IDENTIFICATION OF MULTIDRUG-RESISTANT *ESCHERICHIA COLI* AND *SALMONELLA* SPECIES ISOLATED FROM CHICKEN AND PIGEON IN SOME SELECTED AREAS OF DHAKA CITY

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JUNE, 2022

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REGISTRATION NUMBER: 12-04957

A Thesis
Submitted to the Faculty of Animal Science and Veterinary Medicine
Sher-e-Bangla Agricultural University Dhaka-1207,
In Partial Fulfillment of the Requirements for the degree of

MASTER OF SCIENCE (MS) IN MEDICINE
DEPARTMENT OF MEDICINE AND PUBLIC HEALTH

SEMESTER: JAN-JUN/2022

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This is to certify that the thesis entitled "IDENTIFICATION OF MULTIDRUG-RESISTANT ESCHERICHIA COLI AND SALMONELLA SPECIES ISOLATED FROM CHICKEN AND PIGEON IN SOME SELECTED AREAS OF DHAKA CITY" submitted to the department of MEDICINE AND PUBLIC HEALTH, faculty of Animal Science & Veterinary Medicine, Sher-e-Bangla Agricultural University, Sher- e-bangla nagar, Dhaka-1207, in partial fulfillment of the requirements for the degree of Master of Science (MS) in MEDICINE, embodies the result of a piece of bona fide research work carried out by SHAH JUNGY IBNA KARIM, Registration No.: 12-04957, under my supervision and guidance. No part of thethesis has been submitted for any other degree or diploma.

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DEDICATED TO
MY BELOVED PARENTS

ACKNOWLEDGEMENT

All praises are due to almighty Allah, who has created the universe and who enabled the author to complete this piece of research work and preparation of this thesis successfully. The author with a senseof respect expresses his heartfelt gratitude to his Supervisor Dr. K. B. M. Saiful Islam Chairman & Professor Department of Medicineand Public Health Sher-e-Bangla Agricultural University Sher-e Bangla Nagar, Dhaka-1207, for his untiring and painstaking guidance, invaluable suggestions, continuous supervision, timely instructions, inspirations and constructive criticism throughout the tenure of research work. Heartfelt gratitude and profound respectare due to his Co-Supervisor Dr. Mahfuzul Islam Chairman & Associate Professor Department of Microbiology and Parasitology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207, for his co-operation, constructive criticism and valuable suggestions for the modification and improvement of the research work. The author is also grateful to all the staffs of National Institute of Biotechnology for their logistic support and to the Department of Medicine and Public Health Sher-e-Bangla Agricultural University Sher-e Bangla Nagar, Dhaka-1207, for their co- operation. The author deeply owes his whole hearted thanks to all the relatives, friends, well-wishers for their help and inspiration during the period of the study. Finally author is grateful to the Sher-e-Bangla Agricultural University Research System (SAURES) for partially funding this work.

The author takes the opportunity to express his indebtedness and profound respect to his beloved father Md. Shahidul karim, mother Afroza Begum, for their love, blessings, prayers, sacrifices, moral support and encouragement for his study which can never be forgotten.

The Author

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LIST OF ABBREVIATION AND SYMBOLS

ABBREVIATION	FULL WORD
AMP	Ampicillin
AMX	Amoxicillin
Approx.	Approximately
AZM	Azithromycin
BG	Brilliant Green
CFU	Colony Forming Unit
CFM	Cefixime
CIP	Ciprofloxacin
CLSI	Clinical & Laboratory Standards Institute
COT	CoTrimoxazole
CTR	Ceftriaxone
CNF-1	Cytotoxic necrotizing factor 1
DNA	Deoxyribonucleic acid
EMB	Eosin Methylene Blue
E	Erythromycin
et al.	and others
ESBL	Extended-Spectrum Beta-Lactamase
EX	Enrofloxacin
E. coli	*Escherichia coli*
etc.	Etcetra
Fig.	Figure
GEN	Gentamicin
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen Sulphide
hlyA	Hemolysin A
hrs.	Hours
IN	Intermediate
K-1	Protein K-1
Lbs.	Pound
Ltd.	Limited
LEV	Levofloxacin
MC	MacConkey
Mg	Milligram
MH	Muller Hinton
Ml	Millilitre
Mm	Milimeter
mon.	Month
Min.	Minute
MR	Methyl Red
No.	Number

LIST OF ABBREVIATION AND SYMBOLS (CONT'D)

ABBREVIATION	FULL WORD
PCR	Polymerase chain Reaction
PBS	Phosphate buffered solution
R	Resistant
Rpm	Revolutions Per Minute
S	Sensitive
SAU	Sher-e-Bangla Agricultural University
spp.	Species
SS	Salmonella Shigella
SLT	Shiga-like toxin
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
TET	Tetracycline
TSI	Tripple Sugar Iron
UTI	Urinary tract infection
UV	Ultraviolet
V	Voltage
VP	Voges-Proskauer
yrs.	Years
ºC	Degree Celsius
-	Negative
%	Percentage
~	Tilde
+	Positive
®	Registard trade mark

ABSTRACT

The poultry industry in Bangladesh is considered an important sub-sector for economic growth and employment. Chicken and pigeon both are raised in the farm and sold in the live bird markets. This activity, therefore, may pose potential threats for humans as well as other animals as poultry may carry and spread different pathogens including drug-resistant bacteria. This work concentrated on the isolation and identification of multi-drug resistance *E. coli* and *Salmonella* spp. from chicken and pigeon.Forty five cloacal samples were collected from 45 birds (chicken and pigeon) during the study. *E. coli* and *Salmonella* spp. were isolated and identified on various types of agars. Biochemical tests were also performed. Besides, the presence of *E. coli* and *Salmonella* spp. were further confirmed by polymerase chain reaction (PCR).Moreover, antimicrobial susceptibility testing of the isolates was performed against eleven antibiotics from seven classes on the Kirby–Bauer disk diffusion method. The overall prevalence of *E. coli* and *Salmonella* spp. was 84.45 and 53.33%, respectively. The antibiogram profile of 38 *E. coli* as well as 24 *Salmonella* spp. revealed that all isolates were resistant to more than antibiotics. Hundred percent (100%) of *E. coli* showed resistance against amoxicillin, ampicillin and erythromycin. Furthermore, *E. coli* found sensitive against ceftriaxone and gentamicin. Similarly, around 100% of the *Salmonella* spp. showed resistance against amoxicillin, ampicillin and Co-Trimoxazole, respectively.However, 66.67% and 58.33% *Salmonella* spp. were found to show sensitivity against gentamicin and ceftriaxone. However, all isolated *E. coli and Salmonella* spp. were Multidrug-resistant (MDR). Poultry species carrying MDR *E. coli* and *Salmonella* spp. may contribute to the transmission and spread of these microorganisms. Therefore, stricthygienic measures should be taken during the farming & selling to decrease the potentialtransmission of *E. coli* and *Salmonella* spp. from chicken and pigeon to humans as well as other animals.

CHAPTER 1

INTRODUCTION

The poultry industry in Bangladesh is considered as an important sub-sector in economic growth and creating employment opportunities (Hamid et al., 2017) Chicken is the most popular meat product in both developed and developing countries worldwide. According to the Global Livestock Counts survey, there are approximately 19 billion chickens in the globe (Mpundu et al., 2019). During the previous decades, the poultry business in Bangladesh increased at a pace of 20% per year (Boddington, 2007), and it is today one of Bangladesh's fastest expanding agro-based enterprises (FEB, 2015). Chicken flesh is labeled as white meat because of its low iron level and lack of trans-fat which distinguishes it from other meats such as lamb and cattle. Furthermore, the absence of trans-fats makes it a healthier alternative because they are linked to cardiovascular disease, whereas beef and lamb meat have a large quantity of trans-fat (da Silva, 2019). Broiler chicken, with a current population of 525 million birds, is the most produced and consumed variety in the poultry industry. Sonali chicken is a regional type with a high meat content that has been the fastest growing segment in recent years (Poultry sector study Bangladesh, 2020). Pigeons are a good source of animal protein. Pigeon farming involves relatively little investment, less care, less feed and housing costs, simple and cost-effective husbandry procedures, a short reproductive cycle, and less illness recurrence. Pigeons and decorative birds are employed for natural aesthetics and as a source of pleasure. They provide tasty, delectable, and easily digestible animal protein. However, nowadays, pigeons are mostly raised for family nutrition and recreation (Asaduzzaman et al., 2009). In comparison to other Asia-Pacific markets, Bangladesh's poultry sector is comparatively underdeveloped in practically all stages of the value chain, with the absence of a professional downstream segment (slaughtering, further processing, and cold chain logistics infrastructure standing out). Modern slaughtering is limited to Dhaka and Chittagong (representing only 2-3% of total broiler consumption in Bangladesh), with the other birds butchered manually at wet markets. (Poultry sector study Bangladesh, 2020). Food animals have been identified as a cause of foodborne diseases in humans as well as a reservoir of resistant bacteria (Szmolka and Nagy, 2013). The food chain cycle serves as a conduit for antibiotic-resistant pathogenic pathogens to

spread from agricultural animals to people (Molbak et al., 2002). Poultry environment like soil, faeces, litters and wastes (Hemen et al., 2012; Igbinosa, 2014; Khan et al., 2014),live, and dead chickens (Hossain et al., 2008), meat, carcass, viscera, eggs, and poultry by-products (Haziyamin & Ezureen, 2011; Bhandari et al., 2013; Adeyanju & Ishola, 2014; Laban et al., 2014) could also carry microbes of public and veterinary health importance (antibiogram profile paper). The predominant bacteria found in the intestines of both animals and humans, *Escherichia coli* and *Salmonella*, serve as an indicator of fecal contamination in food and water bodies (Mpundu, 2019). Chicken contaminated with *E. coli* demonstrates poor hygienic practices in slaughterhouses and trading areas (Mpundu, 2019). According to the European Food Safety Authority's (EFSA) assessment, Campylobacter spp. is a major food-borne hazard linked with poultry meat, owing to cross contamination during processing in contaminated broilers and the packaging of ready-to-eat meals (EFSA, 2010). Feral and domestic pigeons (Columba livia) are not harmless birds. Many possible human infections exist silently in pigeons, with the potential to transmit over 30 diseases to humans and another 10 to domestic animals. Pigeon droppings are clearly responsible for environmental degradation. Air pollution is caused by more than only harmful gases emitted by automobiles and smokestacks. Mycotic, bacterial, protozoal, chlamydial, rickettsial, and parasitic illnesses, as well as dermatosis, have been reported in feral pigeons (Weber, 1979). Live Bird markets (LBMs) are the most important terminal hubs for the poultry industry in Asian countries, where people buy live or freshly slaughtered birds (Sarker et al., 2019). Pigeons in LBMs come from many sources and territories, and they are kept in tight places at high densities. Furthermore, clients come into close and direct contact with live or processed chicken at LBMs (Li et al., 2017). *Escherichia coli* (*E. coli*) is regarded as a natural component of the microbiota of all warm-blooded animals, including poultry (Kaper et al., 2004). However, in immunocompromised or debilitated hosts, or when gastro-intestinal barriers are breached, even regular "non-pathogenic" strains of *E. coli* can cause infection in poultry, humans, and animals. Furthermore, certain *E. coli* strains known as avian pathogenic *E. coli* spread into multiple internal organs and cause colibacillosis, a systemic deadly disease (Nakazato et al., 2009). In poultry, diseases caused by *E. coli* include yolk sac infection, omphalitis, respiratory tract infection, septicaemia, polyserositis, enteritis,

cellulitis, and salpingitis (Lutful, 2010). Avian Pathogenic *E. coli* (APEC) is an extra-intestinal pathotype composed of strains that frequently cause economic losses in poultry. However, because of their genetic similarities to Uropathogenic *E. coli* (UPEC) strains, which cause urinary infections in humans, these strains may pose a zoonotic concern (Rodriguez-Siek et al., 2005; Ewers et al., 2007 and Mora et al., 2013). *Salmonella* is the most common foodborne disease and has long been recognized as an important zoonotic bacteria with economic implications in both animals and humans (Carrasco et al., 2012). *Salmonella* agents that cause human infection are more prevalent in chicken than in other animal species (Foley et al., 2011). As a result, poultry products could be a source of a diversified microbial community, such as *Salmonella* enterica, the causative agent of Salmonellosis (Barua et al., 2014). Though there are several contributing factors for a Salmonellosis outbreak in humans, such as consumption of raw or unsafe food, cross-contamination, poor personal hygiene, and so on, consumption of chicken products (e.g. meat, liver, and eggs) is considered the primary route of *Salmonella* transmission into the human food chain (Karim et al., 2017). *Salmonella* ranks first in all cases associated to food consumption in poultry-derived food-borne outcomes (Manoj et al., 2015). From 1999 to 2008, *Salmonella* was responsible for 1335 food-borne outbreaks reported to the Food Disease Outbreak Surveillance System, and poultry products were responsible for a higher percentage of *Salmonella* outbreaks of infection than other food commodities (Nair & Johny, 2019). In poultry farming, the use of antibiotics has expanded productivity by efficiently managing infectious disease and improving bird growth, allowing the industry to meet increased consumer demands while also providing safe and inexpensive products (Kassem et al., 2016). However, unwise antibiotic usage has been linked to food-borne epidemics, where the etiological agents have been identified as resistant clones (Nair et al., 2018). The rise and spread of resistance infections, as well as the corresponding decline in antibiotic efficacy, constitute a direct threat to public health and sustainable agricultural (Kassem et al., 2016). The spread of such resistant strains in food animals is dangerous since they are frequently untreatable with currently available antimicrobials (Davis et al., 2011). The presence of extended-spectrum β-lactamases (ESBL) in *E. coli* and *Salmonella* spp. have exacerbated the worldwide circumstance of AMR in view of their capacity to hydrolyze and inactivate β-lactam antibiotics, including third and fourth

generation cephalosporins, which are generally used to treat serious infections caused by members of the Enterobacteriaceae family (Bush et al., 2018; Su et al., 2003). This study concentrated to determine the AMR pattern of common foodborne bacteria in cloacal swab of broiler, sonali and pigeon are raised in SAU poultry farm and sold in selected live bird markets of Dhaka city

Objective of the Investigation:

1. Molecular identification *Escherichia coli* & *Salmonella* spp. harbors in chicken and pigeon raised in SAU poultry farm and sold in selected live bird markets of Dhaka city.

2. To ascertain the sensitivity pattern of the isolated bacteria having public health significance.

CHAPTER 2

REVIEW OF LITERATURE

Isolation, characterization and drug sensitivity determination of the *Escherichia coli* and *Salmonella* spp. isolated from cloacal swabs of chicken (Broiler & Sonali) and pigeon was performed using the information gained from the following related review of literature.

2.1 Poultry industry in Bangladesh

Bangladesh is seeing the fastest rate of urbanization in Asia, and cities house the majority of the Middle and Affluent Consumer (MAC) population, resulting in a high concentration of disposable income. Consumption of animal-based protein (poultry meat, eggs, beef, milk, and fish) is predicted to rise significantly over the next ten years as disposable incomes rise. Bangladesh's poultry industry is expected to grow at a similar rate. In the next five years, per capita yearly consumption of poultry meat and eggs is predicted to rise by 26% and 41%, respectively. Adding the predicted population growth (CARG 1.2%) to the expected consumption growth rates yields a total estimated poultry meat industry growth of 34% in the Bangladeshi layer sector and 49% in the Bangladeshi meat sector. Broiler chicken, with a current population of 525 million birds, is the most produced and consumed variety in the poultry industry. Sonali chicken is a regional type with a high meat content that has been the fastest growing segment in recent years. The local Deshi chicken has the strongest customer perception and thus the highest price. Bangladesh currently produces roughly 15.5 billion eggs each year. In comparison to other Asia-Pacific markets, Bangladesh's poultry sector is comparatively underdeveloped in practically all stages of the value chain, with the absence of a professional downstream component standing out (slaughtering, further processing and cold chain logistics infrastructure). Modern slaughtering (birds slaughtered in modern slaughterhouses) is limited to Dhaka and Chittagong (representing only 2-3% of total broiler consumption in Bangladesh), with the other birds butchered manually at wet markets. Wet markets are also the most major distribution channel for eggs, with over 90% (about 14 billion eggs sold annually) distributed there, with the remainder marketed through modern methods (Poultry sector study Bangladesh, 2020).

2.2 Domestic & Feral Pigeon

While many of the over 300 species of pigeons and doves (family Columbidae) are kept as pets, the term "domestic pigeon" mainly refers to Columba livia types, commonly known as the rock pigeon. Pigeon breeding is a popular hobby all around the world, with over 350 different breeds officially recognized. Their millennia-long affinity with humans spans literature, visual arts, and religious symbols, and they have also acted as staunch messengers during war and peacetime. Domestic pigeons, like dogs, chickens, and other domesticated animals, reflect a large-scale selection experiment that began thousands of years ago. Domestication entails extensive directional selection for specific phenotypes, followed by stabilizing or purifying selection; comparable evolutionary processes occur in natural selection sweeps and sexual selection.. These processes have resulted in pigeons having the most phenotypic variety of any single avian species. Pigeon face morphology, for example, ranges from the tiny beak of an African owl to the huge, recurved beak of a Scandaroon. Many breeds, like as the Jacobin type preferred by Queen Victoria, have ornate feather embellishments. Body mass extremes differ by an order of magnitude between breeds. Except for Antarctica, the rock pigeon's current geographic range encompasses all continents. Rock pigeons are feral both outside and within their native area. That is, they are free-roaming domestics' offspring, and some Old World feral populations are likely thousands of years old. According to molecular data, the racing homer (Michael and Eric, 2013).

2.3 Microorganisms and Birds

The intricate interactions between birds and microbes are increasingly becoming the focus of ecological research (Maul et al., 2005). Microbial interaction with birds can occur in a variety of ways. Some are commensals, meaning they live in the host as part of the natural feather or gut flora without causing any harm. Several are avian diseases, either by need (e.g., Chlamydia psittaci) or by chance (e.g., Pseudomonas aeruginosa). Some microorganisms, notably fungi like Cladosporium and Epicoccum, may be allergies (Hubalek, 1978). Both pathogenic and allergenic species can impair fitness, rendering individuals more vulnerable to competition and predation, while severe infections/reactions are major causes of death (Nuttall, 1997). Microbes, on the other hand, can be beneficial; for example, Enterococcus faecium has been found to increase

the fitness of pied flycatcher (Ficedula hypoleuca) nestlings (Moreno et al., 2003), while Eupenicillium javanicum contains the cyclic depsipeptide, eujavanicin A, the antifungal properties of which are effective against Aspergillus fumigatus (Nakadate et al., 2008). *Salmonella* species, *Escherichia coli*, Enterobacter aerogenes and Enterobacter cloacae, Klebsiella pneumoniae, Proteus mirablis, and Providencia alcalifaciens are all pathogenic to humans (Vilcins et al., 2002).

