ANTIMICROBIAL RESISTANCE PATTERN OF *ESCHERICHIA COLI* ISOLATED FROM BROILER MEAT

GAZI SOFIUL ALAM

Roll No.: 0119/24

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A thesis submitted in the partial fulfillment of the requirements for the degree of Master of Science in Applied Human Nutrition and Dietetics

Department of Applied Food Science and Nutrition

Faculty of Food Science and Technology

Chattogram Veterinary and Animal Sciences University

Khulshi, Chattogram-4225

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This is to certify that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee have been made.

Professor Mohammad Mahmudul Hassan, PhD	**A/P Md. Ahaduzzaman, PhD**
Supervisor	**Co-supervisor**

Kazi Nazira Sharmin

Associate Professor

Chairman of the Examination Committee

Department of Applied Food Science and Nutrition

Faculty of Food Science and Technology

Chattogram Veterinary and Animal Sciences University

Khulshi, Chattogram-4225

December 2020

Dedicated

To my

Beloved

Friends, Family and

Honorable Teachers

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The Author

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
%	Percent
µg	Microgram
µl	Microliter
ml	Milliliter
mA	Milliampere
gm	Gram
mm	Millimeter
AMR	Antimicrobial Resistance
AMGP	Antimicrobial Growth Promoter
CMA	Chattogram Metropolitan Area
CI	Confidence Interval
CLSI	Clinical and Laboratory Standards Institute
CVASU	Chattogram Veterinary and Animal Sciences University
CFU	Colony Forming Unit
CS	Culture and Sensitivity
DNA	Deoxyribonucleic Acid
DMVPH	Department of Microbiology and Veterinary Public Health
E. coli	*Escherichia coli*
EMB	Eosin Methylene Blue
ESBL	Extended Spectrum β-Lactumase
FAO	Food and Agriculture Organization
hrs	Hours
kb	Kilo Base
LBM	Live Bird Market
MDR	Multi Drug Resistance
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
STEC	Shiga Toxigenic *E. coli*
SS	Super Shop
WHO	World Health Organization

ABSTRACT

Multidrug-resistant *Escherichia coli* is a growing concern all over the world. This study was designed to estimate the prevalence of *E. coli* and their resistance patterns in broiler meat obtained from Live Bird Markets (LBM) and Super Shops (SS) in Chattogram, Bangladesh. A total of 405 samples were collected from super shops and live bird markets consisting of muscle (n=215) and liver (n=190). Isolation and identification of *E. coli* were done using standard bacteriological methods. Later the isolates were screened to reveal the resistance pattern against eight antimicrobials of seven different unrelated groups using the disc diffusion technique. Finally, tetracycline-resistant isolates as obtained were investigated for the presence of *tet*-A, *tet*-B, *tet*-C, and *tet*-D genes using PCR. The results revealed that 229 (56.54%; 95% CI 51.56% - 61.43%) samples were found positive with *E. coli*. Antimicrobial resistance profiling of the isolates showed the highest resistance against sulphamethoxazole trimethoprim 88.65% (95% CI 83.81% - 92.45%) followed by tetracycline 86.90% (95% CI 81.82% - 90.92%), ampicillin 82.53% (95% CI 76.99% - 87.22%), and ciprofloxacin 60.70% (95% CI 54.04%- 67.06%) antimicrobials. The *E. coli* isolates were found to be susceptible to colistin sulfate 79.48% (95% CI 73.66% - 84.51%) followed by Cephalexin 62.88% (95% CI 56.27% - 69.15%), and Gentamycin 58.08% (95% CI 51.40% - 64.55%). Most of the tetracycline resistance isolates encoded *tet*A as 84.4% (95% CI 78.62% - 89.16%). Contrarily, 0.5% (95% CI 0.01% - 2.77%) isolates encoded *tet*C and 6.03% (95% CI 3.15% - 10.3%) isolates encoded containing two genes, while 12.1% (95% CI 7.88% - 17.41%) of the isolates tested negative for all four resistance genes of tetracyclines. This study revealed significant contamination of broiler meat with multidrug resistance *E. coli*. Potential sources of contamination with the alarming prevalence of tetracycline resistance *E. coli* identified in this study would aid in reducing the growing risks of broiler-associated pathogens. Appropriate control measures should be developed and implemented rational use of antimicrobials in poultry farming system and eliminate this multidrug resistance zoonotic pathogen from foods of animal origin to protect public health.

Keywords: poultry meat, AMR, *Escherichia coli*, LBMs, tetracycline resistance, Super shop

INTRODUCTION

Escherichia coli (*E. coli*), a gram-negative bacteria which has a significant impact on human and animal health (Salehi and Bonab, 2006). Most *E. coli* reside in the large intestine as a commensal but can turn into a dangerous pathogen that can promote intestinal and systemic illness under a variety of conditions (Kittana et al., 2018). To resolve these problems antimicrobials are used both in human and veterinary medicine (Moulin et al., 2008). Additionally, antimicrobial agents are routinely fed to poultry especially broilers as antimicrobial growth promoters (AMGP). Indiscriminate use of antimicrobials for therapeutic and preventive purposes could increase the risk of resistance (Hassan, 2020). Development of resistance to antimicrobial is a complex phenomenon that involves bacterial genetic and metabolic mechanisms, and it can be expedited due to antibiotic selection pressure (Zhang et al., 2015; Munita and Arias, 2016). Due to intensive broiler production the antibiotic selection pressure for resistance in microorganisms is high, and consequently, their faecal flora contains a comparatively high proportion of resistant microorganisms (Van den Bogaard et al., 2001; Hassan et al., 2014a). Those resistant organisms can transmit into human, animals, and the environment (McEwen and Collignon, 2018).

It is known that poultry meat can be frequently contaminated with *E. coli* during unhygienic handling and dressing, improper cleaning, and unhygienic practices of selling meat (Hayes and Forsythe, 2013). Poultry meat is considered as a potential source of infection as there is a higher chance of contamination with *E. coli* via direct contact during food preparation (Bélanger et al., 2011). Broiler meat contains antimicrobial-resistant *E. coli*, which is frequently transmitted to people through natural processes such as the food chain, representing a possible risk of infections in human (Osman et al., 2018). Numerous researches have shown that avian *E. coli* is prevalent in broiler meat all over the world, however there is a lack of data in Bangladesh. A review article have shown that the majority of the avian *E. coli* associated AMR study was conducted in some selected metropolitan cities namely Dhaka, Rajshahi, and Mymensingh (Khan et al., 2020). Though there are several veterinary and research laboratory facilities in these parts of the country there is no systemic and structured surveillance on AMR issues because of multiple issues. For example, In Bangladesh,

the majority of the farmers (>60%) use antibiotics without any prescription (Islam et al., 2016c). Besides the farms, the poultry meat sellers in live bird markets (LBMs) also use different types of antibiotic to prevent unwanted mortality (Khan et al., 2018). Among different antimicrobials, tetracycline is the most often used antimicrobials due to its cheaper price and availability, which may lead to emergence of tetracycline resistant strains of *E. coli* in Bangladesh. Several others antimicrobials such as ciprofloxacin, amoxicillin, and gentamycin are also used at different stages of production. Therefore, this study is conducted to understand the current scenario of *E. coli* contamination along with the resistance pattern in chicken meat from the outlets of local super-shops and live bird markets located in Chattogram Metropolitan Area (CMA), Bangladesh.

Objectives:

1. To identify the prevalence of *E. coli* in broiler meat.
2. To assess the antimicrobial resistance pattern against *E. coli* isolated from broiler meat.
3. Investigation on drug-resistant *E. coli* from animal, human and environment carrying *tet*-A, *tet*-B, *tet*-C and *tet*-D genes.

REVIEW OF LITERATURE

2.1 Brief history of the development of antibiotics:

Antibiotics were discovered in the middle of the 19th century, and brought down the threat of infectious diseases, which had devastated the human race. History of antibiotic development started with the discovery of penicillin by Alexander Fleming. In 1928 at St Mar's Hospital of London, Alexander Fleming was working in his laboratory. Fortunately, a piece of mold contaminated his petri-dish. Later he discovered that this could produce a chemical substance (penicillin) which has ability to kill bacteria. Later he found that the mold was *Penicillium notatum*, which inhibited the growth of *Staphylococcus* spp. by producing some chemical substance that is penicillin into the agar medium. This finding had such a great influence in the treatment of infectious diseases that the drug was termed as "magical bullet". Later, this drug saved life of millions of people in world war-II. Later in 1948, the tetracyclines, a large family of antibiotics, were discovered by Benjamin Minge Duggar as natural products, and first prescribed in 1948. The discovery of penicillin together with several other different antimicrobial agents saved millions of humans and animals from infectious disease-causing organisms. The observation of *Staphylococci* spp. that could still grow in the presence of penicillin was the beginning of the era of antimicrobial resistance and the realization that after all the drugs that were described as "magical bullets" were not to last for long due to the selective pressure that was being exerted by the use of these agents. Fortunately, tetracycline is used to treat infections caused by gram negative bacteria mainly *Enterobacteriaceae* both in human and veterinary medicine. This group of drugs is considered to be one of the best options for clinical management of extended spectrum β-lactumase (ESBL) and carbapenemase producing *Enterobacteriaceae* (Morrill et al., 2015).

2.2 Mode of action of antimicrobials against bacterial pathogens:

Knowledge about the mechanism of action of antimicrobials is important to understand the resistance mechanism developed by the microbes. Different antimicrobials target different sites of the microorganisms to exert their effects. Major targets of microorganism are cell wall, cell membrane, ribosomal subunits, and enzymes of DNA synthesis or any other mechanism. Again, bacterial genome has remarkable ability to take up and express new antimicrobial resistance genes. The transposable elements or mobile genetic elements which include transposon, integrons and conjugative transposons play important roles in transfer of resistance genes. Antimicrobial resistance genes in bacteria can be transmitted from each other through horizontal or vertical ways. Mechanism of action of different antimicrobials along with their target sites have been summarized in **Table 2.1**.

Table 2.1: Targets used by commonly used antimicrobial agents (Lange et al., 2007)

Target	Antibacterial class	Example	Principle target
Cell wall biosynthesis	*β-lactams*	Penicillin, Methicillin	PBPs (transpeptidases)
	β-lactamase inhibitors	Clavulanic acid	*β-lactamase*
	Glycopeptidaes	Vancomycin	Terminal D-Ala-D-Ala in lipid II
	Cyclic peptidase	Bacitracin	Undecaprenyl
Bacterial cell membrane	Cationic peptides polymyxins	Colistin	LPS in outer membrane
	Lipopeptides	Daptomycin	Cytoplasmic membrane
Protein biosynthesis:			
30S subunit	Aminoglycosides	Gentamycin	16S rRNA (A-site)
	Tetracyclines	Doxycycline	16S rRNA (A-site)
50S subunit	Phenyl propanoids	Chloramphenicol	23S rRNA
	Macrolides	Erythromycin	23S rRNA
	Ketolides	Azithromycin	23S rRNA
DNA biosynthesis	Fluroquinolones	Ciprofloxacin	A-subunit of DNA gyrase
RNA biosynthesis	Rifamycins	Rifampicin	β-subunit of RNA polymerase
Folate biosynthesis	Diaminopyrimidines	Trimethoprim	Dihydrofolate synthase
	Sulphonamides	Sulfamethoxazole	Dihydropteroate synthase
Fatty acid and mycolic acid biosynthesis	Isoniazid		NADP-dependent enoyl-ACP reductase
Agents exerting pleiotropic or unknown effect	Nitrofurans	Nitrofurantoin	Multiple sites eg. Ribosomal proteins

