Epidemiology and molecular characterization of multi drug-resistant *Escherichia coli* isolated from cow milk

Zarin Tasnim Mim

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A thesis submitted in the partial fulfilment of the requirement for the degree of

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Chattogram veterinary and Animal Sciences University

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STATEMENT OF AUTHOR

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Zarin Tasnim Mim

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This is to certify that we have examined the above masters 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

……	……
(Prof. Dr. Mohammad Mahmudul Hassan, PhD)	**(Prof. Dr. Shahneaz Ali Khan, PhD)**
Supervisor	**Co- Supervisor**

……

(Professor Dr. Sharmin Chowdhury, PhD)

Director, One Health Institute

One Health Institute

Chattogram Veterinary and Animal Sciences University

Chattogram – 4225

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The Author

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LIST OF ACRONYMS AND SYMBOLS

Abbreviations and Symbols	Elaboration
AMR	Antimicrobial Resistance
AMU	Antimicrobial Use
ESBL	Extended-Spectrum Beta-Lactamases
PCR	Polymerase Chain Reaction
UTI	Urinary Tract Infection
QRDR	Quinolone Resistance-Determining Region
DHPS	Dihydropteroate Synthase
DHFR	Dihydrofolate Reductase
ARG	Antibiotic Resistance Gene
CMA	Chattogram Metropolitan Area
STEC	Shiga-toxin Producing *E. coli*
PRSP	Penicillin-Resistant *Streptococcus pneumonia*
MRSA	Methicillin-Resistant *Staphylococcus aureus*
VRE	Vancomycin-Resistant *Enterococci*
MDRGNB	Multiple-Drug-Resistant Gram-Negative Bacilli
EPEC	Enteropathogenic *E. coli*
EHEC	Enterohemorrhagic *E. coli*
ETEC	Enterotoxigenic *E. coli*
EIEC	Entero-invasive *E. coli*
EAEC	Enteroaggregative *E. coli*
DAEC	Diffusely adherent *E. coli*
UPEC	Uro-pathogenic *E. coli*
MNEC	Meningitis/Sepsis-Associated *E. coli*
AAC	Aminoglycoside Acetyltransferases
ANT	Aminoglycoside Nucleotidyltransferase
APH	Aminoglycoside Phosphotransferases
CT	Colistin Sulfate
TE	Tetracycline
CN	Gentamicin
DO	Doxycycline

Abbreviations and Symbols	Elaboration
AMP	Ampicillin
CIP	Ciprofloxacin
NOR	Norfloxacin
KF	Cephalothin
ENF	Enrofloxacin
FFC	Florfenicol
PG	Penicillin G
NE	Neomycin
EMB	Eosin Methylene Blue
FAO	Food and Agriculture Organization

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ABSTRACT

In communities and healthcare settings, *Escherichia coli* is developing antimicrobial resistance (AMR), which is a growing public health crisis. A recent high-profile report estimates that, by 2050, the rise in AMR will cause 10 million deaths each year unless a global response is ordered to the problem. Due to its relatively dense population with extremely unhygienic sanitary facilities and food and water sources, Bangladesh is more likely to suffer from infection than other countries around the world. Raw meat, leafy green vegetables, or fish are most frequently linked to bacterial infections. Milk also acts as a vector for transmitting pathogenic microbes. For this reason, this study focused on molecular characterization of multi drug-resistant *Escherichia coli* isolated from cow milk. To conduct this study milk samples were collected from 18 different farms of Chattogram Metropolitan Area. *E. coli*, enriched culture was streaked on MacConkey agar medium followed by Eosin Methylene Blue (EMB) agar plates and incubated for 24 hours at 37°C in each state. The isolates were then inoculated onto blood agar and incubated for 24 hours at 37°C. After that, blood agar colonies were used to conduct CS tests to find out the samples that are resistant to specific antibiotics and then to extract DNA for PCR to identify the resistant genes. Among 450 milk samples, we found 134 positive isolates for *E. coli* which describes a prevalence of 29.77%. Highest number of *E. coli* isolates were resistant to Ampicillin (69%), Cephalothin (69%), Cephalexin (69%), Amoxicillin + Clavulinic Acid (69%) and lowest number of *E. coli* isolates were resistant to Colistin sulfate (0%). The study was designed using a one-health approach to demonstrate the identification, molecular characterization along with the antimicrobial sensitivity pattern evaluation of multidrug-resistant *E. coli* to determine the status of drug sensitivity and resistance pattern of the *E. coli* isolates from milk samples in Bangladesh and the outcomes indicate that the AMR is increasing in an alarming rate and can cause health threat. Before it manifests in large-scale medical emergencies, it is necessary to identify risks and appropriate mitigation strategies based on scientific evidence and knowledge.

Keywords: Antimicrobial resistance, antimicrobial sensitivity.

CHAPTER-1: INTRODUCTION

Antimicrobial resistance (AMR) is unquestionably one of the greatest health hazards to humanity. It reduces the efficacy of antibiotics, making treatment more complicated, time-consuming, costly, or challenging. It is estimated that AMR will result in a global catastrophe by causing 10 million death per year and a horrifying 100 trillion USD economic cost, and an 11% decline in livestock production by 2050 if adequate steps are not taken to address the challenges (O'Neill, 2019). AMR is defined as the resistance of microorganisms to clinically relevant antimicrobial medications at standard doses (Ganguly and Arora, 2011). Typically, the condition is referred to as multidrug-resistant (MDR) when microorganisms are resistant to at least three types of antibiotics (de Brito *et al.*, 2022). As a result, the consequence is known as the "Silent Pandemic" because it leads to the global spread of multidrug-resistant strains (Sharma *et al.*, 2018).

Since the discovery of the first antibiotic, resistance to antimicrobials has been considered a natural process in which microbes evolve to resist the effects of drugs (Annunziato, 2019). The imbalance between the overuse of antimicrobials and the lack of new antibiotic innovation to combat these new superbugs has caused AMR to progressively deteriorate in recent years (Murugaiyan *et al.*, 2022). AMR is very important and common in animals, though it gets less attention. In general, antimicrobials are used a lot in the dairy industry to treat diseases like mastitis and these have also been used to keep diseases from happening (Abebe *et al.*, 2016). Moreover, subtherapeutic doses of antibiotics are used to prevent disease and boost growth far more than they are used to treat disease. This is one of the things that leads to AMR in animals. By 2030, the Antimicrobial Use (AMU) in food-producing animals will increase by more than 67% to meet this demand (Boeckel *et al.*, 2015). Animals that depend on AMU create a selective pressure that forces bacteria to either change in a way that makes them resistant or get resistance genes (Moudgil *et al.*, 2018). The biggest worry about AMR in animals is that resistant strains of bacteria could spread from animals to people (Loo *et al.*, 2020).

In dairy cattle production, drug-resistant strains can disseminate from animals to humans via the food supply chain (meat and dairy products), direct animal contact, or

environmental routes (Lhermie *et al.*, 2017). Consumers may also be exposed to resistant strains and genes if they consume contaminated food, such as contaminated meat, unpasteurized milk, and milk products, or if resistant strains and genes spread through the environment, such as through animal waste and runoff water from agricultural sites (Ayukekbong *et al.*, 2017). Milk and milk products can harbor diverse microorganisms and serve as significant sources of pathogens that propagate through food. Milk can contain foodborne pathogens due to direct contact with contaminated sources on a dairy farm and because udder debris from an infected animal can enter the milk. Due to dirty conditions and bad udder health, bacteria like *E. coli* can get into milk and cause infections in people (Batabyal *et al.*, 2018). The problem is made worse by the fact that milk contains bacteria that have antimicrobial resistance genes.

The rise of *E. coli* that is resistant to antibiotics is a higher risk and a major concern for global health. They pose a serious challenge to the veterinary and public health fields and dairy cattle producers because thy interfere with treatment (Brouillette and Malouin, 2005). Microbes have already shown resistance to several antimicrobial drugs, such as Ampicillin, Trimethoprim-sulfamethoxazole, Levofloxacin, Cephalothin, and Tetracyclines (Rahi *et al.*, 2020).

E. coli is resistant to antibiotics because it has many antibiotic-resistance genes. These genes include the tet genes (*tetA, tetB, tetC, tetD*) gene for Tetracycline resistance, the bla_{TEM}, bla_{SHV}, *PAmpC*, bla_{OXA}, $bla_{ACC,}$ $bla_{CMY,}$ bla_{CTX-M} genes for Ampicillin, *Sul-1, Sul-2* genes for Trimethoprim-sulfamethoxazole (Skočková *et al.*, 2012; Metzger and Hogan, 2013, Kamaruzzaman *et al.*, 2020).

E. coli is an opportunistic pathogen that can make a lot of people and animals sick. It is also one of the main causes of bovine mastitis, and over the past few years, more and more resistant isolates have been found on dairy farms. These resistance factors have often been linked to resistance to more than one drug. Since the *E. coli* genome can change over time, the exchange of genetic material could lead to more resistance genes being passed on (Bajaj *et al.*, 2016). The spread of MDR *E. coli* is a public health concern because it poses a risk to farm workers and other people who encounter animals (Walther *et al.*, 2017). In *E. coli* resistance to a wide range of β-lactamases is often spread through the horizontal transfer of genes that code for extended-spectrum beta-lactamases (ESBL). Also, ESBL-producing *E. coli* strains are more likely to show

multidrug resistance than non-ESBL-producing strains, which makes it harder to treat infections (Karkaba *et al.*, 2017). Multidrug resistance has been seen in many *E. coli* isolates, and it's especially concerning that the bacteria are resistant to commonly used antibiotics like Ampicillin, Sulfamethoxazole Trimethoprim, Levofloxacin, Cephalothin, and tetracyclines. Many resistant genes, such as, bla_{CTX-M}, bla_{TEM}, *PAmpC*, bla_{OXA}, bla_{CMY}, bla_{ACC-1} have also been found in fecal samples from pigs, cattle, chicken, and sheep (Geser *et al.*, 2012; Ali *et al.*, 2016).

AMR is a growing global public health concern, but it may be even more severe in developing nations with a high prevalence of infectious diseases. This is because a lack of knowledge and uncontrolled access to medicines can lead to more use of medicines that aren't right for the person and more use of medicines that aren't right for the person of fighting back (Kakkar *et al.*, 2017). Bangladesh's livestock industry is using antibiotics and prophylactics in ways that don't make sense. This makes the spread of AMR more likely (Sobur *et al.*, 2019). AMR problems can also happen in developing countries like Bangladesh due to few health care facilities (Khan *et al.*, 2020).

Under these circumstances, the present study was directed to achieve to following objectives:

1. Identification and molecular characterization of *E. coli* isolates from cow milk samples by using culture methods and Polymerase Chain Reaction (PCR).
2. To isolate and identify multidrug-resistant genes of *E. coli* obtained from cow milk.
3. Investigation on drug-resistant *E. coli* from cow milk carrying the ESBLs and Cephalosporins genes (bla_{TEM}, bla_{SHV}, *PAmpC*, bla_{OXA}, bla_{ACC}, bla_{CMY}, bla_{CTX-M}) for Ampicillin, *Sul-1*, *Sul-2* genes for Trimethoprim-sulfamethoxazole, *tet*-A, *tet*-B, *tet*-C and *tet*-D genes for Tetracycline.

CHAPTER-2: REVIEW OF LITERATURE

2.1 Antibiotics and their Developmental History

Infectious diseases were a major contributor to the global increase in illness and mortality at the beginning of the twentieth century. In comparison to modern times, the average lifespan was lower at birth (Blaskovich *et al.*, 2018). There were many kinds of diseases, such as smallpox, malaria, diphtheria, tuberculosis, etc. Antibiotics were discovered in 1928 by Sir Alexander Fleming (1881-1955). For the medical field, this marked the beginning of a revolutionary era. Purifying penicillin G was a 1942 achievement by Ernst Chain and Howard Florey (Durand *et al.*, 2019). The revolutionary invention undoubtedly saved millions of lives, as evidenced by the rise in life expectancy. Antibiotic discovery accelerated greatly from the 1950s through the 1970s. The era we are currently living in has been named the "golden age" of antibiotic discovery. Treatment for infectious diseases was revolutionized after the discovery of antibiotics. Noncommunicable diseases, such as cancer, stroke, and heart disease, account for most deaths in the United States (Banin *et al.*, 2017). But factors such as poor public health, lack of hygiene, bad sewage and sanitation systems, low vaccination rates, etc., mean that the development of antibiotics may not be universally successful.

Table 2.1: Antibiotics according to their mode of action.

Target	**Antibacterial class**	**Example**	**Principle target**
Cell wall biosynthesis	*β-lactam*	Penicillin, Methicillin	PBPs (transpeptidases)
	β-lactamases inhibitors	Clavulanic acid	*β*-lactamases
	Glycopeptides	Vancomycin	Terminal D-Ala-D-Ala in lipid II
	Cyclic peptidase	Bacitracin	Undecaprenyl
Bacterial cell membrane	Cationic peptides polymyxins	Colistin	LPS in the outer membrane

Bacterial cell membrane	Lipopeptides	Daptomycin	Cytoplasmic membrane
Protein biosynthesis: 30S subunit	Aminoglycosides Tetracyclines Phenylpropanoid	Gentamycin Doxycycline Chloramphenicol	16S rRNA (A-site) 16S rRNA (A-site) 23S rRNA
50S subunit	Macrolides Ketolides	Erythromycin Azithromycin	23S rRNA 23S rRNA
DNA biosynthesis	Fluoroquinolones	Ciprofloxacin	A-subunit of DNA gyrase
RNA biosynthesis	Rifamycins	Rifampicin	β-subunit of RNA polymerase
Folate biosynthesis	Diaminopyrimidines	Trimethoprim	Dihydrofolate synthase
	Sulfonamides	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 e.g. Ribosomal proteins

Source: (Reygaert and Reygaert, 2018)

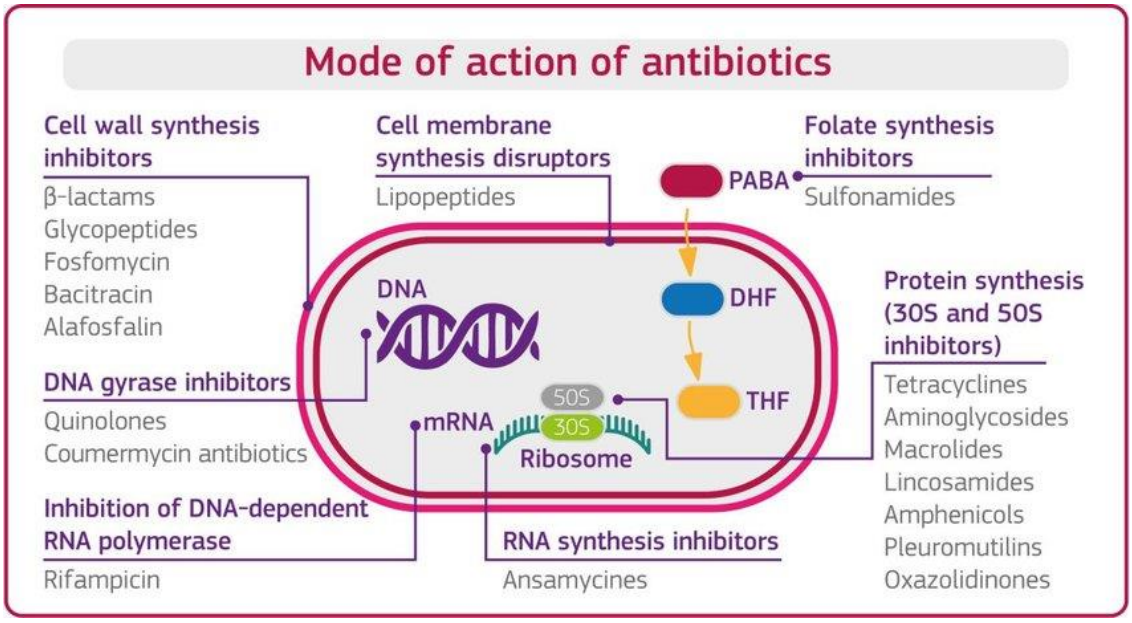


Figure 2.1: Mode of action of antimicrobials with their target sites.

Source: (Isabella *et al.*, 2018)

Table 2.2: Bacterial targets of antibiotics in clinical use.

Target	Type of Antibiotics
Cell-wall biosynthesis	Penicillin, cephalosporins, carbapenems, monobactams, cycloserine, fosfomycin, glycopeptides, lipoglycopeptides
Protein synthesis	Aminoglycosides, tetracyclines (*Subunit 30S*), Oxazolidinones, macrolides, thiopeptides, chloramphenicol, fusidic acid, clindamycin (*Subunit 50S*).
DNA replication and repair	Rifamycin, annamycin, actinomycin (R*NA polymerase*), Fluoroquinolones, aminocoumarins (*DNA gyrase*)
Folic acid metabolism	Sulfonamides-trimethoprim
Membrane structure	Lipopeptides, polymyxins

Source: (Chellat *et al.*, 2016)

2.2 Resistance to Antibiotics

Antibiotic resistance gets worse when antibiotics are used wrong or too much and when people don't take care of infections well. It can be defined as the ability of the bacteria to resist the antibiotic effectiveness which was initially sensitive to those antibiotics. Some resistant pathogens, like Penicillin Resistant *Streptococcus pneumonia* (PRSP), Methicillin Resistant *Staphylococcus aureus* (MRSA), Vancomycin Resistant Enterococci (VRE), and Multiple-Drug-Resistant Gram-Negative Bacilli (MDRGNB), is becoming a global concern (Vestergaard *et al.*, 2019).

2.2.1 Natural Resistance

This type of resistance can be either intrinsic or induced (the genes are naturally occurring in the bacteria but are only expressed to resistance levels after exposure to an antibiotic (González-Bello, 2017). Intrinsic traits are usually shared by all the bacteria in a certain area, are not affected by antibiotics, and have nothing to do with horizontal gene transfer. The most common way that intrinsic resistance works is by making the outer membrane less permeable. Besides that, they can also affect the cellular efflux pumps (Xie *et al.*, 2018).