2.4 *E. coli* as a source of infection

Overview of *E. coli*

Escherichia coli is a Gram-negative, facultative anaerobe Enterobacteriaceae bacterium (Nhung, Chansiripornchai, & Carrique-Mas, 2017). *E. coli*'s primary and secondary habitats are the intestines of warm-blooded animals and the environment. Although many *E. coli* strains are commensals, a fraction has developed the capacity to induce intestinal and extraintestinal illnesses (Stromberg et al., 2017). The innocuous strains are part of the regular flora of the gut and can benefit their hosts by creating vitamin K2 and preventing harmful bacteria from colonizing the intestine, forming a symbiotic connection. *E. coli* is released into the environment through feces.. Under aerobic conditions, the bacteria develops rapidly in new fecal matter for three days before progressively declining. *E. coli* and other facultative anaerobes account for around 0.1% of gut flora. The primary pathway through which pathogenic strains of the bacteria cause disease is fecal-oral transfer. The bacterium can be quickly and cheaply cultivated in a laboratory setting and has been extensively researched for over 60 years. *E. coli* is a chemoheterotrophic organism that requires a carbon and energy source in its chemically specified medium. *E. coli* is the most commonly studied prokaryotic model organism and an important species in biotechnology and microbiology, where it has acted as the host organism for the majority of recombinant DNA research.

Prevalence of *E. coli*

E. coli, like *Salmonella*, is one of the most common pathogens responsible for bacterial illnesses in broilers. Almost always, this pathogen is separated from the environment in which broilers are grown. The overall frequency of *E. coli* was estimated to be 62.5% in

a research conducted on broiler farms in Dinjpur, Bangladesh, with the prevalence in feeds and litter being 37.5% and 87.5%, respectively (Islam et al., 2014).

E. coli Pathogenesis and Virulence

Most *E. coli* strains do not cause disease, but virulent strains do in both animals and humans. *E. coli* is found in both poultry and humans. *E. coli* colonizes the lower digestive tract within the first 24 hours of hatching or birth (Ballou et al., 2016). Despite the fact that many E. Although most *E. coli* are innocuous, some have developed the ability to cause intestinal and extraintestinal illnesses. Extraintestinal pathogenic E. ExPEC strains produce a variety of infections in humans and animals outside the digestive tract (Mellata, 2013a). ExPEC are classified as neonatal meningitis *E. coli* (NMEC), Sepsis associated *E. coli* (SEPEC), Uropathogenic *E. coli* (UPEC), which cause new-born meningitis, sepsis, and urinary tract infections (UTI), and Avian pathogenic *E. coli* (APEC), which primarily causes respiratory and systemic disease in poultry (Stromberg et al., 2017). Although *E. coli* is naturally prevalent in the digestive system and other human mucosal surfaces, strains with specific virulence characteristics known as APEC can cause sickness (Dho-moulin et al., 2016). APEC is most commonly connected with respiratory tract or systemic infections that result in a variety of diseases that cause significant economic losses. APEC are the agents that cause colibacillosis, which is known to inflict significant economic losses in chicken (Kabir, & Lutful, 2010). A range of virulence factors, including adhesions, iron acquisition systems, and hemolysins, have been implicated in the spread of various extraintestinal illnesses in avian species (Cooke & Ewins, 2004). Toxins and antibacterial factors (outer membrane A, protein for improved serum survival, lipopolysaccharide, K-1 capsule, and colicin synthesis) (Silver et al., 1981).

2.5 *Salmonella* as a source of infection

Classification of *Salmonella*

Salmonella is a genus of the Enterobacteriaceae family. *Salmonella* enterica and *Salmonella* bongori are the two species of *Salmonella*. *Salmonella* bongori is only found in cold-blooded animals, primarily reptiles. *Salmonella* enterica is further classified into six subspecies, with about 2,600 serotypes (Gal-Mor, Boyle, & Grassl, 2014). *Salmonella*

enterica subspecies can be found in all warm-blooded animals and the environment globally. *Salmonella* serotypes are classified into two types: typhoidal and nontyphoidal. Non-typhoidal serotypes are more common, causing self-limiting gastrointestinal illness. They affect a variety of species and are zoonotic, which means they can be passed from people to other animals. *Salmonella* typhi and *Salmonella* paratyphi A are typhoidal serotypes that are unique to humans and do not exist in other animals.

Diagnostic and Phenotypic Characteristics of *Salmonella*

Salmonella is a Gram-negative facultative anaerobe with a rod form that belongs to the Enterobacteriaceae family (Barlow & Hall, 2002). *Salmonella* species are motile enterobacteria with peritrichous flagella that do not generate spores. They are facultative aerobes and chemotrophs that get their energy from organic oxidation and reduction reactions.

Prevalence of *Salmonella*

Salmonella is commonly found in poultry and is a major source of human gastrointestinal illnesses. *Salmonella* isolated from the caeca of Ecuadorian broilers at slaughter age was found to be 16%. (VinuezaBurgos et al., 2016). Yet, according to a study conducted in northern Thailand farming villages, the prevalence of *Salmonella* in chicken cloacal swabs was determined to be 1%. (Hanson, Kaneene, Padungtod, Hirokawa, & Zeno, 2002). Furthermore, in a research on broiler farms in Bangladesh's Dinajpur area, the total prevalence of *Salmonella* was observed to be 49.91%, with the prevalence in feed and litter being 29.16% and 66.66%, respectively (Islam, Islam, & Fakhruzzaman, 2014).

Pathogenesis and Virulence

Salmonella serotypes can cause infections in humans as well as poultry (Steve Yan et al., 2004). Salmonellosis is a serious bacterial infection caused by a variety of *Salmonella* serotypes. It is linked to many deaths and economic losses in broiler production (Haider et al., 2009). Many different *Salmonella* serovars can infect chickens; some, like S. Pullorum and S. Gallinarum, are host-specific for chickens, whilst others, like S. Typhimurium, S. Enteritidis, and S. Heidelberg, can infect a wide variety of hosts. *Salmonella*'s pathogenicity is determined by its capacity to penetrate, proliferate, and survive in cells. *Salmonella* bacteria enter the body through the mouth. While inside the

colon, they multiply and some adhere to the mucosal microvilli via adhesion (Humbert & Salvat, 1997). Upon attachment, the microvilli degenerate, resulting in breaches in the cell membrane via which *Salmonella* enters and multiplies. Bacteria then infiltrate the cecal tonsils and Peyer's patches, where they are absorbed by macrophages. Macrophagesgo to other organs in the body such as the liver and spleen via the circulation and/or the lymphatic system. The bacteria multiply even more in these organs (Barrow, Huggins, & Lovell, 2014). *Salmonella*'s main virulence factor is endotoxin generation, and the local reaction is enteritis and gastrointestinal problems (Iushchuk & Tendetnik, 2013).

2.6 Antibiotic Resistance

"Antibiotics" are a class of antimicrobial agents produced by microorganisms such as bacteria or fungus that have the ability to hinder the growth of other microorganisms (bacteria). Although the terms antibiotics and antimicrobials are sometimes used interchangeably, antibiotics refer to naturally occurring biomolecules, whereas antimicrobials include both naturally existing and synthetically generated compounds. Antimicrobials are any substances that work against microorganisms of any type, including bacteria (antibacterial), viruses (antiviral), fungi (antifungal), and protozoa (antiprotozoal). Antibiotics are commonly used in humans and animals to prevent and treat numerous illnesses. Antibiotics are also utilized as growth promoters in the animal food industry, where their inclusion in feed promotes animal growth and product quality (Cheng et al., 2014). As a result, antibiotics are widely employed in the intensive and large-scale farming industries. However, their indiscriminate and irrational use in various fields such as agriculture, fisheries, livestock industry, and so on has resulted in the development of resistant bacteria, which results in the spread of resistance through the transfer of its resistant determinants to other bacteria (Stanton, 2013). The selective evolutionary pressure from the environment or antimicrobial agents is the driving force that induces resistance in microorganisms to specific agents, and it has been observed that there is an association between the indiscriminate use of antimicrobial agents and the occurrence of resistance. Resistance in pathogenic microorganisms renders antimicrobial agents not only ineffective, but also makes it difficult to treat many previously manageable bacterial diseases. As a result, a number of alternatives/replacements have

been recommended to address the increased rate of death and morbidity caused by antibiotic resistance (Seal et al., 2013).

2.7 Mechanism and origin of antibiotic resistance

Antibiotic resistance is the ability of a bacterium or other microorganism to survive and reproduce in the presence of antibiotic doses that were previously thought effective against them. Different mechanisms are known to enhance the antimicrobial resistance. Microbes could be intrinsically resistant and may lack a target for the antibiotics (Bradford, 2001). Chlamydiae do not have peptidoglycan and are not susceptible to the action of penicillins. The antibiotic target may be inaccessible. Membrane changes block antibiotic entrance and penetration into the cell. Peptidoglycan in Gram-negative bacteria is inaccessible to penicillins that cannot penetrate the Gram-negative outer membrane. Efflux pumps can actively pump out antibiotics from cells. Gram-negative bacteria resist the activity of tetracyclines by this important mechanism (Liaw and Hsueh, 2010). The antibiotic target may be modified to prevent the action of the drug: Ribosomes become altered, mutated, and chemical-physical changes prevent antibiotic attachment to those ribosomes. By synthesis of a new metabolic pathway bacteria can produce a new enzyme that is not inhibited by the antimicrobial. Trimethoprim-sulfamethoxazole resistance is due to bacteria that produce a new dihydrofolate reductase not inhibited by trimethoprim and a new dihydropteroate synthetase not susceptible to sulfonamides. Quinolone resistance is affected by point mutations in the DNA gyrase, which prevent binding of the drug to its target (John and Haller, 2007; Hidalgo-Grass and Blondeau, 2004; Jacquet et al., 2008; Marquez, 2005; Alcaide and Esteve, 2010; Liaw and Hsueh, 2010; Wang et al., 2009; Mlynarczyk et al., 2010).

2.8 Antibiotic resistance in bacterial pathogens

It is the ability of bacteria to resist the effect of an antibiotic that it was previously sensitive to. Antibiotic resistance is a challenge due to increased antibiotic-resistant infections in both animals and humans (Prestinaci et al., 2015). The widespread use of antibiotics is one of the main reasons for the occurrence of antibiotic-resistant strains in the environment (Barton, 2000). In poultry production particularly in Wakiso district, there has been intense use of antibiotics where 96.7% of farmers were reported to use antibiotics

on their farms and 33.3% of them used as growth promoters. Tetracycline (73.3%) were the most used followed by sulphonamides (26.7%) (Bashahun GM & Odoch, 2015). Other commonly used antibiotics are ciprofloxacin, gentamicin, chloramphenicol, and nitrofurans. Due to repeated exposure to the antibiotics, bacterial resistant strains have evolved and the use of antibiotics could be one of the reasons for the emergence of bacterial resistant strains. Some bacteria are naturally resistant to bacteria. However, bacteria may become resistant through genetic mutation or by acquiring resistance from other bacteria. Mutations are spontaneous genetic changes that may lead to the evolution of antibiotic-resistant genes within a bacterial genome (Eng et al., 2015). These genes may be responsible for the production of enzymes that inactivate the antibiotic, eliminate the antibiotic target from the bacteria, close up entry ports for the bacteria or even manufacture pumping mechanisms that eliminate the antibiotic from the cell thus the antibiotic never reaches the target. Bacteria may acquire antibiotic-resistant genes from other bacteria through transformation, conjugation and transduction. In transformation, naked DNA is passed to the recipient. Transduction takes place where a bacteriophage transmits the antibiotic-resistant traits into the bacteria. The traits are packed in the head of the virus and then it injects them into the recipient bacteria. In conjugation, the bacteria can transfer genetic material, including antibiotic-resistant genes (found on plasmids and transposons) from bacteria to another via a bridge formed during cell to cell contact. Bacteria like *Salmonella* and *E. coli* have developed different mechanisms by which they can resist to a commonly used antibiotic such as alteration of an antibiotic agent, a mutation in the target site, decreased uptake and increased efflux.

2.9 *Escherichia coli* as the model for antibiotic resistance studies

Role of *E. coli* in relation to its hosts is diverse varying from commensalism to an established pathogen. *E. coli* is classified on the basis of pathogenesis into three major groups: commensal strains, intestinal pathogenic strains, and extra-intestinal pathogenic strains (Russo and Johanson, 2000). It is amongst the first organisms to populate and colonize the gut micro-flora in the neonates (Lozupone et al., 2012; Tenaillon et al., 2010). The primary habitat of *E. coli* is the lower intestinal tract with which it typicallyestablishes commensal associations. It has been estimated that half of the living *E. coli* cells are outside their host, in their secondary habitat (Savageau, 1983). Beside these

habitats, certain strains have the potential to cause a wide spectrum of intestinal and extra-intestinal diseases such as urinary tract infection, septicaemia, meningitis, and pneumonia in humans and animals. With its large range of pathologies, *E. coli* is the major cause of morbidity and mortality around the world. Each year *E. coli* causes more than two million deaths due to infant diarrhoea (Kosek et al., 2003) and extra-intestinal infections (mainly septicaemia derived from urinary tract infection), and is also responsible for approximately 150 million cases of uncomplicated cystitis (Russo and Johnson, 2003). Resistance in *E. coli* is consistently highest for antimicrobial agents that have been in use the longest time in human and veterinary medicine (Tadesse et al., 2012). The past 2 decades have witnessed major increase in emergence and spread of multidrug-resistant bacteria and increasing resistance to newer compounds, such as fluoroquinolones and certain cephalosporins (Levy and Marshall, 2004).

2.10 Public Health Concern for poultry Production Practices

Poultry is considered to be a reservoir of *E. coli* and *Salmonella* capable of causing infections in humans. In an unfortunate linkage, chicken products are suspected to be a source of foodborne ExPEC and *Salmonella* infections in humans. Furthermore, there has been the emergence of multidrug resistance (MDR) (resistance to three or more classes of antimicrobial agents) among avian *E. coli* that have created major economic and health concerns, affecting both human healthcare and poultry industries (Mellata, 2013). The use of antibiotics in poultry production will select for drug-resistant bacteria. Among the various uses for antibiotics, low-dose, prolonged courses of antibiotics among food animals create ideal selective pressures for the propagation of resistant strains. The spread of resistance may occur by direct contact or indirectly, through food, water, and animal waste application to farm fields (Marshall & Levy, 2011). The first evidence was reported by Levy et al., who isolated the same tetracycline resistant *E. coli* strains from the gut flora of chicken caretakers as in the chicken feed on tetracycline supplemented feeds (Levy, Fitzgerald, & Macone, 1976). Another study among US poultry workers revealed the risk of carrying gentamicin resistant *E. coli* was 32 times more in poultry workers than in other people in the community (Price et al., 2007).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Cloacal swab samples were collected three different places namely Sher-e-bangla Agricultural university poultry farm and Townhall market & Krishi market Live Bird Markets (LBM) from July to September 2021. 45 clocal samples (15 from each place) were aseptically from chicken (Broiler, Sonali) and Pigeon. As it is already mentioned in literature section the *Salmonella* spp. and *E. coli* are the predominant microbes of GI tract, thus the cloacal samples were selected. (Figure 1).

Table 1: Samples collected from different species of poultry and places

Sample No. & Name	Collection zone	Sample source	Sample type
1. B1	SAU Poultry farm, Dhaka	Broiler	Cloacal swab
2. B2	SAU Poultry farm, Dhaka	Broiler	Cloacal swab
3. B3	SAU Poultry farm, Dhaka	Broiler	Cloacal swab
4. B4	SAU Poultry farm, Dhaka	Broiler	Cloacal swab
5. B5	SAU Poultry farm, Dhaka	Broiler	Cloacal swab
6. S1	SAU Poultry farm, Dhaka	Sonali	Cloacal swab
7.S2	SAU Poultry farm, Dhaka	Sonali	Cloacal swab
8. S3	SAU Poultry farm, Dhaka	Sonali	Cloacal swab
9. S4	SAU Poultry farm, Dhaka	Sonali	Cloacal swab
10. S5	SAU Poultry farm, Dhaka	Sonali	Cloacal swab
11. P1	SAU Poultry farm, Dhaka	Pigeon	Cloacal swab
12.P2	SAU Poultry farm, Dhaka	Pigeon	Cloacal swab
13.P3	SAU Poultry farm, Dhaka	Pigeon	Cloacal swab
14.P4	SAU Poultry farm, Dhaka	Pigeon	Cloacal swab
15.P5	SAU Poultry farm, Dhaka	Pigeon	Cloacal swab
16. B6	Townhall Market, Dhaka	Broiler	Cloacal swab
17. B7	Townhall Market, Dhaka	Broiler	Cloacal swab
18. B8	Townhall Market, Dhaka	Broiler	Cloacal swab
19. B9	Townhall Market, Dhaka	Broiler	Cloacal swab
20. B10	Townhall Market, Dhaka	Broiler	Cloacal swab
21.S6	Townhall Market, Dhaka	Sonali	Cloacal swab
22.S7	Townhall Market, Dhaka	Sonali	Cloacal swab

Table 1 cont'd			
23.S8	Townhall Market, Dhaka	Sonali	Cloacal swab
24.S9	Townhall Market, Dhaka	Sonali	Cloacal swab
25.S10	Townhall Market, Dhaka	Sonali	Cloacal swab
26.P6	Townhall Market, Dhaka	Pigeon	Cloacal swab
27.P7	Townhall Market, Dhaka	Pigeon	Cloacal swab
28.P8	Townhall Market, Dhaka	Pigeon	Cloacal swab
29.P9	Townhall Market, Dhaka	Pigeon	Cloacal swab
30.P10	Townhall Market, Dhaka	Pigeon	Cloacal swab
31. B11	Krishi Market ,Dhaka	Broiler	Cloacal swab
32. B12	Krishi Market ,Dhaka	Broiler	Cloacal swab
33.B13	Krishi Market ,Dhaka	Broiler	Cloacal swab
34. B14	Krishi Market ,Dhaka	Broiler	Cloacal swab
35. B15	Krishi Market ,Dhaka	Broiler	Cloacal swab
36.S11	Krishi Market ,Dhaka	Sonali	Cloacal swab
37. S12	Krishi Market ,Dhaka	Sonali	Cloacal swab
38. S13	Krishi Market ,Dhaka	Sonali	Cloacal swab
39. S14	Krishi Market ,Dhaka	Sonali	Cloacal swab
40. S15	Krishi Market ,Dhaka	Sonali	Cloacal swab
41. P11	Krishi Market ,Dhaka	Pigeon	Cloacal swab
42.P12	Krishi Market ,Dhaka	Pigeon	Cloacal swab
43.P13	Krishi Market ,Dhaka	Pigeon	Cloacal swab
44.P14	Krishi Market ,Dhaka	Pigeon	Cloacal swab
45.P15	Krishi Market ,Dhaka	Pigeon	Cloacal swab

3.1.2 Bacteriological media

3.1.2.1 Agar media

Agar media used for bacteriological analysis were, MacConkey (MC) agar, Eosin Methylene Blue (EMB) agar, Brilliant Green (BG) agar, Salmonella shigella (SS) agar, and Muller Hinton (MH) agar.