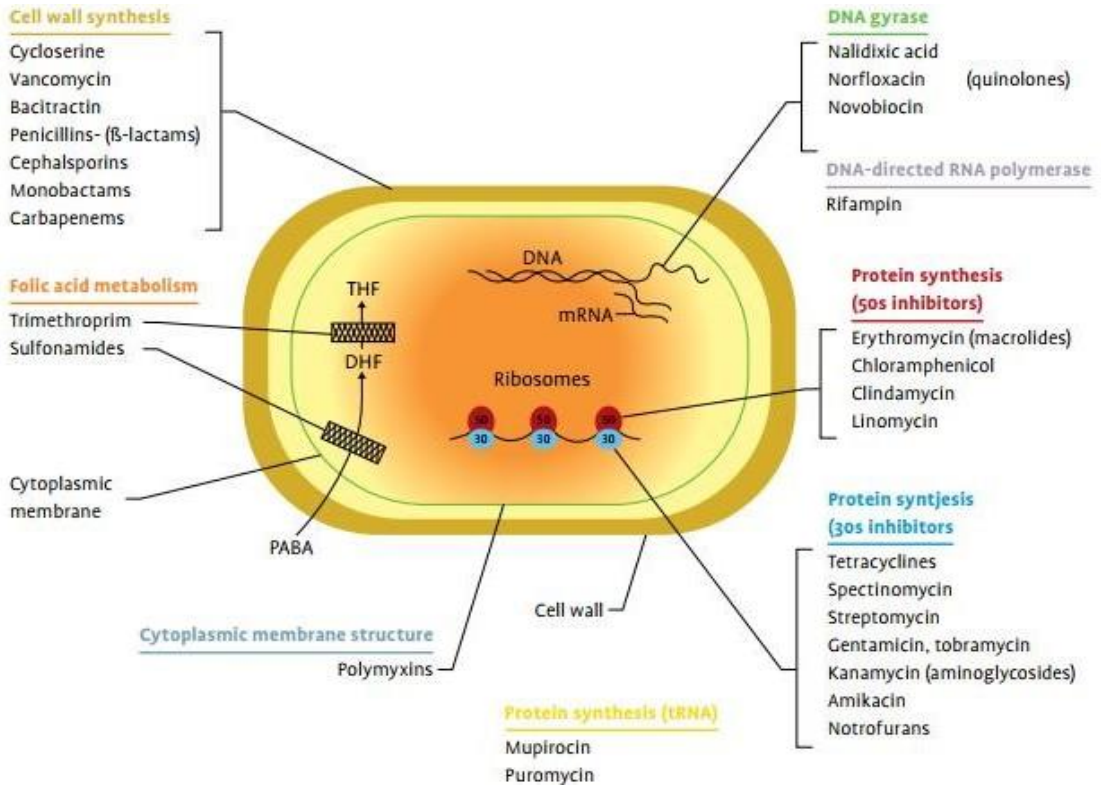


Figure 2.1: Mode of action of antimicrobials with their target sites

2.3 Different categories of *E. coli* with their pathogenic potential:

Though *E. coli* is present as commensal in gastrointestinal tract of many species but pathogenic forms of *E. coli* can cause a variety of diarrheal diseases in hosts due to the presence of specific colonization factors, virulence factors and pathogenicity associated genes which are generally not present in other. Among all the strains that cause diarrheal diseases, following pathotypes are now recognized. **Table 2.2** shows the pathotypes of *E. coli* along with their clinical conditions produced in host.

Table 2.2: Pathotypes of pathogenic *E. coli* and associated clinical conditions

Pathotype	**Associated clinical conditions**
Enteropathogenic *E. coli* (EPEC)	Diarrhea in children and animals
Enterohemorrhagic *E. coli* (EHEC)	Hemorrhagic colitis and hemolytic-uremic syndrome
Enterotoxigenic *E. coli* (ETEC)	Traveler's diarrhea, porcine and bovine diarrhea
Enteroinvasive *E. coli* (EIEC)	Watery diarrhea and dysentery
Enteroaggregative *E. coli* (EAEC)	Persistent diarrhea in humans
Diffusely adherent *E. coli* (DAEC)	Diarrhea in children
Uropathogenic *E. coli* (UPEC)	Urinary tract infections in humans and animals
Meningitis/sepsis-associated *E. coli* (MNEC)	Meningitis and sepsis

Pathogenic strains of *E. coli* are responsible for three types of infections in host. They are intestinal diseases (gastroenteritis), urinary tract infections (UTI) and neonatal meningitis. The diseases caused by a particular strain of *E. coli* depend on distribution and expression of virulence determinants, including adhesins, invasins, toxins, and abilities to withstand host defenses. These are summarized in following:

I. **Adhesins:** CFAI/CFAII, Type 1 fimbriae, P fimbriae, S fimbriae, Intimin (nonfimbrial adhesin), EPEC adherence factor

II. **Invasins:** Hemolysin, Shigella-like "invasins" for intracellular invasion and spread

III. **Motility/ chemotexis:** Flagella

IV. **Toxins:** LT toxins, ST toxin, Shiga toxin, Cytotoxins, Endotoxin (LPS) V. **Anti-phagocytic surface properties**: Capsules, K antigens, LPS

V. **Defense against serum bactericidal reactions:** LPS, K antigens

VI. **Defense against immune responses:** Capsules, K antigens, LPS, Antigenic variation.

VII. **Genetic attributes**: Transmissible plasmids, R factors and drug resistance plasmids, Toxin and other virulence plasmids, Siderophores and siderophore uptake system, Pathogenicity islands, Genetic exchange by transduction and conjugation.

The virulence factors of intestinal pathogenic groups of *E. coli* are summarized in **Table 2.3**.

Table 2.3: Virulence factors of intestinal pathogenic *E. coli* (Kaper et al., 2004)

Pathotype	**Virulence factors**	
	Colonization and fitness factors	**Toxins and effectors**
EPEC	Intimin, Long polar fimbriae (LPF), Paa and Bundle-forming pilus (BFP)	EspC, Cycle inhibiting factor (Cif), EspF, EspH, Map, Tir and Lifa/Efa
EHEC	Intimin, Paa,ToxB, Bundle-forming pilus (BFP), Efa-1/LifA, Saa and OmpA	Shiga toxin (Stx), EspP, Urease, Cycle inhibiting factor (Cif), EspF, EspH, Map, Tir, Lifa/Efa, StcE and Ehx
ETEC	Colonization factor antigens (CFA). More than 20 antigenically diverse CFs, approximately 75% of human ETEC express either CFA/I, CFA/II or CFA/IV	Heat-labile toxin (LT), Shigella enterotoxin 2 (ShET2) and heat-stable toxin (Sta, STb)
EIEC	IcsA (VirG), Aerobactin and Chu (Shu)	Shigella enterotoxin 1(ShET1), Pic, SepA,
EAEC	Aggregative adherence fimbriae and dispersin	SigA, Ipa (A, B, C, H), IpgD and VirA
DAEC	Dradhesins	Shigella enterotoxin 1, Pet and Pic

2.4 Sources of human *E. coli* infections:

E. coli O157:H7 is transmitted to humans primarily through consumption of contaminated foods, such as raw or undercooked ground meat products and raw milk. Faecal contamination of water and other foods, as well as cross-contamination during food preparation (with beef and other meat products, contaminated surfaces and kitchen utensils), will also lead to infection. Examples of foods implicated in outbreaks of *E. coli* O157:H7 include undercooked hamburgers, dried cured salami, unpasteurized fresh-pressed apple cider, yogurt, and cheese made from raw milk. An increasing number of outbreaks are associated with the consumption of fruits and vegetables (including sprouts, spinach, lettuce, coleslaw, and salad) whereby contamination may be due to contact with faeces from domestic or wild animals at some stage during cultivation or handling. STEC has also been isolated from bodies of water (such as ponds and streams), wells and water troughs, and has been found to survive for months in manure and water-trough sediments. Waterborne transmission has been reported, both from contaminated drinking water and from recreational waters.

Person-to-person contact is an important mode of transmission through the oral-faecal route. An asymptomatic carrier state has been reported, where individuals show no clinical signs of disease but are capable of infecting others. The duration of excretion of STEC is about 1 week or less in adults, but can be longer in children. Visiting farms and other venues where the public might come into direct contact with farm animals has also been identified as an important risk factor for STEC infection.

2.5 Antimicrobials used as chemoprophylaxis in animals to control enteric bacterial infections:

Chemoprophylaxis in human and animals using antimicrobials can prevent enteric infections in some extent. Unfortunately, the emergence of antimicrobial resistance has made the decision of how and when to use antibiotic for prophylaxis. Controlled studies have shown that diarrhea attack rates are reduced by 90% or more by the use of antibiotics in human. Fluoroquinolones have been the most effective antibiotics for the prophylaxis and treatment of bacterial traveler's diarrhea in human. Alternative considerations include Azithromycin and Rifaximin, a non-absorbable broad-spectrum

antibiotic. Antimicrobial drugs are widely applied in animal husbandry to increase production, treatment of infectious diseases and as growth promoters (Bien et al., 2015). Tetracycline, β-lactams and macrolides are the most common antibiotic groups which are being used for veterinary purposes (Li et al., 2012). Owing to their lower cost and their higher antimicrobial activity, tetracycline antibiotics are widely used as veterinary drugs for the prevention and treatment of several infectious diseases. An individual relying on prophylactic antibiotics will need to carry an alternative antibiotic to use if severe diarrhea develops despite prophylaxis. The risks associated with the use of prophylactic antibiotics should be weighed against the benefit of using prompt, early self-treatment with antibiotics when moderate to severe clinical state develops, shortening the duration of illness. In poultry and livestock, mass administration of antibiotics is often practiced when transporting or moving young animals, during dry-cow therapy in dairy cows and in preventing respiratory and intestinal maladies when animals have been subjected to severely stressful conditions. Prophylactic antibiotics may be considered for short-term relief for the individuals who are at high risk of those enteric pathogens. When animals are administered an antibiotic that is closely related to an antibiotic used in human medicine, cross-resistance occurs and disease-causing bacteria become resistant to the drug used in human medicine. The consensus of the world's veterinary and medical experts is that it is dangerous and unjustifiable to use antibiotics that are related to drugs of critical importance in human medicine for "preventive" administration to groups of apparently healthy animals.

2.6 Antimicrobial resistance:

Antimicrobial resistance is the resistance of a microbe to an antimicrobial agent that was used effectively in treating or preventing an infection caused by that microbe. When the infectious agent is bacteria then the more specific term is antibiotic resistance or antibiogram. The World Health Organization (WHO) report released on April 2014 stated that, "this serious threat is no longer a prediction for the future, it's happening right now in every region of the world and has the potential to affect anyone, of any age, in any country. This antimicrobial resistance is considered as one of the three greatest threats to public health (WHO report, 2011)." The rising trend of drug resistance can be attributed to three primary areas: use of antibiotics in the human population, use of antibiotics in animal population, and the spread of resistant strains

between human and non-human sources. Any use of antibiotics can increase selective pressure in a population of bacteria, causing vulnerable bacteria to die thereby increasing the relative numbers of resistant bacteria and allowing for further growth.

2.7 Mechanisms involved behind the emergence of antimicrobial resistance in bacterial pathogens:

Prior to the 1990s, the problem of antimicrobial resistance was never taken to such an extent to threat to the management of infectious diseases. Nevertheless, gradually treatment failures were increasingly being seen in health care settings against first-line drugs and second-line drugs or so on. Microorganisms were increasingly becoming resistant against different antimicrobial agents to which they were susceptible before. They achieved this through different ways but primarily based on the chemical structure of the antimicrobial agent and the mechanisms through which the agents acted against those pathogens. Resistance to antimicrobials can be described in two ways:

a) Intrinsic or natural resistance: In this case microorganisms naturally do not possess target sites for the drugs and therefore the drug does not affect them or they naturally have low permeability to those agents because of the differences in the chemical nature of the drug and the microbial membrane structures especially for those that require entry into the microbial cell in order to affect their action.

b) Acquired resistance: Here a naturally susceptible microorganism acquires ways of not being affected by the drugs used to treat infections caused by them.

