CHAPTER-I

INTRODUCTION

Antimicrobial Resistance (AMR) is irrefutably one of the cardinal health threats discerned by mankind. It results in reduced efficacy of antibiotics; making treatment intricate, time-consuming, expensive, or sometimes even unworkable. 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, J., 2016). AMR in microbes is elucidated as their apathy to standard doses of clinically pertinent antimicrobial drugs (Ganguly *et al*., 2011). Usually, the event is called multidrug-resistant (MDR) when the microbes are resistant to at least 3 types of antibiotics (FY Ramírez *et al*., 2018). Thus the consequence is addressed as 'Silent Pandemic' as it leads to the global development of multidrug-resistant strains (Sharma *et al*., 2018).

Since the discovery of the first antibiotic, resistance to antimicrobials is regarded as a natural process whereby microbes evolve in such a way as to withstand the action of drugs (Annunziato *et al*., 2019. But, AMR has progressively become a horrific setback in recent times because of the imbalance between the overuse of antimicrobials and the lack of new antibiotic innovation to challenge these new superbugs (Jackson *et al*., 2018). This results in us facing a growing enemy with a largely curtailed armory.

The problem of AMR is similarly salient and prevalent in animals; although highlighted to a lower extent. In the dairy industry, the use of antimicrobials is highly demanded, on the whole, treating diseases affecting the dairy cattle, such as mastitis. In addition, antimicrobials have also been used in disease prevention, promoting growth in dairy cattle production, as well (Herago *et al*., 2017). Woefully, the use of antibiotics in subtherapeutic doses for disease prevention and growth enhancement is quite excessive than for the treatment of disease (Maron *et al*., 2013). This is one of the intermediaries that prompts AMR in livestock (Bouki *et al*., 2013).

The demand for animal source food is increasing at an expeditious rate. With this growing demand, the utility of veterinary drugs in the global market augmented from 8.65 billion dollars in 1992 to 20 billion dollars in 2010 and is projected to touch the 42.9 billion dollar mark by2018 (Hao*etal*., 2014). Meanwhile, antimicrobial usage (AMU) in food animal production will rise over 67% by 2030 to fulfill this worldwide

demand for animal proteins (Van Boeckel *et al*., 2015). Consequently, this ample dependence over AMU in animals generates a selective compulsion under which bacteria can either develop resistance-mediating mutations or acquire resistance genes (Moudgil *et al*., 2018). The foremost concern of AMR in animals is the chance of the transfer of resistant bacterial strains from animals to humans (Loo *et al*., 2019).

In dairy cattle production, drug-resistant strains of animal origin can spread to humans either through the food supply chain (i.e., Meat and Dairy products); direct animal contact; or through environmental routes (Lhermie *et al*., 2017). Furthermore, consumers may be exposed to resistant strains and genes through consumption of contaminated food products, i.e., meat, unpasteurized milk, and milk products, or through environmental spread including animal sewage and runoff water from agricultural sites (Loo *et al*., 2019; Ayukekbong *et al*., 2017).

Milk and its derivatives can harbor a variety of microorganisms and can be important sources of foodborne pathogens. The presence of foodborne pathogens in milk is due to direct contact with contaminated sources in the dairy farm environment and to excretion from the udder of an infected animal. The harsh consequence is that the bacterial contamination of milk such as *E. coli* and *S. aureus* due to unhygienic conditions and poor udder health can cause infections in humans (Batabyal *et al*., 2018). Belongings of antimicrobial resistance genes by commensal bacteria present in milk make the issue moreserious.

The rise of antibiotic resistant *S. aureus* is a higher risk and great concern to global health possessing a severe challenge to both veterinary and public health professions and dairy cattle producers as they have a negative impact on therapy (Brouillette *et al*., 2005). Against a variety of antimicrobial agents microbes have already shown resistances which include aminoglycosides, macrolides, glycopeptides, fluoroquinolones, and tetracyclines (Rahi *et al*., 2020). Many antibiotic resistance genes play a role in *S. aureus* resistance and these include macrolide resistance encoded by the *erm* (*ermA, ermB, msrA, msrB* and *mefA*) gene, *tet* genes for gentamicin, and tetracycline (*tetK* and *tetM*), *blaZ for* penicillins, *aacA-D for* aminoglycosides, *gyrA* and *grlA* for fluoroquinolones, and *linA* for lincosamides resistance (Hasanpour *et al*., 2017; Momtaz et al., 2013; Qae *et al*., 2015).