3.1.2.2 Liquid media (broth)

The liquid media used for this study were Nutrient broth, Peptone broth, Methyl-Red and Voges-Proskauer broth (MR-VP broth) and Sugar media (dextrose, maltose, lactose, sucrose and mannitol).

3.1.2.3 Phosphate Buffered Saline (PBS)

For preparation of phosphate buffered saline, 8 gm of sodium chloride (NaCl), 2.89 gm of disodium hydrogen phosphate (Na2HPO4.12H2O), 0.2 gm of potassium chloride

(KCl) and 0.2 gm of potassium hydrogen phosphate (KH2PO4) were suspended in 1000 ml of distilled water. The solution was heated to dissolve completely and pH was adjusted with the help of pH meter. The solution was then sterilized by autoclaving and stored at 4ºC for future use.

3.1.3 Chemicals and reagents

The chemicals and reagents used for this study were 0.1% Peptone water, Phosphate buffered saline (PBS), reagents for Gram's staining (Crystal Violate, Gram's iodine, Safranin, Acetone alcohol), 3% Hydrogen peroxide, Phenol red, Methyl red, 10% Potassium hydroxide, Kovac's indole reagent (4-dimethylamino-benzaldehyde, concentrated HCL), Mineral oil, Normal saline and other common laboratory chemicals and reagents.

3.1.4 Glass wares and other appliances

The following glass wares and appliances were used during the course of the experiment. Test tubes (with or without Durham's fermentation tube and stopper), petridishes, conical flask, pipette (1 ml, 2 ml, 5 ml, 10 ml) & micro-pipettes (1ml, 200µl, 100µl, 10 µl) slides and cover slips, hanging drop slides, immersion oil, compound microscope, bacteriologicalloop, sterilized cotton, cotton plug, test tube stand, water bath, bacteriological incubator, refrigerator, sterilizing instruments, thermometer, ice carrier, hand gloves, spirit lamp, match lighter, laminar air flow, hot air oven, centrifuge tubes and machine, PCR machine, thermo scientific nanodrop spectrophotometer, UV transilluminator, Gel documentation machine electronic balance, syringe and needle, tray, forceps, scalpel, scissors etc.

3.1.5 Antimicrobial discs

Commercially available antimicrobial discs (Himedia, India) were used for the test to determine the drug sensitivity and resistance pattern and to interpret their disease potential. This method allowed for the rapid detection of the efficacy of drugs against the test organisms by measuring the diameter of the zone of inhibition that resulted from diffusion of the agent into the medium surrounding the discs inhibiting the growth of the organisms. The following antimicrobial agents with their disc concentration were used to

test the sensitivity and resistance pattern of the selected *E. coli* and *Salmonella* sp. Isolates from oral and cloacal swabs (Table 1) (CLSI, 2020)

Table 2. Drugs with their disc concentration for the Enterobacteriaceae family

Name of drugs	Disc concentration (µg /disc)	Zone Diameter Interpretive Standard (mm)		
		Resistant	Intermediate	Susceptible
AMP	25	≤13	14--17	≥18
AMX	30	≤13	14--17	≥18
GEN	10	≤12	13--14	≥15
CIP	5	≤20	21--30	≥ 31
E	15	≤13	14--17	≥18
CFM	5	≤19	20--22	≥23
CTR	30	≤14	15--18	≥19
AZM	30	≤19	20-24	≥25
LEV	5	≤13	14--16	≥17
EX	5	≤16	17--20	≥21
COT	25	≤10	11--13	≥16

Legend: µg = micro gram

3.2 Methods

3.2.1 Brief description of the experiment

The entire study was divided into two major steps: The first step included selection of sources, collection of samples, isolation, identification and characterization of microorganisms on the basis of their colony morphology, staining properties, motility and biochemical characteristics & molecular identification. In the second step, the current status of drug sensitivity and resistance pattern of microorganism isolated from chicken & pigeons was determined (Figure 2)

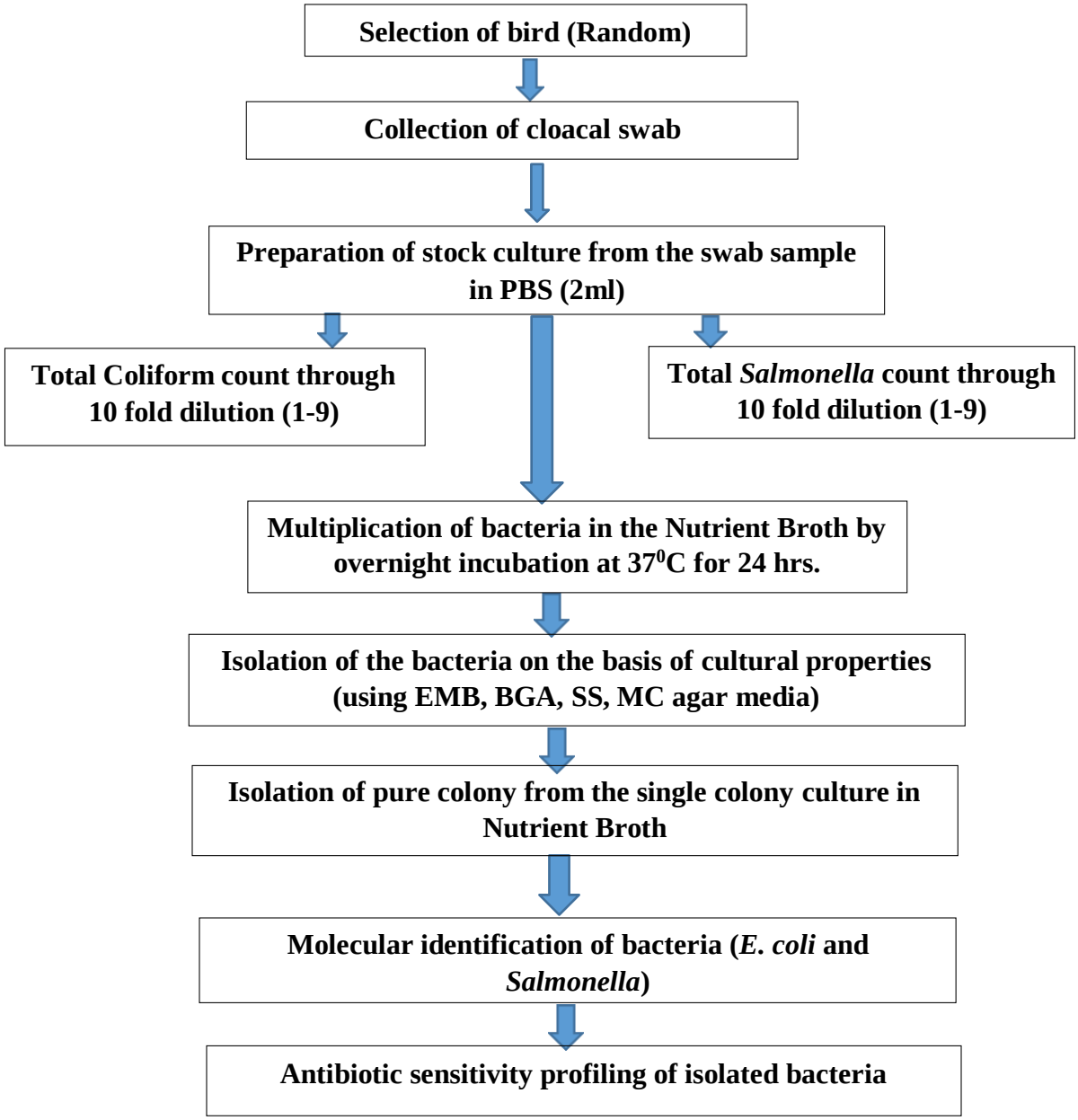

Figure 1: Layout of the Experiment

3.2.2 Preparation of various bacteriological culture media

3.2.2.1 Nutrient Agar

Nutrient agar was prepared by dissolving 28 grams of dehydrated nutrient agar (HiMedia, India) in to 1000 ml of distilled water and was sterilized by autoclaving at 121ºC under 15 lb pressure per square inch for 15 minutes. Then the agar was dispensed into petridish (90 mm and 100 mm) and was incubated at 37ºC for overnight to check their sterility and stored at 4ºC in the refrigerator until used.

3.2.2.2 Nutrient Broth

Nutrient Broth was prepared by Suspended 25 grams in 1000 ml purified/distilled water. Heat if necessary to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 30 minutes. The broth was filled in test tubes & incubated at 37ºC for overnight to check their sterility and stored at 4ºC in the refrigerator until used.

3.2.2.3 MacConkey's agar

49.53 grams of Bacto MacConkey agar (HiMedia, India) was suspended in to 1000 ml of cold distilled water and was heated for boiling to dissolve the medium completely. It was then poured in to sterile petridishes and allowed to solidify. After solidification of the medium in the plates, the plates were then incubated at 37ºC for overnight to check their sterility.

3.2.2.4 Eosine Methylene Blue (EMB) agar

Thirty six grams powder of EMB agar base (HiMedia, India) was suspended in 1000 ml of distilled water. The suspension was heated to boil for few minutes to dissolve the powder completely with water. The medium was autoclaved for 30 minutes to make it sterile. After autoclaving the medium was put in to water bath at 45ºC to cool down its temperature at 40ºC. From water bath 10-20 ml of medium was poured in to small and medium sized sterile petridishes to make EMB agar plates. After solidification of the medium in the plates, the plates were incubated at 37ºC for overnight to check their sterility.

3.2.2.5 Brilliant Green agar

According to the direction of manufacturer (HiMedia, India) 58 grams of dehydrated medium was suspended in 1000 ml distilled water and heated for boiling to dissolve the medium completely. The medium was sterilized by autoclaving. After autoclaving the medium was put in to water bath of 45ºC to decrease its temperature. After solidification of the medium in the petridishes, the petridishes were allowed for incubation at 37ºC for overnight to check their sterility and then stored at 4ºC in a refrigerator for future use.

3.2.2.6 Salmonella-Shigella agar

According to the direction of manufacturer (HiMedia, India) 60 grams of dehydrated medium was suspended in 1000 ml distilled water and heated for boiling to dissolve the medium completely. The medium was sterilized by autoclaving. After autoclaving the medium was put in to water bath of 50ºC to decrease its temperature. After solidification of the medium in the petridishes, the petridishes were allowed for incubation at 37ºC for overnight to check their sterility and then stored at 4ºC in a refrigerator for future use.

3.2.2.7 Mueller Hinton Agar

Suspended 38.0 grams in 1000 ml distilled water & Heated to boiling to dissolve the medium completely. After the sterilization by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cooling was done to 45-50°C. Then it was mixed well and poured into sterile Petridishes. After solidification of the medium in the petridishes, the petridishes were allowed for incubation at 37ºC for overnight to check their sterility and then stored at 4ºC in a refrigerator for future use.

3.2.2.8 Triple Sugar Iron (TSI) agar

In a flask, 65 g of dehydrated medium (Difco, USA) was mixed with 1000 ml cold distilled water and heated to boiling to completely dissolve the medium. The solution was dispensed through cotton-wrapped tubes. The tubes were then sterilized by autoclaving and tilted to provide for a generous butt. Following solidification, tubes were incubated overnight at 37^0C to ensure sterility. For three days, the sugar solutions were sterilized in Arnold's steam sterilizer at 1000C for 30 minutes. In each culture tube containing sterile peptone water, 0.5 ml of sterile sugar solution was added aseptically. To ensure sterility,

the sugar solutions were incubated at 37^0C for 24 hours. These options were employed.for biochemical test.

3.2.2.9 Methyl Red and Voges–Proskauer (MR-VP) broth

A quantity of 3.4 gm of MR-VP medium (HiMedia, India) was dissolved in 250 ml of distilled water, distributed in 2 ml quantities in test tube and then autoclaved. After autoclaving, the tubes containing medium were incubated at 37ºC for overnight to check their sterility and then stored at 4ºC for future use.

3.2.2.10 Sugar solutions

The medium consists of 1% peptone water to which fermentable sugars were added. Peptone water was prepared by adding 1 gram of Bacto peptone (Difco, USA) and 0.5 grams of sodium chloride in 100 ml distilled water, boiled for 5 minutes, adjusted to pH 7.6 by phenol red (0.02%) indicator, cooled and then filtered through filter paper. The solutions were then dispensed in 5 ml amount into cotton plugged test tubes containing invertedly placed Durham's fermentation tubes. Then the sugars, dextrose (MERCK, India), maltose (s.d. fiNE-CHEM Ltd.), lactose (BDH, England), sucrose (MERCK, India) and mannitol (PETERSTOL TENBEG) used for fermentation were prepared separately as 10 percent solutions in distilled water (10 grams sugar was dissolved in 100 ml of distilled water). A little heat was necessary to dissolve the sugar. These were then sterilized by autoclaving for 15 minutes. The sugar solutions were sterilized in Arnold's steam sterilizer at 100ºC for 30 minutes for three consecutive days. An amount of 0.5 ml of sterile sugar solution was added aseptically in each culture tubes containing sterile peptone water. The sugar solutions were incubated at 37ºC for 24 hours to check sterility. These solutions were used for biochemical test.

3.2.3 Isolation of bacteria

3.2.3.1 Collection, transportation and preparation of sample

Cloacal swab A number of 45 samples of cloacal swab were also collected through using sterile swab stick in 2 ml Eppendorf tube filled with PBS from the chicken and pigeon from Sher-e- Bangla Agricultural University poultry farm, Townhall and Krishi market, Dhaka. The collected samples were immediately carried to the laboratory in an ice box containing ice and processed for isolation and characterization of bacteria.

3.2.3.2 Serial dilution for bacterial culture (10 fold dilution method)

The stock sample was serially diluted to reduce the bacterial count for the total viable count (TVC) and total coliform count (TCC). That was accomplished by filling 8 (1-8) Eppendorf tubes with 900 µl of PBS. 100l of stock sample was transferred from the stock tube (2ml) to the adjacent Eppendorf tube. After that, 100l of diluted sample is moved from one Eppendorf tube to the next. The last tube should be diluted in the same way as the previous ones, and 100l of the diluted material should be discarded. To determine the total viable count and total coliform count, transfer 25 µl of liquid sample from the final tube to the nutritional Agar media and MacConkey agar. *Salmonella* were counted by transferring the same amount of liquid sample to Salmomella-Shigella agar.

3.2.3.3 Primary culture of microorganism

Primary growth of all kinds of bacteria present in the collected samples was performed in nutrient broth. The samples were inoculated in nutrient broth and incubated for overnight at 370C for the growth of the organisms.

3.2.3.4 Isolation in culture media

Following primary culture, a small amount of inoculums from Nutrient broth were streaked on MacConkey agar, Brilliant green agar, and Salmonella-Shigella agar to examine the colony morphology of the isolates. The organisms with characteristic colony shape showing *E.coli* were chosen for culturing on selective media such as EMB agar and Salomonella on Salmonella-Shigella agar. Morphological features (shape, size, surface texture, edge and elevation, color, opacity, etc.) of putative colonies grown on different agar media within 18 to 24 hours of incubation were meticulously recorded.

3.2.3.5 Microscopic study for identification of *E. coli* & *Salmonella* spp from the suspected colonies by Gram's staining method

Merchant and Packer (1976) recommended using Gram's staining to identify the size, shape, and arrangement of bacteria. The steps were as follows: A little colony was picked up with a bacteriological loop, smeared on a glass slide, and gently heated to fix it. The smear was then stained with crystal violet solution for two minutes before being cleansed under running tap water. Gram's iodine was then applied as a mordant for one minute before being rinsed with running water again. Acetone alcohol was then added as a

decolorizer. Following washing with water, safranine was used as a counter stain and stained for 2 minutes. The slide was then washed, blotted, and air dried before being inspected under a microscope with a high power objective (100X) and immersion oil. In a positive case, the organism *E. coli* was found to be gram negative, pink in color, and rod shaped, grouped alone or in pairs.

3.2.3.6 Motility test for *E. coli* & *Salmonella* isolates

The motility test was carried out in accordance with Cowan's 1985 approach to distinguish motile bacteria from non-motile bacteria. A pure culture of the test organism was allowed to develop in nutrient broth prior to the test. To prepare the hanging drop, one drop of cultured broth was poured on the cover slip and inverted over the concave depression of the hanging drop slide. To avoid air movement and evaporation of the fluid, Vaseline was applied around the concave depression of the hanging drop slide for better cover slip adhesion. The hanging drop slide was then thoroughly examined using immersion oil under a compound microscope's 100X power objective. The motile and non-motile organisms were identified by observing motility in contrasting with to and from movement of bacteria.

3.2.3.7 Reaction of the organism in TSI agar slant

After the organisms were isolated on the selective media, differential screening media like TSI agar were utilized for further characterisation. The test organisms were grown on TSI agar slant using either the stab or streak method. If the organism ferments solely glucose on An agar slant, the tube will turn yellow within a few hours. The bacteria rapidly deplete their limited supply of glucose and begin oxidizing amino acids for energy, producing ammonia as a byproduct. When amino acids oxidize, the pH rises and the indicator in the slanted portion of the tube turns red.. Because of the enhanced quantity of acid production, if the organism on the TSI agar slant ferments lactose and/or sucrose, the slant will turn yellow and remain yellow for several days. The development of bubbles in the agar indicates that the organisms are producing gas. TSI agar can also be used to determine whether sulphur-containing substances have formed hydrogen sulphide (H2S). H2S combines with the medium's ferrous sulphate to form ferric sulphide, which appears as a black precipitate. Yellow slant, yellow butt, gas bubbles, and the absence of black

precipitate in the butt are all positive for *E. coli*, while black precipitate in the butt is identical for *Salmonella* spp..