Fluit *et al*., (2001) summarized the major mechanisms of acquired resistance as follows:

- The presence of an enzyme that inactivates the antimicrobial agent.
- The presence of an alternative enzyme for the enzyme that is inhibited by the antimicrobial agent.
- Mutation in the antimicrobial agent's target site(s), which reduces the binding of the antimicrobial agent.
- Post-transcriptional or post-translational modification of the antimicrobial agent's target, which reduces binding of the antimicrobial agent.
- Reduced uptake of the antimicrobial agent.

- Active efflux of the antimicrobial agent.
- Overproduction of the target of the antimicrobial agent.
- Expression or suppression of a gene in vivo in contrast to the situation in vitro

2.7.1. Resistance to *β-lactam* antibiotics:

The *β-Lactam* antibiotics are a group of antibiotics characterized by possession of a *β-lactam* ring and they include penicillins, cephalosporins, carbapenems, oxapenams, and cephamycins. The penicillins are one of the most commonly used antibiotics in developing countries because of their ready availability and relatively low cost. The *β-lactam* ring is important for the activity of these antibiotics which results in the inactivation of a set of trans-peptidases that catalyze the final cross-linking reactions of peptidoglycan synthesis in bacteria. The effectiveness of these antibiotics relies on their ability to reach to the penicillin-binding protein (PBP) and to intact, to bind with the PBPs. Resistance to *β-lactams* in many bacteria is usually due to the hydrolysis of the antibiotic by a *β-lactamase* or the modification of PBPs or cellular permeability. *β-lactamase* constitute a heterogeneous group of enzymes which are classified according to different ways including their hydrolytic spectrum, susceptibility to inhibitors, genetic localization (plasmidic or chromosomal), and gene or amino acid sequence in protein. The functional classification scheme of *β-lactamase* proposed by Bush et al., (1995) defines four groups according to their substrate and inhibitor profiles:

i. Group 1 are cephalosporinases that are not well inhibited by clavulanic acid;
ii. Group 2 are penicillinases, cephalosporinases, and broad-spectrum β-lactamases that are generally inhibited by active site-directed β-lactamase inhibitors;
iii. Group 3 are metallo-β-lactamases that hydrolyze penicillins, cephalosporins, carbapenems and that are poorly inhibited by almost all β-lactam containing molecules;
iv. Group 4 are penicillinases that are not well inhibited by clavulanic acid.

2.7.2. Resistance to tetracyclines:

Tetracyclines are another of the very commonly used antimicrobial agents in both human and veterinary medicine in developing countries because of their availability and low cost as well as low toxicity and broad spectrum of activity. They are broad-

spectrum agents, exhibiting activity against a wide range of gram-positive and gram-negative bacteria, atypical organisms such as chlamydia, mycoplasmas, rickettsia and protozoan parasites. Examples of these include tetracycline, doxycycline, minocycline, and oxytetracycline. Resistance to these agents occurs mainly through three mechanisms (Roberts, 1996), namely

i. Efflux of the antibiotics,
ii. Ribosome protection, and
iii. Modification of the antibiotic

Efflux of the drug occurs through an export protein from the major facilitator superfamily (MFS). These export proteins are membrane-associated proteins, which are coded for by *tet* efflux genes and export tetracycline from the cell. Export of tetracycline reduces the intracellular drug concentration and thus protects the ribosomes within the cell (Levy, 1988).

2.7.3. Resistance to aminoglycosides:

Resistance to aminoglycosides such as gentamicin, tobramycin, amikacin, and streptomycin is widespread, with more than 50 aminoglycoside-modifying enzymes described. Most of these genes are associated with gram-negative bacteria. Depending on their type of modification, these enzymes are classified as aminoglycoside acetyltransferases (AAC), aminoglycoside adenyltransferases (also named aminoglycoside nucleotidyltransferases; ANT), and aminoglycoside phosphotransferases (APH) (Shaw et al., 1993). Aminoglycosides are modified at amino groups by AAC enzymes or at hydroxyl groups by ANT or APH enzymes and thus lose their ribosome-binding ability resulting no longer inhibition of protein synthesis. Besides aminoglycoside-modifying enzymes, efflux systems and rRNA mutations have been described for aminoglycoside resistance (Byarugaba, 2010).

2.7.4. Resistance to quinolones and fluroquinolones:

Mechanisms of bacterial resistance to quinolones as described by Hooper (2001) fall into two principal categories:

i. Alterations in drug target enzymes and
ii. Alterations that limit the permeability of the drug to the target.

In gram-negative organisms, DNA gyrase seems to be the primary target for all quinolones. In gram-positive organisms, topoisomerase-IV or DNA gyrase is the primary target depending on the fluroquinolones considered. In almost all instances, amino acid substitutions within the quinolone resistance-determining region (QRDR) involve the replacement of a hydroxyl group with a bulky hydrophobic residue. Mutations in *gyr*A induce changes in the binding-site conformation that may be important for quinolone– DNA gyrase interaction. Changes in the cell envelope of gram-negative bacteria, particularly in the outer membrane, have been associated with decreased uptake and increased resistance to fluroquinolones, and this has not been demonstrated in gram-positive bacteria.

2.7.5 Resistance to sulfonamides and trimethoprim:

Resistance in sulfonamides is commonly mediated by production of drug-resistant forms of dihydropteroate synthase (DHPS). Sulfonamide resistance in gram-negative bacilli generally arises from the acquisition of either of the two genes *sul*1 and *sul*2, encoding for the production of enzyme dihydropteroate synthase that are not inhibited by the drug (Enne et al., 2001). The *sul*1 gene is normally found linked to other resistance genes in class-1 integrons, while *sul*2 is usually located on small non-conjugative plasmids or large transmissible multi-resistance plasmids. Trimethoprim is an analog of dihydrofolic acid, an essential component in the synthesis of amino acid and nucleotides that competitively inhibits the enzyme dihydrofolate reductase (DHFR). At least 15 DHFR enzyme types are known based on their properties and sequence homology.

Trimethoprim resistance is caused by a number of mechanisms (Thomson, 1993) including:

i. Over production of the host DHFR enzyme.
ii. Mutations in the structural gene for DHFR.
iii. Acquisition of a gene (*dfr*) encoding a resistant DHFR enzyme which is the most resistant mechanism in clinical isolates.

2.8. Mechanisms of resistance to tetracyclines:

Tetracyclines interfere with the initiation step of protein synthesis by inhibiting the binding of aminoacyl tRNA to the A-site of the ribosome (Chopra et al., 2001). The 7S protein and the 16S RNA show the greatest affinity for tetracyclines and are therefore the main targets. This binding inhibits the fixation of a new aminoacyl tRNA on the ribosome. In addition, tetracyclines bind, or at least protrude, in the P-site by alteration in ribosome conformation in the posttranslocational state, and may modify the ribosome conformation at the level of the head of the 30S subunit and the interface side of the 50S subunit.

Resistance to tetracyclines is widespread. Some 29 genes on mobile elements have been identified in the so-called 'tetracycline'(*tet*) family and three in the 'oxytetracycline resistance' gene family (*otr*). Two main mechanisms have been described, namely efflux and ribosomal protection. Resistance by enzymatic inactivation has been described but remains uncommon. Low levels of resistance can also result from mutations or decreased expression of porins. As tigecycline increases the number of bonds to the target 16S RNA, the drug is unaffected by the ribosome protection mechanism (Olson et al., 2006). Together with the vicinal hydrophobic moiety, it makes the molecule less susceptible to efflux, with the noticeable exception of resistance nodulation cell division (RND)-type efflux pumps constitutively expressed by *P. aeruginosa* and Proteae, against which it is inactive (Zhanel et al., 2004). Moreover, mutants (related to efflux) have already been reported in Acinetobacter spp.

2.9. WHO's prioritization of multi-drug resistant bacterial pathogens:

Recently, in 27th February, 2017 World Health Organization (WHO) has published its first ever list of antibiotic resistant "priority pathogens" a catalogue of 12 families of bacteria that pose the greatest threat to human health. The list was drawn up to guide and promote research and development (Research and Development) of new antibiotics, as part of WHO's efforts to address growing global resistance to antimicrobial medicines. The list highlights in particular the threat of gram-negative bacteria that are resistant to multiple antibiotics. These bacteria have built-in abilities to find new ways to resist treatment and can pass along genetic material that allows other bacteria to become drug-resistant as well. The list was developed in collaboration

with the Division of Infectious Diseases at the University of Tübingen, Germany. The team used a multi-criteria decision analysis technique vetted by a group of international experts. The criteria for selecting pathogens on the list were:

- How deadly the infections they cause are?
- Whether their treatment requires long hospital stays?
- How frequently they are resistant to existing antibiotics when people in communities catch them?
- How easily they spread between animals, from animals to humans, and from person to person?
- Whether they can be prevented (e.g. through good hygiene and vaccination)?
- How many treatment options remain and finally?
- Whether new antibiotics to treat them are already in the Research and Development pipeline?

The WHO list is divided into three categories according to the urgency of need for new antibiotics: critical, high and medium priority.

A) Priority 1: Critical

a. *Acinetobacter baumannii*, carbapenem-resistant
b. *Pseudomonas aeruginosa*, carbapenem-resistant
c. *Enterobacteriaceae*, carbapenem-resistant, ESBL-producing

B) Priority 2: High

a. *Enterococcus faecium*, vancomycin-resistant
b. *Staphylococcus aureus*, methicillin-resistant, vancomycin-intermediate and resistant
c. *Helicobacter pylori*, clarithromycin-resistant
d. *Campylobacter* spp., fluoroquinolone-resistant
e. *Salmonellae*, fluoroquinolone-resistant
f. *Neisseria gonorrhoeae*, cephalosporin-resistant, fluoroquinolone-resistant

C) Priority 3: Medium

a. *Streptococcus pneumoniae*, penicillin-non-susceptible
b. *Haemophilus influenzae*, ampicillin-resistant
c. *Shigella* spp., fluoroquinolone-resistant

2.10. Prevalence of multi-drug resistant (MDR) *E. coli* in animals in different parts of the world:

2.10.1. MDR *E. coli* in poultry and poultry farm environment:

The problem of antimicrobial drug resistance in veterinary pathogens mainly in poultry is augmented by the uncontrolled use of un-prescribed antimicrobial drugs in developing countries like Bangladesh. A high prevalence of resistant phenotypes has recently been reported in poultry isolates of Bangladesh. Hassan *et al*. (2014) isolated *E. coli* and *Salmonella* spp. from commercial layer and stated that 100% of *E. coli* isolates were resistant to Tetracycline, Ciprofloxacin, Enrofloxacin and Pefloxacin followed by Amoxicillin (84.62%), Kanamycin (69.24%), Colistin (63.75%), Doxycycline (53.75%), and Neomycin (23.08%). In case of *Salmonella* spp. 100% resistance were found for Amoxicillin and Tetracycline followed by Enrofloxacin (87.5%), Ciprofloxacin (87.5%), Pefloxacin (87.5%), Doxycycline (50%), Colistin (50%) and Kanamycin (50%) (Hassan et al., 2014b). A study published by Center for Science and Environment (CSE), New Delhi stated that 100% *E. coli*, 92% *Klebsiella pneumoniae* and 78% *Staphylococcus lentus* of 12 randomly selected broiler farms from 4 states with heavy poultry production were resistant to 3 or more classes of antibiotics. Even some *E. coli* and *K. pneumoniae* isolates were resistant to 10 or more antibiotics.

