demographic/Socioeconomic characteristics of the interviewee

(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 If no (All birds are of the same age), then THE SHED TO BE SAMPLED will be selected randomly.	0=Shed 1 3= Shed 4	1= Shed 2 4= Shed 5	2=Shed 3 5=Shed 6 6=Other shed (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 is involved in poultry keeping go to other commercial poultry farms?	0=No	1=Yes	
If yes in question 23, then how frequently does he/they visit in the last month?	0=daily	1=consecutive days	2=once in a week
	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 and 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. Do 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 poultry 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 interviewer ticks egg type)	0=Novogen Brown 1=White Hyline Brown 2=White Shaver 579	3=ISA Brown 4=Hi-Sex Brown	5=White Bovine White 6= Others_____
What type of breed/strain you have in the farm currently? (THIS QUES will come if interviewer ticks meat type)	1=Cobb 500 2=Ross 308	3=Indian River Meat 4=Tiger Sasso	5=Habbard and Arber acre

Appendix-II

Description of the study area

The Chattogram region in Bangladesh's southeast (21º54´ and 22º59´N and 91º17´ and 92º13´E), was chosen as the study site because it is one of the country's most important chicken-producing districts. The area of Chattogram is about 5284.92 sq. km. The city is situated between the Chattogram Hill Tracts and the Bay of Bengal on the banks of the Karnaphuli River. With a population density of 1442 square kilometers, this city has a population of roughly 7,616,352.(BBS,2013).The district of Chattogram is made up of 15 upazilas and 3 metro thanas. Literacy rates in this district are 58.9% (BBS, 2013). The main occupations of the people in this district include fishing, wholesale and retail trade, manufacturing, hotel/restaurant management, and education. Chattogram is habitat to around 3.5 million poultry, despite of production type, accounting for 0.95%t(N=365 million) of Bangladesh's total population (DLS,20).In Chattogram, there are 4882 broiler farms, 295 Sonali farms, 559 layer farms, 20 breeder farms, and 20 household farms (DLS, 20). Therefore, the Chattogram was selected for estimating AMR patterns in commercial chicken farms.

Studytype and duration

A cross-sectional study was conducted on commercial poultry farms (broiler and layer) in Chattogram, Bangladesh for 6 months (February to July 2019).

Population:

Reference population

All commercial poultry (broiler and layer) farms under Chattogram district were treated as the reference population.

Source population

To maximize wider coverage of Chattogram district, Gupta et al. (2021) selected eight upazilas (sub-districts) by using the following criteria: presence of water bodies, forests, hills and distance from Chattogram city. These were Anowara, Chandanaish, Fatickchari, Lohagara, Patiya, Rangunia, Raozan and Sitakunda. Poultry farms under these upazilas of the district were selected as the source population for the present study.

Epidemiology unit and sampling frame

Regardless of the production type a farm consisting at least 500 birds was considered as the smallest unit of the sampling frame. According to the sampling unit, there were total of 1748 commercial poultry farms (1493 broiler and 255 layer farms). Distribution of the farms in the sampling frame by upazillas is displayed in **Table 3.1**. The sampling frame was constructed by Gupta et al.,2021 with the support of relevant stakeholders or offices: Chattogram Livestock Services, government and private poultry practitioners, feed and chick dealers and pharmaceuticals representatives. Then Gupta et al.,2021 selected farms (as per sample size calculation given below) from the list by using proportionate probability of random sampling.

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
Anwara	234	500-4000	9	500-5000
Chandanaish	199	500-5500	18	1000-6500
Fatickchari	180	500-4800	36	500-5500
Lohagara	180	500-3500	40	1000-13000
Patiya	199	500-5000	40	500-5000
Rangunia	231	500-3000	40	500-7000
Raozan	144	500-3500	27	500-6000
Sitakunda	126	500-7000	45	500-8000
Total	**1493**	**500-7000**	**255**	**500-13000**

Sample size calculation

Considering a prevalence of 90%(If 50% of routinely used antibiotics become resistant on a farm, the farm is classed as an AMR farm) ±10 precision(since there was no published assessment of ARM prevalence at the farm level), 139 farms were required, 95% Confidence Interval (CI) and 1 design effect (Formula: N = Design effect * $p(1-p)/E^2$)) (OpenEpi, 2013).