3.2.4 Identification of isolated *E. coli* & *Salmonella* spp. by using specific biochemical tests

Several biochemical tests were performed for confirmation of *E. coli* & *Salmonella* isolates.

3.2.4.1 Carbohydrate fermentation test

The carbohydrate fermentation test was performed by inoculating 0.2 ml of nutrient broth culture of the isolated organisms into the tubes containing different sugar media (five basic sugars such as dextrose, maltose, lactose, sucrose and mannitol) and incubated for 24 hours at 37ºC. Acid production was indicated by the color change from red to yellow and gas production was noted by the accumulation of gas bubbles in the inverted Durham's tube (Cheesbrough, 2006).

3.2.4.2 Catalase test

For this study 3 ml of catalase reagent (3% H_2O_2) was taken in a test tube. Single colony from the pure culture of *E. coli* was taken with a glass rod and merged in the reagent. The tube was observed for bubble formation. All of the isolates were catalase positive; formation of bubble within few seconds was the indication of the positive test, while the absence of bubble formation indicated negative result (Cheesbrough, 2006).

3.2.4.3 Methyl Red test

The test was conducted by inoculating single colony from the pure culture of the test organism in 5 ml sterile MR-VP broth. After 5 days incubation at 37ºC, 5 drops of methyl red solution was added and observed for color formation. Development of red color was positive and indicated an acid pH of 4.5-6 resulting from the fermentation of glucose. Development of yellow color indicated negative result (Cheesbrough, 2006).

3.2.4.4 Voges-Proskauer (V-P) test

The test *E. coli* organisms were grown in 3 ml of sterile MR VP broth at 37ºC for 48 hours. Then 0.6 ml of 5% alpha-napthol and 0.2 ml of 40% potassium hydroxide containing 0.3% creatine was added per ml of broth culture of the test organism. Then

shaking well and allowed to stand for 5-10 minutes to observe the color formation. Positive case was indicated by the development of a bright orange red color. In negative cases there was no development of pink color (Cheesbrough, 2006).

3.2.4.5 Indole test

The test organisms were cultured in test tubes having 3 ml of peptone water containing tryptophan at 37ºC for 48 hours. Then 1 ml of diethyl ether was added, shaked well and allowed to stand until the ether rises to the top. Then 0.5 ml of Kovac's reagent was gently run down the side of the test tube so that it forms a ring in between the medium and the ether layer and observed for the development of color of the ring. Development of a brilliant red colored ring indicated indole production. In negative case there is no development of red color (Cheesbrough, 2006).

3.2.4.6 Molecular identification of the isolated organism (*Escherichia coli* and *Salmonella* spp.):

3.2.4.7 Bacterial DNA isolation:

a) Materials:

i. TE buffer 10% (w/v) sodium dodecyl sulfate (DSS)

ii. 20 mg/ml proteinase K (stored in small single use aliquots -200C)

iii. 3 M sodium acetate, pH 5.2

iv. 25:24:1 phenol/chloroform/Isoamyl alcohol

v. Isopropanol

vi. 70% ethanol

vii. 90% ethanol

viii. 1.5 ml microcentrifuge tube

b) Procedure:

-Inoculate a 25 ml of liquid culture with the bacterial strain of interest. Grow in conditions appropriate for that strain until the culture is saturated.

-Spin 1.0 ml of overnight culture in a microcentrifuge tube for 5 minutes at 10000 rpm.

-Discard the supernatant.

-Repeat the step.

-Resuspended the supernatant in 467µl TE Buffer by pipetting. Add 30 µl of 10% SDS & 3µl of proteinase K to give a final concentration of 100µg/ml proteinase K in 0.5% SDS. Mix thoroughly and incubate 30min. to 1hr. at 370C.

-Add and approximately equal volume of (500µl) of phenol/chloroform/Isoamyl alcohol. Mix thoroughly but carefully to avoid the mixing of DNA, by inverting the tube until the phases are completely mixed.

-Then the centrifuge the tubes at 12000 rpm for 10 minutes.

-Remove aqueous, viscous supernatant (~450 µl) to a fresh microcentrifugetube, leaving the interface behind. Add an equal volume of phenol/chloroform/Isoamyl alcohol, extract thoroughly and spin in a micro centrifuge at 10000 rpm for 5 min.

-Transfer the supernatant to a fresh tube (~400 µl).

-Add 1/10th volume of 3m sodium acetate and mix.

-Add 0.6 volumes of isopropanol to precipitate the nucleic acids, keep on ice for 10 minutes. Centrifuge at 13500 rpm for 15 minutes.

-Decant the supernatant.

-Wash the obtained pellet with 1ml of 95% ethanol for 5 minutes. Then centrifuge at 12000 rpm for 10 minutes.

-Decant the supernatant.

-Dry the pellets as well as there are no alcohol.

-Resuspend the pellet in 50µl of TE and stored DNA at 4^0C for short time and -20^0C for long term.

c) Polymerase Chain Reaction (PCR):

d) Primer used for PCR (*E. coli* and *Salmonella* spp.)

A genus specific PCR was to perform to amplify 16S rRNA of E.coli using previously published primers (Tsen et al., 1998) the list of primers are shown in table The reverse primer TTCCCGAAGGCACATTCT is used with the forward GGGAGTAAAGTTAATCCTTTGCTC to identify the pathogenic organism. *Salmonella* specific primers described previously [Rahn et al., 1992], the forward primer GTG AAA TTATCGCCACGTTCGGGCAA and reverse primer TCATCGCACCGTCAAAGGAACC based on the invA gene of *Salmonella* were used.

Table 3: PCR primers with sequence

Primer	Sequence(5´-3´)	Size (bp)	Reference
E.coli 16E1(F) *E.coli* 16E2(R)	GGGAGTAAAGTTAATCCTTTGCTC TTCCCGAAGGCACATTCT	**584**	Tsen et al., 1998
S139 (F) S141 (R)	GTG AAA TTA TCG CCA CGT TCG GGC AA TCATCGCACCGTCAAAGGAACC	**284**	Rahn et al., 1992

e) Preparation for a PCR mixture

PCR mixture (25 µl) was prepared as follows: Nuclease free water: 12.5µl

2x master PCR mix: 9.5µl

Forward primer: 0.5µl

Reverse primer: 0.5µl

DNA template: 2.0µl

f) Thermal profile

i.* For *E. coli

PCR reaction profile was prepared as follows:

Initial denaturation: 95^0C for 5 minute 1 cycle

Denaturation: 94^0C for 30 sec 35 cycle

Annealing: 60^0C for 30 sec

Extension: 72^0C for 30 sec

Final extension: 72^0C for 5 min 1 cycle

ii. For *Salmonella* spp.

PCR reaction profile was prepared as follows:

Initial denaturation: 94^0C for 1 minute 1 cycle

Denaturation: 94^0C for 1 minute 35 cycle

Annealing: 64^0C for 30 sec

Extension: 72^0C for 30 sec

Final extension: 72^0C for 7 min 1 cycle

g) Electrophoresis

2% agarose (Sigma) gel was used for electrophoresis of the PCR products. The procedure of gel electrophoresis is given below:

The gel casting tray was made using a gel comb with the right tooth size and number. A 2% agarose solution in TBE buffer was made by melting it in a microwave oven. The casting tray was poured with molten agarose and allowed to solidify on the bench. The solidified gel was transferred to an electrophoresis tank containing enough TBE buffer to cover the gel by 1mm. The comb was removed with care. 7 l of each PCR product was combined with 2-3 l of loading buffer before loading the sample into the appropriate well of the gel. A well was filled with 5 l of DNA marker. The electrophoresis apparatus's leads were linked to the power supply, and the electrophoresis was performed at 100V. The power supply was turned off once DNA had migrated sufficiently, as determined by the migration of bromphenicol blue from the loading buffer. In a dark environment, they were stained with ethidium bromide (0.5 g/ml) for 10 minutes. For 10 minutes, the gel was destained in distilled water. The stained gel was placed on the UV transilluminator in the image documentation system's dark chamber. The system's UV light was turned on, and the image was viewed on the monitor, focussed, acquired, and stored to a USB flash drive.

3.2.7 Maintenance of stock culture

Stock culture was prepared by adding 1ml of 80% sterilized glycerol in 1 ml of pure culture in nutrient broth and it was stored in -20^0C.

3.2.8 Antimicrobial sensitivity pattern of *E. coli* and *Salmonella* spp. isolated from cloacal samples

There were 38 *E. coli* isolates and 24 *Salmonella* spp. were examined for disc diffusion sensitivity testing, 45 cloacal samples from Broiler, Sonali, and Pigeon were collected. The Kirby-Bauer disc diffusion method was used to test the antibiotic sensitivity of each isolate in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines. GEN (Gentamicin), AZM (Azithromycin), LEV (Levofloxacin), AMP (Ampicillin), E (Erythromycin), AMX (Amoxicillin), CIP (Ciprofloxacin), EX (Enrofloxacin), CTR (Ceftriaxone), CFM (Cefixime) and COT (Co-Trimoxazole) antibiotic sensitivity discs were employed. This method allows for thequick measurement of a drug's efficacy by measuring the diameter of the zone of

Inhibition caused by the agent's diffusion in the media surrounding the disc. The test organism suspension was made in a test tube containing 5 mL of nutrient broth by overnight incubation in a shaking incubator. 100l of broth culture of the test organism was placed onto Muller-Hinton agar plate using a micropipette. A sterile glass spreader was used to evenly distribute the culture on the medium. After inoculating the plates, they were closed and left to dry for around 3-5 minutes. The antibiotic discs were then put aseptically to the surface of the inoculated agar plates using a sterile forceps in a specific arrangement. After that, the plates were inverted and incubated at 37^0C for 24 hours. The plates were checked after incubation, and the diameter of the zone of complete inhibition was determined on a mm scale. Using an interpretation table, the zone diameters of individual antimicrobial drugs were classified as sensitive, moderate, or resistant.

CHAPTER 4

RESULTS & DISCUSSION

The results presented below, demonstrated the identification of bacterial isolates obtained from the cloacal swab samples of chicken (Broiler & Sonali) & pigeon from SAU poultry farm and selected Live Bird Markets of Dhaka district. Result also concentrated on the sensitivityand resistance pattern of the isolates to the different drugs.

4.1 Total Coliform Count & Total Salmonella Count from the isolated sample

Table 4: Total Viable count and Total Coliform Count from the isolated sample

Serial no & Name of Sample (CFU/g)	Total Coliform Count (TCC) (CFU/g)	Total Salmonella Count (TSC)(CFU/g)
1. B1	Nil	Nil
2. B2	Nil	Nil
3. B3	4.6×10^{6}	3.3×10^{6}
4. B4	3.4×10^{6}	4.7×10^{6}
5. B5	8.0×10^{6}	3.7×10^{6}
6.S1	2.9×10^{6}	2.2×10^{6}
7. S2	1.1×10^{6}	1.3×10^{6}
8. S3	3.2×10^{5}	3.4×10^{5}
9. S4	Nil	Nil
10. S5	3.7×10^{5}	2.7×10^{5}
11. P1	5.7×10^{8}	Nil
12.P2	6.7×10^{8}	3.1×10^{6}
13.P3	6.3×10^{8}	Nil
14.P4	6.5×10^{6}	Nil
15.P5	5.8×10^{8}	Nil
16. B6	8.7×10^{4}	4.3×10^{4}
17. B7	7.7×10^{4}	3.4×10^{4}
18. B8	5.7×10^{4}	3.7×10^{4}
19. B9	6.7×10^{4}	3.3×10^{4}
20. B10	4.7×10^{4}	3.1×10^{2}
21.S6	1.2×10^{5}	3.1×10^{2}
22.S7	1.1×10^{5}	3.3×10^{2}
23.S8	1.3×10^{5}	5.3×10^{2}
24.S9	1.4×10^{5}	3.5×10^{2}

Table 4 cont'd		
25.S10	1.4×10^5	4.5×10^2
26.P6	7.7×10^6	Nil
27.P7	6.7×10^6	Nil
28.P8	1.4×10^5	Nil
29.P9	1.3×10^5	Nil
30.P10	9.7×10^6	Nil
31. B11	1.3×10^7	Nil
32. B12	1.3×10^7	3.3×10^2
33.B13	1.1×10^7	Nil
34. B14	1.3×10^7	3.7×10^2
35. B15	1.1×10^7	Nil
36.S11	0.8×10^5	Nil
37. S12	Nil	1.3×10^5
38. S13	Nil	1.7×10^5
39. S14	2.96×10^7	Nil
40. S15	1.9×10^7	Nil
41. P11	Nil	Nil
42.P12	3.3×10^4	3.9×10^4
43.P13	3.1×10^4	Nil
44.P14	Nil	Nil
45.P15	7.7×10^4	3.5×10^4

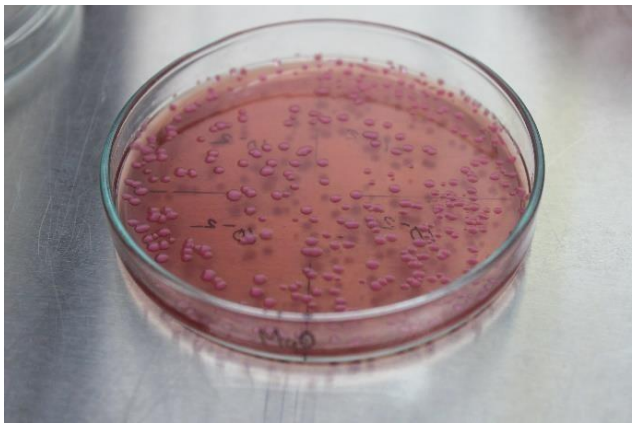

Plate 1: Total coliform count by10 fold Dilution method

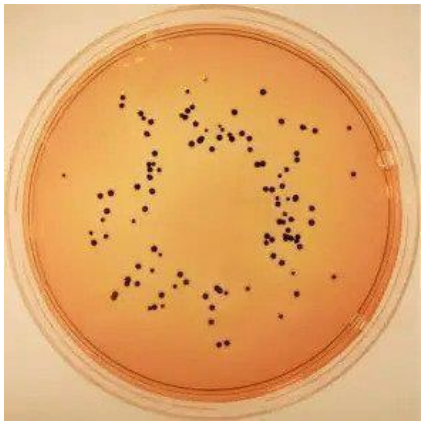

Plate 2: Total *Salmonella* count by10 fold Dilution method

4.2 Prevalence of microorganism in chicken and pigeon:

Among 45 cloacal swab samples which were collected from chickens (Broiler, Sonali) & pigeon found with 38 *E. coli* & 24 *Salmonella* spp., which were 84.45% and 53.33% respectively. E.coli isolates were found among the species in the manner of Broiler 13, sonali 12 & pigeon 13. Prevalence percentage were 86.67, 80 & 86.67% respectively. In turns to Salmonella it was 10(66.67%) in broiler, 11(73.33%) in sonali and 3 (20%) in pigeon (Table 5).

Table 5: Overall Prevalence of E. coli and Salmonella spp. in chicken and pigeon

Sources	Number of sample tested	Number of sample positive	Prevalence (%)
E. coli			
Broiler	15	13	86.67
Sonali	15	12	80
Pigeon	15	13	86.67
Overall	**45**	**38**	**84.45**
***Salmonella* spp.**			
Boiler	15	10	66.67
Sonali	15	11	73.33
Pigeon	15	3	20
Overall	**45**	**24**	**53.33**

4.3 Percentage of Prevalence with specific organism from sample:

Based on the collection site of samples from farm & LBM available of *E. coli* samples were 12 (80%) & 26 (86.67%) respectively. Regarding *Salmonella* it was 8 (53.33%) & 16 (53.33%) respectively (Table 6).

Table-6: Prevalence of *E. coli* and *Salmonella* spp. in Cloacal swabs of chicken and Pigeon according to site of sample collection

Site of collection	Number of sample tested	Number of sample positive	Prevalence (%)
E. coli			
Farm	15	12	80
Live Bird Market	30	26	86.67
Salmonella			
Farm	15	8	53.33
Live Bird Market	30	16	53.33

4.4 Prevalence percentage of *E. coli* & *Salmonella* spp. and bacterial load from different location

Table-7: *Escherichia coli* count, and *Salmonella* spp. count in the swab samples of Broiler, Sonali and Pigeon (n=45)

Category	Sample type	Number of sample tested	Microorganism count (CFU/ml)			
			Total *Escherichia coli*		Total *Salmonella* spp.	
			Number of positive (%)	Bacterial load	Number of positive (%)	Bacterial load
Farm sample(SAU poultry farm)	Cloacal sample	15	12 (80)	1.1×10^{6} - 6.7×10^{8}	8 (53.33)	3.4×10^{5}- 4.7×10^{6}
Live bird market (Townhall & krishi market)	Cloacal sample	30	26 (86.67)	3.1×10^{4} - 2.96×10^{7}	16 (53.33)	3.1×10^{2}- 1.7×10^{5}

4.5 Results of isolation and identification of *E. coli*

4.5.1 Results of cultural examination

4.5.1.1 Culture in nutrient broth

All the *E. coli* isolates produced turbidity in nutrient broth.

4.5.1.2 Culture on MacConkey agar

After overnight incubation Bright pink colored colonies on MacConkey agar produced by the organisms were presumptively selected as *E. coli* (shown in Table 8, Plate 3).

4.5.1.3 Culture on Eosine Methylene Blue (EMB) agar

Greenish colonies with metallic sheen produced by the organisms on EMB agar after overnight incubation were tentatively confirmed as *E. coli*. (shown in Table 8, Plate 4).

4.5.1.4 Culture on Brilliant green (BG) agar

Intense yellow green zone surround the Yellowish green colonies on BG agar produced by the organisms after overnight incubation were tentatively chosen as *E. coli* (shown in Table 8, Plate 5).