2.10.2. MDR *E. coli* in different food animals:

Antimicrobial resistant pathogens are easily transmitted from food animals to human through food chain. Sanjukta *et al*. (2016) isolated *E. coli* and *Salmonella* spp. of different animal species (cattle, goat, swine, yak and avian) from seven states of India. They found 85.2% samples were positive for *E. coli* and 7.03% for *Salmonella* spp. About 45.07% *E. coli* isolates were MDR while 6% were resistant to all 20 antimicrobials tested (Sanjukta et al., 2016). As stated by Adenipekun *et al*. (2015) the prevalence of *E. coli* is 88.7% in cattle, 81% in chicken, 89.5% in swine. The resistance patterns were 58.8%, 39.8% and 34.1% for Tetracycline, Sulphamethoxazole-trimethoprim and Ampicillin individually. Among the isolates 26 were multi-drug resistant (resistant to ≥2 antimicrobials) (Adenipekun et al., 2015). Islam et al. (2016) isolated *E. coli* from goat of Cox's Bazar, Bangladesh. The overall prevalence that was

reported is 52% and higher prevalence was found in goat having diarrhea (diarrheic: 62%, non-diarrheic: 38%). Among the isolates 39.74% showed multi-drug resistance (resistant to 3 to 8 classes of antimicrobials) (Islam et al., 2016b). Shiga toxin-producing *E. coli* (STEC) O157:H7 is a well-recognized cause of hemolytic uremic syndrome and hemorrhagic colitis in human, which can be transmitted from domestic food animals. About 2.1% samples from goat cecal contents is positive for *E. coli* O157 which is resistant to 2 to 18 antimicrobials (Dulo et al., 2015b). Iweriebor *et al*. (2015) identified that about 31.7% isolates of cattle were *E. coli* O157 that harbored genes for shiga toxin production (*stx*1 and or *stx*2). Distribution of resistance genes among the isolates were bla_{ampC} 90 %, bla_{CMY} 70 %, $bla_{CTX\text{-}M}$ 65 %, bla_{TEM} 27 % and *tet*A 70 % and *str*A 80 % (Iweriebor et al., 2015). A study to characterize the genotype of MDR *E. coli* strains recovered from cattle and farm environment in Ireland showed that the most prevalent antimicrobial resistance identified is to Streptomycin (100%) followed by Tetracycline (99%), Sulfonamides (98%), Ampicillin (82%) and Neomycin (62%) (Karczmarczyk et al., 2011). Investigation of healthy food animals (cattle, chicken and swine) showed that *E. coli* is positive for 88.7% in cattle, 81% in chicken and 89.5% in swine. *E. coli* were resistant to Tetracycline (58.8%), Sulphamethoxazole-trimethoprim (39.8%), Ampicillin (34.1%) and among the resistant isolates less than 50% were MDR (resistant to ≥ 2 antimicrobials) (Adenipekun et al., 2015).

2.11. Prevalence of multi-drug resistant *E. coli* in humans in different parts of the world:

The increasing rate of antibiotic resistance among bacterial pathogens causing both hospital and community acquired infections is a serious threat to human worldwide. Typically, ESBL-producing strains, carbapenem resistant *Enterobacteriaceae* (CRE), and other multidrug resistant (MDR) *Enterobacteriaceae* possess several additional resistance mechanisms to other classes of popular antibiotics such as phenicols, sulfonamides, fluroquinolones, tetracyclines, and aminoglycosides (Leski et al., 2012; Tada et al., 2013). This makes them extremely difficult and, in some cases, virtually impossible to treat. Leski *et al*. (2016) isolated 70 stains of *Enterobacteriaceae* family from human samples and tested for susceptibility for different groups of antimicrobials. They showed that 85.7% of the isolates were multi-drug resistant where 64.3% produced extendedspectrum ß-lactamase (ESBL) (Leski et al., 2016). Resistance

pattern to Sulphonamides, Chloramphenicol, Gentamycin, Ampicillin with Sulbactam and Ciprofloxacin were 91.4%, 72.9%, 72.9%, 51.4% and 47.1% respectively. Study by Purohit et al. (2017) showed that *E. coli* isolates from human (n=127), animals (n=21), waste (n=12), common water source for human and animals (n=12) and household drinking water (n=122) carried 70%, 29%, 41%, 30%, and 30% multi-drug resistance, respectively. Extended spectrum beta-lactamase producers were 57% in human and 23% in environmental isolates. Co-resistance was frequent for penicillin, cephalosporin, and quinolone (Purohit et al., 2017). Balkhair et al. (2014) conducted a retrospective study to find out the prevalence of MDR pathogens in a teaching hospital in Oman. The overall prevalence for MDR patient was 10.8 and MDR isolates was 11.2 in per 1000 admissions in the hospital (Balkhair et al., 2014). Drinking water contains 64.29% *E. coli* isolated in Hyderabad and 62.96% isolates showed MDR (resistant to 3 to 6 antimicrobials) (Patoli et al., 2010). They observed maximum resistance against Nalidixic acid (92.6%) followed by Ampicillin (88.89%), Ceftriaxone (40.74%), Ciprofloxacin (37.04%), Ceftazidime (25.23%), Cefotaxime (18.52%), and Gentamicin (18.52%) whereas none of the *E. coli* isolates showed resistance against Amikacin (Patoli et al., 2010). Chellapandi *et al*., (2017) conducted a study to determine the prevalence of MDR diarrhoeagenic *E. coli* isolated from children with or without diarrhea in India. About 41.40% diarrhoeagenic isolates were MDR (resistant against ≥5 antimicrobials) (Chellapandi et al., 2017). People working in livestock farms are also in critical condition. The prevalence of antimicrobial resistant *E. coli* was higher in livestock farm worker compared to non-livestock worker (restaurant workers) (Cho et al., 2012). The rates of livestock workers in association with multi-drug resistance were also higher than the rates in restaurant workers (Cho et al., 2012). Street foods contain variable types and number of microorganisms, which cause food-borne illness in human. The percentage of resistance of theses isolates ranged from 13.3% to 100% (Ogidi et al., 2016). Aly *et al*., (2012) showed that in Eygpt 35% *E. coli* isolates from food and clinical samples showed multi-drug resistance pattern (≥3 groups of antibiotics) and interestingly the arte of MDR *E. coli* isolated from foods was higher than those of clinical isolates (Aly et al., 2012). Campos *et al*., (2014) in Portugal who showed that about 55% food samples were positive for *E. coli* investigated street-vending foods. The presence of different resistance determinants pattern were 30% *tetA*/*tetB* gens (for Tetracycline), 23% bla_{TEM} (for Ampicillin), 20% *strA-strB/aadA* (for Streptomycin), 20% *sul1*/*sul2* (for Sulphamethoxazole), 20%

catA/*catB3*/*floR* (for Chloramphenicol), 13% *dfrA1* (for Trimethoprim), 17% Nalidixic acid, 13% Ciprofloxacin. About 23% isolates were multi-drug resistant (Campos et al., 2014). According to Kibret and Tadesse (2013) overall multiple antimicrobial resistance rate was 75% in *E. coli* from street-vended white lupin in Ethiopia (Kibret and Tadesse, 2013). About 3.2% goat carcasses contain *E. coli* O157 which is resistant to at least to 2 to 18 antimicrobials tested (Dulo et al., 2015a).

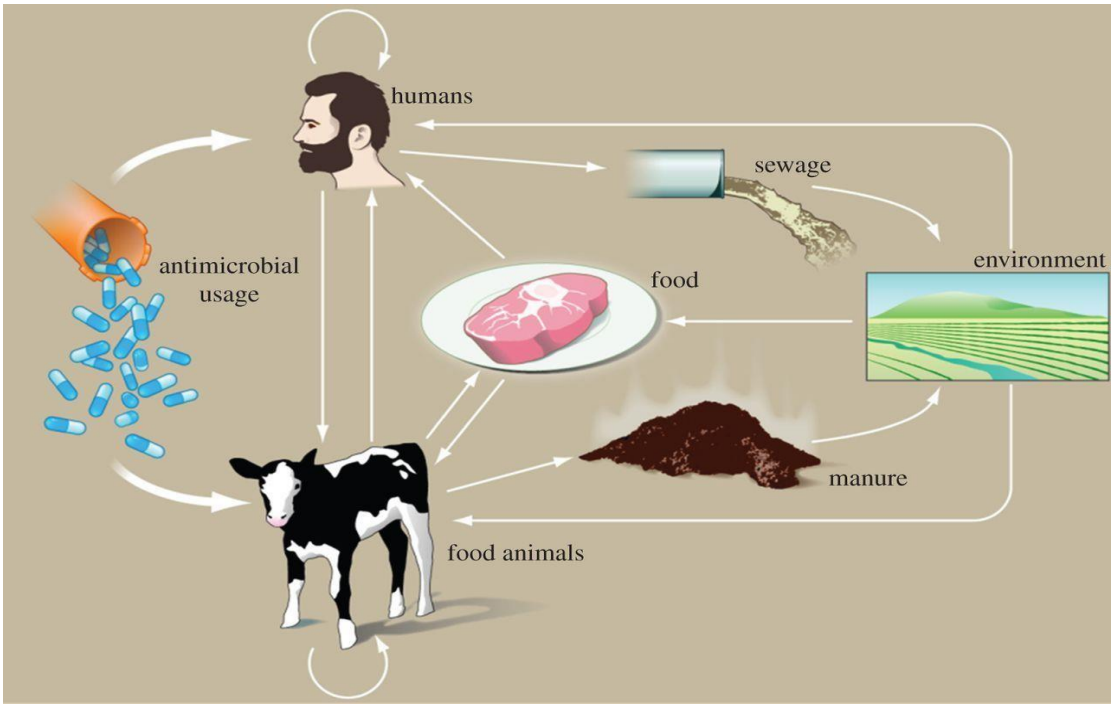


Figure 2.2: Relationship among human, animal and environment health in relation to transmission of antimicrobial resistance (Woolhouse and Ward, 2013)

2.12. Prevalence of multi-drug resistant *E. coli* of environmental origin in different parts of the world:

Another important concern in poultry practice is the ESBL producing *E. coli*. ESBL positive isolates make the treatment extremely difficult. ESBL-producing *E. coli* were detected in in layer and broiler farms at the rate of 65% and 81% respectively (Blaak et al., 2015). In case of farm environment, the percentage of ESBL producing *E. coli* were 81%, 79%, 60%, 57%, 55%, 15% and 6% in rinse and run-off water, other farm animals, dust, surface water adjacent to farms, soil, on flies and barn air correspondingly (Blaak et al., 2015). Adelowo et al., (2014) conducted a study in Nigeria to investigate antibiogram of poultry farm isolates. *E. coli* was isolated from

farm waste, litter, soil and water of poultry farms and resistance profile was Tetracycline 81%, Sulphamethoxazole 67%, Streptomycin 56%, Trimethoprim 47 %, Ciprofloxacin 42%, Ampicillin 36%, Spectinomycin 28%, Nalidixic acid 25%, Chloramphenicol 22%, Neomycin 14%, Gentamicin 8% and surprisingly 0% for Colistin, Amoxicillin-clavulanate, Ceftiofur, Cefotaxime, Florfenicol and Apramycin (Adelowo et al., 2014). Zakaria *et al*., (2015) isolated *E. coli* from human, animal and environment samples. The findings show that the prevalence of *E. coli* was 31.4% in diarrheic human, 17.3% in stools of sheep, cattle and chicken with diarrhea, 17.3% in surface water, 6.4% in sea foods, 6% in processed meat products, 3.9% in dairy products and 1.1% in poultry products (liver). About 93% isolates were resistant to 4 to 13 types of antimicrobials tested (ARABIA and Vol, 2015). Resistance to Ampicillin, Tetracycline and Sulfonamides are frequent in *E. coli* originated from fattening pig and calves less than 1 year of age from several countries of Europe (European Food Safety Authority report, 2017). About 7.1% isolates from abattoir water samples are positive for *E. coli* O157 and resistant for 2 to 18 antimicrobials (Dulo et al., 2015a).