2.2.2 Acquired Resistance

Acquired resistance is the process of getting resistant genes through any of the three ways: transformation, transposition, or conjugation. Horizontal gene transfer and mutations in the organism's chromosomal DNA are the key parts of these three processes. This resistance could be short-term or long-term. Plasmid-mediated transmission is the most common way, while bacteriophage-mediated transmission is rare. There are many ways to become resistant, such as being exposed to chemical or physical stressors or having a change in your genes (substitution, deletion etc.). On average, a bacterial mutation happened once every 106 to 109 cell divisions, and this change was usually bad for the cell. Certain genes, like drug targets or drug transporters, regulator genes or antibiotic-modifying enzymes genes, etc., had mutations that helped bacteria become resistant to antibiotics (Aanen Debets, 2019).

2.2.3 Mechanism of Antibiotic Resistance

Mechanism of antimicrobial resistance mechanisms falls into four main groups: Limiting uptake of a drug, modifying a drug target, inactivating a drug, and active drug efflux (Reygaert and Reygaert, 2018). Bacteria may manifest antibacterial drug resistance through a variety of mechanisms. Certain bacterial species are naturally resistant to one class of antimicrobial agents. In these instances, all strains of that bacterial species are resistant to all members of these antibacterial classes. Cases of acquired resistance, in which initially susceptible populations of bacteria become resistant to an antibacterial agent and proliferate and spread because of the selective pressure exerted using that agent are of greater concern.

Many bacterial species can quickly acquire and spread a variety of resistance mechanisms to antimicrobials. To begin, the organism might pick up genes for lactamases and other enzymes that neutralize antibiotics before they can exert their effect. Second, bacteria may evolve efflux pumps that remove the antibacterial agent before they can exert their effect. Finally, bacteria can acquire mutations that reduce the amount of antimicrobial agent that can reach the intracellular target site by downregulating porin genes, or they can acquire several genes for a metabolic pathway that results in altered bacterial cell walls that no longer contain the antimicrobial agent's binding site (McManus, 1997). Thus, normally susceptible bacterial populations can acquire antimicrobial resistance through mutation, natural selection, or genetic transfer from other bacteria. Transformation, conjugation, or transduction may cause the final event.

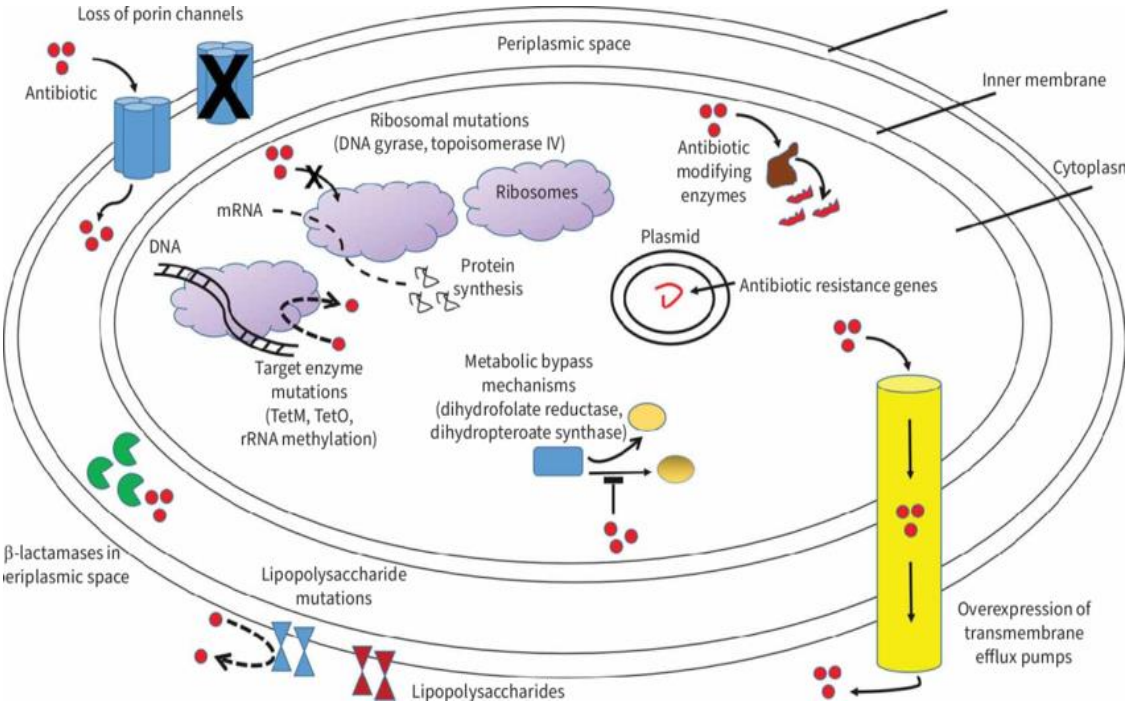


Figure 2.2: Antibiotics, effects, and mechanisms of resistance.

Source: (Reynolds *et al.*, 2022)

Several bacteria have become resistant to multiple antibacterial agents through genetic exchange, making them a major concern in hospitals and other healthcare facilities. New mutations can give susceptible bacteria antimicrobial resistance. Such spontaneous mutations can cause resistance in multiple ways. Firstly, modifying the target protein to which the antibacterial agent binds by changing the binding site, for example, penicillin-binding protein 2b in pneumococci, which causes penicillin resistance (Barcus *et al.*, 1995). Secondly, upregulating the production of enzymes that inactivate the antimicrobial agent, such as, erythromycin ribosomal methylase in staphylococci (Eady *et al.*, 1993). Thirdly, downregulating or altering an outer membrane protein channel that the drug requires for cell entry, for instance, OmpF in *E. coli* (Wang *et al.*, 2017) or lastly upregulating pumps that expel the drug from the cell (efflux of fluoroquinolones in *S. aureas* (Eady *et al.*, 1993).

Antimicrobials select bacteria with resistance-conferring mutations, killing susceptible strains but allowing resistant strains to survive and multiply. Vertical evolution is chromosomal mutation and natural selection-induced resistance. Bacteria also acquire

resistant genes from other organisms. Horizontal evolution occurs between strains of the same species or between genera of bacteria. Conjugation, transduction, and transformation exchange genes. Acquired resistance genes are transferred and integrated into the host's genome or plasmids by transposons. During conjugation, Pilus helps a gram-negative bacterium to transfer plasmids with resistance genes to an adjacent bacterium. Sex pheromones from mating gram-positive bacteria start conjugation. These pheromones clump donor and recipient organisms to transfer DNA. Bacteriophages transfer resistance genes between bacteria during transduction (bacterial viruses). Transformation, where bacteria acquire and incorporate DNA segments from other bacteria that have released their DNA complement into the environment after cell lysis, can finally transfer resistance genes into previously susceptible strains.

Many bacterial species quickly adapt to antibacterial agents by mutation, natural selection, and genetic exchange. A single mutation in a key bacterial gene may only slightly reduce the host bacteria's susceptibility to that antibacterial agent, but it may be enough to allow the bacteria to survive until it acquires additional mutations or genetic information resulting in full resistance. Rarely, a single mutation can give an organism clinically significant, high-level resistance, such as, rifampin resistance in *S.aureus* or fluoroquinolone resistance in *Campylobacter jejuni*. Case studies of three bacterial species illustrate the evolution of bacterial resistance to antibacterial drugs and the interactive effects of multiple resistance mechanisms (Tenover, 2006).

2.3 Leading Resistant Pathogens

Infections in humans and animals can be caused by a vast array of microorganisms; therefore, prevention and treatment strategies must be adaptable enough to account for varying infection risks and therapeutic options. Most pathogenic species have become resistant to at least one class of antimicrobials over the past several decades. The following are examples of species where resistance has the greatest public health concern: *Escherichia coli, Staphylococcus aureus, Mycobacterium tuberculosis, Neisseria gonorrhoeae, Salmonella Typhi, Streptococcus pneumonia.*

MDR *E. coli* has become problematic due to its increased virulence and resistance to an increasingly broad spectrum of antibiotics. *E. coli* causes pneumonia, urinary tract

infections, and bloodstream infections in hospitalized patients. *E. coli* is one of the most common causes of healthcare-associated Infections (HAIs) (Dadi *et al.*, 2021).

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 2.3 shows the pathotypes of *E. coli* along with their clinical conditions produced in host.

Table 2.3: 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
Entero-invasive *E. coli* (EIEC)	Watery diarrhea and dysentery
Enteroaggregative *E. coli* (EAEC)	Persistent diarrhea in humans
Diffusely adherent *E. coli* (DAEC)	Diarrhea in children
Uro-pathogenic *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 the 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. The virulence components of intestinal pathogens of *E. coli* are summarized in Table 2.4.

Table 2.4: Virulence factors of intestinal pathogenic *E. coli.*

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), IAMPD and VirA*
DAEC	Dradhesins	Shigella enterotoxin 1, *Pet* and *Pic*

2.4 Sources of Human *E. coli* Infections

People usually get *E. coli* O157:H7 from eating contaminated foods, like raw or undercooked ground meat products and raw milk (Alhadlaq *et al.*, 2023). Infections can

also happen when feces get into water and other foods, or when beef and other meat products, contaminated surfaces, and contaminated kitchen tools are used to make. Some foods that have been linked to *E. coli* O157:H7 outbreaks are undercooked hamburgers, dried cured salami, yogurt, and cheese made from raw milk (Chaleshtori *et al.*, 2017). Fruits and vegetables, like sprouts, spinach, lettuce, coleslaw, and salad, are linked to a growing number of outbreaks (Lim *et al.*, 2010). Contamination may be caused by contact with the feces of domestic or wild animals at some point during growing or handling. People have gotten sick from drinking water which can be contaminated by precipitation and sur- face runoff caused by landfills. The oral-fecal route relies heavily on person-to-person contact as a mode of transmission. It has been reported that some people can be infectious despite showing no outward signs of illness (a "carrier state"). STEC is typically eliminated from the body within a week or less in adults, though in children this period may be extended. STEC infection risk also increases when people visit farms and other places where they might encounter farm animals (Dulo *et al.*, 2015).

2.5 Antimicrobial Resistance

Antimicrobial resistance is when a microbe is resistant to an antimicrobial agent that was used to treat or prevent an infection caused by that microbe. When the infectious agent is bacteria, the more specific term is antibiotic resistance or antibiogram. In an April 2014 report, the World Health Organization (WHO) said, "This serious threat is no longer a prediction for the future; it's happening right now in every part of the world and could affect anyone, of any age, in any country. According to the 2011 WHO report, antibiotic resistance is one of the three greatest hazards to public health. Most of the rise in drug resistance is attributable to three factors: the use of antibiotics by humans, the use of antibiotics by animals, and the transmission of resistant strains between humans and animals. Any use of antibiotics can increase selective pressure in a bacterial population, causing weaker bacteria to die off and resistant bacteria to proliferate.

2.6 Mechanisms Involved Behind the Emergence of AMR

Prior to the 1990s, antimicrobial resistance was never considered a major threat to infectious disease management. Despite this, treatment failures against first-line, second line, etc. drugs were progressively increasing in prevalence in healthcare

settings. Antimicrobial agents to which microorganisms were previously susceptible were losing their effectiveness. This was achieved in a variety of ways but was primarily dependent on the chemical structure of the antimicrobial agent and the mechanisms by which the agents acted against pathogens. Resistance to antimicrobials can be described in two ways:

Intrinsic or natural resistance: In this case, microorganisms do not have target sites for the drugs, so the drugs have no effect on them. Alternatively, microorganisms have naturally low permeability to these agents due to differences in the chemical nature of the drug and the microbial membrane structures, particularly for those that require entry into the microbial cell to exert their effect.

Acquired resistance: Here, a microorganism that is normally susceptible acquires resistance to the drugs used to treat infections caused by it. Acquired resistance is a major mechanism by which microbes develop resistance to antimicrobial agents. This phenomenon can occur through various means, including the presence of enzymes that inactivate the antimicrobial agent or alternative enzymes that can substitute for the inhibited enzyme. Additionally, mutations in the target site(s) of the antimicrobial agent, post-transcriptional or post-translational modifications of the target, reduced uptake of the agent, active efflux of the agent, overproduction of the target, and differential gene expression in vivo compared to in vitro can all contribute to acquired resistance. These mechanisms highlight the complex and adaptable nature of microbes in response to selective pressures and emphasize the importance of continued research and development of new antimicrobial strategies (Fluit *et al.*, 2001).

2.6.1 Resistance to *β*-lactam Antibiotics

The *β*-lactam antibiotics consist of penicillin, cephalosporins, carbapenems, oxapenams, and cephamycin, and are characterized by the presence of a *β*-Lactam ring. Due to its ready availability and relatively low cost, penicillin is one of the most used antibiotics in developing nations. The *β*-Lactam ring is crucial to the activity of these antibiotics, which results in the inactivation of trans-peptidases that catalyze the final cross-linking reactions of peptidoglycan synthesis in bacteria. The effectiveness of these antibiotics depends on their ability to reach and bind with intact penicillin-binding proteins (PBPs). Numerous bacteria develop resistance to *β*-lactam because of the

hydrolysis of the antibiotic by *β*-lactamases or the modification of PBPs or cellular permeability. *β*-lactamases are classified according to their hydrolytic spectrum, susceptibility to inhibitors, genetic localization (plasmidic or chromosomal), and gene or protein amino acid sequence. According to their substrate and inhibitor profiles, the functional classification scheme of *β*-lactamase defines four groups according to their substrate and inhibitor profiles (Bush and Jacoby, 2010).

The functional classification scheme of *β*-lactamases categorizes these enzymes into four groups based on their substrate and inhibitor profiles. Group 1 comprises cephalosporinases that are not easily inhibited by clavulanic acid. Group 2 includes penicillinases, cephalosporinases, and broad-spectrum *β*-lactamases that can typically be inhibited by active site-directed *β*-lactamases inhibitors. Group 3 contains cephalosporinases that are not inhibited by clavulanic acid, and Group 4 encompasses penicillinases that are not effectively inhibited by clavulanic acid. Lastly, Group 3 also includes metallo-lactamases that can hydrolyze a broad range of *β*-lactam-containing molecules, including penicillin, cephalosporins, and carbapenems, and are poorly inhibited by nearly all *β*-lactam-containing molecules. This classification system is crucial for understanding the mechanisms of resistance to *β*-lactam antibiotics and guiding the development of novel therapeutic strategies to overcome them.

2.6.2 Resistance to Tetracyclines

Tetracyclines are widely used in both human and veterinary medicine in developing countries due to their accessibility, low cost, low toxicity, and wide spectrum of activity. They have activity against a wide variety of gram-positive and gram-negative bacteria, as well as atypical organisms such as chlamydia, mycoplasmas, rickettsia, and protozoan parasites. Tetracycline, doxycycline, minocycline, and oxytetracycline are some examples. Resistance to these agents is primarily mediated by three mechanisms (Roberts, 1996) — efflux of the antibiotics, ribosome protection, and modification of the antibiotic.

Drug efflux is mediated by an export protein from the major facilitator superfamily. These export proteins are membrane-associated, *tet* efflux gene-encoded proteins that export tetracycline from the cell. Export of tetracycline lowers the intracellular

concentration of the drug, thereby protecting the ribosomes within the cell (Marshall and Levy, 2011).

2.6.3 Resistance to Aminoglycosides

More than 50 aminoglycoside-modifying enzymes have been identified, indicating widespread resistance to aminoglycosides like gentamicin, tobramycin, amikacin, and streptomycin. Gram-negative bacteria are the primary hosts for most of these genes. Aminoglycoside acetyltransferases (AAC), aminoglycoside nucleotidyltransferase (ANT), and aminoglycoside phosphotransferases (APH) are three examples of these enzymes (Shaw *et al.*, 1993). Aminoglycosides lose their ability to inhibit protein synthesis when they are modified by AAC enzymes at the amino group or by ANT or APH enzymes at the hydroxyl group. Resistance to aminoglycosides can be caused by a few mechanisms, including aminoglycoside-modifying enzymes, efflux systems, and rRNA mutations.

2.6.4 Resistance to Quinolones and Fluoroquinolones

There are two main types of mechanisms by which bacteria develop resistance to quinolones: changes in drug target enzymes, and changes in membrane permeability (Hooper *et al.*, 2001). It appears that DNA gyrase is the primary target of all quinolones in gram-negative bacteria. Among gram-positive bacteria, fluoroquinolones primarily target either topoisomerase-IV or DNA gyrase. Almost always, a hydroxyl group is exchanged for a bulky hydrophobic residue when an amino acid is changed in the quinolone resistance-determining region (QRDR). It is possible that the binding site conformation between quinolones and DNA gyrase is altered by *gyrA* mutations. Reduced uptake and increased resistance to fluoroquinolones have been linked to alterations in the cell envelope of gram-negative bacteria, specifically the outer membrane, but this has not been demonstrated in gram-positive bacteria.

2.6.5 Resistance to Sulfonamides and Trimethoprim

Sulfonamide resistance is commonly mediated by the expression of dihydropteroate synthase (DHPS) mutants that are resistant to the drugs. Gram-negative bacterial sulfonamide resistance typically results from the acquisition of one of two genes, *sul1* or *sul2*, that code to produce dihydropteroate synthase, an enzyme that is not inhibited

by sulfonamide (Enne *et al.*, 2001). *Sul2* is typically found on small non-conjugative plasmids or large transmissible multi-resistance plasmids, while sul1 is typically found linked to other resistance genes in class-1 integrons. Trimethoprim is a competitive inhibitor of the enzyme dihydrofolate reductase (DHFR), which is necessary for the synthesis of amino acids and nucleotides. The properties and sequence homology of at least 15 distinct DHFR enzyme types are known. There are several mechanisms that lead to trimethoprim resistance (Thomson, 1993) and these include: The host's increased DHFR enzyme production DHFR gene structural mutations. The most common mechanism of resistance in clinical isolates is the acquisition of a gene (*dfr*) encoding a resistant DHFR enzyme.