Table 8. Demonstration of the cultural characteristics of *Escherichia coli* isolated from oral and cloacal samples of pigeon in different agar media

Samples positive with *E. coli*	**Colony characteristics in different agar media**		
(B3-B5) , (S1-S3), S5, (P1-P5), (B6-B10), (S6-S10) (P6-P10), (B11-B15), S11, S14, S15, P12, P13, P15	MC agar	EMB agar	BG agar
	Bright pink colored colonies	**Greenish colonies with metallic sheen**	**Yellowish green colored colonies**

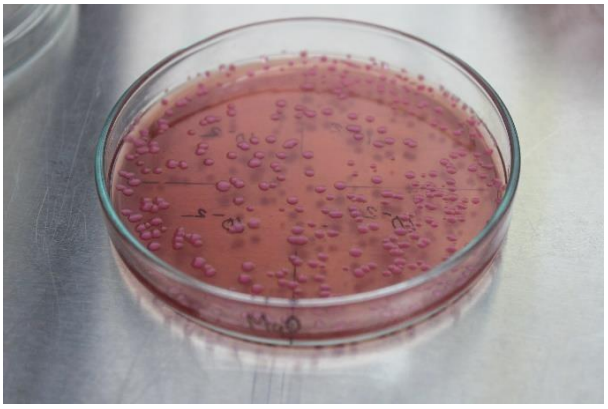

Plate 3*: E. coli* in MC agar media

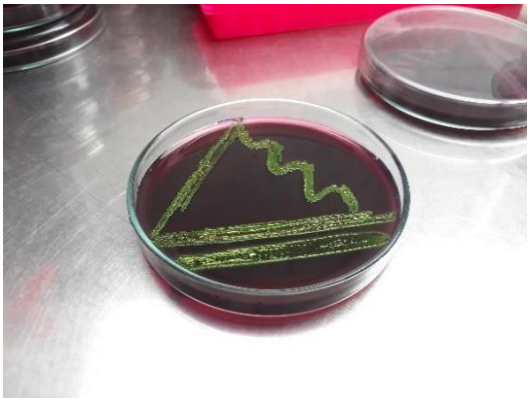

Plate 4*: E. coli* in EMB agar media

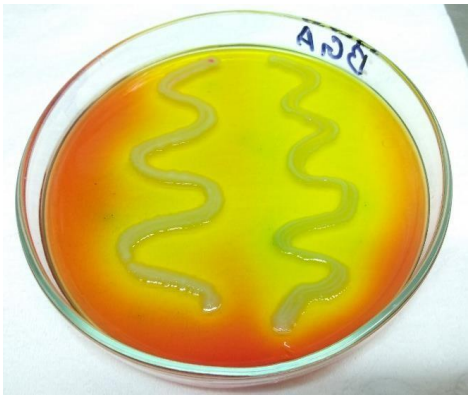

Plate 5*: E. coli* in BGA media

4.5.1.5 Results of Gram's staining technique

Light microscopic examination after Gram's staining revealed Gram-negative, pink colored, rod shaped organisms arranged as single or in pair (shown in table 9, Plate 7).

4.5.1.6 Results of Hanging drop technique

All the *E. coli* isolates were found to be motile with hanging drop slide preparation under microscopy.

4.5.1.7 Results of reactions in TSI agar slant

After inoculation and overnight incubation of the suspected E. coli organisms on TSI agar slant, yellow colored slant and yellow colored butt with buildup of gas bubbles in the butt were discovered. There was no generation of hydrogen sulphide, hence there was no blackening of the bottom (shown in Table 9, Plate 6).

Table 9. Demonstration of the morphology and staining characteristics, motility and reactions in TSI agar slant of *Escherichia coli* isolated from cloacal samples of chicken and pigeon in different agar media

Morphology and staining characteristics	Motility	Reactions in TSI agar slant
Gram negative rod shaped organism arranged as single or in pair	+	**Yellow slant and butt with gas but no H2S production**

Plate 6: *E. coli* in TSI agar slant

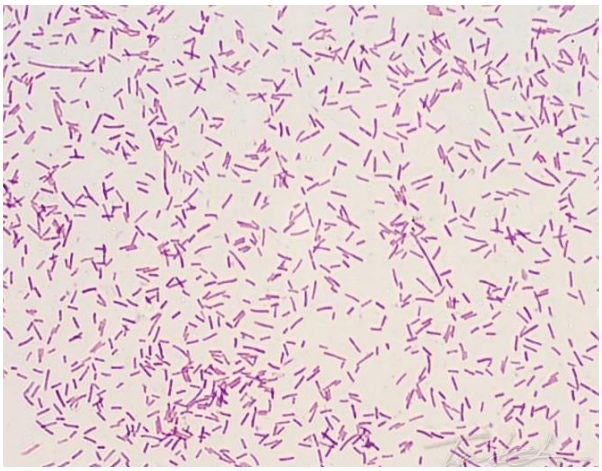

Plate 7: Gram negative rod shape organism (*E. coli*) at 100X magnification

4.5.1.8 Results of the biochemical tests

Tentatively confirmed *E. coli* isolates by colony characteristics, morphology and staining characteristics and by motility and reaction in TSI agar slant were subjected to different biochemical tests for identification.

4.5.1.9 Sugar fermentation test

All of the E. coli isolates fermented five basic carbohydrates, producing acid and gas in the process. During sucrose fermentation, the generation of acid and gas in pigeon swab isolates was reduced. The color change of the sugar media from reddish to yellow showed acid generation, and the formation of gas bubbles in the inverted Durham's tube indicated gas production (shown in Table 10, Plate 8 & 9).

Table 10. Demonstration of the biochemical reactivity pattern of *E. coli* isolated cloacal swabs of chicken & pigeon

Sources of *E. coli*	**Fermentation properties with five basic sugars**					**M R test**	**V- P Test**	**Indole test**	**Catalase test**	**Urease test**	**Citrate test**
Cloacal swab samples	**DX**	**ML**	**L**	**S**	**MN**	+	-	+	+	-	-
	AG	**AG**	**AG**	**A↓G↓**	**AG**						

Legends: DX = Dextrose; ML = Maltose; L = Lactose; S = Sucrose; MN=Mannitol; A = Acid production; G = Gas production; A↓ = Less acid production; G↓ = Less Gas production; + = Positive reaction; - = Negative reaction.

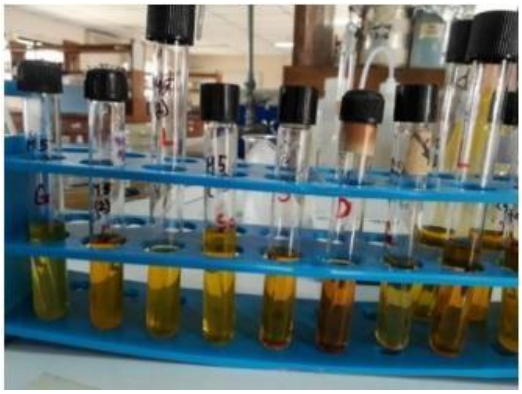

Plate 8: Production of acid and gas in sugar fermentation test for *E.coli*

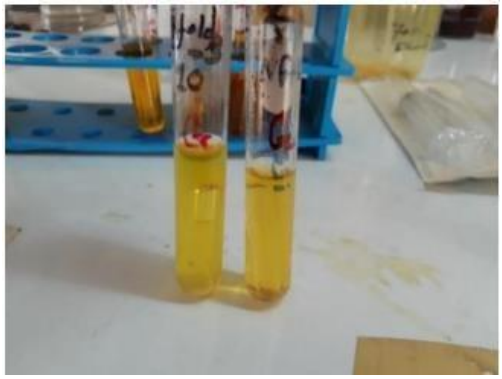

Plate 9: Production of acid and gas in sugar Fermentation test positive for *E. coli*

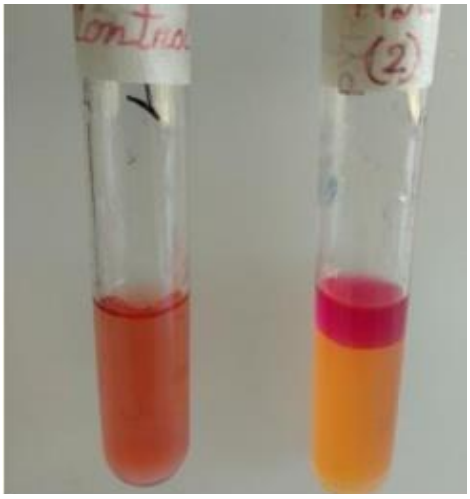

Plate 10: Indole production test (positive) for *E. coli*

Plate 11: Urease test (negative) for *E. coli*

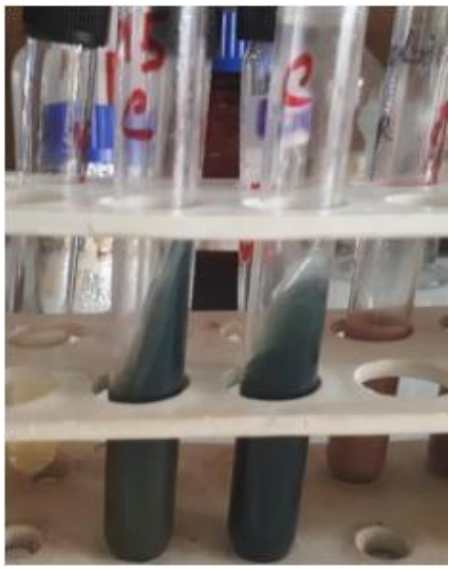

Plate 12: Citrate utilization test (negative) for *E. coli*

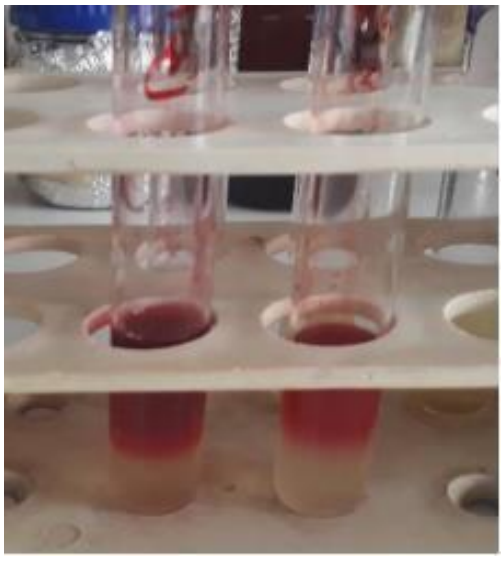

Plate 13: Methyl red test positive for *E.coli*

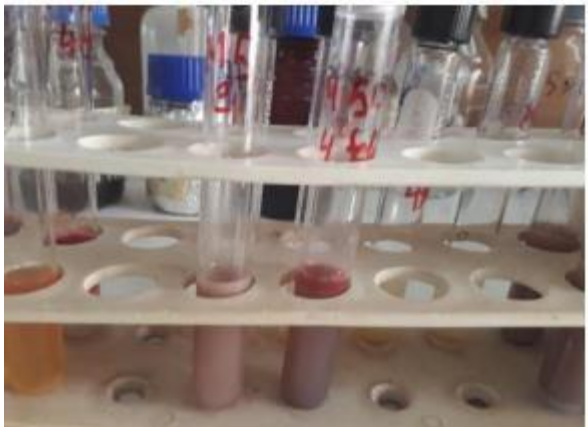

Plate 14: Voges-proskauer test (negative) for *E.coli*

4.5.1.10 other biochemical tests

All the isolates were Catalase test positive, Indole test positive (Table 10, Plate 10), Urease test (Table 10, Plate 11), Citrate test (Table 10, Plate 12), Methyl red (MR) test positive (Table 10, Plate 13), Voges- Proskauer (V-P) test negative (Table 10, Plate 15) and The above mentioned pattern of biochemical reactions were considered as *E. coli*.

4.5.1.11 **Molecular Identification of *E. coli***

DNA was extracted from isolated *E coli* for molecular identification. Afterwards, PCR was done, followed by agarose gel electrophoresis. For the detection of pathogenic *E.coli* organisms, 584bp DNA was amplified from the 16S rRNA gene using the primers GGGAGTAAAGTTAATCCTTTGCTC as forward and TTCCCGAAGGCACATTCT as reverse. PCR results showed that all of the isolates were positive. A typical illustration is provided below (Plate 15). The pathogenic organism yielded a positive result, but the other reverse primer yielded a negative result.

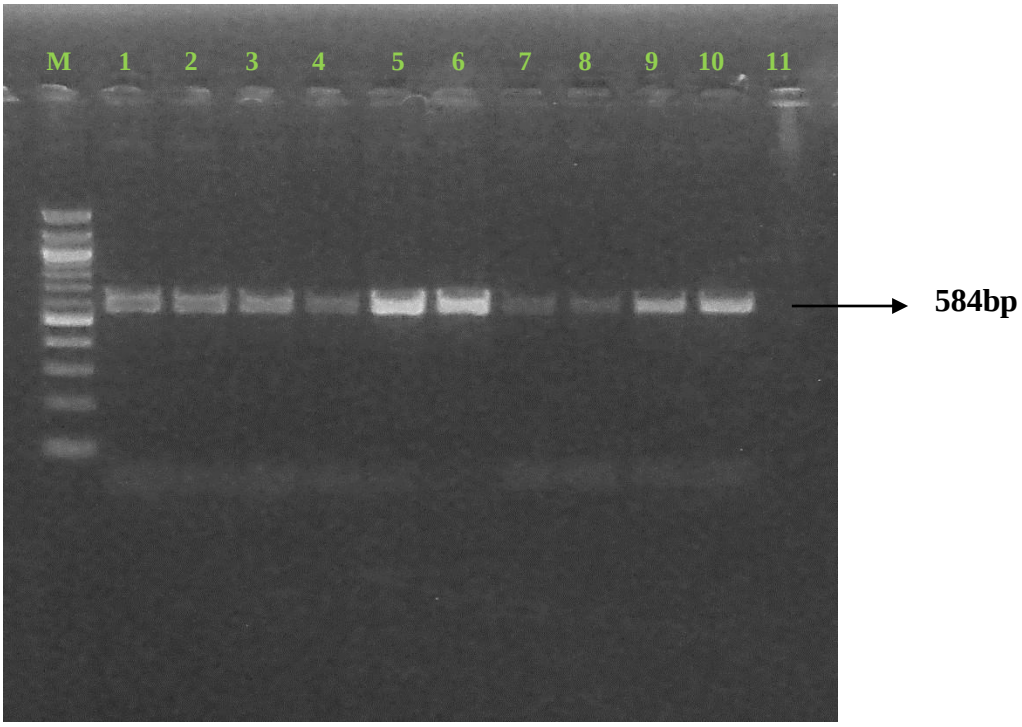

Plate 15: Amplification of 584bp DNA from 16S rRNA gene of *E coli*. Lane M: 100bp DNA Marker, Lane 1-10: Test sample, Lane 11: Negative control

4.6 Results of isolation of *Salmonella* Spp.

4.6.1 Identification of *Salmonella* Spp.: The isolated samples are arranged as follows.

Plate 16: Streak plate technique for *Salmonella* isolation in SS agar media

4.6.2 Results of isolation and identification of *Salmonella* spp.

4.6.3 Results of cultural examination

4.6.3.1 Culture in nutrient broth

All *Salmonella* spp. isolates produced turbidity in nutrient broth.

4.6.3.2 Culture on MacConkey agar

Red to pink-white colonies surrounded by brilliant red zones after overnight incubation were presumptively selected as *Salmonella* spp. (shown in Table 11, Plate 17).

4.6.3.3 Culture on Eosine Methylene Blue (EMB) agar Grey color colonies produced by the organisms on EMB agar after overnight incubation were tentatively confirmed as *Salmonella* spp. (shown in Table 11, Plate 18).

Table 11. Demonstration of the cultural characteristics of *Salmonella* Isolated from chicken and pigeon in different agar media

Sources of *Salmonella* spp.	**Colony characteristics in different agar media**		
(B3-B5), (S1-S3), S5, P2, (B6-B10), (S6-S10), B12, B14, S12, S13, P12, P15	**MC agar**	**EMB agar**	**SS agar**
	Red to pink-white colonies surrounded by brilliant red zones	Grey colour colony	Colonies with black centers

Plate 17: *Salmonella* isolation in MC agar media

Plate 18: *Salmonella* isolation in EMB agar media

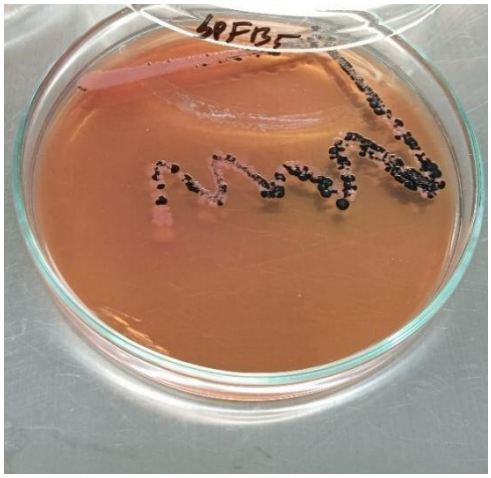

Plate 19: *Salmonella* isolation in SS agar media

4.6.3.4 Culture on Salmonella-Shigella agar

Colonies with Hydrogen Sulphide production after overnight incubation. Produced by the *Salmonella* spp. isolated from oral and cloacal samples (shown in Table 11, Plate 19).

4.6.3.5 Results of Gram's staining technique

Light microscopic examination after Gram's staining revealed Gram-negative, pink colored, rod shaped organisms arranged as single or in pair.

4.6.3.6 Results of Hanging drop technique

All the *Salmomella* spp. isolates were found to be motile with hanging drop slide preparation under microscopy.

4.6.3.7 Results of reactions in TSI agar slant

Yellow colored slant and black colored butt with accumulation of gas bubbles in the butt were found after inoculation and overnight incubation of the suspected *Salmonella* spp. organisms in TSI agar slant. There was presence of Hydrogen Sulphide production that's why blackening of the butt (Plate 20).

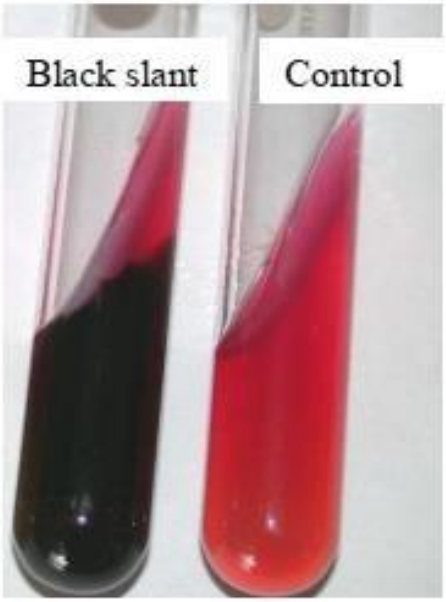

Plate 20: TSI agar slant positive test for *Salmonella* spp.