2.13. FAO's goals on AMR

AMR threatens progress in meeting the SDGs as more agriculture producers may struggle to prevent and manage infections that threaten to disrupt food supply chains and thrust tens of millions more people into extreme poverty (Bank, 2017). To respond to this challenge and realize the four betters: better production, better nutrition, a better environment, and a better life, FAO (Food and Agriculture Organization) has established two main goals for its work on AMR:

1. Reduce AMR prevalence and slow the emergence and spread of resistance across the food chain and for all food and agriculture sectors.
2. Preserve the ability to treat infections with effective and safe antimicrobials to sustain food and agriculture production.

Through the achievement of these goals, FAO will work with stakeholders to increase the capacities of the food and agriculture sectors in managing AMR risks and building resilience to AMR impacts. By working together, FAO and partners will better protect food systems, livelihoods and economies from the destabilizing forces caused by AMR.

METHODOLOGY

3.1 Study area:

The study was conducted in Chattogram Metropolitan Area of Bangladesh from October 2020 to February 2021. Samples were collected from five super shops and nine live bird markets.

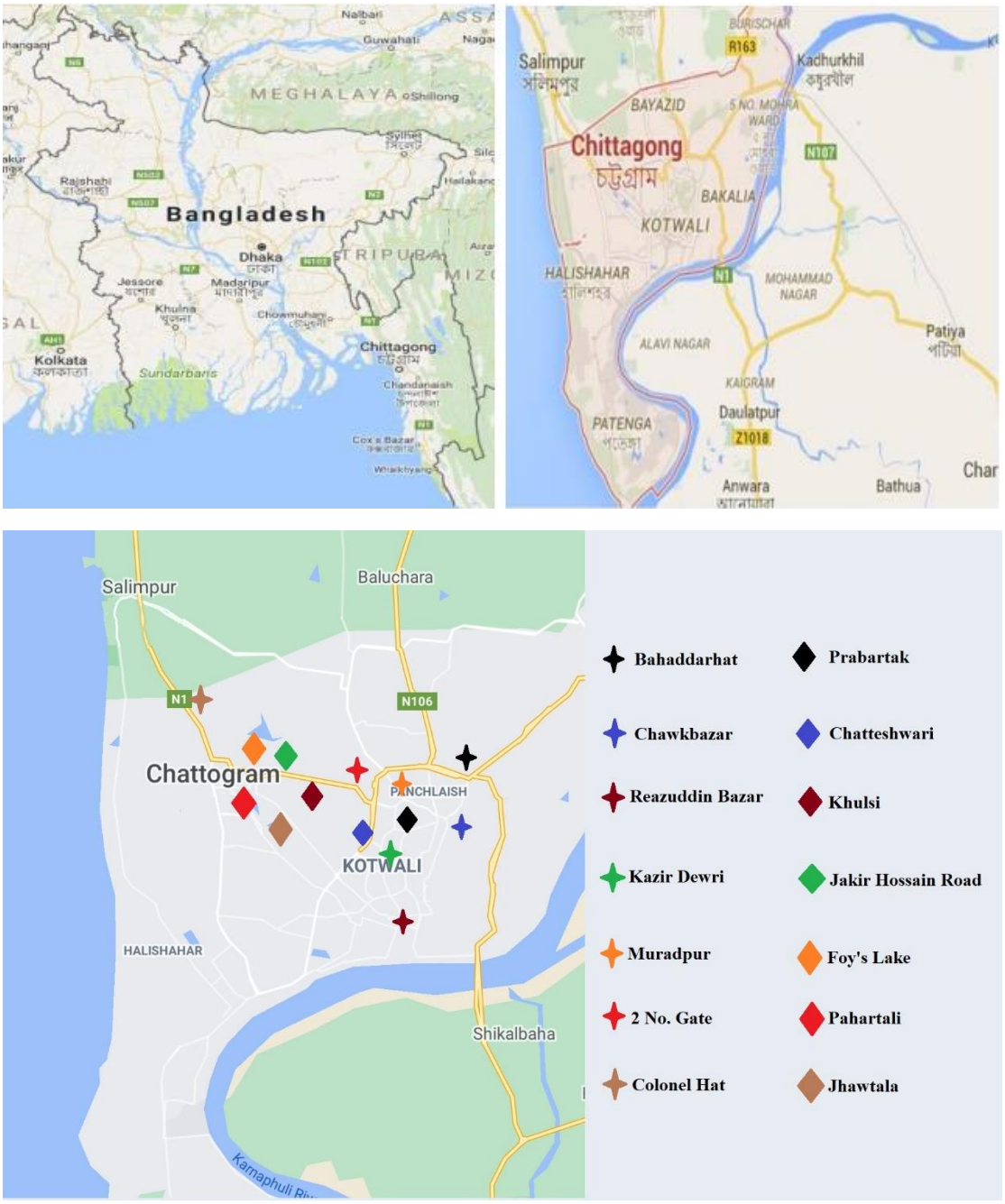


Figure 3.1: Locations of selected markets of Chattogram Metropolitan Area

3.2 Sample size:

A total of 405 meat samples were collected consisting of 215 breast muscle and 190 liver of which 225 samples were collected from super shops and 180 samples were from live bird market. Details of sampling location and sample size are presented in **Table 3.1.**

3.1: Sample source and type

Sources of samples	Type of meat samples	No. of Samples
SS	Muscle	125
	Liver	100
LBM	Muscle	90
	Liver	90
Total		**405**

SS: Super shops; LBM: Live bird markets

3.3 Sample collection, transportation, and processing procedure:

Samples were collected in separate zipper bags maintaining proper hygiene procedures. After collection, samples were shifted to the Department of Microbiology and Veterinary Public Health (DMVPH), CVASU for further investigation through maintaining a cool chain. Samples were processed into small pieces by using sterile scissors and transferred to separate sterile test tube containing buffer peptone water (BPW) (HIMEDA, p^H:7.0±0.2, Mumbai, India) and incubated at 37°C overnight for primary enrichment.

3.4 Preservation of the isolates:

All *E. coli* isolates were cultured in brain heart infusion (BHI) broth, incubated overnight at 37°C. For each isolate 700 µl BHI broth culture was added to 300 µl 15% glycerol in an eppendorf tube. Tubes were properly leveled and stored at -80°C for further investigation.

3.5 Microbiological isolation

3.5.1 Isolation and identification of *E. coli*:

For the isolation of *E. coli*, enriched culture was streaked on MacConkey agar medium (HIMEDIA, p^H: 7.1±0.2, Mumbai, India) and incubated at 37°C for 24 hours. Bright pink-colored large colonies yielded on a MacConkey agar plate were suspected as the growth of *E. coli*. Such colonies were streaked on Eosin Methylene Blue (EMB) agar plate (Merck, p^H: 7.1±0.2) and incubated at 37°C for 24 hours. Based on the "green metallic sheen" colony morphology yielded on this medium *E. coli* was confirmed. Thereafter the isolates were inoculated on blood agar and incubated at 37°C for 24 hours. After completion of the incubation period, colonies from blood agar were used for DNA extraction to be used for polymerase chain reaction (PCR).

3.5.2 Molecular identification of *E. coli*:

All phenotypically positive isolates in blood agar were subjected to molecular identification with species-specific multiplex PCR in the thermal cycler (DLAB, USA) using primers for the *uid*A gene and flanking region of the *usp*A gene. The primer sequences are listed in **Table 3.2**.

Table 3.2: Oligonucleotide primer sequences used for detection and confirmation of *E. coli*

Primer	Primer Sequence (5′-3′)	Annealing	Fragment size (bp)	Reference
*usp*A Up	CCGATACGCTGCCAAT CAGT	55.2°C	884	(Godambe et al., 2017)
*usp*A Down	ACGCAGACCGTAGGC CAGAT			
*uid*A Up	TATGGAATTTCGCCGA TTTT		164	
*uid*A Down	TGTTTGCCTCCCTGCT GCGG			

PCR reactions were conducted with a final volume of 15 µl. Proportions of different reagents used for PCR for two different resistance genes are given in **Table 3.3.**

Table 3.3: Contents of each reaction mixture of PCR assay

Serial No	Name of the contents	Amount
1	OneTaq Quick-load 2X MM w/Std Buffer (Biolabs Inc., New England)	7.5µl
2	*usp*A Up	0.5µl
3	*usp*A Down	0.5µl
4	*uid*A Up	0.5µl
5	*uid*A Down	0.5µl
6	DNA template	1µl
7	Nuclease free water	4.5 µl
Total Volume		**15 µl**

All PCR reactions were performed on a thermal cycler (DLAB Scientific Inc., USA) in Molecular Microbiology lab under DMVPH, CVASU following the cyclic conditions mentioned in **Table 3.4**.

Table 3.4: Cycling conditions used during PCR for detection of *E. coli*

Serial No	Steps	Temperature and time
1	Initial denaturation	94°C for 5 minutes
2	Final denaturation (35 cycles)	94°C for 10 seconds
3	Annealing	55.2°C for 10 seconds
4	Initial extension	72°C for 1 minute
5	Final extension	72°C for 10 minutes
6	Final holding	4°C

3.5.3 Screening of antimicrobial resistance pattern of *E. coli* isolates against a panel of antimicrobials:

The *E. coli* positive isolates in PCR were screened for antimicrobial susceptibility against a panel of antimicrobials using Kirby-Bauer disc diffusion method (Bauer, 1966). Eight antimicrobials of seven different groups (β-lactam antibiotics,

tetracyclines, polymyxins, aminoglycosides, quinolones, sulfonamides and penicillins) of drugs having public health significance were selected for the CS testing. The following anti-microbial agents (with respective disc potencies) were used: CT: Colistin sulfate (10µg), TE: Tetracycline (30µg), CN: Gentamycin (10µg), DO: Doxycycline (30µg), AMP: Ampicillin (10µg), CL: Cephalexin (30µg), SXT: Sulfamethoxazole-trimethoprim (25µg), CIP: Ciprofloxacin (5µg). To interpret the result of CS test the CLSI-2018 standards are given in **Table 3.5**.

Table 3.5: Concentrations and diffusion zone breakpoints for resistance against antimicrobials standard for *E. coli* isolates (CLSI, 2018)

Group of anti-microbial agents	**Anti-microbial agent (code)**	**Disc content**	**Diffusion zone breakpoint (diameter in mm)**		
			R	**I**	**S**
β-lactam antibiotics	Cephalexin (CL)	30µg	≤14	-	≥15
Tetracyclines	Tetracycline (TE)	30µg	≤15	12-14	≥11
	Doxycycline (DO)	30 µg	≤10	11-13	≥14
Polymyxins	Colistin sulfate (CT)	10µg	≤10	-	≥11
Aminoglycosides	Gentamycin (CN)	10µg	≤12	13-14	≥15
Quinolones	Ciprofloxacin (CIP)	5µg	≤15	16-20	≥21
Sulfonamides	Sulphamethoxazole trimethoprim (SXT)	25µg	≤10	11-15	≥16
Penicillins	Ampicillin (AMP)	10 µg	≤13	14-16	≥17

Manufacturer of disc: Oxoid Limited, Basingstoke, Hampshire, England.