2.7 AMR Bacteria in Livestock

Current worries about AMR transmission from livestock to humans' center on resistant bacteria that are either zoonotic or harbor mobile genetic elements (primarily plasmids) encoding AMR and are known to enter the food chain or otherwise transmit to humans. *Salmonella enterica, Campylobacte*r, and methicillin-resistant *Staphylococcus aureus* (MRSA) are three zoonotic bacteria that warrant attention. *E. coli*, other members of the Enterobacteriaceae (especially *Klebsiella spp*.), and Enterococcus spp. are examples of less zoonotic organisms that still pose a threat to susceptible individuals and may carry mobile resistance determinants. Healthy cattle, sheep, and goats all carry *E. coli* O157 in their guts to varying degrees. Animals can be shedding *E. coli* O157 in their stool but still give the impression of being healthy and clean. It doesn't take long for the germs to spread all over the animals and their habitats.

2.8 Diseases Caused by *E. coli*

E. coli can cause disease in both humans and animals. In humans, pathogenic *E. coli* strains cause diarrhea, hemorrhagic colitis, hemolytic uremic syndrome, and other indications in humans and diseases like meningitis, bacteremia or urinary tract infect6ion can occur in animals.

2.8.1 Human Diseases Caused by *E. coli*

Escherichia coli, or *E. coli*, is a bacterium that can cause a variety of human diseases. Enterohemorrhagic strains, such as serotype O157:H7, produce cytotoxins,

neurotoxins, and enterotoxins that result in bloody diarrhea (Ahsan *et al.*, 2020) and hemolytic-uremic syndrome in a small percentage (2%-7%) of cases (Loos *et al.*, 2017). These strains are often transmitted through the consumption of raw or undercooked ground beef or via the fecal-oral route when sanitation is inadequate. Enterotoxigenic strains can cause diarrhea, particularly in infants and travelers (traveler's diarrhea) (Mirhoseini *et al.*, 2018; Farajzadeh-Sheikh *et al.*, 2020) while entero-invasive strains can cause diarrhea in infants. Enteropathogenic strains primarily cause watery diarrhea in infants (Moxley and Smith, 2010). Enteroaggregative strains can lead to persistent diarrhea in patients with AIDS or children living in tropical regions (Asea *et al.*, 2010). *E. coli* can also cause urinary tract infections, prostatitis, and pelvic inflammatory disease, which usually represent ascending infections from the perineum via the urethra (Forsyth *et al.*, 2020). Understanding the different strains of *E. coli* and their associated diseases is crucial for developing effective treatment and prevention strategies, such as proper food handling and sanitation practices.

Extra intestinal infection if normal intestinal anatomic barriers are disrupted (e.g., by ischemia, inflammatory bowel disease, or trauma), in which case the organism may spread to adjacent structures or invade the bloodstream. Hepatobiliary, peritoneal, cutaneous, and pulmonary infections also occur. *E. coli* bacteremia may also occur without an evident portal of entry. In neonates, particularly preterm infants, *E. coli* bacteremia and meningitis (caused by strains with the K1 capsule, a marker for neuro invasiveness) are common (Russo and Johnson, 2000).

2.8.2 Cattle Diseases Caused by *E. coli*

Infections of the urinary tract typically represent ascending infection (i.e., from the perineum to the urethra). If normal intestinal anatomic barriers are disrupted (e.g., by ischemia, inflammatory bowel disease, or trauma), the organism may spread to adjacent structures or enter the bloodstream and cause extraintestinal infection. There are also instances of hepatobiliary, peritoneal, cutaneous, and pulmonary infections. *E coli*. bacteremia may also occur in the absence of an obvious entry point. *E. coli* bacteremia and meningitis (caused by strains with the K1 capsule, a marker of neuro invasiveness) are common in neonates, particularly preterm infants (Russo and Johnson, 2000).

2.9 Importance of Cow Milk as Sample

Milk is considered as a perfect food produced from the secretion of mammary gland of mammals. Colostrum is the early lactation milk and contains a considerable number of antibodies in comparison of the normal lactation secretion. Cow milk is the main dairy product having major portion of dairy industry. From the milk there are different by products that are made and have large impact for the livestock industry. Milk contains both protein and carbohydrates along with other necessities. In microbiological perspective there is containing number of bacteria irrespective their clinical abnormality (Bowler *et al.*, 2001). Until now many zoonotic pathogenic bacteria were isolated from the raw milk and people are encouraged to drink boiled or pasteurized milk for the preventive point of view (Abdeen *et al.*, 2020). The nutritional components make this milk a perfect media for microbial growth (Porcellato *et al.*, 2018).

In developed nations, up to 5% of food-borne infections were associated with the consumption of milk and dairy products (Ahmed and Shimamoto, 2014). The case scenario could be worse in developing nations where high rates of milk contamination are associated with unhygienic milk production and inadequate preservation (Garedew *et al.*, 2012). *E. coli* pathogens are frequently used as an indicator of fecal contamination of milk and can indicate the presence of human pathogenic serotypes (Ahmed Shimamoto, 2014; Garedew *et al.*, 2012). *E. coli* contamination of milk is either associated with milkers or milk handlers, particularly those with poor hygienic practices such as wheezing or sneezing during milking or milk handling or with infected cows as reservoirs of *E. coli* infection (Abebe *et al.*, 2016). *E. coli* is excreted in milk regardless of milk consistency or udder shape; as a result, consuming or processing milk from these cases may have a negative effect on human health.

Table 2.5: Worldwide prevalence of AMR gene in *E. coli* isolated from cow milk.

Antibiotic	Resistant gene	Prevalence area and study	Reference
Ceftriaxone	bla_{CTX-M}	22.6% in Nottingham, UK.	(Ibrahim *et al.*, 2016)
		54.54% in West Bengal, India.	(Batabyal *et al.*, 2018)
		66.7% in PutrA, Malaysia.	(Kamaruzzaman *et al.*, 2020)
Ampicillin	bla_{TEM}	7.5% in Nottingham, UK.	(Ibrahim *et al.*, 2016)
		83.1% in Northern, Southern China.	(Yu *et al.*, 2020)
Sulphur drug	*sul1*	83% in West Bengal, India.	(Mahanti *et al.*, 2020)
		15.7% in Northern, Southern China	(Yu *et al.*, 2020)
	sui2	30.4% in West Bengal, India.	(Mahanti *et al.*, 2020)
Tetracycline	*tetA*	44.2% in Algeria	(Tahar *et al.*, 2020)
		10% in West Bengal, India.	(Das *et al.*, 2017)
		93% in Irbid, Jordan	(Ismail and Abutarbush, 2020)
	tetB	16% in West Bengal, India.	(Das *et al.*, 2017)
	tetC	57% Irbid, Jordan	(Ismail and Abutarbush, 2020)
	tetD	71% Irbid, Jordan	(Ismail and Abutarbush, 2020)
Colistin	*mcr-1*	2.0% in Beijing, China.	(Liu *et al.*, 2020)
	mcr-3	2.6% in Lyon, France	(Lupo *et al.*, 2018)

Microorganisms containing AMR genes spread throughout the environment and animals incurring diseases (Baker *et al.*, 2018). The pictorial presentation reveals the spread of antibiotic resistance bacteria. Use of antimicrobial agents in veterinary practices is considered as one of the basic routes in the transmission of AMR and antibiotic resistance. For the spreading of resistance from animals to humans, some pathogens follow direct. The environment and fauna become a reservoir of antibiotic resistance and serve as the source of proliferation of antibiotic-resistant bacteria and their spread among humans and animals. It is because antibiotic residues and bacteria get released from food-animal production with manure and reenter into the environment where they promote the development of resistance. Use of livestock manure as a fertilizer and the overuse of antibiotics in aquaculture are two important ways of spreading antibiotic resistance (Magouras *et al.*, 2017).

Antibiotics used in food-producing animals are like those used in humans and can be selected for resistance by animals. Cross transmission of resistant bacteria and resistance genetic elements can also occur easily (Tang *et al.*, 2017). One of the experimental studies in United States confirmed the presence of gentamycin-resistance genes in Enterococci isolated from animals, and the same genes were also present in the food products of the same animals. It was observed that similar resistance patterns were also shown by Enterococci isolated from human and retailed food of different regions (Donabedian *et al.*, 2003). A study from Nigeria confirms the presence of resistant *E. coli* isolated from poultry forms. Various resistance genes were found in the isolates, including bla_{TEM}, *sul2, sul3*, and *tetA, tetB*, etc. These results provide evidence that livestock production farms are important reservoirs of antimicrobial resistant genes (ARGs) (Adelowo *et al.*, 2014).

2.10 Animal-To-Human AMR Transmission Pathways

2.10.1 Direct Exposure

Farmworkers, veterinarians, slaughterhouse workers, and animal food handlers who have direct contact with animals and their biological substances are at a high risk of becoming colonized with antibiotic-resistant bacteria from animals and animal farms, which may allow the spread of ARGs/ARBs into local communities and health care settings (Marshall and Levy, 2011).

E. coli strains isolated from turkey and chicken farmers in the Netherlands were found to be virtually resistant to all tested antibiotics. These ARGs/ARBs associated with livestock could further transmit from farmers to their families and the local community via human-to-human transmissions, leading to an increase in colonization and infections in individuals with/without contact with livestock (Larsen *et al.*, 2015).

2.10.2 Animal-To-Human Transmission Via Environmental Compartments

The environment plays a crucial role in the global transport of ARGs from sustenance animals to humans (Graham *et al.*, 2019). This environmental dissemination from animal farms involves primarily two processes: the selection of antibiotic residues and the dispersal of ARGs/ARBs. Antibiotics and antibiotic-resistant bacteria have been detected in farm sediment, air current within feeding operations, and groundwater impacted by animal husbandry and feedlots in general (McEachran *et al.*, 2015). Exposure scenarios involving contaminations in agricultural environments also serve as transmission routes for ARGs (Marshall and Levy, 2011). The proximity to animal feeding operations or areas with fecal contamination increases the risk of MRSA (Casey *et al.*, 2013). However, animal sourced ARG transmissions are not limited to animal husbandry environments. To reveal the complexities of AMR across animals, environments, and humans, we propose the animal-to-human AMR transmission pathways by concentrating on the environmental transmission pathways from the perspective of One Health.

2.10.3 Manured Farms and Soils

Manure is used as a fertilizer all over the world because it is full of nutrients and organic matter. This helps to improve the soil and make crops grow better. Manure, on the other hand, has become a major source of antibiotic chemicals, ARBs, ARGs, and MGEs. ARGs in manure-affected soils could get into the food chain, and eating organic veggies and fruits grown in manured-soils is likely another way for ARGs to get to people (Zhang *et al.*, 2019).

The soil microbes have a big effect on the growth of bacterial communities in both below-ground and above-ground parts of plants (Rahman *et al.*, 2021). This is shown by the fact that bacteria from leaves and roots share a lot of the same functions. There is a strong link between endophytic bacteria and bacteria in the rhizosphere, and many

endophytic bacteria that can live outside of plants can also live in the rhizosphere. The rhizosphere is also known as a place where genes move from one plant to another (Badri *et al.*, 2009). Another research showed that soil bacteria could live inside the root and turn into root endophytes (Frank *et al.*, 2017). Because of this, the bacterial community of plants growing in soil with dung can be affected by the bacterial community of the soil. (Zhang *et al.*, 2020) and from soils to the veggie microbiome.

Root endophytes, leaf endophytes, and phyllo-sphere are all types of ARGs that have been found in veggies and fruits that grew in manure-amended soil (Zhang *et al.*, 2019). Using high-throughput quantitative PCR (HT-qPCR), a total of 134 ARGs were found in both conventionally and organically grown lettuce. The phyllo sphere of organically grown lettuce had more ARGs than the phyllo sphere of conventionally grown lettuce (Zhu *et al.*, 2017). Because of this, vegetables and fruits grown in manured soils, especially those that are eaten raw, could be a major way that antibiotic resistance spreads to people. In this case, controlling the spread of ARGs requires a better understanding of how ARGs work in the soil-plant system when waste is used as fertilizer. A study showed that ARGs only went up temporarily after manure fertilization in Finland, where antibiotic use in animals is limited (Muurinen *et al.*, 2017). This suggests that the negative effects of manure application on ARG contamination in soils might be lessened by the limited use of antibiotics in animals.

2.10.4 Aquatic Environments

Aquatic habitats are the best places to find and spread ARGs. A study says that genetic exchange and recombination can happen often in aquatic settings to shape how aquatic microbial communities evolve (Watts *et al.*, 2017). Because the genes of aquatic microbes are flexible, ARGs can move quickly through bacterial populations and communities to protect against drug pollution and/or make the bacteria more competitive. Also, once bacteria got ARGs (or MGEs that carried ARGs), the ARGs they got were more likely to stick around in water than on land, even when there wasn't any selection pressure. Another study says that aquaculture is a gateway to the growth and spread of AMR in aquatic habitats (Cabello *et al.*, 2017; Watts *et al.*, 2017). Large amounts of selective agents and feces that contain ARGs/ARBs from aquaculture get into our water bodies. This encourages bacterial mutation, recombination, and

horizontal gene transfer, which raises the level of ARGs in natural aquatic environments and the risk of spreading ARGs from aquaculture to humans (Watts *et al.*, 2017).

Different aquaculture systems had different ways of spreading ARGs that came from aquaculture. In open systems, between 70% and 80% of the antibiotics that are used end up as residues. These residues stay in the water and help ARBs grow, while ARGs that are excreted by fish and other seafood animals have a lasting effect on marine microbial communities (Watts *et al.*, 2017). Most farming systems are kept separate from the rest of the world in closed systems. In near zero-discharge recirculating aquaculture closed systems, there isn't much exchange between the aquaculture water systems and the environment. However, antibiotics build up in the systems, which helps ARBs grow on the RAS biofilter (Yin *et al.*, 2017).

In integrated aquaculture systems, fish farming is generally linked to farming crops or livestock to make better use of resources. However, they also cause a lot of ARG pollution and gene transfer in the soil, water, and plants, which raises the risk of resistomes spreading to people. In other regular closed systems, antibiotics and ARGs can be cut down by treating trash and controlling how much is released into the environment. After the wastewater is treated, antibiotic residues and ARGs typically flow into waterways or are treated to make aquaculture sludge, which is used as an organic fertilizer. Some treated aquaculture effluents are used to water crops or urban parks. This, along with the land application of aquaculture sludge as organic fertilizer, creates a pathway for ARGs to move from animals to soils and crops, which could affect workers, people who eat crops, and people who live in cities (Fahrenfeld and Bisceglia, 2016). But it should be noted that most aquaculture systems in developing countries don't treat waste or have few regulations. This means that untreated wastewater from aquaculture can flow directly into nearby water bodies, which could increase the risk of ARGs spreading from aquaculture to humans through water (U.S. Centers for Disease Control and Prevention, 2018).

2.10.5 The Horizontal Gene Transfer Promotes the Transmission

The plasmids, integrative conjugative elements, integrons and transposons, significant genetic exchange and recombination can occur for various purposes (Soucy *et al.*, 2015; Wintersdorff *et al.*, 2016). The horizontal movement of ARGs could help spread ARGs

from animals to people. As many environmental microbes especially aquatic bacteria from aquaculture share many MGEs (Marti *et al.*, 2014; Wintersdorff *et al.*, 2016). Also, it has been shown that there is a strong link between antibiotic resistance in soil settings and human clinical pathogens. This is because MGE-mediated HGT puts together tandem arrays of different ARGs into integrons, transposons, and plasmids and then makes them mobile. These mobile ARGs and bacteria can spread into the environment and move up our food chains. Transduction, bacterial conjugation, and bacterial uptake of "free" DNA can also spread ARGs to human pathogens (Zhu *et al.*, 2017). Because of this, the Class 1 integrons, which are often physically linked to multiple antibiotic-resistant determinants, are thought to be the most important and widespread agents of ARGs and a good stand-in for ARGs with human-made causes, such as the animal food industry.

Conjugation is the transfer of DNA from a donor cell to a recipient cell using bacterial pili or adhesins. Compared to transformation and transduction, it has been shown to have a much bigger effect on the spread of ARGs among bacteria (Wintersdorff *et al.*, 2016). Another study says that ARGs are often linked to conjugative plasmids, integrons, or transposons in animal systems, especially aquaculture (Watts *et al.*, 2017). Once ARG exchange events have happened in environmental bacteria, the ARGs can be spread among local bacterial populations, including human pathogens, and then spread globally through the international transport of food products and global travelers (Cabello *et al.*, 2016; Zhu *et al.*, 2017).

Several studies have shown that livestock environments may have helped spread the plasmid-encoded qnrA gene, which gives bacteria low-level resistance to quinolones. The qnrA gene is also linked to the waterborne species *Shewanella spp,* which are found in both marine and freshwater environments (Poirel *et al.*, 2005). Another study found that suggests that most plasmid-borne *mcr* genes may have come from aquatic systems (Cabello *et al.*, 2017). This is because aquaculture activities move *mcr* genes from aquatic bacteria to terrestrial bacteria. One piece of proof is that the amino acid sequences of *mcr-3* and *mcr-4* were very similar to phosphoethanolamine transferases found in *Aeromona Salmonicida* (84%) and *Shewanella frigidimarina* (99%) (Yin *et al.*, 2017; Carattoli *et al.*, 2017).

A recent study also showed that aquaculture is a major source of the *mcr-1* gene, and it was found that the chances of being *mcr-1* positive are much smaller in areas with low aquaculture activity (odds ratio = 0.5, 95% confidence interval = 0.3–0.7). So far, *mcr* genes have been found in animal farms, animal food items, vegetables, imported reptiles, the environment (sewage and soils), and people (Ali *et al.*, 2016; Xie *et al.*, 2018; Wang *et al.*, 2018). This gene family has caused a new risk to public health, which has since spread around the world (Wang *et al.*, 2018). This is a scary situation because colistin is the last drug that can stop MDR Gram-negative bacteria from spreading around the world.