4.6.3.8 Results of the biochemical tests

Tentatively confirmed *Salmonella* spp. isolates by colony characteristics, morphology and staining characteristics and by motility and reaction in TSI agar slant were subjected to different biochemical tests for identification.

Table 12. Demonstration of the biochemical reactivity pattern of *Salmonella* spp. isolated from chicken & pigeon

Sources of *Salmonella* spp.	**Fermentation properties with five basic sugars**					**M R test**	**V- P Test**	**Indole test**	**Citrate test**
Cloacal swab samples	**DX**	**ML**	**L**	**S**	**M N**	**+**	**-**	**-**	**+**
	AG	**AG**	**NF**	**NF**	**AG**				

Legends: DX = Dextrose; ML = Maltose; L = Lactose; S = Sucrose; MN=Mannitol; A = Acid production; G = Gas production; A↓ = Less acid production; NF =No Fermentation G = Gas production; + = Positive reaction; - = Negative reaction.

4.6.3.9 Sugar fermentation test

The result carbohydrate fermentation test of *Salmonella* spp. was performed by inoculating a loopful of thick bacterial culture into the tubes containing five basic sugars (dextrose, maltose, sucrose, lactose, and mannitol) and incubated at 37^0C for 24 hours. Acid production was indicated by the change of media from pink to yellow color and gas production was indicated by the appearance of gas bubbles in the inverted Durham's fermentation tubes (Table 13, Plate 20).

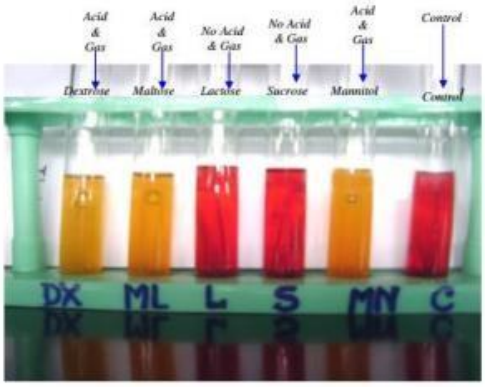

Plate 20: Sugar fermentation test of *Salmonella* spp.

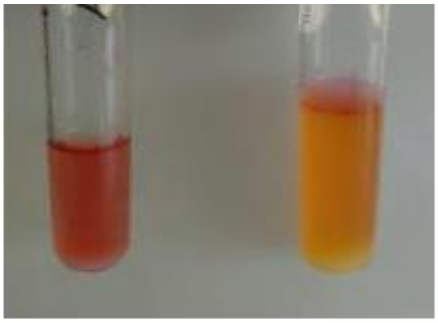

Plate 21: Indole test positive for *Salmonella* spp.

Plate 22: Citrate test positive (on left) for *Salmonella* spp.

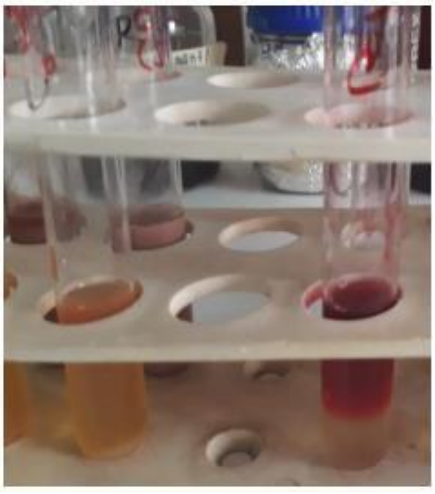

Plate 23: Methyl Red positive for *Salmonella* spp.

4.6.3.10 Result of Biochemical test

All the isolates were, Indole test positive (Table 13, Plate 21), Citrate test (Table 13 Plate 22), Methyl red (MR) test positive (Table 13, Plate 23).The above mentioned pattern of biochemical reactions were considered as *Salmonella* spp.

4.6.3.11 Result of Biochemical test

For molecular identification, DNA was extracted from isolated *Salmonella* spp. Then PCR was performed followed by electrophoresis in agarose gel. About 284bp DNA was amplified from S139 and S141 primers. All the isolates were found positive in PCR. A representative figure is shown below (Plate 24).

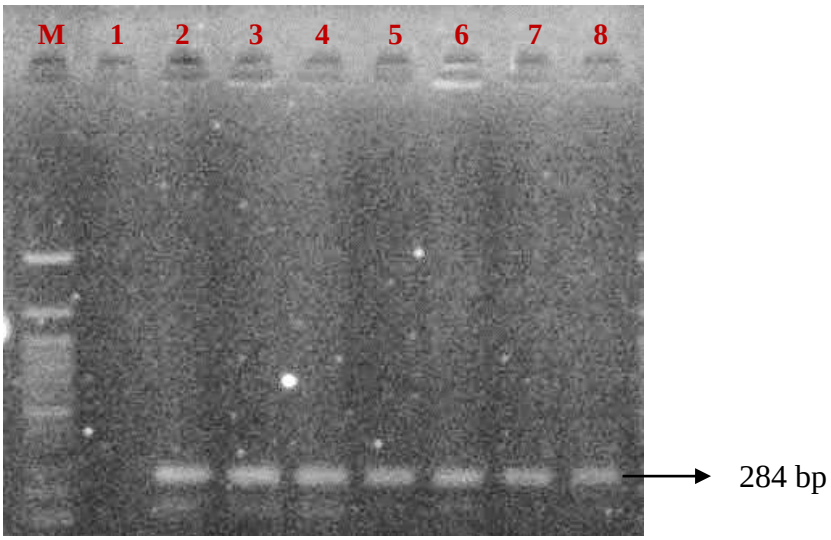

Plate 24: Amplification of 284bp DNA from *invA* gene of *Salmonella* spp. Lane M: 100bpDNA Marker, Lane 2-8: Test sample, Lane 1: Negative control

4.7 Results of drug sensitivity and resistance pattern of *E. coli* & *Salmonella* spp. isolated from chicken & pigeon sample

A total of thirty eight (38) different *E. coli* isolated from forty five (45) different samples taken from cloaca of chicken (Broiler & Sonali) and pigeon were used for drug sensitivity testing. Eleven different drugs were used for disc diffusion method test.

A large number of *E. coli* isolates from chicken and pigeon samples were found sensitive to CTR (68.42%), GEN (57.89%), CIP (31.58%) and LEV (18.42%). A little number was

sensitive to AZM (10.53%), EX (7.89%) & CFM (2.63%). None of the isolate showed sensitivity to AMP, AMX, COT & E. The highest resistance was against AMP, AMX and E (100%) Without any kinds sensitivity (either complete or intermediate). They showed comparatively higher resistance against COT (97.37%), CFM (39.48%), CTR (21.05%) & EX (18.42%). Comparatively lower resistance was showed by them against GEN, CIP, and LEV 5.26%, 5.26%, and 2.63%, respectively. Highest level of intermediate sensitivity OBSERVED against AZM (89.47%). Against LEV & EX they have shown 78.94% & 73.64% intermediate sensitivity. About (63.16%) and (57.89%) intermediate sensitivity found against CIP & CFM. a little portion of the isolates shown intermediate sensitivity against CTR & COT which were 10.53 & 2.63 % respectively. (33%). (Table 13, Figure 2).

Twenty four (24) different *Salmonella* isolates extracted from forty five (45) different cloacal samples of chicken and pigeon. A large number of Salmonella isolates found to be sensitive to GEN (66.67%), CIP (58.33%), CTR (58.33%), LEV (25%) and AZM (16.67%). A little number were sensitive to E & EX (8.33%). No resistance was found against GEN & CIP. The highest resistance was against AMP, AMX & COT (100%) E (91.67%) & LEV (66.67%) with a lower proportion against them found with intermediately sensitive. They showed comparatively lower resistance against CFM, CTR, AZM, and EX which were 25, 25, 20.83, & 12.50% respectively. Most of the isolates showed intermediate sensitivity against EX (79.17%) & intermediate sensitivity against CFM was 70.83%. Against AZM & CIP they have shown relatively higher intermediate sensitivity about 62.5% & 41.67%. Intermediate sensitivity against GEN, CTR and LEV were 33.33, 16.67 & 8.33% respectively. Against E they didn't shown any intermediate sensitivity. (Table 14, Figure 3) Compared to the *E.coli* isolated from the farm & live bird market samples, *E.coli* from the farm samples showed higher sensitivity against CIP & CTR which were 66.67& 66.66% respectively and isolates from the LBM were sensitive to GEN & CTR (73.08% &69.24%). The farm isolates of *E.coli* showed comparatively higher sensitivity against LEV, AZM & GEN (41.67%, 33.33 & 25%). Whereas the LBM isolates founds to sensitiveto CIP were 15.38%. Lower sensitivity (8.33%) found against both CFM & COT in caseof farm isolates, whereas (7.69%) of the isolates were sensititve to LEV & EX isolated

from the farm (Figure 4) .Isolates from both location found with complete resistance (100%) TO AMP, AMX & E. COT found with 100% resistant in case of LBM samples and 91.67% to farm samples.Higher resistance found against CFM, EX & CTR in the LBM isolates 50%, 26.92% and 23.07% respectively. lower resistance of (16.67) % found in farm isolates against GEN, CFM, & CTR. on the other hand lower resistance found against CIP & LEV IN 7.69% & 3.85% of LBM isolates. Complete intermediate sensitivity (100%) found against AZM among LBM *E. coli* isolats without any resistance or sensitivity. higher intermediate sensitivity found against LEV, CIP, EX & CFM which were 88.64%, 76.93%, 65.39% & 50% respectively in LBM. farm isolated showed higher intermediate resistance against EX, AZM, LEV, GEN & CIP WERE 91.67%, 66.67%, 58.33%, 58.33% & 33.33% respectively. any isoltes were not intermediately sensitive to AMP, AMX & E from farm & LBM. (Figure 5)

In comparison between the *Salmonella* spp. isolated from cloacal swab samples of farm and LBM, *Salmonella* spp. from the farm samples showed highest sensitivity to CIP (87.5%) and the LBM samples showed highest against GEN (75%). The farm isolates of *Salmonella* spp. showed comparatively higher sensitivity against CTR, GEN, AZM, LEV, E and EX was 62.5, 50, 50, 50, 25, & 25% respectively & lower sensitivity against CFM (12.5%). (Figure 6).

Higher sensitivity among the LBM isolates found in case of CTR and CIP was 56.25% and 43.75% respectively, lower sensitivity found to LEV was (12.5%). Hundred percent (100%) *Salmonella* isolates were resistance against AMP, AMX & COT from both location farm and LBM. The resistance found higher in *Salmonella* spp. isolated from farm samples against E, CFM, CTM, AZM, & LEV which were 75%, 50%, 25%, 25%, & 25% respectively. Conversely lower Resistance to LBM *Salmonella* isolates were found against CTR (25%), AZM (18.75%), EX (18.75%) & CFM (12.5%). Higher intermediate resistance found against EX, GEN, CFM, AZM & LEV which were 75%, 50%, 37.5% , 25% & 25% respectively in case of farm *Salmonella* isolates & lower to CTR (12.5%).The LBM *Salmonella* isolates found to be highly intermediate sensitive to CFM (87.5%), LEV (87.5%), AZM (81.25%), EX (81.25%), & CIP (56.25%). Lower

intermediate sensitivity found in LBM isolates against GEN (25%) & CTR (18.75%). (Figure 7)

4.8 Multidrug resistance bacteria (MDR) Hundred percent (100%) isolates were resistant to more than one antibiotics. Among which 18.42% of the *E. coli* isolates were resistant to 5 different antibiotics from four different classes & one isolate was resistant to seven antibiotics with the inclusion of macrolide. In terms of *Salmonella* spp. 8.33% of the total isolates were resistant to 5 different antibiotics and 16.67% of the isolates were resistant to 6 antibiotics with the inclusion of macrolides. (Table 15)

Table 13: Demonstration of the sensitivity and resistance pattern of different *E. coli* isolates to different drugs in percentage

Antimicrobial agents	No. of *E. coli* isolates (%)		
	R	IN	S
AMP	38 (100%)	0 (0%)	0 (0%)
AMX	38 (100%)	0 (0%)	0 (0%)
GEN	2 (5.26%)	14 (36.84%)	22 (57.89%)
CIP	2 (5.26%)	24 (63.16%)	12 (31.58%)
E	38 (100%)	0	0
CFM	15 (39.48%)	22 (57.89%)	1 (2.63%)
CTR	8 (21.05%)	4 (10.53%)	26 (68.42%)
AZM	0 (0%)	34 (89.47%)	4 (10.53%)
LEV	1 (2.63%)	30 (78.94%)	7 (18.42%)
EX	7 (18.42%)	28 (73.89%)	3 (7.89%)
COT	37 (97.37%)	1 (2.63%)	0 (0%)

Legends: GEN = Gentamicin; AZM = Azithromycin; LEV = Levofloxacin; AMP = Ampicillin; E = Erythromycin; AMX = Amoxicillin; CIP = Ciprofloxacin; EX= Enrofloxacin; CTR= Ceftriaxone; CFR= Cefixime; COT= Co-Trimoxazole; S = sensitive; IN = intermediate; R = resistant

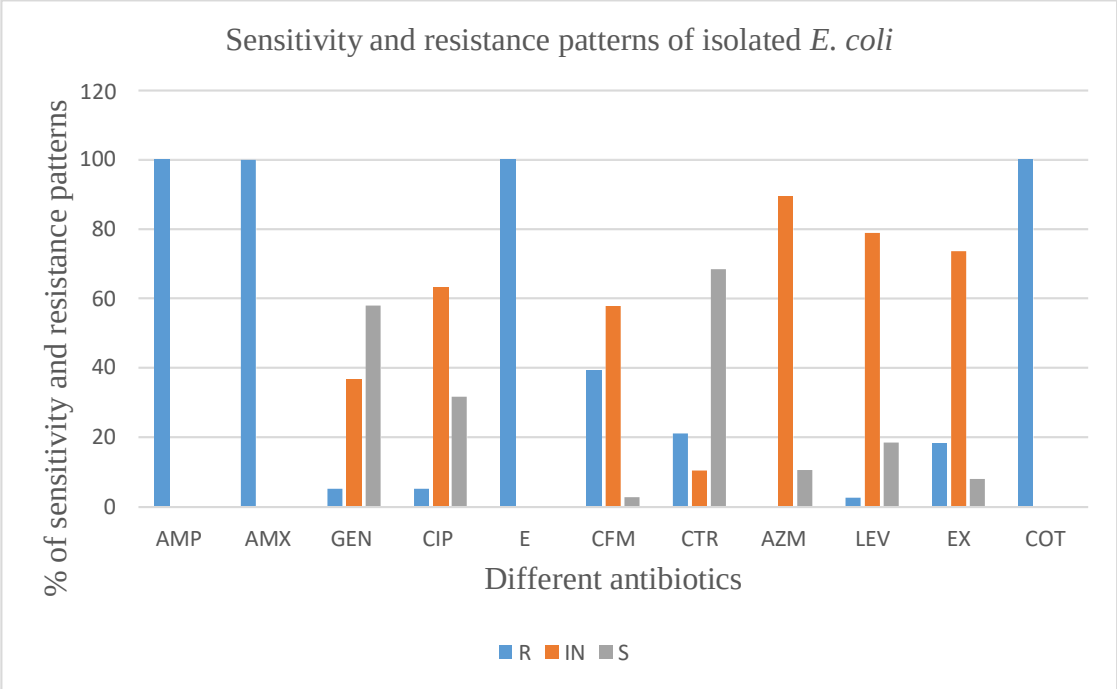

Figure 2: Diagram showing the antibiotic sensitivity pattern of *E. coli* isolated from cloacal swab samples of chicken and Pigeon

Table 14: Demonstration of the sensitivity and resistance pattern of different *Salmonella* spp. isolates to different drugs in percentage

Antimicrobial agents	**No. of *Salmonella* isolates (%)**		
	R	**IN**	**S**
AMP	24(100%)	0 (0%)	0 (0%)
AMX	24 (100%)	0 (0%)	0 (0%)
GEN	0 (0%)	8 (33.33%)	16 (66.67%)
CIP	0 (0%)	10 (41.67%)	14 (58.33%)
E	22 (91.67%)	0 (0%)	2 (8.33%)
CFM	6 (25%)	17 (70.83%)	1 (4.17%)
CTR	6 (25%)	4 (16.67%)	14 (58.33%)
AZM	5(20.83%)	15 (62.50%)	4 (16.67%)
LEV	16 (66.67%)	2 (8.33%)	6 (25%)
EX	3 (12.50%)	19 (79.17%)	2 (8.33%)
COT	24 (100%)	0 (0%)	0 (0%)

Legends: GEN = Gentamicin; AZM = Azithromycin; LEV = Levofloxacin; TET = Tetracycline; AMP = Ampicillin; E = Erythromycin; AMX = Amoxicillin; CIP = Ciprofloxacin; EX= Enrofloxacin; CTR= Ceftriaxone; CFR= Cefixime; COT= Co-Trimoxazole; S = sensitive; IN = intermediate; R = resistant