3.6 Procedure of CS test:

At first sub-culturing of the preserved organism was done on blood agar and incubated at 37° for 24 hours to obtain a pure growth. Using sterile inoculating loop 3 or 4 individual colonies from the blood agar were transferred into a tube containing 3ml of sterile phosphate buffer saline solution (0.85% w/v NaCl solution). Emulsification of

the inoculums was done to avoid clumping of the cells inside test tube using vortex machine. Then the bacterial suspension was adjusted to the turbidity of 0.5 McFarland standard (equivalent to growth of 1-2×108CFU/ml). Within 15 minutes of preparing the inoculums, a pre-sterile cotton swab was dipped into the inoculums and rotated against the side of the tube with firm pressure to remove excess fluid. Then the swab was streaked over the entire dry surface of Mueller Hinton agar for three times rotating the plate approximately at 60 degrees. After 15 minutes of inoculation the discs were placed on the agar surface using sterile forceps. After dispensing all the discs, the agar plates were incubated at 37°C for 18 hours. After incubation the size of zone of inhibition (in mm) around a disc including the diameter of the disc was measured using a ruler and the result was interpreted according to Clinical Laboratory Standards Institute guidelines (CLSI, 2018).

3.7 Polymerase chain reaction (PCR) to test for the presence of tetracycline resistant isolates:

All tetracycline resistant *E. coli* isolates were further investigated by PCR. The detailed procedure that was followed is given below:

3.7.1. Sub-culturing on blood agar:

The preserved isolates were removed from the freezer and thawed at room temperature. Thereafter the isolates were inoculated on blood agar and incubated at 37°C for 24 hours. After completion of incubation period colonies from blood agar were used for DNA extraction to be used for PCR.

3.7.2. DNA extraction from the isolates:

For the extraction of DNA from the recovered isolates, boiling method was used. Briefly, the procedure is mentioned below:

I. A loop full of fresh colonies (about 3-4) was picked from each blood agar and transferred to 1.5 ml eppendorf tubes containing 100µl de-ionized water. The tubes were then vortexed to make a homogenous cell suspension. A ventilation hole was made on the lid of each tube.

II. Then the tubes were boiled at 99°C for 15 minutes in water bath. Immediately after boiling, the tubes were placed into the ice pack for 5 minutes. The process

of high temperature boiling and immediate cooling allowed the cell wall to break down to release DNA from the bacterial cell.

III. Finally, the tubes with the suspension were centrifuged at 15000 rpm for 5 minutes. Then 50 µl of supernatant containing bacterial DNA from each tube was collected in another sterile eppendorf tubes and preserved at -20°C until used.

3.7.3. PCR reactions:

All the molecular investigation of the isolates for *tet* genes were conducted with PCR machine name DLAB Scientific, USA in DMPH-CVASU. The primer sequences used for the PCR are shown in **Table 3.6**

Table 3.6: Primers used for PCR detection of tetracycline genes

Target gene	Primer Name	Primer sequence (5′-3′)	Annealing temperature	Amplicon size (bp)	References
*tet*A	*tet*A-F	CGCCTTTCCTTTGG	55°C	182	(Koo and Woo, 2011)
	*tet*A-R	GTTCTCTATATC CAGCCCACCG AGCACAGG			
*tet*B	*tet*B-F	GCCAGTCTTG	55°C	975	
	*tet*B-R	CCAACGTTAT ATAACACCGG TTGCATTGGT			
*tet*C	*tet*C-F	TTCAACCCAG	55°C	560	
	*tet*C-R	TCAGCTCCTT GGGAGGCAGAC AAGGTATAGG			
*tet*D	*tet*D-F	GAGCGTACC	55°C	780	
	*tet*D-R	GCCTGGTTC TCTGATCAGCA GACAGATTGC			

Table 3.7: Reagents used for PCR amplifications of the resistance genes

Serial No	Name	Manufacturer
1	Master Mix	Thermo Scientific
2	Molecular marker	Thermo Scientific O‟ GeneRuler 1 kb plus
3	Ethidium bromide solution (1%)	Fermantas
4	Electrophoresis buffer 50x TAE	Fermantas
5	Agarose powder	Seakem® Le agarose-Lonza
6	Nuclease free water	Thermo Scientific

PCR reactions were conducted with a final volume of 15 µl. Proportions of different reagents used for PCR for two different resistance genes are given in **Table 3.8**.

Table 3.8: Contents of each reaction mixture of PCR assay

Serial no	Name of the contents	Amount
1	Thermo Scientific Dream Taq PCR Master Mix (2x) ready to use	7.5 µl
2	Forward primer	2 µl
3	Reverse primer	2 µl
4	DNA template	1 µl
5	Nuclease free water	2.5 µl
	Total	**15 µl**

PCR was run on a thermocycler (DLAB TC1000-G thermal cycler, China) following the cycling conditions mentioned in **Table 3.9.**

Table 3.9: Cycling conditions used during PCR for detection of resistance genes

Serial no	Steps	Temperature and time
1	Initial denaturation	94°C for 5 minutes
2	Final denaturation (35 cycles)	94°C for 30 seconds
3	Annealing	55°C for 30 seconds
4	Initial extension	75°C for 30 seconds
5	Final extension	72°C for 5 minutes
6	Final holding	4°C

3.7.4. Visualization of PCR products by Agar Gel Electrophoresis:

Agarose gel (W/V) (1.5%) was used to visualize the PCR product. Briefly, the procedure is given below:

i. 0.75 gm of agarose powder and 50 ml of 1X TAE buffer was mixed thoroughly in a conical flask and boiled in a microwave oven until agarose dissolved.
ii. Then the agarose mixture was cooled at 50°C in a water bath and one drop of ethidium bromide was added to the mixture.
iii. The gel casting tray was assembled by sealing the ends of gel chamber with tape and placed appropriate number of combs in gel tray.
iv. The agarose-TAE buffer mixture was poured into the gel tray and kept for 20 minutes at room temperature for solidification then combs were removed and the gel was shifted into an electrophoresis tank filled with 1X TAE buffer and kept until the gel is drowned completely.
v. An amount of 5 µl of PCR product for a gene was loaded into a gel hole.
vi. 5 µl of 1 kb DNA marker (O'GeneRular 1 kb plus) was used to compare the amplicons size of a gene product and the electrophoresis was run at 110 volts and 80 mA for 30 minutes.
vii. Finally, the gel was examined by using a UV trans-illuminator for image acquisition and analysis.

3.8 Statistical analysis:

All the data from CS test results were recorded and sorted (according to sample and market type) in Microsoft excel 2019 for statistical analysis. Then the data was analyzed in STATA-13 to get the prevalence and 95% confidence interval (CI). Descriptive statistics was performed to identify the resistance and sensitivity of the samples. Univariate analysis was performed for different antimicrobials tested in broiler samples for different markets. Prevalence along with *p*-value of resistant tetracycline were analyzed according to CS test performed in broiler samples. Different values of prevalence of resistant tetracycline were arranged in tables according to sample, market type, and different antimicrobials.

RESULTS

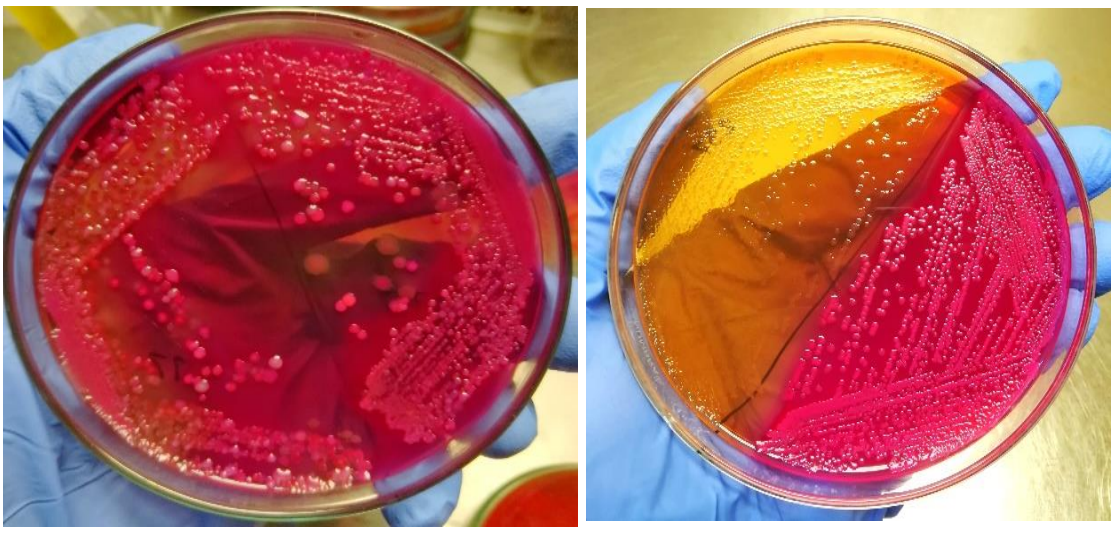
4.1 Prevalence of *E. coli* in different sources:

Among the 405 samples 229 (56.54%; 95% CI 51.56% - 61.43%) were confirmed as *E. coli*. Characteristics growth of *E. coli* strain on MacConkey agar plates, on EMB agar plate and on blood agar plate are shown in **Figure 4.1** to **Figure 4.3,** respectively. Prevalence estimate of *E. coli* in different sources: SS and LBM according to muscle and liver are summarized in **Table 4.1**.

Table 4.1: Prevalence of *E. coli* in relation to different sources

Source of samples		**No. of samples**	**No. of positive samples**	**Prevalence (%) (95% CI)**
SS	Muscle	125	68	54.40 (45.25-63.33)
	Liver	100	56	56.00 (45.72-65.92)
LBM	Muscle	90	48	53.33 (42.51-63.93)
	Liver	90	57	63.33 (52.51-73.25)
Total		**405**	**229**	56.54 (51.56- 61.43)

LBM=Live Bird Market; SS=Super-shop, CI = Confidence Interval



(a) **(b)**

Fig 4.1: Growth of *E. coli* on MacConkey agar plate (large pink color colony)

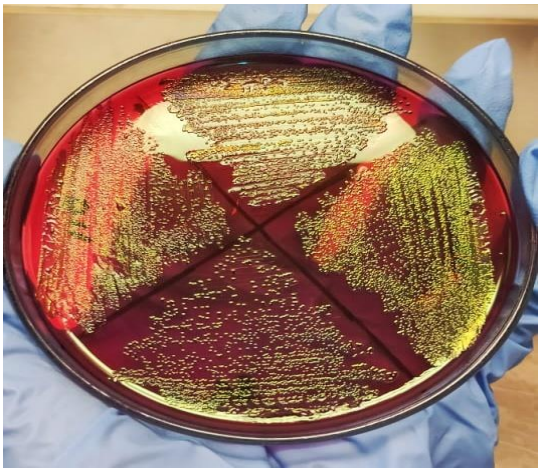


Fig 4.2: *E. coli* on EMB agar plate (green metallic sheen)

Fig 4.3: *E. coli* on blood agar plate

4.2 Antimicrobial resistance pattern of *E. coli* isolates of different sources:

An isolate of *E. coli* showing sensitivity to tetracycline and another one showing resistance to tetracycline along with sensitivity patterns to other antimicrobials are shown in Figure 4.4(a) and Figure 4.4(b), respectively. According to the guidelines of CLSI-2018 breakpoints, a significant percentage of resistance to the tested antimicrobials was observed. The resistance rates of *E. coli* isolates (n=229) in sulphamethoxazole trimethoprim (88.65%), tetracycline (86.90%), ampicillin (82.53%), and ciprofloxacin (60.70%) antimicrobials were detected. The tested *E. coli* isolates were found to be susceptible to some antibiotics, with susceptibility rates for colistin sulfate (79.48%), cephalexin (62.88%), gentamycin (58.08%). The susceptibility patterns of the isolates are shown in **Table 4.2**.