Sampling technique

The required number of farms was recruited from the farms studied by Gupta et al. (2020) by applying a proportionate probability of random sampling technique. Some farms were excluded due to not in operation or having no birds during the field visits, and neighboring farms were substituted in this circumstance. If a farm only had one shed, data and samples were obtained from there. If a farm had multiple sheds and the same antimicrobial was used in all of them, data and samples were recorded from the shed with the oldest chickens. If a farm had more than one shed and multiple antimicrobials were used in different sheds, data and samples were recorded from the shed with the most antimicrobials used.

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

Upazilla	**Broiler farm**		**Layer farm**	
	No of farms	Size: Min-Max	No of farms	Size: Min-Max
Anwara	13	500-4000	2	500-5000
Chandanaish	11	500-5500	4	1000-6500
Fatickchari	10	500-4800	8	500-5500
Lohagara	10	500-3500	9	1000-13000
Patiya	11	500-5000	9	500-5000
Rangunia	13	500-3000	9	500-7000
Raozan	8	500-3500	6	500-6000
Sitakunda	7	500-7000	10	500-8000
Total	83	500-7000	57	500-13000

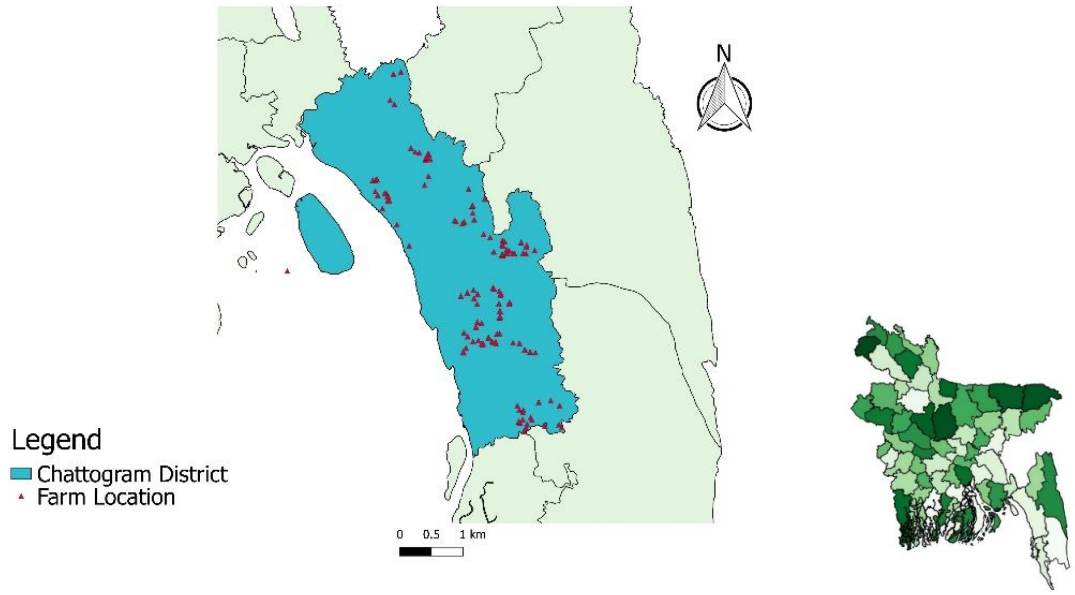

Figure I. Location of selected poultry farms in Chattogram district

Data collection:

Questionnaire development, validation and administration

As per set objectives, a questionnaire was developed. A thorough literature review and some peer-consultation before drafting a questionnaire were conducted to identify the areas to be covered. The draft questionnaire was peer-reviewed to determine gaps and revised as needed. The questionnaire was then piloted on five broiler and five layer farms to ensure consistency and timeliness of administration. The questionnaire was then modified based on the results of the pilot study.