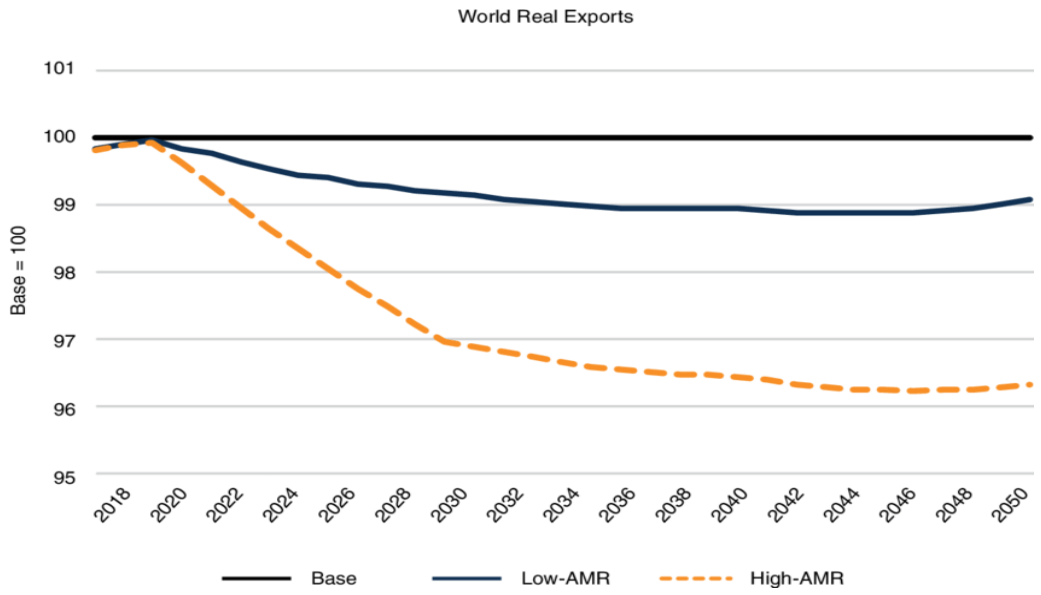
2.11 Public Health Importance of Antimicrobial Resistance

Prior to the discovery and widespread use of antimicrobials, infectious diseases were humanity's leading cause of death. In much of the developing world that lacks access to high-quality pharmaceuticals, infections continue to be the leading cause of death, and in all nations, healthcare-associated infections caused by resistant microorganisms are a leading cause of death (Ferri *et al.*, 2017). Depending on the scenario, it is estimated that if AMR is not addressed, the global population in 2050 will be between 11 million and 444 million fewer than it would be without AMR. The lower bound from a scenario in which resistance rates have been kept at a comparatively low rate, whereas the upper bound reflects a world without effective antimicrobial drugs (Taylor, 2019).

2.12 Antimicrobial Resistance — Unusual Public Health Threat

Antimicrobial resistance is not a "disease" There is typically no difference in disease severity between susceptible and resistant strains. Resistance is typically not a disease pathogenesis issue, but rather a consequence of limited treatment options. Our dependence on antimicrobials to treat infections is the central issue. If alternative methods of treating infections were available, antimicrobial resistance would still exist in the world, but it would no longer be a public health concern. Antimicrobial resistance is a hazard to public health caused by healthcare practices, specifically the excessive use of antimicrobials for conditions in which they are ineffective. Moreover, it has been assessed that AMR will lead to a global catastrophe by resulting in 10 million deaths per year and endow with a terrifying economic cost of 100 trillion USD along with an

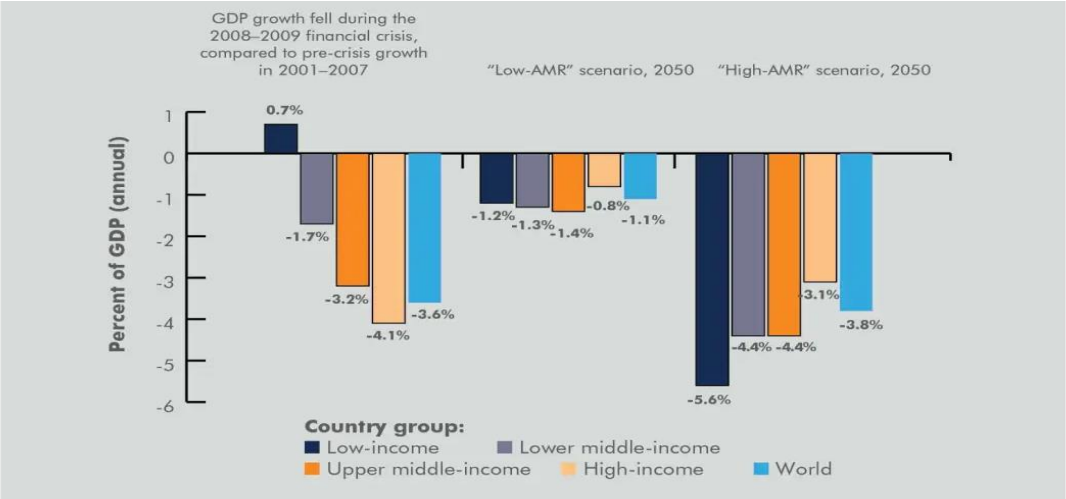
11% fall in livestock productions by 2050 if adequate measures will not be taken to address the challenges (O'Neill, 2019).



Source: (Banco Mundial, 2017)

Figure 2.3: Global AMR consequence in near future.

Resistance is a characteristic of numerous pathogens that cause various diseases. Thus, containment strategies must be tailored to the requirements of disease prevention and treatment initiatives (Ferri *et al.*, 2017).



Source: (Barıs *et al.*, 2017)

Figure 2.4: Worldwide economic loss due to AMR

2.13 Managing Resistance in Farm Animals

2.13.1 Surveillance

Determining the levels of resistance in these populations is a crucial step in assessing any hazard to public health posed by AMR in farm animals. At the national level, AMR in farm animals is typically reported through passive surveillance. Alternatives to passive surveillance have been considered for AMR in humans. As has been suggested in the context of emerging zoonotic diseases in general (Keusch *et al.*, 2010). These types of approaches could theoretically be extended to agricultural animals.

2.13.2 Reducing Antimicrobial Usage in Farm Animals

As demonstrated by the experience of the EU-wide prohibition on growth promoters, reducing the consumption of antimicrobials by farm animals has proven difficult. Outside of Europe, the adoption of voluntary codes and the formulation of guidelines for drug use, while commendable in and of themselves, do not appear likely to significantly reduce drug use. There may be potential for a more effective use of antimicrobials in farm animals, particularly if this generated measurable cost savings or increased productivity. These include the same strategies proposed for human medicine, including overload strategies, combination therapies, and drug reuse and recycling. As with humans, there would be clear benefits of rapid diagnosis of bacterial infections and real-time profiling of resistance determinants using whole genome sequence data to determine treatment strategies more rapidly and precisely (Gordon *et al.*, 2014).

A total prohibition on the use of antimicrobials in farm animals would have unavoidable negative effects on animal health, welfare, and productivity, and consequently on food prices. However, reduced antimicrobial consumption in farm animals could be part of an industry-wide coordinated strategy. If viable alternatives to antimicrobials were available, any negative effects on the agricultural industry would be mitigated at least in part.

2.13.3 Alternatives to Antimicrobials for Farm Animals

Several prebiotics and probiotics are currently on the market, even though their efficacy is unknown and presumably variable. Also proposed are so-called 'symbiotics' that combine the two organisms. This necessitates rapid phage selection and administration, as well as elevated bacterial concentrations. It may be possible to use purified phage lysins instead of the phage itself, thereby preventing the unintended transfer of genetic material from the phage. However, none of these options are close to being commercially available for use against the complete spectrum of microbial diseases in farm animals.

Increasing the variety of vaccines that can be administered to animals may be a more immediately applicable suggestion. Despite the availability of vaccines against the most prevalent viral diseases of livestock, the routine use of vaccines that protect against bacterial infection and disease is currently restricted. Even when a vaccine is available, it is not always adopted by producers; for example, one trial of a live oral Lawsonia vaccine in piglets resulted in 80% less oxytetracycline consumption and increased productivity (Bak and Rathkjen, 2009) but the vaccine is not extensively utilized. As long as antibiotics remain accessible and effective, there is arguably little commercial incentive to use existing antibacterial vaccines or develop new ones for farm animals.

Long-term objectives for reducing antimicrobial use in farm animals could entail the utilization of livestock that are genetically resistant to infection or disease, most likely through the application of genetic modification technologies. Early progress in this direction includes the development of transgenic poultry incapable of transmitting avian influenza (Lyall *et al.*, 2011). Before any of the above approaches to disease control in agricultural animals could serve as effective alternatives to antimicrobials, it is evident that substantial investment in research and development would be required.

2.14 One-Health Approaches to Check the AMR Issue

The complex epidemiology and socioeconomic determinants of AMR make this topic the quintessential One-Health concern. Trans-sectoral and transdisciplinary approaches are required to effectively combat AMR. Reducing the spread and transmission of resistant microorganisms within and between animal and human populations is essential for combating antimicrobial resistance. It is difficult to explain with certainty

the origin of resistant bacteria strains due to the ability of bacteria to spread from one environment to another, sometimes over large geographic distances and between diverse populations. Therefore, the reservoirs and transmission pathways of antimicrobial-resistant bacteria merit additional research, preferably utilizing a One-Health approach.

Therefore, it is essential to increase our understanding of how animal contacts and trade (direct transmission), farm management, and the broader farm environment (indirect transmission) contribute to the spread of antimicrobial resistance and to identify potential countermeasures to this phenomenon.

Farm management studies may include all practices that may facilitate the spread of resistant bacteria within and between farms and from farms to the environment, including farm hygiene and biosecurity, animal waste management, structure (and construction material) of holdings, and animal production intensity. One-Health approaches should always be supported by molecular epidemiological data, which can provide information about the relationships between resistance genes observed in various samples, such as those from animals of different origins. Not only should resistance genes be examined in animal samples, but also in the larger farm environment, including farmers, other livestock species, farm pets, wildlife, manure, and water.

These ecological data can provide the molecular link necessary to characterize reservoirs of resistant bacteria and could support studies on transmission pathways between animal populations, as well as between animals and humans. Source attribution can aid in shedding light on the contribution of AMR from livestock to the public health resistance burden. In addition, it can be a crucial piece of evidence in the development of targeted interventions against AMR. In addition, genomic data may provide additional insight into the evolution of microbes during transmission within the studied populations. In addition, molecular epidemiology data can cast light on the proportion of resistance reservoirs that can be attributed to the spread of resistant bacteria or de novo emergence because of AMU selection pressure in the studied farms.

2.15 WHO's Prioritization of Multi-Drug Resistant Bacterial Pathogens

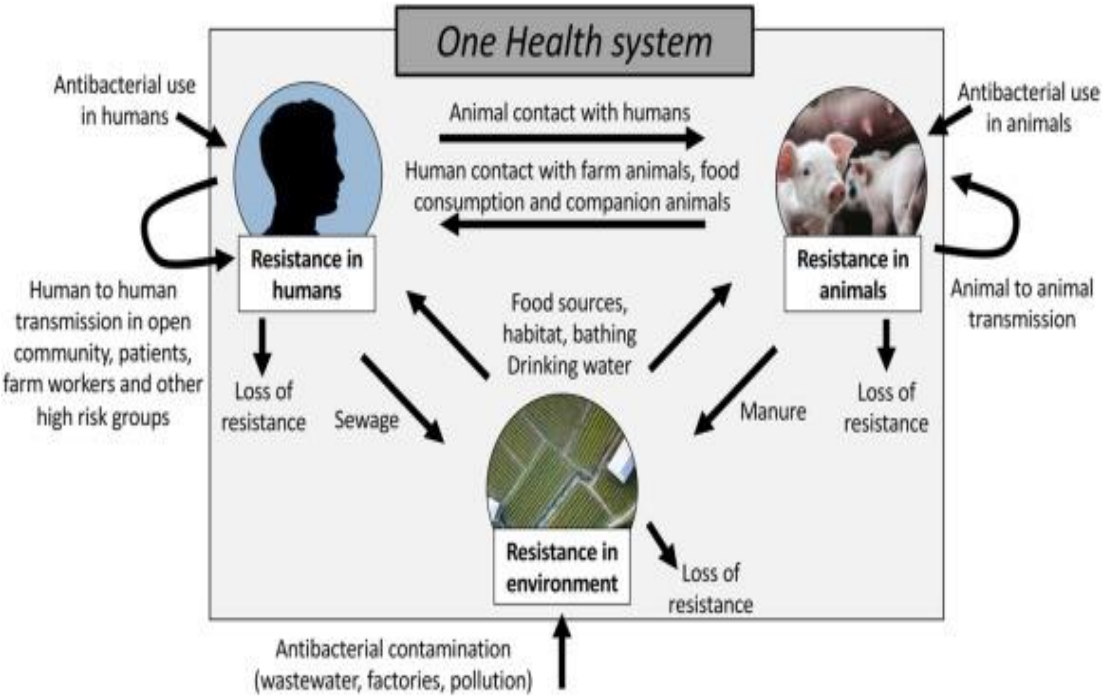
Recently, on February 27, 2017, the World Health Organization (WHO) published its first-ever list of antibiotic-resistant "priority pathogens" — a catalogue of 12 bacterial families that pose the greatest hazard to human health. As part of WHO's efforts to combat the escalating global resistance to antimicrobial drugs, the list was compiled to guide and encourage research and development of new antibiotics. The list emphasizes the threat posed by gram-negative bacteria resistant to multiple antibiotics. These bacteria have innate abilities to discover new ways to resist treatment and can transmit genetic material that enables other bacteria to develop drug resistance. The roster was compiled in collaboration with the University of Tübingen's Division of Infectious Diseases. The team utilized a technique for multi-criteria decision analysis that was reviewed by a group of international specialists. The selection of pathogens for the list was based on the lethality of the infections they cause.

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

A) Priority 1 (Critical)- Carbapenem-resistant- *Acinetobacter baumannii* Carbapenem-resistant- *Pseudomonas aeruginosa*, Carbapenem-resistant ESBL-producing- *Enterobacteriaceae*.

B) Priority 2 (High)- Vancomycin-resistant- *Enterococcus faecium*, Methicillin-resistant Vancomycin-intermediate and resistant- *Staphylococcus aureus*, Clarithromycin-resistant- *Helicobacter pylori*, Fluoroquinolone-resistant- *Campylobacter* spp., Fluoroquinolone-resistant- *Salmonellae*, Cephalosporin-resistant Fluoroquinolone-resistant- *Neisseria gonorrhoeae*.

C) Priority 3 (Medium)- Penicillin-non-susceptible- *Streptococcus pneumoniae*, Ampicillin-resistant- *Haemophilus influenzae*, Fluoroquinolone-resistant- *Shigella* spp.



Source: (Booton *et al.*, 2021)

Figure 2.5: Relationship among human, animal and environment health in relation to transmission of antimicrobial resistance

2.16 Prevalence of Multi-Drug Resistant *E. coli* in Animals

Antimicrobial resistant pathogens are easily transmitted from food animals to human through the food chain. *E. coli* was isolated from different animal species (cattle, goat, swine, yak and avian) from seven states of India. They found 85.2% of samples were positive for *E. coli*. 45.07% *E. coli* isolates were MDR while 6% were resistant to all 20 antimicrobials tested. A total of 668 fecal samples randomly collected from swine, cattle, goat, yak and avian scattered in India (Sanjukta *et al.*, 2016). The prevalence of *E. coli* is 88.7% in cattle, 81% in chicken, and 89.5% in swine. The resistance patterns were 58.8%, 39.8% and 34.1% for Tetracycline, Sulfamethoxazole-trimethoprim and Ampicillin individually and among the isolates 26 were multi-drug resistant (resistant to ≥2 antimicrobials) (Adenipekun *et al.*, 2015). *E. coli* was isolated from goat of Cox's Bazar, Bangladesh where the overall prevalence was reported 52% and higher prevalence was found in goats having diarrhea (diarrheic: 62%, non-diarrheic: 38%) and among the isolates 39.74% showed multi-drug resistance (resistant to 3 to 8 classes of antimicrobials) (M Saiful Islam *et al.*, 2016).

Shiga toxin-producing *E. coli* (STEC) O157:H7 is a well-recognized cause of hemolytic uremic syndrome and hemorrhagic colitis in humans, which can be transmitted from domestic food animals. About 2.1% samples from goat fecal contents is positive for *E. coli* O157 which is resistant to 2 to 18 antimicrobials (Dulo *et al.*, 2015). About 31.7% of 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 %, $bla_{\text{CTX-M}}$ 65 %, bla_{TEM} 27 % and *tetA* 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).

2.17 Prevalence of Multi-Drug Resistant *E. coli* in Humans

The growing number of bacteria that are resistant to antibiotics and cause both hospital- and community-acquired infections is a major threat to people all over the world. Typically, ESBL-producing strains, carbapenem resistant Enterobacteriaceae (CRE), and other 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 it very hard, and in some cases almost impossible, to treat them. A study found that 85.7% of the isolates were resistant to more than one drug and that 64.3% of them made an enzyme called extended-spectrum β-lactamases (ESBL) out of 70 Enterobacteriaceae stains from people (Leski *et al.*, 2016). 57% of human isolates and 23% of outdoor isolates were β-lactamases producers with a wide range of activity. Penicillin, cephalosporin, and quinolone were often resistant to all three drugs at the same time (Purohit *et al.*, 2017).

In 2014, a retrospective study to find out how often MDR bacteria were found in an Oman teaching hospital. There were 10.8 MDR patients and 11.2 MDR cases for every 1000 hospital admissions (Balkhair *et al.*, 2014). Another research found that 64.29% of the *E. coli* found in drinking water in Hyderabad was isolated there, and 62.96% of the isolates showed MDR, which means they were resistant to 3 to 6 antimicrobials. They found the most resistance to 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%). A study to find out how often MDR diarrhoeagenic *E. coli* was found in children in India who had or didn't have diarrhea. About 41.40% of isolates that cause diarrhea were MDR, which means they were resistant to at least 5 antibiotics (Chellapandi *et al.*, 2017). The amount of resistance in these isolates ranged from 13.3% to 100% (Ogidi *et al.*, 2016). 35% of *E. coli* isolates from food and clinical samples in Egypt had a pattern of resistance to more than three groups of antibiotics (Aly *et al.*, 2012). 3.2% of goat bodies have *E. coli* O157 that is resistant to at least 2 to 18 antimicrobials (Dulo *et al.*, 2015; Booton *et al.*, 2021).