Table 4.2: Antimicrobial susceptibility profiles of *E. coli* isolates (n=229) in the present study

Antimicrobial Agent	Susceptible (S)		Intermediate (I)		Resistance (R)		I+R %
	Number of Isolates	%	Number of Isolates	%	Number of Isolates	%	
β-lactam antibiotics Cephalexin (CL, 30µg)	144	62.88	0	0	85	37.12	37.12
Penicillins Ampicillin (AMP, 10µg)	28	12.23	12	5.24	189	82.53	87.77
Tetracyclines Tetracycline (TE, 30µg)	16	6.99	14	6.11	199	86.90	93.01
Doxycycline (DO,30µg)	42	18.34	65	28.38	122	53.28	81.66
Aminoglycosides Gentamycin (CN,10 µg)	133	58.08	22	9.61	74	32.31	41.92
Quinolones Ciprofloxacin (CIP,5µg)	48	20.96	42	18.34	139	60.70	79.04
Polymyxins Colistin sulfate (CT,10µg)	182	79.48	0	0	47	20.52	20.52
Sulfonamides Sulphamethoxazole trimethoprim (SXT, 25µg)	22	9.60	4	1.75	203	88.65	90.40

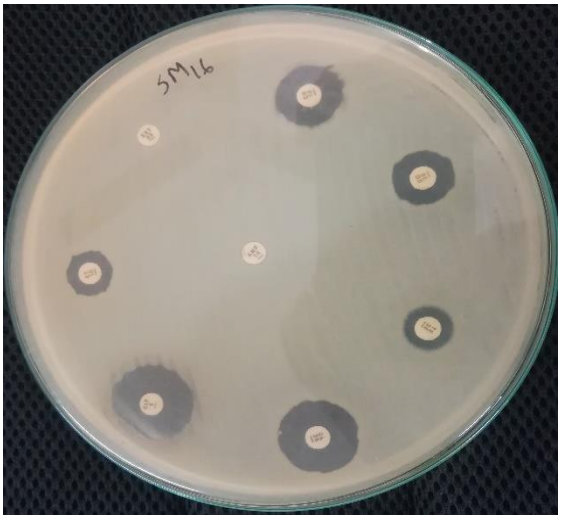


Fig 4.4(a): Tetracycline sensitive *E. coli*

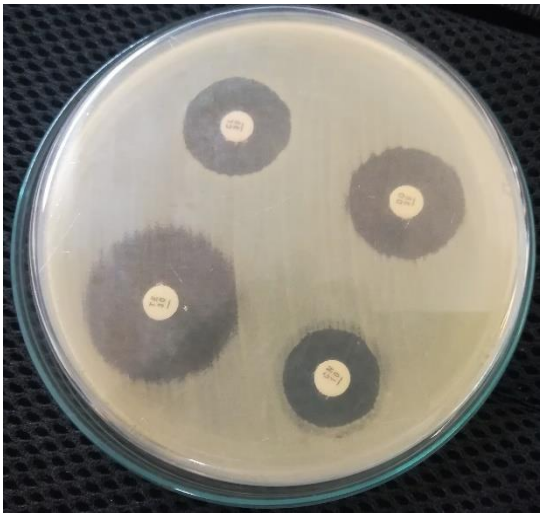


Fig 4.4(b): Tetracycline resistant *E. coli*

Figure 4.4: Antimicrobial resistance pattern of *E. coli* against tetracycline

All of the samples that found positive for *E. coli* in the culture also tested positive in the PCR is shown in **figure 4.5**.

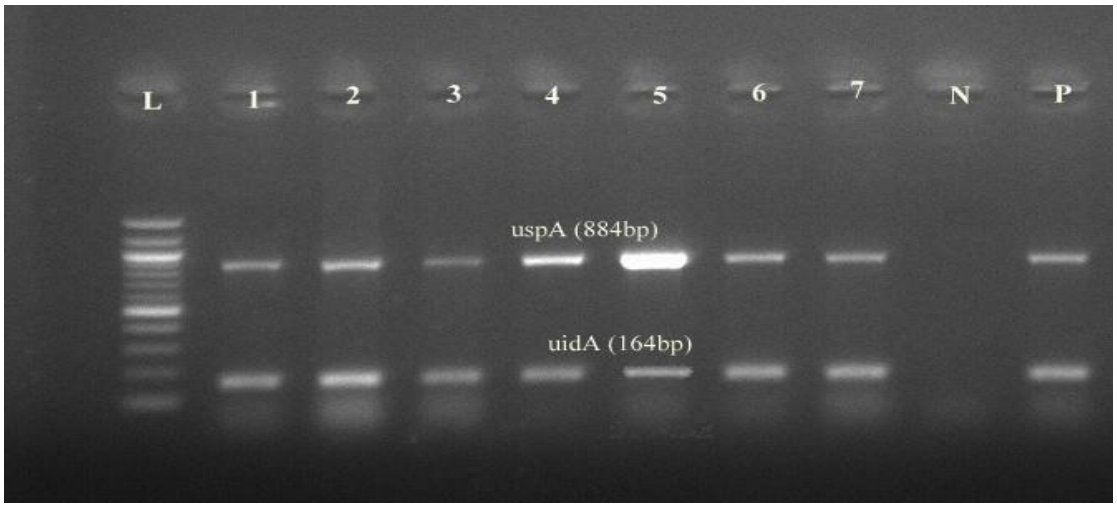


Fig 4.5: Molecular confirmation of *E. coli* isolates. Lane L: DNA ladder; Lane P: Positive control; Lane N: Negative control

The *E. coli* was found in 59.47% (n=113) of liver samples and 53.95% (n=116) of muscle samples (p=0.2634). There was a higher trend in the prevalence of *E. coli* in meat samples collected from live bird market 58.33% (n=105) than those from the super shops 55.11% (n=124) (p=0.5157). The majority of *E. coli* isolates shown resistance to multiple antimicrobials (**Table 4.3**). Overall, the percentage of resistance (R) profile for all tested antimicrobials was similar for samples collected from both super shops and live bird market except for ciprofloxacin which was substantially higher in samples obtained from live bird markets (p=0.0065).

Table 4.3: Antimicrobial resistance pattern against *E. coli* in meat

Source	**Organ**		**Antimicrobial resistance pattern, n (%)**							
			CL	CT	CIP	DO	CN	SXT	AMP	TE
Super shop	Liver	S	41(73.2)	49(87.5)	19(33.9)	12(21.4)	39(69.7)	4(7.1)	9(16.1)	6(10.7)
		I	0(0.0)	0(0.0)	9(16.1)	15(26.8)	4(7.1)	0(0.0)	2(3.6)	2(3.6)
		R	**15(26.8)**	**7(12.5)**	**28(50.0)**	**29(51.8)**	**13(23.2)**	**52(92.9)**	**45(80.3)**	**48(85.7)**
	Muscle	S	43(63.2)	54(79.4)	16(23.5)	19(27.9)	38(55.9)	11(16.2)	11(16.2)	7(10.3)
		I	0(0.0)	0(0.0)	16(23.5)	23(33.8)	4(5.9)	1(1.5)	5(7.4)	3(4.4)
		R	**25(36.8)**	**14(20.6)**	**36(53)**	**26(38.3)**	**26(38.2)**	**56(82.3)**	**52(76.4)**	**58(85.3)**
Live bird market	Liver	S	28(49.1)	44(77.2)	6(10.5)	6(10.5)	33(57.9)	3(5.3)	4(7)	1(1.8)
		I	0(0.0)	0(0.0)	10(17.5)	14(24.6)	9(15.8)	2(3.5)	4(7)	3(5.3)
		R	**29(50.9)**	**13(22.8)**	**41(72)**	**37(64.9)**	**15(26.3)**	**52(91.2)**	**49(86)**	**53(92.9)**
	Muscle	S	32(66.7)	35(72.9)	7(14.6)	5(10.4)	23(47.9)	4(8.3)	4(8.3)	2(4.2)
		I	0(0.0)	0(0.0)	7(14.6)	13(27.1)	5(10.4)	1(2.1)	1(2.1)	6(12.5)
		R	**16(33.3)**	**13(27.1)**	**34(70.8)**	**30(62.5)**	**20(41.7)**	**43(89.6)**	**43(89.6)**	**40(83.3)**

PCR detection of tetracycline resistance genes in phenotypically tetracycline-resistant isolates indicated no significant differences in the prevalence when sample types and sources were considered (**Table 4.4**). Among the isolates, 84.4% (n=168) encoded *tet*A, 5.0% (n=10) encoded *tet*B, 3.0% (n=6) encoded *tet*D, and 0.5% (n=1) encoded *tet*C, 3.0% (n=6) encoded *tet*A+*tet*B, 0.5% (n=1) encoded *tet*A+*tet*C, 2.5% (n=5) encoded *tet*A+*tet*D, 0% encoded for *tet*B+ *tet*C, *tet*B+*tet*D, and *tet*C+*tet*D, and none encoded for more than two resistance genes. While 12.1% (n=24) of the isolates tested negative for all four resistance genes.

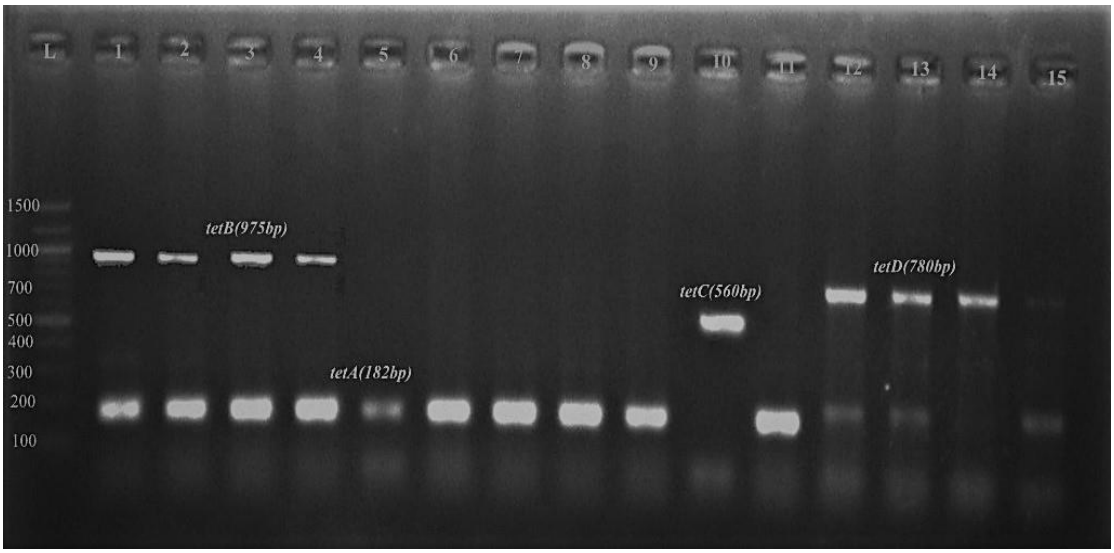


Figure 4.6: Presence of *tet*A (182bp), *tet*B (975bp), *tet*C (560bp), and *tet*D (780bp) genes of tetracycline resistance *E. coli* in PCR assay.