The questionnaire composed of the following information: i) poultry farm information such as farm location, type of production system, number of sheds, bird population, ii) husbandry practices such as farm hygiene, biosecurity, water bath facility, cleaning and disinfection, isolation of sick birds, cleaning of egg trays, disposal of dead birds, manure, and farm waste. The questionnaire included closed-ended, open-ended, and mixed-type questions. The complete questionnaire is presented as Appendix-I. During the study period, a team of three members went on field trips, visiting 4-5 farms per day. One team member conducted the interview, another collected biological samples, and a third photographed and closely examined the antimicrobials used on farms. Before traveling to the field, the team contacted the local veterinarian, who then contacted the farmers to schedule an interview for data

collection and biological sampling. Before administering the questionnaire and collecting samples, each participant farmer provided verbal consent. All of the farmers had been incentivized with soap and liquid hand-wash.

Sample collection, transportation, preservation and storage

Cloacal and environmental swab samples were obtained from each selected poultry farms. Samples were taken from a single flock for single-housed farms. Samples were obtained from older or oldest flocks on farms with more than one house. Cloacal samples were taken from five birds at random and combined in a 5 ml sterile falcon tube with Stuart transport media (Neogen, Lansing MI). Environmental swab samples were taken from the middle and four corners of each farm, then combined in a 15 mL sterile falcon tube containing buffered peptone water (BPW) (Neogen, Lansing MI) with a unique identity number. After that, all tubes were placed in an insulated box with ice packs and transported to the lab within 4-6 hours.

Laboratory evaluation:

Sample preparation

E. coli was isolated using standard microbiological procedures from both sample types (cloacal and environmental pools) (Markey et al., 2013).Each sample pooled swab was diluted in a 1:10 ratio with BPW (full) and incubated at 37ºC for 24 hours for enrichment before beginning laboratory work.

Bacteriological test

The surface of Mac Conkey (MAC) agar (Neogen, Lansing MI) was streaked with 10 µl of pre-enriched cultured broth and incubated aerobically overnight at 37°C. On MC agar, any brilliant, pink-colored translucent smooth elevated colonies were suspicious colonies, which were streaked on eosin methylene blue (EMB) agar (Neogen, Lansing MI).The plates were incubated for 18-24 hours at 37°C.Following incubation, the plates were examined for the presence of characteristic *E. coli* colonies with a yellow-green metallic sheen on EMB agar. The triple sugar iron (TSI) agar (Neogen, Lansing MI) slant reaction (Yellow slant, yellow butt, presence of gas bubbles, and absence of black precipitate in the butt), indole reaction, and citrate utilization test were employed to verify and confirm suspicious colonies.

After that, the colonies were transferred to 5% blood agar (BA) (Blood agar base, Oxoid®, Oxoid Ltd, Basingstoke, United Kingdom).These were produced in brain heart infusion (BHI) broth after being incubated overnight at 37°C (Neogen, Lansing MI). For future use, all positive isolates were preserved at -80°C in 50% glycerol.

Cultural sensitivity test

162 *E.coli* isolates were initially screened for antimicrobial sensitivity pattern by cultural sensitivity test. Antibiotic sensitivity testing was done through broth micro-dilution assay using 96 wells Thermo-fisher Sensititre EUVSEC plates containing a dilution series (ranging from 0.12 µg/ml to 128 µg/ml) of each of the following antimicrobials: ampicillin, azithromycin, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, colistin, gentamicin, meropenem, nalidixic acid, sulfamethoxazole, tetracycline, tigecycline and trimethoprim. *Escherichia coli* ATCC 25922 was used for quality control purpose .The Sensititre plate was sealed and incubated for 16-20 hours at 34-36ºC in a non-CO_2 incubator. The lowest concentrations of antibiotics that inhibited a color change (EGWU et al., 1994) or prevented growth in the wells of the micro-dilution trays after incubation were used to calculate the MICs of the test solutions. Based on the interpretation guidelines by CLSI guideline (2020) for dilution susceptibility testing, the results were categorized as susceptible or resistant (CLSI 2020).