2.18 Prevalence of Multi-Drug Resistant *E. coli* of Environmental Origin

The *E. coli* that makes ESBL is also a big problem in the chicken industry. Treatment is very hard when ESBL-positive isolates are found. 65% of layer farms and 81% of broiler farms had *E. coli* that could make ESBLs and there were 81%, 79%, 60%, 57%, 55%, 15%, and 6% of ESBL-producing *E. coli* in rinse and run-off water, other farm animals, dust, surface water next to farms, dirt, flies, and barn air, respectively (Blaak *et al.*, 2015). *E. coli* was isolated from farm waste, litter, soil and water of poultry farms and resistance profile was Tetracycline 81%, Sulfamethoxazole 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 in Nigerian poultry farm (Adelowo *et al.*, 2014). Resistance to Ampicillin, Tetracycline, and Sulfonamides is common in *E. coli* that came from fattening pigs and calves younger than 1 year old in several European countries, according to a 2017 study from the European Food Safety Authority. About 7.1% of *E. coli* O157-positive isolates from slaughterhouse water samples are resistant to 2 to 18 antimicrobials (Dulo *et al.*, 2015).

2.19 FAO’s Goals on AMR

AMR jeopardizes the achievement of the Sustainable Development Goals because more agricultural producers may struggle to prevent and manage infections that threaten to disrupt food supply chains and push tens of millions of additional people into extreme poverty. FAO (Food and Agriculture Organization) has established two primary objectives for its work on AMR to resolve this challenge and realize the four betters: better production, better nutrition, a better environment, and a better life. To sustain food and agricultural production, we must continue to have access to antimicrobials that are both effective and harmless. Through the attainment of these objectives, FAO will collaborate with stakeholders to improve the food and agriculture sectors' capacity to manage AMR risks and establish resilience to AMR's effects. If FAO and its partners collaborate, they will be better able to protect agricultural systems, livelihoods, and economies from the destabilizing effects of AMR.

CHAPTER-3: MATERIALS AND METHODS

3.1 Study Area

Eighteen dairy farms of seventeen locations under Chattogram Metropolitan Area (CMA), Bangladesh (Patenga Thana, Akbershah, Dewanhut, Foys Lake, Sadarghat road, Pahartali, Wireless area) were selected randomly for sample collection. These locations were pointed out in Figure 3.1.

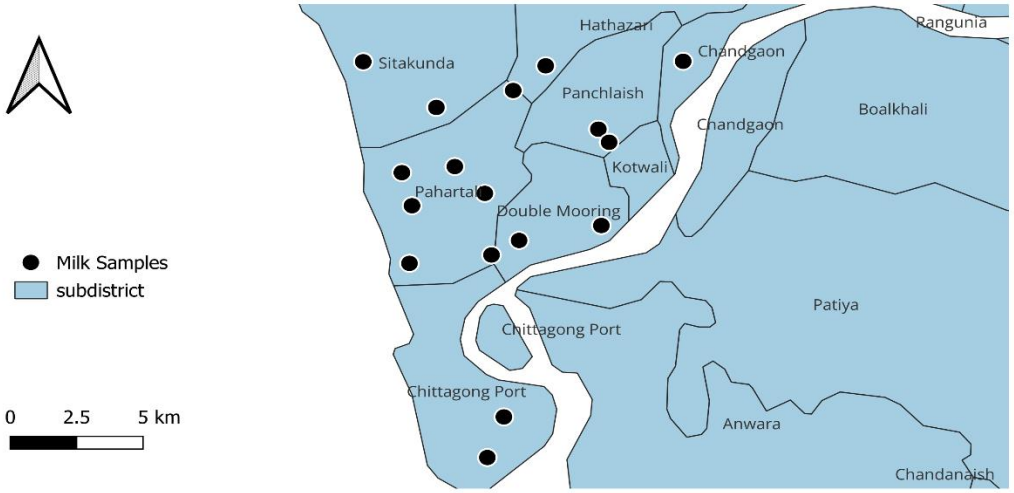


Figure 3.1: Geographical locations of the sites of different kinds of samples collected for the study.

3.2. Sample Collection Duration

The samples were collected between September 2021 and August 2022.

3.3 Study Population

A total of 450 cow raw milk samples were collected from different geographical locations. All the eighteen dairy farms were clustered based on farm size: Large farm (farms having more than 50 cows), Medium farm (farms having 20 to 50 cows), Small farm (farms having less than 20 cows). Then the total samples were collected randomly from each cluster based on proportion.

3.4 Sample Collection, Transportation, and Processing Procedure

Samples were collected in separate falcon tubes maintaining proper hygiene procedures. After collection, samples were shifted to the Department of Physiology Biochemistry and Pharmacology, CVASU for further investigation through maintaining a cool chain. For primary enrichment, samples were diluted with buffer peptone water (BPW) (HIMEDA, pH: 7.0±0.2, Mumbai, India) maintaining a ratio of 9:1 (Buffer-peptone: cow milk sample) and incubated at 37°C overnight.

3.5 Preservation of The Isolates

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

3.6 Microbiological Isolation

3.6.1 Isolation and Identification of *E. coli*

To isolate *E. coli*, enriched culture was streaked on MacConkey agar medium (HIMEDIA, pH: 7.10.2, Mumbai, India) and incubated for 24 hours at 37°C. On a MacConkey agar plate, large, bright pink colonies were suspected to be the proliferation of *E. coli*. These colonies were streaked on Eosin Methylene Blue (EMB) agar plates (Merck, pH: 7.10.2) and incubated for 24 hours at 37°C. *E. coli* was confirmed based on the "green metallic sheen" of the colony morphology on this medium. The isolates were then inoculated onto blood agar and incubated for 24 hours at 37°C. After the incubation period had concluded, blood agar colonies were used to extract DNA for PCR.

3.6.2 Molecular Identification of *E. coli*

All phenotypically positive blood agar isolates were subjected to species-specific multiplex PCR using primers for the *uidA* gene and adjacent region of the *uspA* gene on a thermal cycler (DLAB, USA). The primer sequences are listed in Table 3.1.

Table 3.1: Oligonucleotide primer sequences used for detection and confirmation of *E. coli.*

Primer	Primer Sequence (5′-3′)	Annealing	Fragment size (bp)	Reference
*usp*A Up	CCGATACGCTGCCAATCAGT	55.2°C	884	(Godambe *et al.*, 2017)
*usp*A Down	ACGCAGACCGTAGGCCAGAT			
*uid*A Up	TATGGAATTTCGCCGATTTT		164	
*uid*A Down	TGTTTGCCTCCCTGCTGCGG			

With a final volume of 15 µl, PCR reactions were conducted. Proportions of different reagents used for PCR for two different resistance genes are given in Table 3.2.

Table 3.2: 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)	7.5 µl
2	*usp*A Up	0.5 µl
3	*usp*A Down	0.5 µl
4	*uid*A Up	0.5 µl
5	*uid*A Down	0.5 µl
6	DNA template	1 µl
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 the Research lab under the Department of Physiology Biochemistry and Pharmacology, CVASU following the steps mentioned in Table 3.3.

Table 3.3: Steps 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

3.6.3 Screening of Antimicrobial Resistance Pattern of *E. coli* Isolates

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, sulfonamides, polymyxins, aminoglycosides, quinolones, and penicillin) of drugs having public health significance were selected for the CS testing. The following antimicrobial agents (with respective disc potencies) were used: CT: Colistin sulfate (10 µg), TE: Tetracycline (30 µg), CN: Gentamicin (10 µg), DO: Doxycycline (30 µg), AMP: Ampicillin (10 µg), CL: Cephalexin (30 µg), SXT: Sulfamethoxazole-trimethoprim (25 µg), CIP: Ciprofloxacin (5 µg), NOR: Norfloxacin (10 µg), KF: Cephalothin (30 µg), ENF: Enrofloxacin (5 µg), FFC: Florfenicol (30 µg) , AMP1: Penicillin G (10 µg),, NE: Neomycin (30 µg). To interpret the result of the CS test, the CLSI-2018 standards are given in Table 3.4.

Table 3.4: Concentrations and diffusion zone breakpoints for resistance against antimicrobials standard for *E. coli* isolates.

Group of antimicrobial agents	Anti-microbial agent (code)	Disc content	Diffusion zone breakpoint (diameter in mm)		
			R	I	S
β-lactam antibiotics/ Penicillin Derivatives	Amoxicillin+ Clavulinic Acid	30 µg	≥18	14-17	≤13
	Ampicillin	10 µg	≤13	14-16	≥17
	Penicillin G	10 µg	≥19	15-18	≤14
1st gen cephalosporins	Cephalexin	30 µg	≤14	-	≥15
	Cephalothin	30 µg	≥18	15-17	≤14
2nd gen cephalosporins	Cefoxitin	30 µg	≥15	13-14	≤12
3rd gen cephalosporins	Cefotaxime	30 µg	≥26	23-25	≤22
	Ceftazidime	30 µg	≥21	18-20	≤17
Tetracyclines	Tetracycline	30 µg	≤15	12-14	≥11
	Doxycycline	30 µg	≤10	11-13	≥14
Polymyxins	Colistin sulfate	10 µg	≤10	-	≥11
Aminoglycosides	Gentamicin	10 µg	≥18	14-17	≤13
	Neomycin	10 µg	≥17	13-16	≤12
Fluoroquinolones	Ciprofloxacin	5 µg	≤15	16-20	≥21
	Levofloxacin	5 µg	≥18	15-17	≤13
Quinolones	Enrofloxacin	5 µg	≥21	17-20	≤16
	Norfloxacin	10 µg	≥17	13-16	≤12

Sulfonamides	Sulfamethoxazole trimethoprim	25 µg	≤10	11-15	≥16
Amphenicols	Florfenicol	30 µg	≥18	13-17	≤12

Manufacturer of disc: Oxoid Limited, UK. Mast Group Limited, UK.

3.7 Procedure of CS Test

To acquire a pure growth, the preserved organism was sub-cultured on blood agar for 24 hours at 37 degrees Celsius. Using a sterile inoculating loop, three or four distinct colonies from the blood agar were transferred into a tube containing 3 ml of sterile phosphate buffer saline solution (0.85% w/v NaCl solution). Using a vortex machine, inoculums were emulsified to prevent cells from clumping within test containers. The bacterial suspension was then adjusted to a turbidity level of 0.5 McFarland standard, which corresponds to a growth of 1-2 108 CFU/ml. To drain excess fluid, a pre-sterile cotton swab was inserted into the inoculums and rotated against the tube's side with firm pressure within 15 minutes of the inoculums' preparation. The Mueller Hinton agar plate was then rotated three times as the swab was splattered across the entire desiccated surface. After 15 minutes of inoculation, the discs were placed on the agar surface using sterile instruments. The agar plates were then loaded with all the discs and incubated for 18 hours at 37°C. Following incubation, the diameter of the disc and the extent of the inhibition zone (measured in millimeters) around it were recorded, and the results were interpreted according to Clinical Laboratory Standards Institute guidelines (CLSI, 2018).

3.8. PCR to Test for The Presence of different resistant genes

All resistant *E. coli* isolates were further investigated by PCR. The detailed Procedure That Was Followed Is Given Below:

3.8.1. 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.

3.8.2. DNA Extraction from the Isolates

For the extraction of DNA from the recovered isolates, a boiling method was used. 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 deionized water. The tubes were then vortexed to make a homogenous cell suspension. A ventilation hole was made on the lid of each tube. Then the tubes were boiled at 99°C for 15 minutes in a 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. 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 tube and preserved at -20°C until used.

3.8.3. PCR Reactions

All the molecular investigation of the isolates for *tet, sul,* ESBL, *β*-lactamases genes were conducted with PCR machine name DLAB Scientific, USA in Department of Physiology Biochemistry and Pharmacology, CVASU. The primer sequences used for the PCR are shown in Table 3.5.

Table 3.5: Primers used for PCR detection of tetracycline genes.

Antibiotic Resistance	**Target gene**	**Primer Name**	**Primer sequence (5′-3′)**	**Annealing temp**	**Amplicon size (bp)**	**References**
Tetracyclines	*tetA*	*tetA*-F	CGCCTTTCCTTTGGGTTCTCTATATC	55°C	182	(Koo and Woo, 2011)
		tetA-R	CAGCCCACCGAGCACAGG			
	*tet*B	*tet*B-F	GCCAGTCTTGCCAACGTTAT	55°C	975	
		*tet*B-R	ATAACACCGGTTGCATTGGT			

	*tet*C	*tet*C-F	TTCAACCCAGTCAGCTCCTT	55°C	560	
		*tet*C-R	GGGAGGCAGACAAGGTATAGG			
	*tet*D	*tet*D-F	GAGCGTACCGCCTGGTTC	55°C	780	
		*tet*D-R	TCTGATCAGCAGACAGATTGC			
Sulphonamides	**Sulfamethoxazole-trimethoprim**	*Sul-1-F*	CGGCGTGGGCTACCTGAACG	55°C	779	(Lanz *et al.*, 2003)
		Sul-1- R	GCCGATCGCGTGAAGTTCCG			
		Sul-2-F	CCTGTTTCGTCCGACACAGA	55°C	721	
		Sul-2-R	GAAGCGCAGCCGCAATTCAT			
***β*-lactamases**	bla_{TEM}	bla_{TEM} -*F*	ATAAAATTCTTGAAGACGAAA	54°C	1080	(Baker *et al.*, 2018)
		bla_{TEM} -*R*	GACAGTTACCAATGCTTAATC			
	bla_{SHV}	bla_{SHV} -F	GCTTTCCCATGATGAGCACC	60°C	854	(Hasman *et al.*, 2005)
		bla_{SHV} -R	AGGCGGGTGACGTTGTCGC			
	PAmpC	*PAmpC*-F	GTGAATACAGAGCCAGACGC		343	(Hasman *et al.*, 2005)
		PAmpC-R	GTTGTTTCCGGGTGATGC			
	bla_{OXA}-1	bla_{OXA-1}-R	GTGTGTTTAGAATGGTGATCGCATT	56°C	619	(M. Sharma

		bla$_{OXA-1}$ -R	GTGTGTTTAGAATGGTGATCGCATT			*et al.*, 2013)
	bla$_{OXA}$-2	*bla*$_{OXA-2}$ -F	ACGATAGTTGTGGCAGACGAAC	53°C	602	(Hasman *et al.*, 2005)
		bla$_{OXA-2}$ -R	ATYCTGTTTGGCGTATCRATATTC			
ESBLs	*bla*$_{CTX-M}$	*bla*$_{CTX-M}$-F	ATGTGCAGYACCAGTAARGTKATGGC	57°C	593	(Hasman *et al.*, 2005)
		bla$_{CTX-M}$ -F	TGGGTRAARTARGTSACCAGAAYCAGCGG			
	bla$_{CMY-1}$	*bla*$_{CMY-1}$-F	GTGGTGGATGCCAGCATCC	61°C	915	(Hasman *et al.*, 2005)
		bla$_{CMY-1}$-R	GGTCGAGCCGGTCTTGTTGAA			
	bla$_{CMY-2}$	*bla*$_{CMY-2}$-F	GCACTTAGCCACCTATACGGCAG	61°C	758	(Hasman *et al.*, 2005)
		bla$_{CMY-2}$-R	GCTTTTCAAGAATGCGCCAGG			
	bla$_{ACC-1}$	*bla*$_{ACC-1}$-F	ATYCTGTTTGGCGTATCRATATTC		818	(Hasman *et al.*, 2005)
		bla$_{ACC-1}$-R	AGCCTCAGCAGCCGGTTAC			

Table 3.6: 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%)	Fermentas
4	Electrophoresis buffer 50x TAE	Fermentas
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 3.7.

Table 3.7: Contents of each reaction mixture of PCR assay.

Serial No	Name of the contents	Amount
1	Thermo Scientific DreamTaq 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 3.8.

Table 3.8: 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

3.8.4. 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 summarized as such- 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. Then the agarose mixture was cooled at 50°C in a water bath and one drop of ethidium bromide was added to the mixture. The gel casting tray was assembled by sealing the ends of the gel chamber with tape and placing an appropriate number of combs in the gel tray. 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 was completely drowned. An amount of 5 µl of PCR product for a gene was loaded into a gel hole.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. Finally, the gel was examined by using a UV trans-illuminator for image acquisition and analysis.

3.9 Statistical Analysis

All the data from CS test results were recorded and sorted (according to sample and farm 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 were performed to identify the resistance and sensitivity of the samples. Univariate analysis was performed for different antimicrobials tested in cow milk samples for different farms. Prevalence along with *p*-value of resistant tetracycline were analyzed according to CS test performed in cow milk samples. Different values of prevalence of resistant tetracycline were arranged in tables according to sample, farm type, and different antimicrobials.

CHAPTER-4: RESULTS

4.1 Demographic Information of The Present Study

This research's demographic information is summarized in Table 4.1, which lists variables, sample category, sample size for each category, and the prevalence of *E. coli* has been shown.

Table 4.1: Descriptive demography of the present study.