Table 4.4: Prevalence of tetracycline resistance gene of *E. coli* in broiler meat samples

Source	**Organ**	**Tetracycline resistance isolates**	**Prevalence of tetracycline resistance gene, n (%) (95% CI)**			
			***tet*-A**	***tet*-B**	***tet*-C**	***tet*-D**
SS	Liver	48	43(89.6) (77.34-96.53)	4(8.3) (02.31-19.99)	0(0)	2(4.2) (0.5-14.25)
	Muscle	58	49(84.5) (72.58-92.65)	4(6.9) (1.91-16.73)	1(1.7) (.04-9.24)	1(1.7) (.04-9.24)
	p-value		0.4364	0.7804	0.3607	0.4504
LBM	Liver	53	41(77.4) (63.8-87.72)	2(3.8) (0.5-13.00)	0(0.0)	3(5.7) (1.18-15.66)
	Muscle	40	35(87.5) (73.2-95.8)	0(0.0)	0(0.0)	0(0.0)
	p-value		0.2103	0.2142	1.0000	0.1261
SS*LBM	*p*-value		0.3249	0.0821	0.3477	0.8706

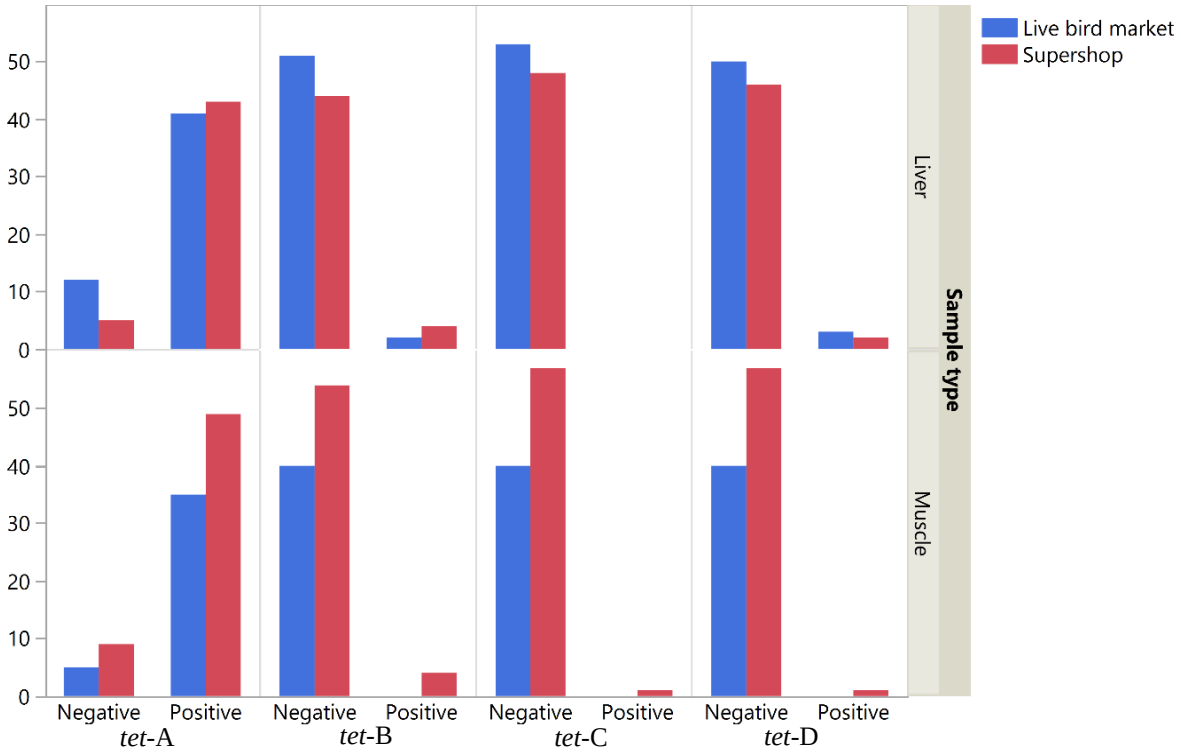


Figure 4.7: Prevalence of tetracycline resistance gene (*tet*A, *tet*B, *tet*C, *tet*D) of *E. coli* in broiler meat samples

DISCUSSION

Antimicrobial resistance is a burning issue worldwide nowadays. Antimicrobials are losing its sensitivity gradually. The findings of the study revealed that overall prevalence of *E. coli* from breast muscle and liver of broilers collected from super shops and live bird markets is high and exhibit resistance to multiple antimicrobials.

In this study, the overall prevalence of *E. coli* in broilers from LBMs is almost similar with the finding of Hossain et al. (2008) who reported the prevalence was 63.6%, whereas Jakaria et al. (2012) found 82% prevalence and Bashar et al. (2011) found 100% prevalence of *E. coli* in poultry.

Resistances that observed against tetracycline is less similar with Islam et al. (2008), they showed 96.6% resistance to tetracycline of *E. coli* isolated from poultry at Chattogram District in Bangladesh. Schroeder et al. (2002) stated comparatively lower resistance (71%) to tetracycline of *E. coli*. Parvin et al. (2020) stated that resistances to ampicillin (89.5%), Sulphamethoxazole trimethoprim (88.4%), and tetracycline (84.9%) are comparatively more devastating which are similar with the present study.

Obeng et al. (2012) have found that 67.2% *E. coli* isolated from commercial poultry possess *tet* gene which is comparatively lower than present study. Among these *tet* gene *tet*A were found with highest number which is similar with this finding. Adelowo et al. (2014) have found that *tet*A were present 21% in *E. coli* which were lower than present study whereas *tet*B were present 17% which were comparatively higher than this study.

The culture and sensitivity test of isolates showed highest resistance against combination of sulphamethoxazole-trimethoprim followed by tetracycline, and ampicillin. Highest sensitivity was found against colistin followed by gentamycin, and cephalexin. Li et al. (2014) stated that about 70.9% (N=219) isolates were multi-drug resistant (MDR: resistant to at least 3 groups of antimicrobials) while only 6.5% (N=20) isolates showed no observable resistance to the different groups of antimicrobials tested.

The presence of MDR *E. coli* was observed in this study which was agreed by Hassan et al. (2014b) where 100% prevalence of MDR *E. coli* was found in poultry. This result

is alarming since the MDR pathogen will emerge as superbug in near future, which will be inevitable.

Different studies have shown that poultry farms and their environment, such as manure, and waste water (Hasan et al., 2011; Hosain et al., 2012). Moreover, vegetables, and animal products collected from wet market and shops (Islam et al., 2016a; Sobur et al., 2019) have been identified as hot spots of antibiotic residues, which are responsible for AMR as microorganisms get exposure of these antimicrobials frequently (Hassan et al., 2021a). The high prevalence of these residues in tissues and environments of the farms enhance the issue of AMR in developing countries like Bangladesh. Horizontally transferred resistance bacteria and genes have emerged in those farms from antimicrobial residues (Sattar et al., 2014; Ferdous et al., 2019).

Antimicrobials are used in both human and veterinary medicines (Couper, 1997). Lack of knowledge on antimicrobial use and indiscriminate practices of antimicrobials (Hassan et al., 2021b) might have the sources of AMR bacteria in the meat of LBM and super shop birds. Random application of antimicrobial drugs without prescriptions from responsible person are encouraged by drug sellers and medical representatives (Okeke et al., 1999; Kalam et al., 2021). As accurate dose and dosage cannot be maintained according to age and body weights, human bacteria get exposure to these antimicrobials. When commensals get exposed with a low dose, they acquire resistance against the particular antimicrobial (Barbosa and Levy, 2000). If these are administered with a higher dose, then the residues remain in tissues for a longer period of time, so resistance may also be acquired (Levy, 1998). In poultry sector, antimicrobials are used in whole flock at the time of infection or to prevent infections (Hofacre et al., 2013). Even they are used on regular basis as a growth promoter at a lower dose to increase profitability (Shroha et al., 2019). Although the profit is increased by reducing mortality, there is a huge impact on human health. If people intake poultry meat that is treated with antimicrobials recently and the withdrawal period is not maintained, they easily acquire antimicrobial residues (Hassan et al., 2021a). This is another important cause of antimicrobial resistance as this fraction amount gets exposed to the commensals. So resistant genes can be developed which will be transferred to other pathogens both horizontally and vertically (Summers, 2006). These pathogens are responsible for further resistance.

Generally, pathogenic bacteria are not present in muscle tissues in healthy living birds (Gill, 2007). If there is any fault during slaughtering and meat processing, there can be contamination with bacteria from the ingesta and surroundings (Barnes, 1979; Lin et al., 2020). These gut microflorae may be resistant to particular antimicrobials which they already get exposure at a continuously lower dose.

Antimicrobial resistance is posing the threat to both veterinary and public health. Antimicrobials are getting resistant more and more gradually. So, World Health Organization (WHO) categorized antimicrobials in three categories: Access group, watch group, and Reserve groups of antibiotics to mitigate the situation (Gandra et al., 2019). Access group of antimicrobials are available for the physicians to prescribe for the patients. If these group fails due to resistant genes present in microbes for that particular patient watch group is recommended to be used. Reserve group of antimicrobials are for future application if others get resistant (Hsia et al., 2019).

To mitigate the AMR issue before they become large-scale emergencies scientific knowledge and science-based evidence are needed. Profitability can be increased through more effective agriculture practices along with reduced AMR problem by achieving rationale use of antimicrobials (Moellering Jr, 1983; Barnett, 2006).

CONCLUSION

Much of the interest in AMR of bacteria that reside in poultry and other food animals are sparked by a concern for human health that is threatened by zoonotic pathogens and by selection for AMR determinants. More than half of the meat samples of LMBs and SSs were showed a positive presence of *E. coli* and a high prevalence of resistance against sulfonamides and tetracycline antimicrobials. Among the resistance genes of tetracycline, *tet*-A showed higher resistance in broiler meat samples of both markets in the study area. Increasing AMR also threatens agriculture, as bacterial diseases in animals become more difficult to treat. However, poultry meat is one of the protein food chains of humans, AMR will be developed in humans and increase the infection burden in the community through the poultry food chain. Prudent use of antimicrobials will be necessary to preserve these valuable drugs for use in poultry farms. Awareness buildup and training programs are highly recommended for poultry handlers to maintain strict proper use of antimicrobials in the poultry farms.

RECOMMENDATIONS

Antimicrobial resistance has become a global burden for which inappropriate antimicrobial use is an important contributing factor Antimicrobial resistance has become a global burden for which inappropriate antimicrobial use is an important contributing factor. Spread of antibiotic resistance to different environmental niches and development of superbugs have further complicated the effective control strategies. International, national and local approaches have been advised for control and prevention of antimicrobial resistance. To minimize the devastating consequences for human health few recommendations are suggested through this study. These are applicable for the public health authorities, commercial poultry farm owners, veterinarians, and consumers.

a) Increased collaboration between governments, nongovernmental organizations, professional groups and international agencies.
b) Establishment of national committee to monitor impact of antibiotic resistance and provide intersectoral co-ordination is required.
c) Establishing and implementing national standard treatment guidelines, having essential drug list (EDL), enhancing coverage of immunization are other essential strategies desired at national level.
d) Use of alcohol-based hand rubs or washing hands has proven efficacy in prevention of infection. This factor can restrict the spread of infection and thereby the AMR.
e) The public health authorities should set up a permanent national poultry quality control program (for antimicrobial residues and AMR). They should arrange seminars on public health hazard due to antimicrobial residues and resistance. Laboratories should be established to control the veterinary drug residues in foods from animal origin.
f) Commercial poultry farm owners should not use antimicrobials without suggestions from registered veterinarians. They should follow the withdrawal periods before harvesting.
g) Veterinarians should prescribe the actual dose of antimicrobials and suggest about withdrawal periods.

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BRIEF BIOGRAPHY

Gazi Sofiul Alam passed the Secondary School Certificate Examination in 2011 from Govt. Science College Attached High School, Dhaka, and then Higher Secondary Certificate Examination in 2013 from Tejgaon College, Dhaka. He obtained his B.Sc. (Honors) in Food Science and Technology from the Faculty of Food Science and Technology at Chattogram Veterinary and Animal Sciences University, Chattogram, Bangladesh. Now, he is a candidate for the degree of Masters of Science in Applied Human Nutrition and Dietetics under the Department of Applied Food Science and Nutrition, Chattogram Veterinary and Animal Sciences University **(**CVASU**)**. He has an immense interest to work in improving the health status of people through proper guidance and suggestions and to create awareness among people about food safety and nutrition.