Appendix-III

Biosecurity checklist and scoring

SL No.	Parameter	Biosecurity/hygienic score	
		Layer	**Broiler**
1	Is the farm surrounded by a protective fence?	Yes=1 No/Other (partially fenced) =0	Yes=1 No/Other (partially fenced) =0
2	In addition to the people involved in rearing poultry (listed in ques 23), who has access to your farm?	No=1 Yes=0	No=1 Yes=0
3	Does anyone who are involved in poultry keeping go to other commercial poultry farms?	No=1 Yes=0	No=1 Yes=0
4	Do you isolate the sick birds in a separate shed?	Yes=1 No/corner in the shed (others)=0	Yes=1 No/corner in the shed (others)=0
5	What do you do with dead birds?	Deep burial/Burial/Pitting=1 Dispose in river/pond/canal/distance place=0	Deep burial/Burial/Pitting=1 Dispose in river/pond/canal/distance place=0
6	Do the employees change clothes and shoes before entering the farm/shed?	Yes/other (apron, spray on shoe) =1 No=0	Yes/other (apron, spray on shoe) =1 No=0
7	Do the visitors/employees use washing facility before entering farm/shed?	Yes/other =1 No=0	Yes/other =1 No=0
8	Do the visitors change clothes and shoes before entering the farm/shed?	Yes/other (apron, spray on shoe) =1 No/NA (no changing room) =0	Yes/other (apron, spray on shoe) =1 No/NA (no changing room) =0
9	Are the vehicles checked and decontaminated before entering farm?	Yes/ NA (vehicle not allowed) =1 No/Others (cleaning with only water) =0	Yes/ NA (vehicle not allowed) =1 No/Others (cleaning with only water) =0

10	Are the vehicles decontaminated when leaving the farm?	Yes/ NA (vehicle not allowed) =1 No/Others (cleaning with only water) =0	Yes/ NA (vehicle not allowed) =1 No/Others (cleaning with only water) =0
11	Do you have footbaths available and used, and disinfectant water changed within 6 hours?	Yes/other (not changed within 6 hours) =1 No=0	Yes/other (not changed within 6 hours) =1 No=0
12	What types of water you allow for cooling at the farm?	Deep Well/Shallow well=1 Ponds=0	Deep Well/Shallow well=1 Ponds=0
13	Do you weekly disinfect and clean the farm surfaces and equipment?	Yes=1 No/other (monthly disinfect) =0	Yes=1 No/other (monthly disinfect) =0
14	Are egg trays washed when bringing back from market?	Yes/NA (do not bring back) =1 No/Other (wiped only) =0	*Not applicable for broiler*
15	Are farm employees given training on biosecurity measures?	Yes=1 No/Other/NA =0	Yes=1 No/Other/NA =0
16	Are farm workers live within the farm premises?	Yes/NA (no staff) =1 No=0	Yes/NA (no staff) =1 No=0
17	If yes, do they rear their own poultry birds within the farm premises?	Yes=0 No/NA (rear neighbor's poultry) =1	Yes=0 No/NA (rear neighbor's poultry) =1
	Total score	**17**	**16**

Short biography

Nasreen Sultana passed the Secondary School Certificate examination from Ispahani Adarsha High School in 2009 and then the Higher Secondary School Certificate examination from Bangladesh Mahila Samiti Girl's High School and College in 2011. She completed her Doctor of Veterinary Medicine (DVM) from Chattogram Veterinary and Animal Sciences University, Chattogram, Bangladesh in 2018. She is now a candidate for the degree of MS in Epidemiology under the Department of Medicine and Surgery, Faculty of Veterinary Medicine, CVASU. She has an immense interest in working in the field of veterinary epidemiology and the betterment of humanity in every way possible, particularly by devoting herself to scientific research.