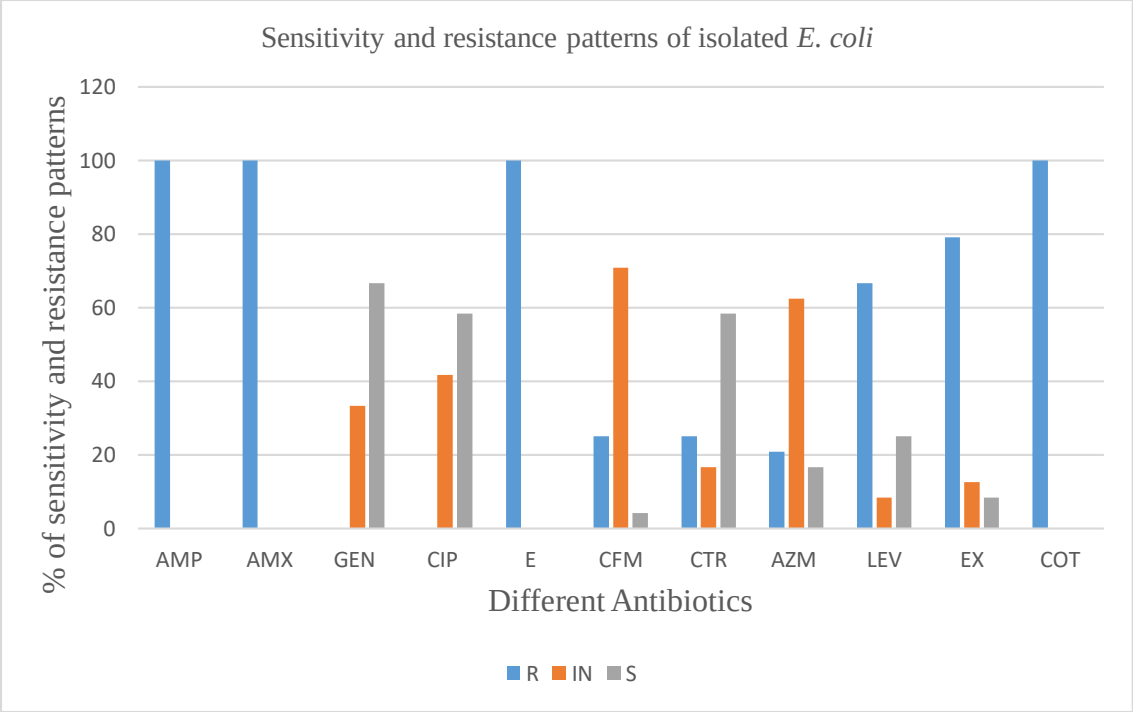

Figure 3: Diagram showing the antibiotic sensitivity pattern of *Salmonella* spp. isolated from cloacal swab samples of chicken and Pigeon

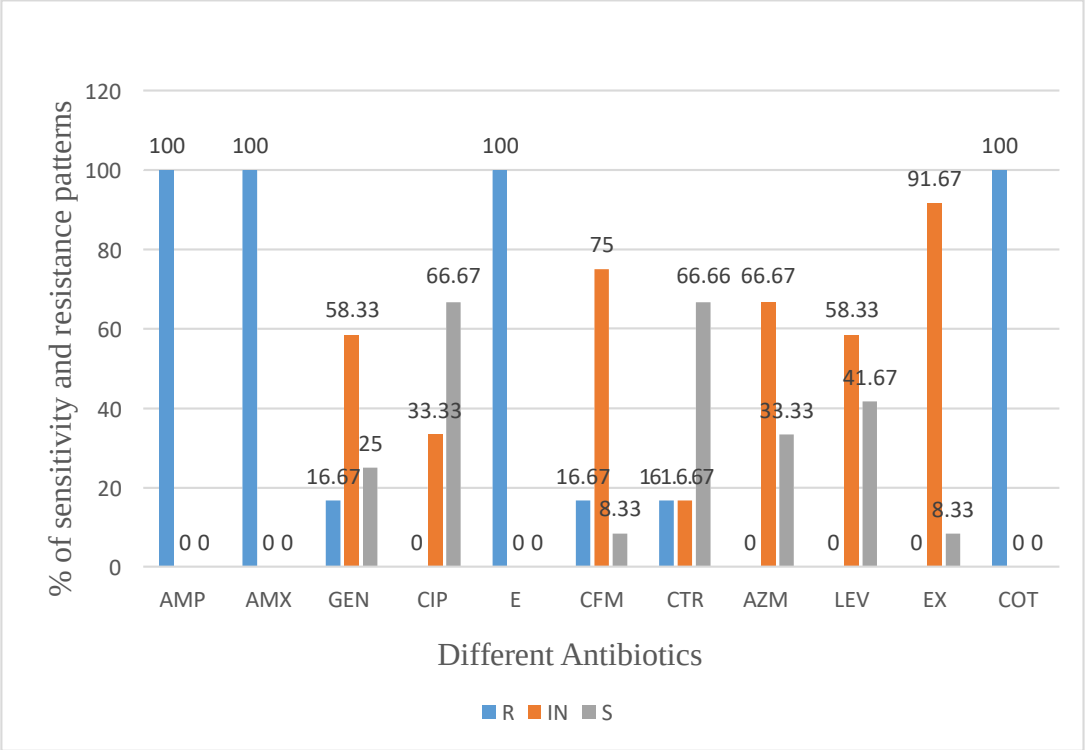

Figure 4: Diagram showing the antibiotic sensitivity pattern of *E*. coli isolated from cloacal swab samples of chicken and Pigeon from farm

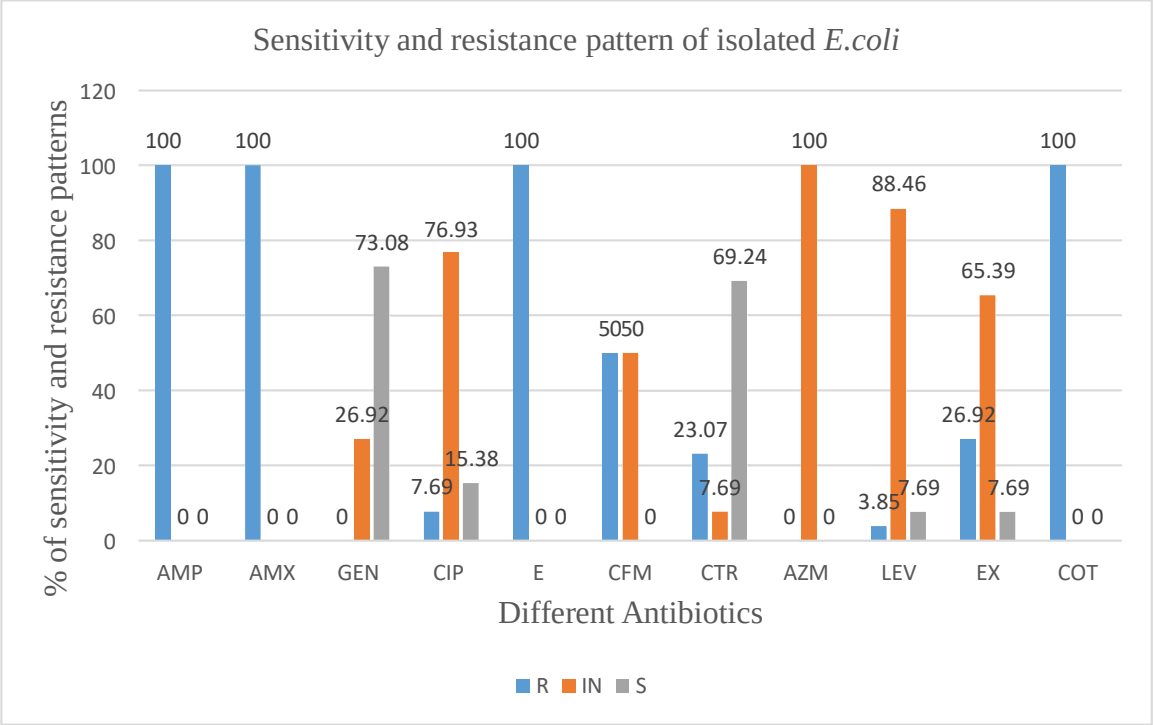

Figure 5: Diagram showing the antibiotic sensitivity pattern of *E*. coli isolated from cloacal swab samples of chicken and pigeon from LBM

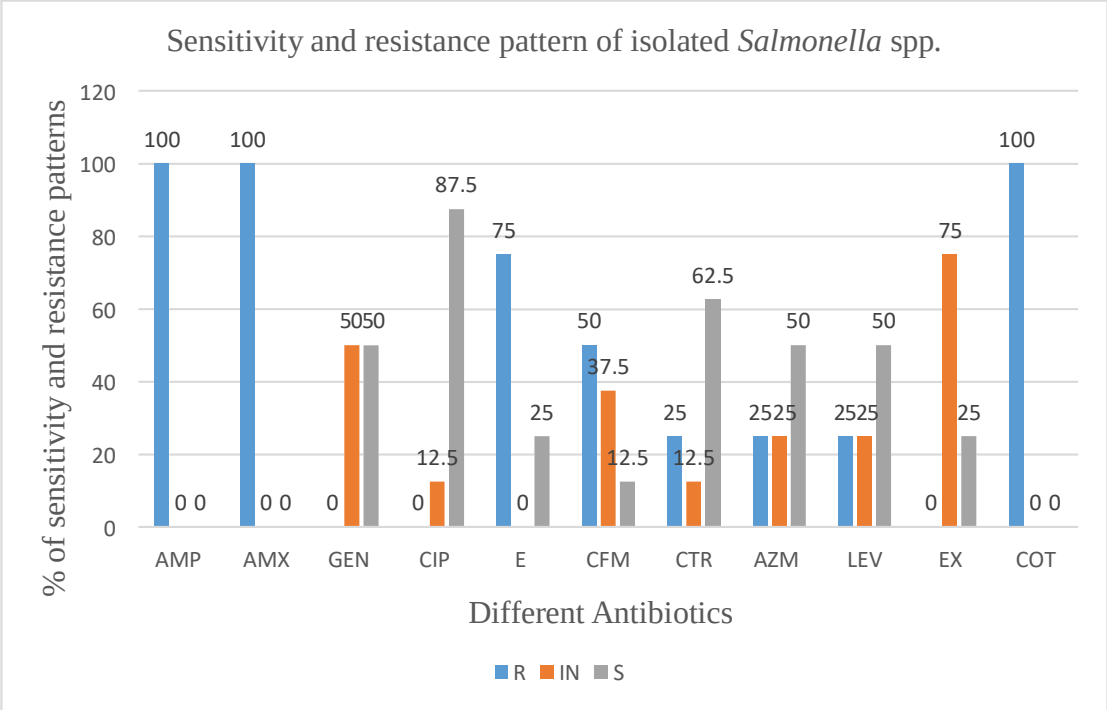

Figure 6: Diagram showing the antibiotic sensitivity pattern of *Salmonella* spp. isolated from cloacal swab samples of chicken and Pigeon from farm

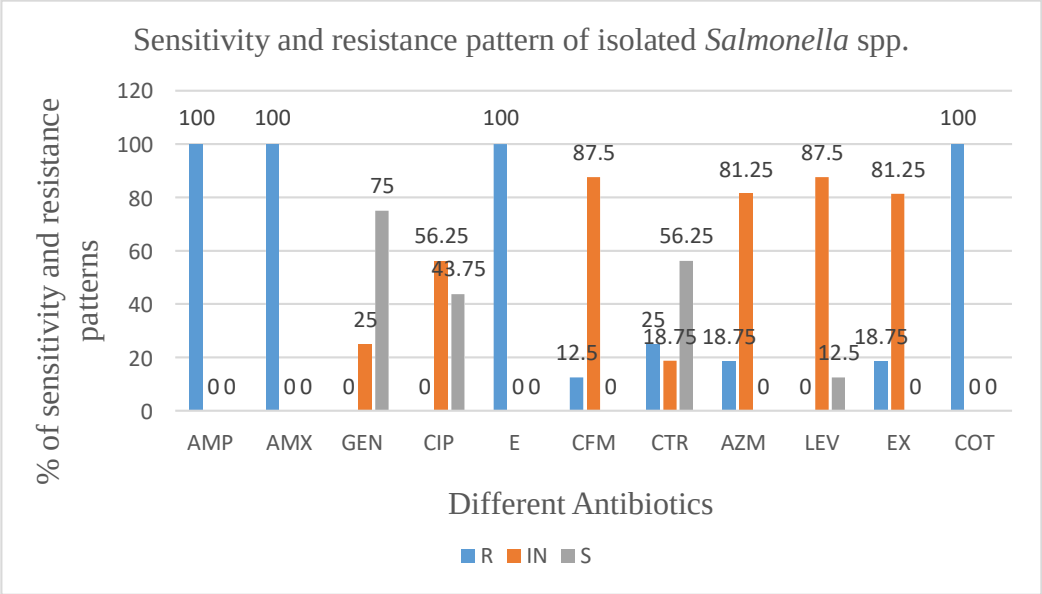

Figure 7: Diagram showing the antibiotic sensitivity pattern of *Salmonella* spp. isolated from cloacal swab samples of chicken and pigeon from LBM

Table 15: Demonstration of MDR resistant *E. coli Salmonella* spp. isolates to different drugs in percentage

MDR calculation

Antimicrobial compound	**Antibiotic class**	**Number of MDR isolates (%)**
E.coli (n=38)		
AMP-AMX--COT	Pen-Bet-Sul	14 (36.84)
AMP-AMX--CFM	Pen-Bet-Cef	1 (2.63)
AMP-AMX--CFM-COT	Pen-Bet-Cef-Sul	6 (15.79)
AMP-AMX-GEN--COT	Pen-Bet-Ami-Sul	2 (5.26)
AMP-AMX--EX-COT	Pen-Bet-Flu-Sul	5 (13.16)
AMP-AMX-CIP--EX-COT	Pen-Bet-Flu-Sul	2 (5.26)
AMP-AMX--CFM-CTR-COT	Pen-Bet-Cef-Sul	7 (18.42)
AMP-AMX-CFM-CTR-LEV-COT	Pen-Bet-Cef-Flu-Sul	1 (2.63)
Total		38 (100)
Salmonella (n=24)		
AMP-AMX-COT	Pen-Bet-Sul	16 (66.67)
AMP-AMX-EX-COT	Pen-Bet-Flu-Sul	2 (8.33)
AMP-AMX--CFM-LEV-COT	Pen-Bet-Cef-Flu-Sul	2 (8.33)
AMP-AMX-CFM-CTR-COT	Pen-Bet-Cef-Sul	4(16.67)
Total		24 (100)

4.9 Discussion

This study aimed Isolation and identification of *E. coli* and *Salmonella* spp. isolated from chicken (Broiler and Sonali) and pigeon from SAU poultry farm and selected Live Bird Markets in Dhaka city, as well as to determine the current status of drug sensitivity and resistancepattern of the isolates in order to determine the drug of choice for therapeutic use againstinfection caused by these organisms. The study's isolation and identification results revealed that the selected samples contained Gram negative and motile organisms (*E.coli*& *Salmonella* spp.).The colony properties of *E.coli* in three different agar media and fermentation ability with five basic sugars were identical. Remarkable colony properties of the isolates were also discovered. On EMB agar, all *E. coli* isolates produced a metallicsheen colony, a brilliant pink colony on MacConkey agar, and yellowish green colonies surrounded by an intense yellow green zone on BG agar. The isolated bacteria's morphology in Gram's staining revealed pink, tiny rod-shaped Gram negative bacilli, and all of the isolates were motile in the hanging drop technique. Which was previously identified by (Merchant , 1967; Cowan , 1985). In MacConkey agar media, all Salmonella isolates produced red to pink-white colonies surrounded by brilliant red zones, grey colonies in EMB agar, and colonies with black centers in SS agar. The isolated bacteria's morphology in Gram's staining revealed pink, tiny rod-shaped Gram negative bacilli, and all of the isolates were motile in the hanging drop technique. Similar result found by (Merchant , 1967; Cowan, 1985). These findings were supported by several authors such as (Karim et al., 2020) (Sobur et al., 2019) (Buxton and Fraser, 1977), (Freeman, 1985) and (Jones, 1987).

Another important basis for identifying *E. coli* and Salmonella organisms was analyzing the capacity or inability of five basic sugars to ferment with acid and gas generation. However, species identification and classification by fermentation reaction proved problematic (Freeman, 1985), with identical reactions observed in different sugars (OIE Manual, 2000). According to (Mckec et al., 1995), (Shandhu and Clarke, 1996), all *E.coli* isolates from pigeons showed complete fermentation of five basic sugars (Beutin et al., 1997). However, based on their sugar fermentation style, it was difficult to differentiate *Salmonella* into species. All of the isolates in this investigation fermented dextrose,

maltose, and mannitol, producing acid and gas, but not sucrose or lactose.which satisfied the statement of (Karim et al., 2020), (Sobur et al., 2019), (Buxton and Fraser, 1977), (Hossain, 2002) and (Han et al., 2011).

The isolated *E. coli* organisms in this investigation fermented dextrose, maltose, lactose, sucrose, and mannitol, producing both acid and gas. It was discovered that sucrose fermentation produced less acid and gas. (Ewing et al., 1973) and (Ali et al., 1998) investigated the biochemical properties of various strains of *E. coli* derived from various sources. They found little to no difference in these biochemical properties and speculated that this closeness among isolates could be attributable to the existence of certain common genetic components. Catalase, MR, and indole tests of *E. coli* isolates were positive, while the V-P test was negative, as reported by (Buxton and Fraser, 1977). The Indole test for *Salmonella* came out negative (formation of yellow ring), MR positive & result of V-P test was negative which satisfy the statement of (Karim et al., 2020), (Sobur et al., 2019) (Buxton and Fraser, 1977).

Molecular identification for *E.coli* yielded a positive result with a 16S rRNA identification band at 584bp.similar result was found in a previous study by (Rawal et al., 2013) from lake water. For molecular identification of *Salmonella* spp., S139 & S142 primer was used and obtained a final result with band 284bp. This finding was similar with (pal et al., 2017).

Current study concentrated on 45 samples collected from chicken (Broiler & Sonali) and pigeon cloacal swab samples. Among 45 samples 15 were collected from SAU poultry farm and 30 samples were collected from Live Bird Markets (LBM). 38 samples found positive with *E.coli* among 45 samples & 26 samples found positive with *Salmonella*. The overall prevalence of *E.coli* was (84.45%). Infection with *E.coli* from farm & LBM samples were respectively 80% & 86.67%. According to the species species of bird, in broiler, sonali and pigeon the prevalence percentage of *E.coli* were 86.67%, 80% & 86.67% respectively. In a previous study (jakaria et al., 2012) found overall prevalence of *E. coli* in cloacal swab samples from layers, broilers and indigenous chickens were 78.67, 82 and 70%, respectively from. (Afsal et. al., 2021) determine the occurrence of *E. coli* in cloacal samples of broiler chicken from different farms of Kollam and Kottayam districts

Kerala, India. The occurrence of *E. coli* in cloacal samples from broiler chicken was 76.5 per cent from Kollam and 79 per cent from Kottayam through culture techniques. Out of the total 400 cloacal swab samples collected from broiler chicken, 77.8 per cent were positive for *E. coli*. Which were slightly lower than the present lower than result. Conversely (Sarker et al., 2019) found only (61.67%) prevalence of *E.coli* from the cloacal samples of Broiler from LBM.(Dey et al., 2013) in a study found with prevalence of *E. coli* in cloacal swabs, foot pads and feces samples of pigeon were 86.11%, 44.44% and 77.50%, respectively. The overall prevalence of *E. coli* in pigeon was 69.64% (78 of 122 samples were found positive for *E. coli*). Which satisfy the present study the prevalence o of *E. coli* in the cloacal samples of pigeon. In another study by (Karim et al., 2020) found overall prevalence of *E.coli was* 52.5% in cloacal samples of pigeon collected from household and farm. Present result was much higher than the previous study.