Variable	Category	Sample Size	Number of positive samples	Prevalence (%) (95% CI)
Sample type	Milk	450	134	29.77
Market Location	F1 - Aribah Dairy	25	5	20% (CI: 6.83% – 40.7%)
	F2 - Bondhu dairy	25	6	24% (CI: 9.36% – 45.13%)
	F3 - Dewanhut	25	8	32% (CI: 14.95% – 53.5%)
	F4 - Foys lake	25	7	28.% (CI: 12.07% – 49.39%)
	F5 - Jorif Dairy	25	4	16% (CI: 4.54% – 36.08%)
	F6 - Josim dairy Farm	25	5	20% (CI: 6.83% – 40.7%)
	F7 - Khorshed dairy	25	3	12% (CI: 2.55% – 31.22%)
	F8 - Mashrifa Food Products	25	5	20% (CI: 6.83% – 40.7%)
	F9 - Molla	25	9	36% (CI: 17.97% – 57.48%)

	F10 - Pahartali 1	25	3	12% (CI: 2.55% – 31.22%)
	F11 - Pahartali 2	25	6	24% (CI: 9.36% – 45.13%)
	F12 - Potenga Agro	25	10	40% (CI: 21.13% – 61.33%)
	F13 - Raza Badsha Dairy	25	8	32% (CI: 14.95% – 53.5%)
	F14 - Saifuddin Dairy	25	10	40% (CI: 21.13% – 61.33%)
	F15 - Smart dairy	25	8	32% (CI: 14.95% – 53.5%)
	F16 - Sodesh dairy	25	8	32% (CI: 14.95% – 53.5%)
	F17 - Wireless 1	25	13	52% (CI: 31.31% – 72.2%)
	F18 - Wireless 2	25	16	64% (CI: 42.52% – 82.03%)

*F= Farm

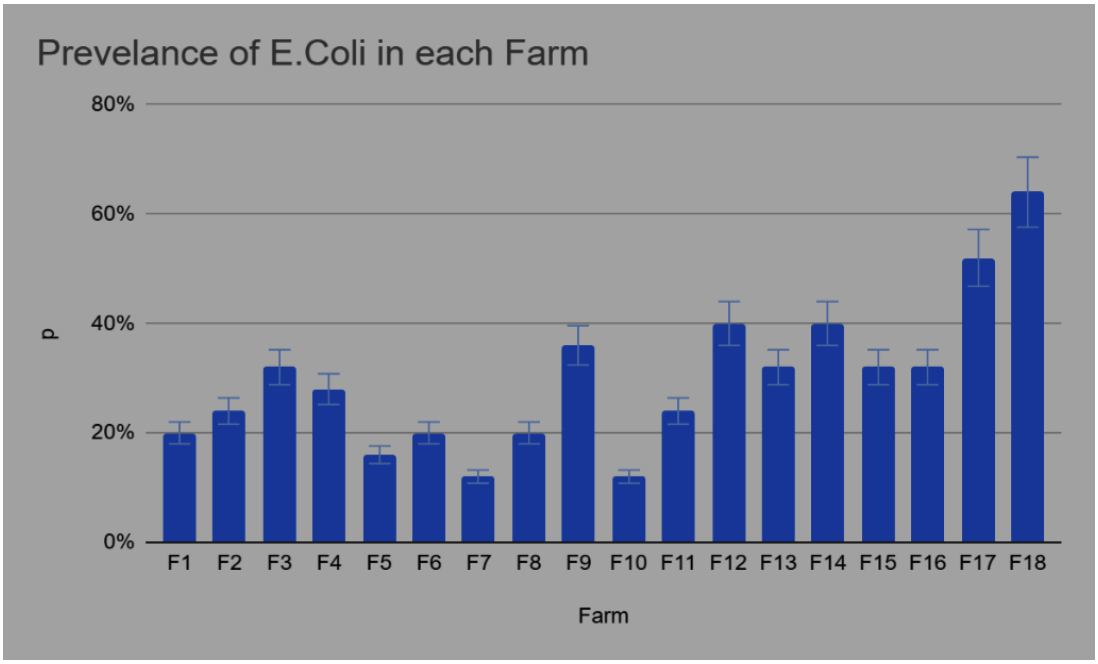


Figure 4.1: *E. coli* Prevalence at farm level.

Figure 4.1 describes the prevalence at farm Level. The maximum positive isolates have been found at farm 18 which is wireless 2 (16 out of 25) with a highest prevalence value of 64.00% (95% CI: 42.52% – 82.03%). Whereas least study prevalence of 12% (95% CI: 2.55% – 31.22%) was observed from positive isolates (n= 3) of both Khorshed dairy Pahartali 1. Besides, the second highest results of positive isolates (13 out of 25) were found in wireless 1 with a prevalence value of 52% (95 % CI: 31.31% – 72.2%). In total, 29.78% (95 % CI: 25.59% – 34.24%) was noticed in the total milk sample of 450.

4.2. *E. coli* Isolates from Milk Samples

A total of 450 milk samples from eighteen prominent farms of the Chattogram city area were investigated in this study. Among those, 134 (29.77%; 95% CI: 25.59% – 34.24%) isolates were phenotypically confirmed as *E. coli* based on the characteristic growth on a petri dish with MacConkey agar medium EMB agar medium in Figure 4.2 to Figure 4.4, respectively. Also, Figure 4.5, represents the PCR assay of molecular confirmation of the culture positive isolates.

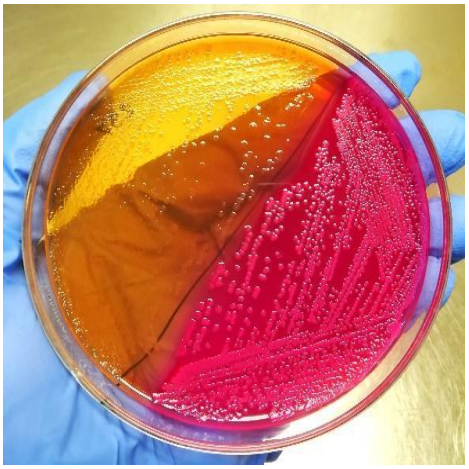


Figure 4.2: Growth of *E. coli* on MacConkey agar plate (large pink color colony).

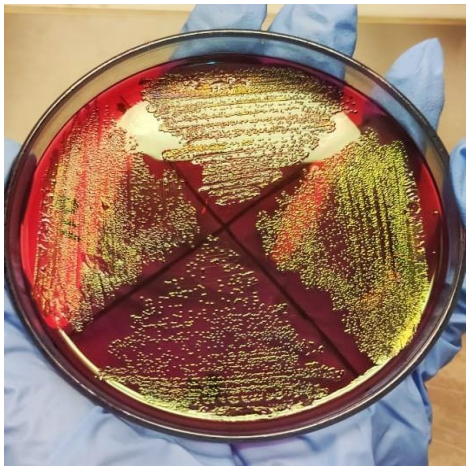


Figure 4.3: *E. coli* on EMB agar plate (green metallic sheen).

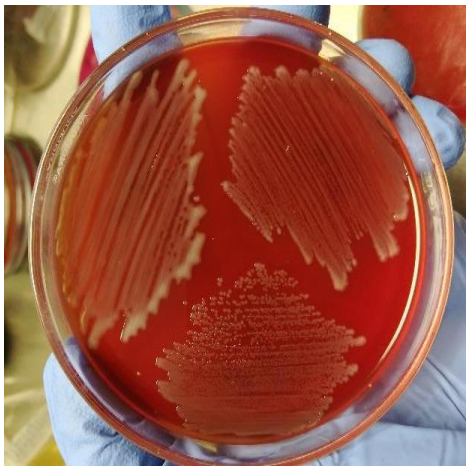


Figure 4.4: *E. coli* on Blood agar plate (greyish white moist colonies)

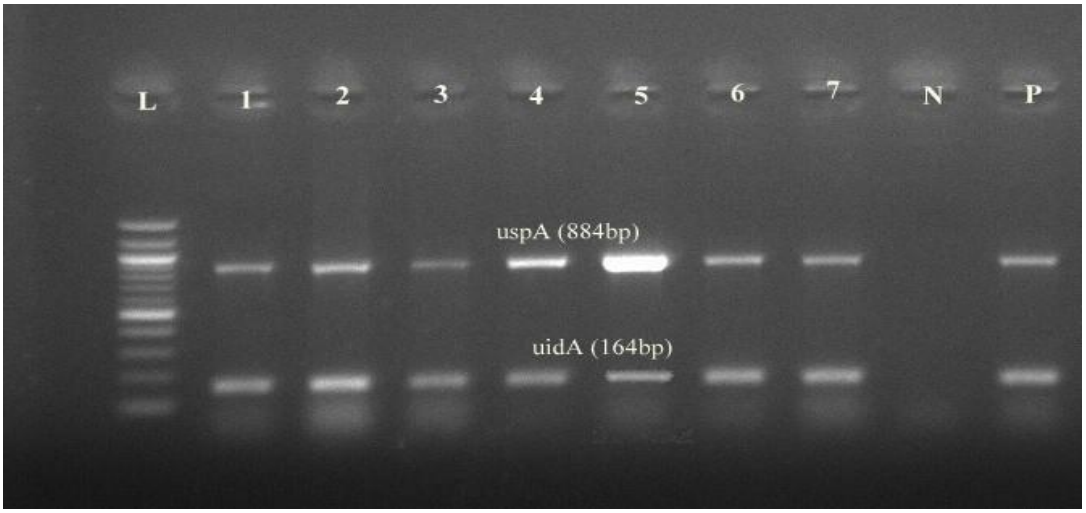


Figure 4.5: Molecular confirmation isolates using *E. coli* specific molecular markers (uidA and flanking region of uspA)

Lane L: DNA ladder; Lane P: Positive control; Lane N: Negative control

4.3 Antimicrobial Resistance Pattern of *E. coli* Isolates of Different Sources

An isolate of *E. coli* showing sensitivity to some antibiotics are shown in Figure 4.6 and resistance to some antibiotics are shown in Figure 4.7. 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=134) in sulfamethoxazole trimethoprim (67.16%), tetracycline (51.49%), ampicillin (69.40%) antimicrobials were detected. The tested *E. coli* isolates were found to be susceptible to some antibiotics, with susceptibility rates for Colistin sulfate (100%), Norfloxacin (63.43%). The susceptibility patterns of the isolates are shown in Table 4.2

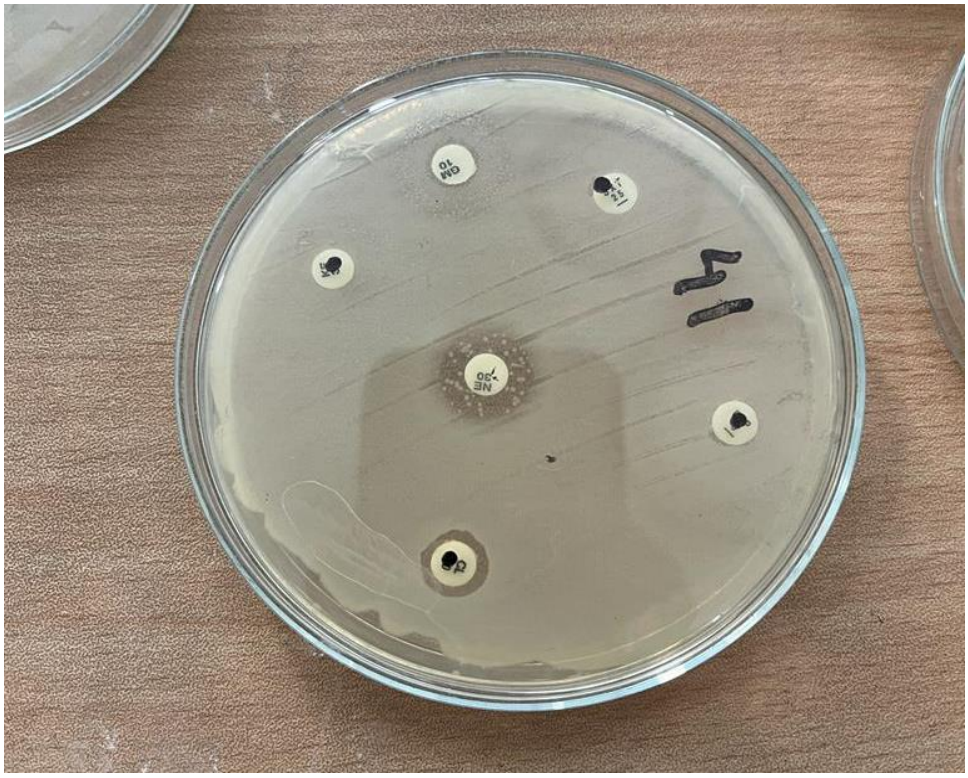


Figure 4.6: Antibiotic sensitivity of isolated *E. coli* on Mueller Hinton Agar

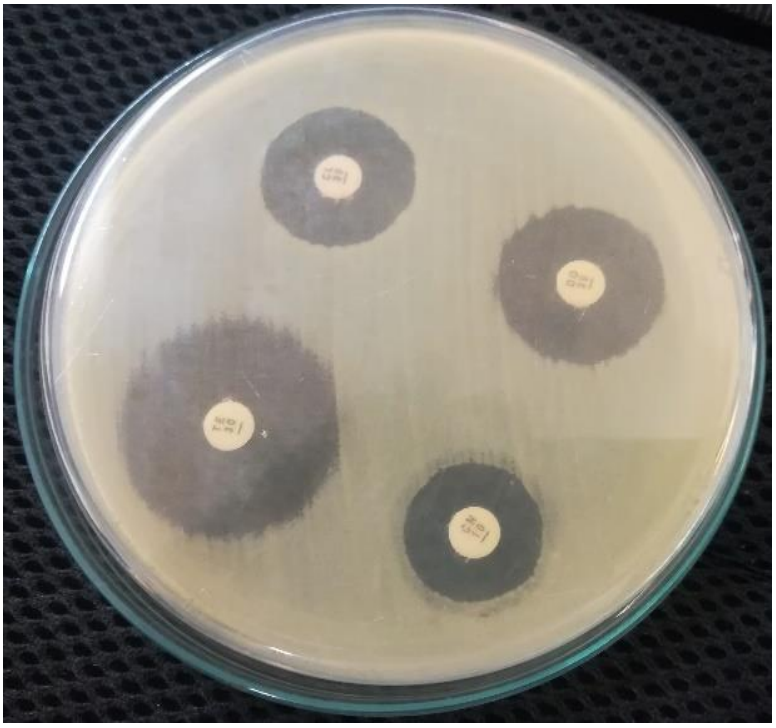


Figure 4.7: Antibiotic resistance of isolated *E. coli* on Mueller Hinton Agar

Table 4.2: Antimicrobial susceptibility profiles of *E. coli* isolates in the present study.

Antimicrobial Agent Group	**Anti microbial Agent**	**Susceptible (S)**		**Intermediate (I)**		**Resistance (R)**	
		No of Isolates	**%**	**No of Isolates**	**%**	**No of Isolates**	**%**
β-lactam antibiotics	(AMP, 10µg)	36	26.86% (CI: 19.58% – 35.2%)	5	4.00% (CI: 1.22% - 8.49%)	93	69.00% (CI: 60.86% – 77.07%)
1st gen cephalosporins	(KF, 30µg)	40	29.85% (CI: 22.26%-38.36%)	1	1.00% (CI: 0.02%-4.09%)	93	69.00% (CI: 60.86% – 77.07%)
	(CL, 30µg)	41	30.59% (CI: 22.93%-39.14%)	0	0.00% (CI: 0%-2.72%)	93	69.00% (CI: 60.86% – 77.07%)
2nd gen cephalosporins	(FOX, 30 µg)	34	25.37% (CI: 18.26%-33.61%)	44	33.00% (CI: 24.97%-41.47%)	56	42.00% (CI: 33.33% – 50.62%)
	(AUG, 30µg)	36	26.86% (CI: 19.58% – 35.2%)	5	4% (CI: 1.22% – 8.49%)	93	69% (CI: 60.86% – 77.07%)
3rd gen cephalosporins	(CTX, 30 µg)	53	39.55% (CI: 31.22%- 48.36%)	49	37.00% (CI: 28.42%- 45.32%)	32	24.00% (CI: 16.94% – 32.01%)
	(CAZ, 30 µg)	53	39.55% (CI: 31.22%- 48.36%)	49	37.00% (CI: 28.42%-45.32%)	32	24.00% (CI: 16.94% – 32.01%)
Amphenicols	(FFC, 30µg)	49	36.56% (CI: 28.42% – 45.32%)	10	7% (CI: 3.64% – 13.3%)	75	56% (CI: 47.14% – 64.53%)
Tetracyclines	(TE, 30µg)	40	29.85% (CI: 22.26% – 38.36%)	25	19% (CI: 12.45% – 26.3%)	69	51% (CI: 42.71% – 60.21%)
	(DO, 30µg)	52	38.80% (CI: 30.52% – 47.6%)	13	10% (CI: 5.27% – 16.02%)	69	51% (CI: 42.71% – 60.21%)

Aminoglycosides	(GM, 10 µg)	5	3.73% (CI: 1.22% – 8.49%)	45	34% (CI: 25.66% – 42.25%)	44	33% (CI: 24.97% – 41.47%)
	(NE, 30µg)	25	18.65% (CI: 12.45% – 26.3%)	40	30% (CI: 22.26% – 38.36%)	69	51% (CI: 42.71% – 60.21%)
Fluoroquinolones	(CIP, 5µg)	53	39.55% (CI: 31.22% – 48.36%)	49	37% (CI: 28.42% – 45.32%)	32	24% (CI: 16.94% – 32.01%)
	(LEV, 5µg)	80	59.70% (CI: 50.89% – 68.08%)	22	16% (CI: 10.58% – 23.8%)	32	24% (CI: 16.94% – 32.01%)
Quinolones	(ENF, 5µg)	34	25.37% (CI: 18.26% – 33.61%)	44	33% (CI: 24.97% – 41.47%)	56	42% (CI: 33.33% – 50.62%)
	(NOR, 10µg)	85	63.43% (CI: 54.68% – 71.58%)	20	15% (CI: 9.36% – 22.11%)	29	22% (CI: 10.58% – 23.8%)
Polymyxins	(CT,10µg)	134	100% (CI: 97.28% – 100%)	0	0% (CI: 0% – 2.72%)	0	0% (CI: 0% – 2.72%)
Sulfonamides	(SXT, 25µg)	41	30.59% (CI: 22.93% – 39.14%)	2	1% (CI: 0.18% – 5.29%)	90	67% (CI: 58.53% – 75.03%)

Table 4.2 represents, highest number of *E. coli* isolates were resistant to Ampicillin, Cephalothin, Cephalexin, Amoxicillin+Clavulinic Acid (93, 69% (CI: 60.86% – 77.07%)) following Sulfamethoxazole trimethoprim (90, 67% (CI: 58.53% – 75.03%) and Florfenicol (75, 56% (CI: 47.14% – 64.53%) followed by Neomycin (69, 51% (CI: 42.71% – 60.21%). Over against, Lowest number of *E. coli* isolates were resistant to Colistin sulfate (0, 0% (CI: 0% – 2.72%) following Norfloxacin (29, 22% (CI: 10.58% – 23.8%).