The overall prevalence of *Salmonella* was (53.33%) & which is also similar as farm and LBM. In terms of species the prevalence of *Salmonella* in Broiler, Sonali and Pigeon were 66.67%, 73.33% & 20% respectively. In previous studies (Islam et al., 2016) found *Salmonella* spp. in 48% swab samples of healthy broiler and 66.7% diarrheic broiler raised in farm., (Al Mamun et al., 2017) found (20.4%) samples have shown positive for *Salmonella* species & samples collected from live bird markets of Gazipur district were (100%) positive for *Salmonella* species in broiler meat, floor swab and water samples. In a previous study with cloacal samples of chicken (Naurin et al., 2012) found (52%) prevalence of *Salmonella* spp. & Prevalence of *Salmonella* was 71.11% in broiler, 38.89% in layer and 25% in indigenous chicken. Broilers showed significantly higher prevalence of *Salmonella* as compared to layer and indigenous chickens which satisfy the present findings. (Nidaullah et al., 2017) found prevalence of 88.46% in poultry wet market of among which 91.67%, 83.33%, and 66.67% of defeathering machines, drain swabs, and apron, respectively.

Bacteria can counteract the effects of medications used in therapy by creating enzymes and metabolites that either breakdown the antimicrobial agents or aid the bacteria survive through various ways. As a result, the current sensitivity and resistance pattern of E. coli and Salmonella isolates to various medications should be established in order to select the

optimal antibiotic for treatment purposes. To carry out this investigation, 38 *E. coli* isolates 24 *Salmonella* isolates from 45 chicken and pigeon (cloacal) samples were exposed to an antibiotic sensitivity test using the disc diffusion method. Eleven different drugs were used for this study. The susceptibility test exhibited that most of the *E. coli* isolates, from chicken and pigeon's cloacal samples were sensitive to CTR and GEN followed by CIP and LEV. In terms of resistance, most of the isolates were resistant to AMP, AMX & E followed by COT, CFM, CTR and EX. In the present study a high percentage of *E. coli* isolates, from chicken and pigeon samples were sensitive to CTR (68.42%) and GEN (57.89%) followed by CIP and GM showing 31.58% and 18.42% sensitivity respectively. On the other hand, 100% of the isolates were resistant to AMP, AMX & E, followed by COT (97.37%), CFM (39.48%), CTR (21.05%) and EX (18.42%). A previous study of (Dameanti et al., 2022) showed in broiler antibiotic resistance, 100% of *E. coli* positive samples were found resistant to Erythromycin, Bacitracin, and Amoxicillin, 96.6% to Enrofloxacin, 92.6%. Previous study about antimicrobial resistant *E. coli* strains in broilers and local chickens were studied by (Ejeh & Kwanashie, 2017), in nigeria the highest levels of resistance by *E. coli* were against Ampicillin (100%), Erythromycin (85.7%) and Sulphamethoxazole-trimethoprim (85.7%). While all *E. coli* isolates were sensitive to Enrofloxacin, present study have a lower range of sensitivity. In another study conducted by (sarker et al., 2019) clocal samples of broiler from LBM, antibiogram study showed that the isolates were 100% resistant to ampicillin and tetracycline followed by sulfomethoxazole-trimethoprim (94.59%) and 56.76% isolates were sensitive to both ceftriaxone and gentamicin. In this study a number of isolates also showed intermediate reaction to AZM (89.47%), LEV (78.94%) & EX (73.64%), CIP (63.16%), & CFM (57.89%). According to (karim et al., 2020) Around 61.90%, 71.43%, 23.81%, 61.90%, 23.81%, 19.05%, and 52.38% of *E. coli* showed resistance against amoxicillin, ampicillin, azithromycin, erythromycin, nalidixic acid, gentamicin, and tetracycline, respectively & *E. coli* resistance was not observed in case of ciprofloxacin and levofloxacin isolated from pigeon. The Resistance rate is much higher in the present study and complete resistance were not found against ciprofloxacin and Levofloxacin. Intermediate Sensitivity drugs could not be compared due to lack of relevant literature.

In this Present investigation, all the *E. coli* isolates from farm samples found to be sensitive to CIP (66.67%), CTR (66.67%) followed by LEV (41.67%), AZM (33.33%), GEN (25%), CFM and EX (8.33%) showing marked resistance to AMP (100%), AMX (100%) & COT (91.67%). In a previous study (Rafiq et al., 2022) found AST (Antimicrobial Sensitivity Testing) pattern of *E. coli* showed the highest resistance to penicillin (P) (96.19%) followed by ampicillin (AMP) (90.71%), amoxicillin (AMX) (86.87%), oxytetracycline (O) (78.32%), cloxacillin (COX) (70.37%), and sulfamethoxazole-trimethoprim (COT) (70.01%). Among the antimicrobials, gentamicin (GEN) (66.46%) was found to be the most susceptible from poultry product. Specific sensitivity pattern of all the *E. coli* isolates from Live Bird Market (LBM) found to be sensitive to GEN (73.08%) and CTR (69.24%) followed by CIP (15.38%), LEV & EX (7.69%). showing marked resistance to AMX (100%), AMP (100%) , E (100%) & COT (100%). Previous study by (sarker et al., 2019) & (Rafiq et al., 2022) almost similar with current study.

24 *Salmonella* isolates from 45 chicken and pigeon (oral + cloacal) samples weresubjected to antimicrobial sensitivity test which was done by disc diffusion method. Eleven different drugs were used for this study. The sensitivity test revealed that most ofthe *Salmonella* isolates, from cloacal samples were sensitive to GEN followed by CIP, CTR, LEV, EX, E and AZM. In terms of resistance, most of the isolates were resistant toAMP, AMX & COT followed by E, LEV, CTR, AZM and EX. In the present study a highpercentage of *Salmonella* isolates, from cloacal samples were sensitive to GEN (66.67%)followed by CIP, CTR, & LEV showing 58.33%, 58.33 % and 58.33% sensitivity respectively. On the other hand, 100% of the isolates were resistant to AMP, AMX, COTand 91.67% to E followed by CFM (25%), CTR (25%), AZM (20.83%) & EX (12.50%).

(Rafiq et al., 2022) in a previous study identified that AST result of *Salmonella* showed the highest resistance to penicillin (96.15%), followed by ampicillin (AMP) (91.48%), oxytetracycline (O) (82.2%), amoxicillin (73.1%), and cloxacillin (67.85%) whilst the highest susceptibility was recorded to gentamicin (82.91%), followed by ceftriaxone (CTR) (58.88%). The sensitivity level among the isolates toward gentamicin and ceftriaxone was much higher and the resistance pattern in almost similar in case of similar antibiotics. In a previous study (Ejeh & Kwanashie, 2017) revealed *Salmonella* spp.

showed highest resistance against sulphamethoxazole-trimethoprim (100%), ampicillin (100%), cefuroxime (100%), ceftazidime (100%), oxacillin (100%) and chloramphenicol (100%). Highest level of susceptibility of *Salmonella* were to enrofloxacin (100%), neomycin (75.0%) and streptomycin (75.0%). In this section, the variation was found in the sensitivity pattern of Salmonella isolates against Enrofloxacin. Which was higher in the previous study than present one. In another study (Al Mamun et al., 2017) found sensitivity against ciprofloxacin, gentamicin and norfloxacin & Resistant profile of *Salmonella* species were recorded 36% multi-drug resistant *Salmonella* species. (karim et al., 2020) found around 36.36%, 27.27%, 27.27%, 45.45%, 81.82%, 100%, and 18.18% of the *Salmonella* spp. showed resistance against amoxicillin, ampicillin, azithromycin, erythromycin, nalidixic acid, tetracycline, and levofloxacin, respectively. However, all *Salmonella* spp. (100%) were found to show sensitivity against ciprofloxacin and gentamicin. Resistance to different drugs are similar in the present study but the proportion is much higher and the sensitivity pattern is similar but proportion is lower than the previous study.

In the present study, all the *Salmonella* isolates from farm samples found to be sensitive to CIP (87.5%), CTR (62.5%) followed by GEN (50%), AZM (50%), LEV(50), & EX (25%), Presence of resistance to AMP (100%), AMX (100%), COT (100%) & E (75%) which were similar with the findings of (Islam et al., 2016). They showed that *Salmonella* isolates from broiler farm were resistant to (Penicillin-G, Erythromycin, Ampicillin and Bacitracin) & found sensitivity against gentamicin. This study didn't include all antibiotics which were used in the present study. (Zhao et al., 2021) in a previous study 86 *Salmonella* isolates from hatcharies were tested for resistance to 12 common antibiotics, revealing resistance rates as follows: ampicillin (66.3%), nalidixic acid (59.3%), tetracycline (47.7%), chloramphenicol (40.7%), sulfamethoxazole (38.4%), streptomycin (29.1%), and fosfomycin (2.3%). All *Salmonella* strains exhibited susceptibility or intermediate susceptibility to other tested antibiotics. These findings were much lower than the present study in aspects of ampicillin and sulfamethoxazole.

In the present study, all the *Salmonella* isolates from LBM found to be sensitive to GEN (75%) and CTR, CIP, LEV was 56.25, 43.75, & 12.5% respectively. resistance pattern against AMP, AMX, E, & COT were 100%. In a previous study by (Islam et al., 2022) exhibited 100% resistance to vancomycin and cephalexin, followed by ampicillin (75%), nalidixic acid (58.33%), chloramphenicol (41.66%), doxycycline (50%), and neomycin (50%). On the other hand, ciprofloxacin showed 83.33%, ceftazidime and amoxicillin showed 91.6% sensitivity respectively. A considerably high proportion of isolates (11/12, 91.67%) was resistant to three or more antibiotics and 6 multidrug profiles were observed. The ampicillin-chloramphenicol-nalidixic acid-neomycin-cephalexin, doxycycline-vancomycin (4/12) was more frequently observed phenotype in multidrug profiles. This result was contradictory to the present result.

Presence of multidrug resistance among the isolated *E. coli* and *Salmonella* spp. were upto 100%. All samples were resistant more than one antibiotics and 18.42% of the total E.coli isolates were resistant to 5 different antibiotics from four different classes & one isolate was resistant to seven antibiotics with the inclusion of macrolide. In terms of *Salmonella* spp. 8.33% of the total isolates were resistant to 5 different antibiotics and 16.67% of the isolates were resistant to 6 antibiotics with the inclusion of macrolides. In a previous study in Nepal (Koju et al., 2022) found among 159 *E.coli* samples, 113 (71%) were resistant to more than 3 antibiotics which were collected from ceacal samples of chicken. (Isalm et al., 2022) found considerably high proportion of *Salmonella* isolates (11/12, 91.67%) were resistant to three or more antibiotics and 6 multidrug profiles were observed. The ampicillin chloramphenicol-nalidixic acid-neomycin-cephalexindoxycycline-vancomycin collected from chicken in chattagram district. (Ali et al., 2020) found in a study *Salmonella* spp. isolated in Ethiopia from the ceacal samples of chicken. Among 50 isolates, 48 were resistant to at least one drug. Multidrug resistance was recorded in 43 (86.0%) of the isolates.

The importance of antibiotic resistance in food-borne pathogens has grown dramatically, and it is likely linked to the widespread use of antimicrobial drugs in veterinary medicine and human medicine (Bronzwaer et al., 2002). Many Salmonella spp. species are known to possess multidrug resistant genes (Gebreyes and Altier, 2002), which is cause for

concern. Based on the findings of this study, it is possible to conclude that GEN and CTR will be the first medications of choice, followed by CIP, to combat E. coli and Salmonella infections in chickens and pigeons, as well as humans, cattle, sheep, goats, and ducks. It should be highlighted that determining drug sensitivity and resistance patterns may be useful as baseline information for the usage of future treatments to effectively control bacterial infections. Alternatively, indiscriminate use of antimicrobial medications may result in the emergence of drug-resistant mutants, posing major health risks to various animals and birds, including humans. Routine laboratory isolation and drug sensitivity testing of the microbes, however, is impractical. As a result, periodic testing of medication sensitivity and resistance patterns in organisms is critical in order to identify the best medicine of choice for the treatment of infectious diseases.

CHAPTER 5

SUMMARY AND CONCLUSION

The present study was conducted for the isolation and identification (molecular) of multi-Drug *E.coli* and *Salmonella* spp. from chicken (Broiler & Sonali) and Pigeon. After collection, the samples were subjected to various tests and experiments for isolation and identification of bacteria which possess zoonotic importance. It is reported that *E.coli* and *Salmonella* spp. Primary isolation was performed by 10 fold dilution of the organism using phosphate buffer saline from the clocal swab samples and enrichment of the isolates in nutrient broth, followed by culture on different agar media such as MacConkey agar, EMB agar, BG agar and SS agar for the determination of their colony characteristics. A total of 45 cloacal samples were collected asepically from 45 birds. Total Coliform Count (TCC) and Total *Salmonella* Count (TSC) was done by 10 fold dilution method. Among the isolates 38 were was *E.coli* and 24 were *Salmonella* spp. in this study They were identified using colony morphology. Gram's staining and the hanging drop technique were used, and the reaction in TSI agar slant was also observed. The biochemical properties of the isolates were investigated using a fermentation test with five basic sugars, as well as the Catalase test, the MR test, the V-P test, and the Indole synthesis test. Molecular identification of the isolates were done to confirm the genus by following previously described methods by scientists.

The study was also extended to investigate in vitro sensitivity and resistance pattern of the *E. coli* & *Salmonella* spp. isolates to different drugs. Study revealed that there were considerable variations among the isolates of different sources in respect of drug sensitivity and resistance pattern.

A high percentage of *E. coli* isolates from the chicken & pigeon were sensitive to CTR and GEN followed by CIP and LEV while most of the *E. coli* isolates were resistant to AMP, AMX, E, COT and CFM. In case of *Salmonella* isolates good sensitivity found against GEN & CIP followed by CTR, LEV, and AZM while most of the *Salmonella* spp. isolates were resistant to AMP, AMX, COT and E. Multidrug resistance pattern is also identified from the isolates. Hundred percent isolates were resistant to more than one antibiotics. Presence of isolates resistant to more than 7 antibiotic in case of *E.coli* &

Resistance to 6 antibiotic *Salmonella* isolates were also identified. It is assumed that one or more drug resistant clones have gradually acquired resistance to other drugs by conjugation with multi-drug resistant strains.

From the present study it may be concluded that

(a) Cloacal samples collected from chicken and pigeon from the SAU poultry farm and selected live bird markets in Dhaka city, which were detected with multi-drug resistant *E.coli* and *Salmonella* spp..

(b) *E. coli* infections of birds may be treated effectively with CTR and GEN followed by CIP and LEV. Infection with *Salmonella* spp.can be treated with GEN followed by CIP & CTR. Indiscriminate use of antimicrobial agents should be avoided in order to prevent the development of multi-drug resistant mutant in nature.

Drawback of this study was, the sample size were not fare enough to draw any concrete conclusion .Previous medication history cannot be taken while sampling.

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APPENDIX I

Composition of different media

1. Nutrient broth

peptic digest of animal tissue	5.0 gm
Sodium chloride	5.0 gm
Beef extract	1.5 gm
Yeast extract	1.5 gm
Distilled water	1000 ml
Final pH (at 25ºC)	7.4 ± 0.2

2. Nutrient Agar

Peptone	5.000 gm
Sodium chloride	5.000 gm
HM peptone B#	1.500 gm
Yeast extract	1.500 gm
Agar	15.000 gm
Final pH (at 25°C)	7.4±0.2

3. MacConkey Agar

Peptones (meat and casein)	3.000 gm
Pancreatic digest of gelatin	17.000 gm
Lactose monohydrate	10.000 gm
Bile salts	1.500 gm
Sodium chloride	5.000 gm
Crystal violet	0.001 gm
Neutral red	0.030 gm
Agar	13.500 gm
pH after sterilization(at 25°C)	7.1±0.2

4. Eosin Methylene Blue Agar

Peptic digest of animal tissue	10.000 gm
Dipotassium phosphate	2.000 gm
Lactose	5.000 gm
Sucrose	5.000 gm
Eosin - Y	0.400 gm
Methylene blue	0.065 gm
Agar	13.500 gm
Final pH (at 25°C)	7.2±0.2

5. Brilliant Green Agar Medium

Peptone	5.000 gm
Tryptone	5.000 gm
Yeast extract	3.000 gm
Lactose	10.000 gm

Sucrose	10.000 gm
Sodium chloride	5.000 gm
Phenol red	0.080 gm
Brilliant green	0.0125 gm
Agar	20.000 gm
pH after sterilization (at 25°C)	6.9±0.2 gm

6. Salmonella-Shigella agar

Proteose peptone	5.000 gm
Lactose	10.000 gm
Bile salts mixture	8.500 gm
Sodium citrate	8.500 gm
Sodium thiosulphate	8.500 gm
Ferric citrate	1.000 gm
Brilliant green	0.00033 gm
Neutral red	0.025 gm
Agar	13.500 gm
Final pH (at 25°C)	7.0±0.2

7. Mueller Hinton Agar

HM infusion B from	300.000
Acicase	17.500
Starch	1.500
Agar	17.000
Final pH (at 25°C)	7.4±0.1

8. Methyl Red Indicator

Methyl red	0.200 gm
Ethyl alcohol	60.000 ml
Distilled water	40.000 ml

9. Voges–Proskauer (MR-VP) broth

Buffered peptone	7.000
Dextrose	5.000
Dipotassium phosphate	5.000
Final pH (at 25°C)	6.9±0.2

10. Phosphate buffer saline

Sodium chloride	8.0 gm
Disodium hydrogen phosphate	2.8 gm
Potassium chloride	0.2 gm
Potassium hydrogen phosphate	0.2 gm
Distilled water to make	1000 ml