4.4 Multi-Drug Resistance of *E. coli* Isolates

Table 4.3 and Table 4.4 narrate MDR pattern of antimicrobial classes (≥ 3 groups of antimicrobials). In table 4.4, an aggregated 134 MDR patterns were displayed where the multiple antibiotic resistance phenotypes and multiple antibiotic resistance index of *E. coli* isolates with associated factors were revealed.

Table 4.3: Multidrug resistance of antimicrobial classes.

No. of multidrug resistance antimicrobial classes	Number of observed patterns n (%)	95% CI
3	4 (2.99%)	0.82% – 7.47%
4	8 (5.97%)	2.61% – 11.42%
5	12 (8.96%)	4.71% – 15.12%
6	16 (11.94%)	6.98% – 18.67%
7	32 (23.88%)	16.94% – 32.01%
8	16 (11.94%)	6.98% – 18.67%
9	10 (7.46%)	3.64% – 13.3%
10	12 (8.96%)	4.71% – 15.12%
11	9 (6.72%)	3.12% – 12.37%
12	9 (6.72%)	3.12% – 12.37%
13	5 (3.73%)	1.22% – 8.49%
14	1 (0.75%)	0.02% – 4.09%
Total number of MDR Patterns observed	134 (100%)	97.22%-100%

The discrete Multiple Antibiotic Resistance Phenotypes (MARPs) and the Multiple Antibiotic Resistance Index (MARIs) displayed by *E. coli* isolates are arrayed in table 4.3. The generated multiple antibiotic resistance indices ranged from 0.15 to 1.00, amongst all the highest value noticed in one MDR *E. coli* isolates from milk which displayed resistance against all the antibiotics assayed (MAR index is calculated as the ratio between the number of antibiotics that an isolate is resistant to and the total number of antibiotics the organism is exposed to). Isolates having a MAR index ≥ 0.2 originate from a high-risk source of contamination where several antibiotics are used desperately. In this study, 97.76%, **(**N=134) of *E. coli* isolates having MAR index of 0.2 and above is worrisome.

Table 4.4: The multiple antibiotic resistance phenotypes and multiple antibiotic resistance index of *E. coli* isolates with associated factors.

No. of resistant Antibiotics	Multiple antibiotic resistance phenotypes (MARPs)	No. of MARPs observed, n(%) 95% CI	MAR index (MARIs)
3	CL, KF, AUG	2	0.16
	DO, CL, AUG	1	
	SXT, CL, KF	1	
4	AMP, DO, CL, AMP	1	0.21
	DO, CL, ENF, NOR	1	
	DO, CL, TE, AUG	2	
	SXT, CL, TE, AUG	1	
	SXT, DO, CL, TE	3	
5	AMP, CL, AMP, KF, AUG	5	0.26
	AMP, CL, AMP, TE, KF	1	
	AMP, DO, CL, AMP, TE	1	
	AMP, SXT, CL, AMP, KF	1	
	AMP, SXT, DO, AMP, KF	1	
	SXT, DO, CL, TE, AUG	3	
6	AMP, CL, AMP, TE, KF, AUG	2	0.32

	AMP, DO, CL, AMP, TE, KF	1	
	AMP, SXT, CL, AMP, ENF, NOR	1	
	AMP, SXT, CL, AMP, KF, AUG	1	
	AMP, SXT, DO, CL, AMP, KF	3	
	AMP, SXT, DO, CL, AMP, TE	1	
	GM, AMP, CL, NE, AMP, KF	1	
	GM, AMP, NE, AMP, KF, AUG	2	
	GM, FFC, CL, NE, TE, AUG	1	
	GM, FFC, SXT, CL, NE, TE	2	
	SXT, CL, ENF, TE, NOR, AUG	1	
7	AMP, DO, CL, AMP, ENF, NOR, KF	1	
	AMP, DO, CL, AMP, ENF, TE, NOR	1	
	AMP, DO, CL, AMP, TE, KF, AUG	1	
	AMP, SXT, CL, AMP, TE, KF, AUG	2	
	AMP, SXT, DO, CL, AMP, ENF, KF	1	
	AMP, SXT, DO, CL, AMP, ENF, NOR	1	
	AMP, SXT, DO, CL, AMP, KF, AUG	8	
	AMP, SXT, DO, CL, AMP, TE, KF	1	
	GM, AMP, NE, AMP, TE, KF, AUG	4	0.37
	GM, AMP, SXT, CL, NE, AMP, ENF	1	
	GM, AMP, SXT, NE, AMP, KF, AUG	4	
	GM, AMP, SXT, NE, AMP, TE, KF	1	
	GM, DO, CL, NE, ENF, TE, AUG	1	
	GM, FFC, AMP, SXT, NE, AMP, KF	1	
	GM, FFC, DO, CL, NE, TE, AUG	1	
	GM, FFC, SXT, CL, NE, TE, AUG	1	
	GM, SXT, CL, NE, ENF, TE, AUG	1	
	SXT, DO, CL, ENF, TE, NOR, AUG	1	
8	AMP, SXT, DO, CL, AMP, ENF, NOR, KF	1	
	AMP, SXT, DO, CL, AMP, TE, KF, AUG	4	0.42
	GM, AMP, DO, CL, NE, AMP, ENF, TE	1	
	GM, AMP, DO, CL, NE, AMP, KF, AUG	2	

	GM, AMP, SXT, CL, NE, AMP, TE, KF	2	
	GM, AMP, SXT, DO, CL, NE, AMP, ENF	1	
	GM, AMP, SXT, NE, AMP, TE, KF, AUG	1	
	GM, CL, NE, ENF, TE, NOR, KF, AUG	2	
	GM, FFC, AMP, SXT, NE, AMP, KF, AUG	2	
9	AMP, DO, CL, AMP, ENF, TE, NOR, KF, AUG	1	0.47
	GM, AMP, CIP, NE, LEV, AMP, ENF, NOR, KF	1	
	GM, AMP, SXT, CL, NE, AMP, ENF, TE, KF	1	
	GM, AMP, SXT, DO, CL, NE, AMP, KF, AUG	1	
	GM, FFC, AMP, SXT, DO, NE, AMP, KF, AUG	2	
	GM, FFC, AMP, SXT, NE, AMP, TE, KF, AUG	1	
	GM, SXT, CL, NE, ENF, TE, NOR, KF, AUG	2	
	GM, SXT, DO, CL, NE, ENF, TE, NOR, KF	1	
10	GM, AMP, CIP, NE, LEV, AMP, ENF, NOR, KF, AUG	1	0.53
	GM, AMP, CIP, SXT, NE, LEV, AMP, ENF, NOR, KF	3	
	GM, CIP, DO, CL, NE, LEV, ENF, TE, NOR, AUG	1	
	GM, CIP, SXT, CL, NE, LEV, ENF, TE, NOR, AUG	2	
	GM, FFC, AMP, DO, CL, NE, AMP, ENF, NOR, KF	1	
	GM, FFC, AMP, SXT, DO, CL, NE, AMP, TE, AUG	1	
	GM, FFC, AMP, SXT, DO, NE, AMP, TE, KF, AUG	1	

	GM, SXT, DO, CL, NE, ENF, TE, NOR, KF, AUG	2	
11	GM, AMP, CIP, SXT, NE, LEV, AMP, ENF, NOR, KF, AUG	2	
	GM, CIP, SXT, DO, CL, NE, LEV, ENF, TE, NOR, AUG	2	
	GM, FFC, AMP, CIP, NE, LEV, AMP, ENF, NOR, KF, AUG	2	
	GM, FFC, AMP, CIP, SXT, NE, LEV, AMP, ENF, NOR, KF	1	
	GM, FFC, CIP, DO, CL, NE, LEV, ENF, TE, NOR, AUG	1	
	GM, FFC, CIP, SXT, CL, NE, LEV, ENF, TE, NOR, AUG	1	
12	GM, AMP, CIP, SXT, DO, NE, LEV, AMP, ENF, NOR, KF, AUG	2	
	GM, FFC, AMP, CIP, SXT, DO, NE, LEV, AMP, ENF, NOR, KF	1	
	GM, FFC, AMP, CIP, SXT, NE, LEV, AMP, ENF, NOR, KF, AUG	2	0.63
	GM, FFC, CIP, SXT, DO, CL, NE, LEV, ENF, TE, NOR, AUG	3	
	GM, FFC, CIP, SXT, DO, NE, LEV, ENF, TE, NOR, KF, AUG	1	
13	GM, FFC, AMP, CIP, DO, NE, LEV, AMP, ENF, TE, NOR, KF, AUG	1	
	GM, FFC, AMP, CIP, SXT, DO, NE, LEV, AMP, ENF, NOR, KF, AUG	2	0.68
	GM, FFC, AMP, CIP, SXT, NE, LEV, AMP, ENF, TE, NOR, KF, AUG	2	
14	GM, FFC, AMP, CIP, SXT, DO, CL, NE, LEV, AMP, ENF, TE, NOR, AUG	1	0.74

4.5 Detection of AMR Genes in *E. coli* Isolates from Milk Samples

All of the phenotypically resistant isolates (n=134) were tested to detect the presence of antimicrobial resistant genes i.e., bla_{TEM}, bla_{SHV}, bla_{CTX}, bla_{OXA-1}, bla_{OXA-2}, bla_{CMY-1}, bla_{CMY-2}, blaACC-1, *PAmpC*, *tetA, tetB, tetC, tetD*, *Sul-1 and Sul-2* via uniplex, duplex and multiplex PCR assays (Figure 4.8 to Figure 4.13).

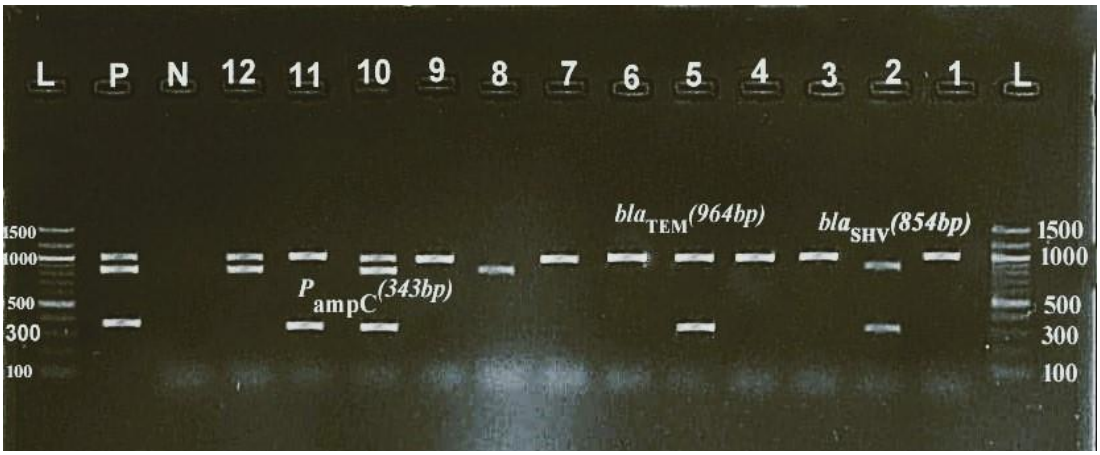


Figure 4.8: Presence of bla_{TEM} (964bp), bla_{SHV} (854bp) PAmpC (343bp) genes with MDR *E. coli* isolates in multiplex PCR assay of agarose gel electrophoresis. Lane L: DNA ladder; Lane P: Positive control; Lane N: Negative control.

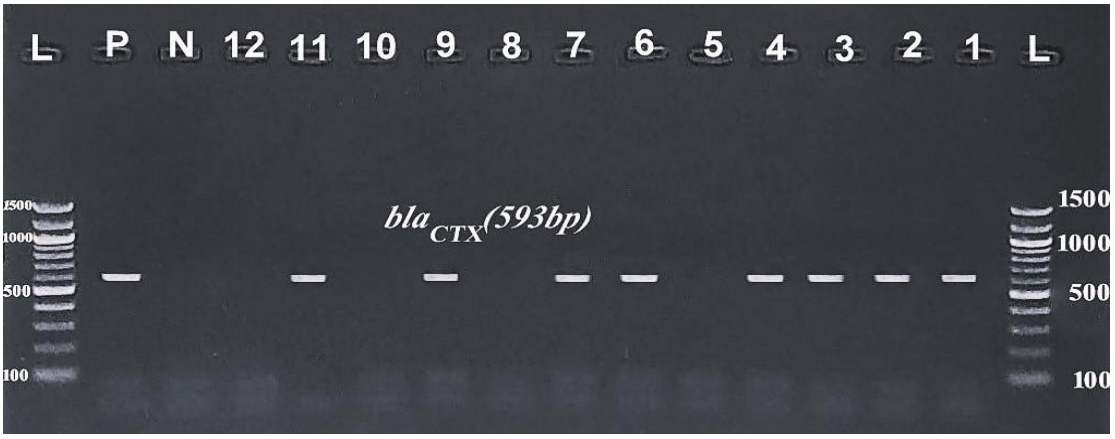


Figure 4.9: Presence of bla_{CTX} (721bp) with MDR *E. coli* isolates in uniplex PCR assay of agarose gel electrophoresis.
Lane L: DNA ladder; Lane P: Positive control; Lane N: Negative control.

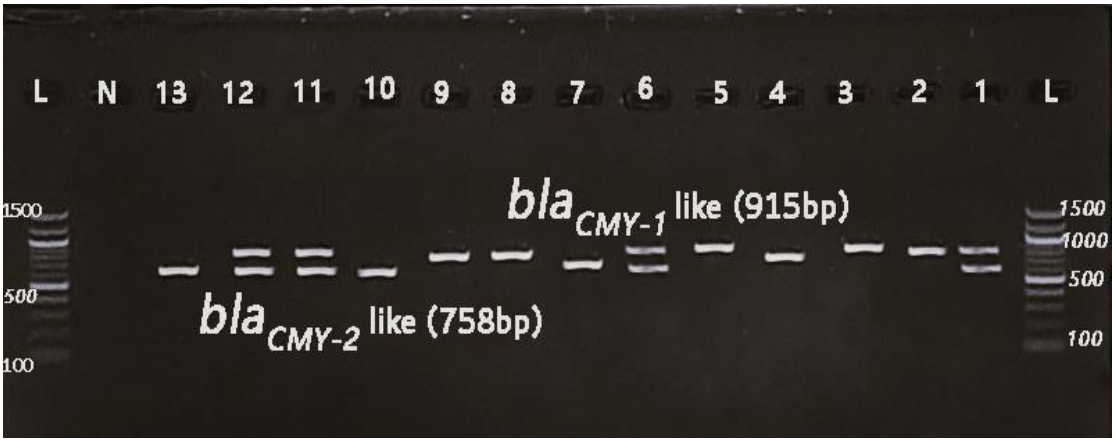


Figure 4.10: Presence of *bla*$_{CMY\text{-}1\ LIKE}$ (915bp), *bla*$_{CMY\text{-}2\ LIKE}$ (758bp) *bla*$_{OXA\text{-}1\ LIKE}$ (820bp), *bla*$_{OXA\text{-}2\ LIKE,}$ (602bp) genes with MDR *E.coli* isolates in Duplex PCR Assay of agarose gel electrophoresis.

Lane L: DNA ladder; Lane P: Positive control; Lane N: Negative control.

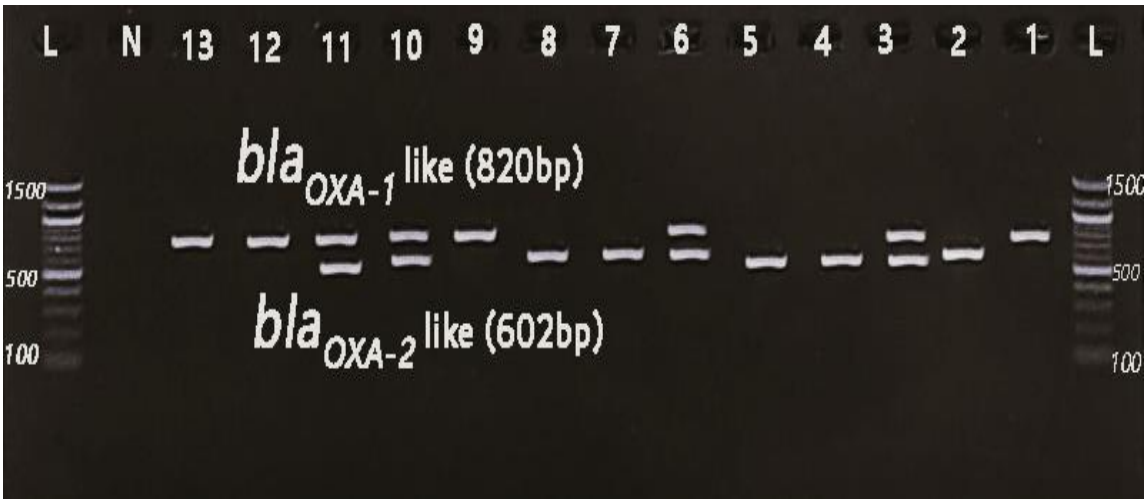


Figure 4.11: Presence of *bla*$_{OXA\text{-}1\ LIKE}$ (820bp), *bla*$_{OXA\text{-}2\ LIKE,}$ (602bp) genes with MDR *E. coli* isolates in Duplex PCR Assay of agarose gel electrophoresis.

Lane L: DNA ladder; Lane P: Positive control; Lane N: Negative control.

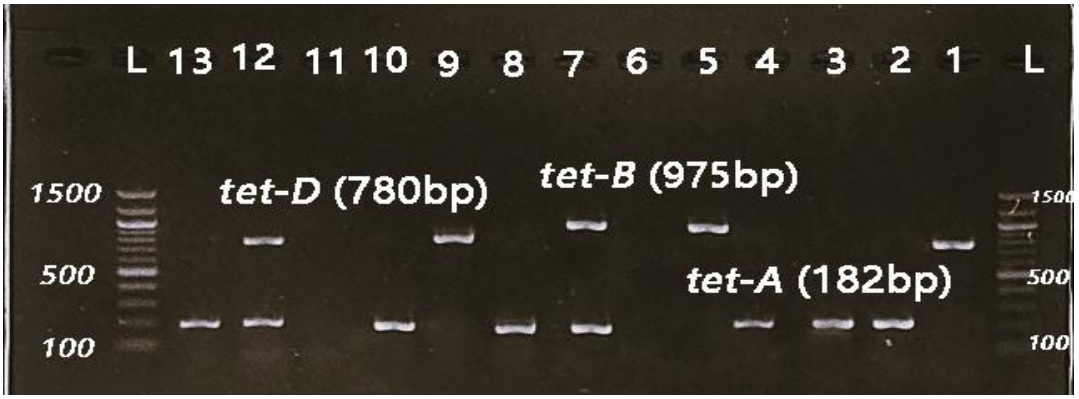


Figure 4.12: Presence of *tetA* (182bp), *tetB* (975bp), and *tetD* (780bp) with MDR *E. coli* isolates in multiplex PCR Assay of agarose gel electrophoresis.
Lane L: DNA ladder; Lane P: Positive control; Lane N: Negative control.

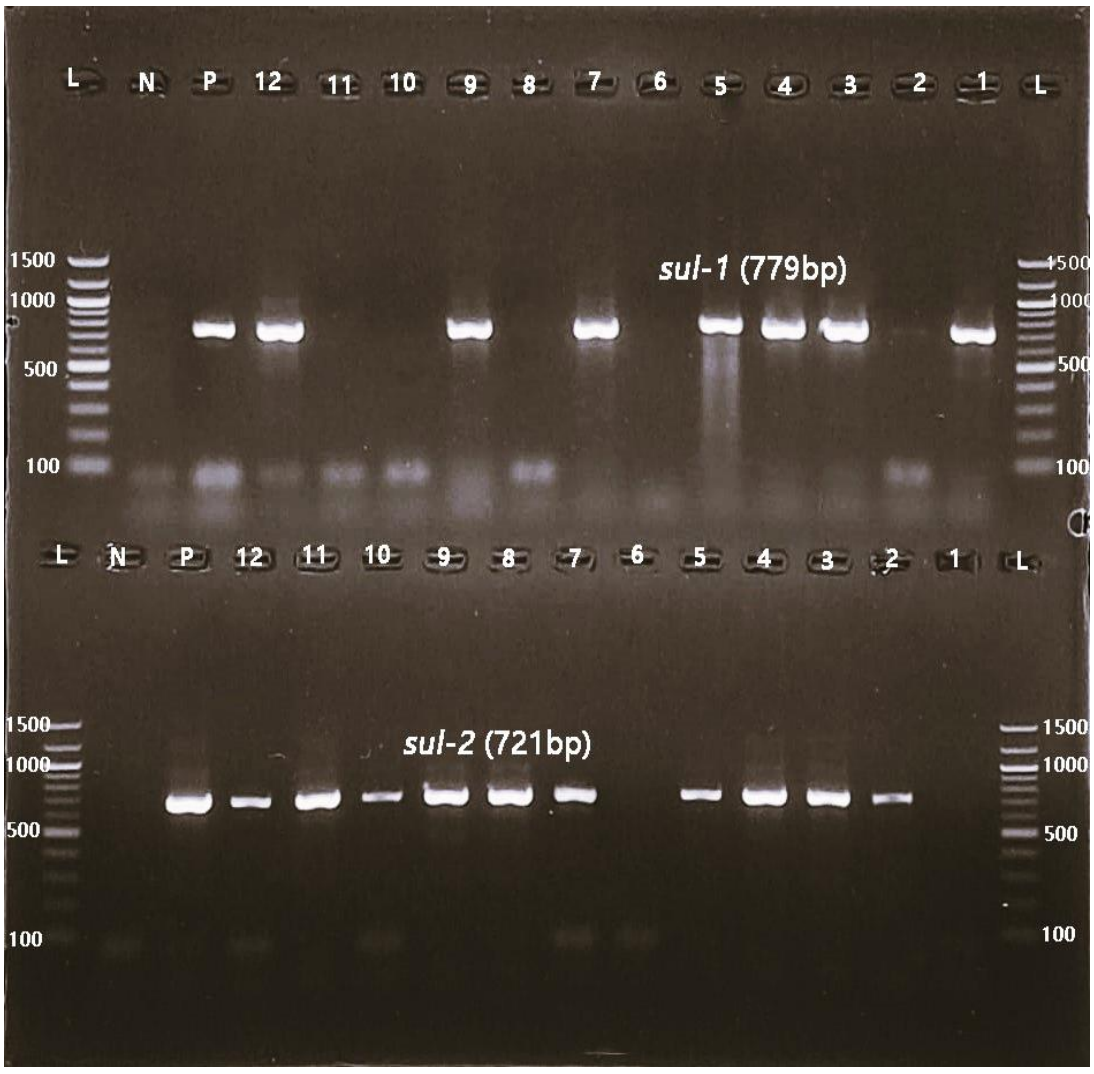


Figure 4.13: Presence of *sul-1* (779bp) and *sul-2* (721bp) with MDR *E. coli* isolates in simplex PCR Assay of agarose gel electrophoresis.
Lane L: DNA ladder; Lane P: Positive control; Lane N: Negative control.

Table 4.5: Prevalence of antimicrobial resistance gene detected in resistant isolates.

Resistant Gene Determinants	**No. of Resistant Gene Present**	**Number of isolates**	**Resistant gene, % (n), 95%CI**
tetA	21	69	30.43% (CI: 19.92% – 42.69%)
tetB	4	69	5.8% (CI: 1.6% – 14.18%)
tetC	2	69	2.9% (CI: 0.35% – 10.08%)
tetD	2	69	2.9% (CI: 0.35% – 10.08%)
Sul-1	11	90	12.22% (CI: 6.26% – 20.82%)
Sul-2	23	90	25.56% (CI: 16.94% – 35.84%)
bla_{TEM}	33	93	35.48% (CI: 25.83% – 46.09%)
bla_{SHV}	8	93	8.6% (CI: 3.79% – 16.25%)
PAmpC	17	93	18.28% (CI: 11.02% – 27.65%)
bla_{OXA-1}	15	93	16.13% (CI: 9.32% – 25.2%)
bla_{OXA-2}	18	93	19.35% (CI: 11.89% – 28.85%)
bla_{CTX-M}	31	93	33.33% (CI: 23.89% – 43.87%)
bla_{CMY-1}	3	93	3.23% (CI: 0.67% – 9.14%)
bla_{CMY-2}	9	93	9.68% (CI: 4.52% – 17.58%)
bla_{ACC-1}	2	93	2.15% (CI: 0.26% – 7.55%)

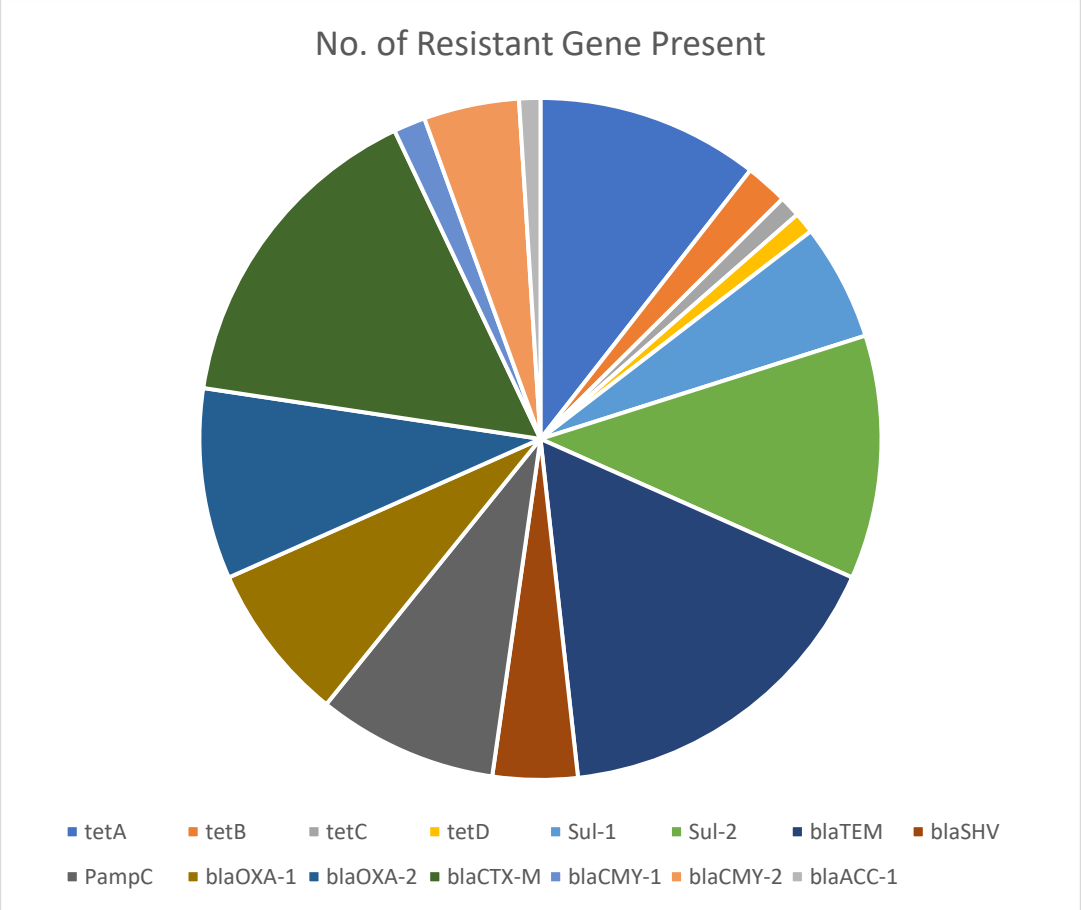


Figure 4.14: Pie chart representing the detection of all the resistance gene determinants of MDR *E. coli* isolates is portrayed by the color codes.

Among all 134 resistant isolates, the prevalence of ampicillin resistant genes of *β*-lactam resistance determinants (n=93) highest share was contributed by *blaTEM* gene (n=33, 635.48% (CI: 25.83% – 46.09%) Among the ESBLs resistance determinants bla_{OXA-2} (n=18, 19.35% (CI: 11.89% – 28.85%) genes was the utmost and no bla_{ACC-1} gene was very low in number. (n=2, 2.15% (CI: 0.26% – 7.55%). Moreover, in non *β*-lactam resistance determinants *tetA*, (n=21, 30.43% (CI: 19.92% – 42.69%) was detected most following *Sul-2* (n=23, 25.56% (CI: 16.94% – 35.84%) while no *tet C tetD* gene both were detected a few (n=2, 2.9% (CI: 0.35% – 10.08%)

CHAPTER-5: DISCUSSION

AMR is a global hazard to human and animal health, resulting in the progressive loss of antimicrobials' efficacy in response to an increasing exposure to resistant bacteria. The current study revealed a high prevalence of *E. coli* in the milk of Bangladeshi farms collected from various dairies, with the isolates frequently displaying resistance to multiple antimicrobial classes. In this study, the overall prevalence of *E. coli* in farm milk is 29.77%, which is less than the 42.0% prevalence reported by (Vahedi *et al.*, 2013). Another study reported the same 42 percent prevalence of *E. coli* in milk (Megersa *et al.*, 2019).

In this study, 19 antibiotics were tested during cultural sensitivity test and 15 resistant genes were tested against those antibiotics. The culture sensitivity testing of the isolates revealed that *E. coli* resistance to sulfamethoxazole–trimethoprim was the most prevalent, followed by tetracycline and ampicillin. Most of the *E. coli* isolated from milk were colistin-sensitive. Isolates exhibited varied resistance to the combination of sulfamethoxazole and trimethoprim, as determined by culture and sensitivity tests. Out of 34 samples, 5 (15%) were resistant to sulfonamide and 3% were resistant to trimethoprim, according to a recent study (Agatha *et al.*, 2023). Another study discovered that the prevalence of *E. coli* in calves from a dairy farm was 37.5% (Astorga *et al.*, 2019). In contrast, this study revealed that out of 90 sulfamethoxazole-trimethoprim isolates, 11 were resistant for the sul-1 gene and 23 were resistant for the sul-2 gene.

A recent study found, 13.4% of *E. coli* isolated from milk were resistant to tetracycline (Liu *et al.*, 2021), whereas in this study, 51% of *E. coli* were resistant to tetracycline. Among 69 tetracycline-resistant isolates, *tetA* was the most frequently detected gene, accounting for 21 (86.5%), while *tetB* was detected in only 4 (8.1%) isolates. Only two resistant genes for *tetC* and *tetD* were discovered.

According to, Skočková *et al.*, 2012, only one (1) (0.4%) of the 229 collected fecal samples was positive for ESBL-producing *E. coli* isolated from a lactating bovine, while five (6.5%) of the 77 collected farm environment samples were positive. According to, Kamaruzzaman *et al.*, 2020, five (62.50%) of eight milk samples contained ESBL *E. coli* with the gene combination bla_{TEM} + $bla_{\text{CTX-M}}$. In this study, 33

isolates produced the *bla*$_{TEM}$ gene, 8 isolates produced the *bla*$_{SHV}$ gene, 17 isolates produced the *PAmpC* gene, 31 isolates produced the *bla*$_{\text{CTX-M}}$ gene, 15 isolates produced the *bla*$_{OXA}$-1 gene, 18 isolates produced the *bla*$_{OXA}$-2 gene, 3 isolates produced the *bla*$_{CMY-1}$ gene, 9 isolates produced the *bla*$_{CMY-2}$ gene, and 2 isolates produced the *bla*$_{ACC-1}$ gene.

The occurrence of AMR in insects, rodents, and pets. Insects (e.g., houseflies, cockroaches), rodents (rats, mice), and pets (dogs, cats) act as reservoirs of AMR for first line and last-resort antimicrobial agents. AMR proliferates in insects, rodents, and pets, and their skin and gut systems. Subsequently, insects, rodents, and pets act as vectors that disseminate AMR to humans via direct contact, human food contamination, and horizontal gene transfer. Thus, insects, rodents, and pets might act as sentinels or bioindicators of AMR (Gwenzi *et al.*, 2021). From antimicrobial residues, horizontally transferred resistant microbes and genes have emerged in these farms (Sattar *et al.*, 2014; Khan *et al.*, 2019).

Antimicrobials are utilized in human and animal medicine. Lack of knowledge regarding antimicrobial use and indiscriminate use of antimicrobials (Hassan, 2021) could be the origins of AMR bacteria in milk. Drug sellers and medical representatives encourage the use of antimicrobials without prescriptions from a responsible person (Okeke *et al.*, 1999; Kalam *et al.*, 2021). As accurate dose and dosage cannot be maintained based on age and body weight, these antimicrobials are exposed to human pathogens. When commensals are exposed to a low dose of an antimicrobial, they develop resistance (Barbosa and Levy, 2000). If these are administered at a higher dose, then the residues remain in the tissues for a prolonged period, and resistance may also develop (Levy *et al.*, 1989).

To mitigate the situation, the World Health Organization (WHO) categorized antimicrobials into three groups: Access group, monitor group, and Reserve group (Gandra and Kotwani, 2019). Access group antimicrobials are available to prescribe to patients by physicians. If this group fails due to the presence of resistant genes in organisms, it is recommended that the patient be monitored in a separate group (Hsia *et al.*, 2019). Reserve categories of antimicrobials are for future use if others become resistant. To mitigate AMR issues before they become widespread crises, scientific knowledge, and scientific evidence are required. AMR is among the deadliest threats

to human and animal health. To alleviate the AMR health threat before it manifests in large-scale medical emergencies, it is necessary to identify risks and appropriate mitigation strategies based on scientific evidence and knowledge.

CHAPTER-6: CONCLUSION

Much of the interest in AMR of bacteria that reside in milk and other food animals are sparked by a concern for human health that is threatened by zoonotic pathogens and by selection for AMR determinants. One-third of the total samples showed a positive presence of *E. coli* with a high prevalence of resistance against ESBL and cephalosporins 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, milk is one of the protein food chains of humans, AMR will be developed in humans and increase the infection burden in the community through milk. Prudent use of antimicrobials will be necessary to preserve these valuable drugs for use in dairy farms. Awareness buildup and training programs are highly recommended for poultry handlers to maintain strict proper use of antimicrobials in dairy farms.

CHAPTER-7: RECOMMENDATIONS AND FUTURE PERSPECTIVES

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. The spread of antibiotic resistance to different environmental niches and the development of superbugs has further complicated effective control strategies. International, national, and local approaches have been advised for the control and prevention of antimicrobial resistance. To minimize the devastating consequences for human health, a few recommendations are suggested in this study. These apply to public health authorities, commercial poultry farm owners, veterinarians, and consumers.

- Establishment of a national committee to monitor the impact of antibiotic resistance and provide intersectoral coordination is required.
- Establishing and implementing national standard treatment guidelines, having an essential drug list, and enhancing coverage of immunization are other essential strategies desired at the national level.
- Use of alcohol-based hand rubs or washing hands has proven efficacy in the prevention of infection. This factor can restrict the spread of infection and thereby AMR.
- 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 hazards due to antimicrobial residues and resistance. Laboratories should be established to control the veterinary drug residues in foods of animal origin.
- Commercial poultry farm owners should not use antimicrobials without suggestions from registered veterinarians. They should follow the withdrawal periods before harvesting.
- Veterinarians should prescribe the actual dose of antimicrobials and suggest withdrawal periods.
- Increased collaboration between governments, nongovernmental organizations, professional groups, and international agencies.

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CHAPTER-9: BIOGRAPHY

Zarin Tasnim Mim passed Secondary School Certificate (SSC) examination from Bangladesh Mahila Samity Girls High School in 2012 and then Higher Secondary School Certificate (HSC) examination from Chattogram Government Women College, Chattogram in 2014. She B.Sc. in Food Science and Technology from Chattogram Veterinary and Animal Sciences University, Chattogram, Bangladesh in 2020 with CGPA of 3.59 out of 4.00. She has been studying Masters of Public Health at the One Health Institute of Chattogram Veterinary and Animal Sciences University, Chattogram, Bangladesh. She has a great interest in working in Epidemiology and the One Health field.