Expression of resistance to penicillinase-stable antibiotics to penicillinase commonly referred to as “methicillin resistance” or “oxacillin resistance”, and expressed by the mecA gene in *S. aureus* bacteria (Stapleton et al., 2002; Zapun et al., 2008). The

emergence of methicillin-resistant *S. aureus* (MRSA) infection in dairy farms is of great concern for animal and public hygiene. Livestock products contaminated with MRSA, including bovine milk, may become causal agents for human MRSA infection (Lee *et al*., 2003). MRSA can be found both in mastitis cases in dairy cows and in healthy cows (Pu *et al*., 2014). But it indicates an alarming situation when many reports reveal that MRSA can be transmitted between farmers working in dairy pens and people working in the dairy industry and colonization of dairy cows acts as a risk factor for veterinarians, breeders, employees of the dairy processing industry(Antoci *et al*., 2013; Schnitt *et al*., 2020). *S. aureus* causes diseases in humans and animals which include toxic syndrome and staphylococcal food poisoning (SFP) (Ateba *et al*., 2010) whereas, Livestock associated Methicillin-resistant *S. aureus* (LA-MRSA) infections in humans, originating from milk and milk products, can include mild to severe skin and soft tissue infections in humans (Layer *et al*.,2012).

E. coli is an opportunistic pathogen, which can cause massive health disorders in humans and animals. *E. coli* is also among the major causes of bovine mastitis, and resistant isolates from dairy farms have been increasingly reported over the past years. These resistance determinants have frequently been associated with multidrug resistance. As it is known that the *E. coli* genome is able to evolve constantly, the exchange of genetic material may lead to further transmission of resistance genes (Bajaj *et al*., 2016). Multidrug-resistant (MDR) *E. coli* spread is a public health concern representing a zoonotic risk for farm workers and contact people (Walther *et al*., 2017). In *E. coli*, horizontal transfer of genes coding for extended- spectrum β-lactamases (ESBL) is a common mechanism of dissemination of resistance to a broad range of β-lactams. Furthermore, ESBL-producing *E. coli* strains have a higher inclination to express multidrug resistance than non-ESBL-producing strains, therefore complicating infection management (Karkaba *et al.*, 2017). Multidrug resistance has been widely observed in *E. coli* isolates more disturbingly with coresistance to commonly used antibiotics such as aminoglycosides, fluoroquinolones, tetracycline phenicols, and potential tedsulfonamides, and a wide variety of resistant genes such as *bla*CTX-M, *blaTEM*, *mcr* have been isolated (Geser *et al*., 2012; Xu*et al*., 2015; Ali *et al*., 2016).

Antimicrobial resistance (AMR) is an emerging public health issue globally, but the problem may even be more serious in developing countries with a high burden of infectious diseases, lack of awareness and uncontrolled access to medicines may lead to higher consumption and inappropriate use, and subsequently result in higher levels

of resistance (Kakkar *et al*., 2017). Unhygienic animal husbandry practices, poor sanitation, widespread misuse, and irrational antibiotics and prophylactics used in the livestock industry in Bangladesh are increasing the threat of extensive AMR development (Sobur *et al*., 2019). Also due to poor surveillance in health care facilities, developing countries such as Bangladesh are at risk of AMR issues (Khan *et al.,* 2020). Under these circumstances the present study was directed to achieve to followingobjectives:

Objectives:

1. To identify and characterize the AMR genes in *S. aureus* and *E. coli* isolated from bovine milk within the studyarea.
2. To investigate the prevalence of AMR genes in the strainsisolated.
3. To assess the risks associated with the circulation of *S. aureus* and *E.coli* acquiring resistance to antimicrobials.

CHAPTER- II

REVIEW OF LITERATURE

2.1 Antibiotics and their developmental history

At the beginning of 20th century diseases particularly infectious one caused higher morbidity and mortality irrespective to the location. Average life expectancy at birth was lower in comparison to this time (Blaskovich *et al*., 2018). Different diseases such as smallpox, malaria, diphtheria, tuberculosis etc. were rampant. Sir Alexander flaming (1881-1955) in 1928 discovered the very first antibiotic start a revolution in the medicine. Penicillin G was purified in 1942 by Ernst Chain and Howard Florey (Durand *et al*., 2019). The breakthrough invention saved millions of life and the change of the average life expectancy showed the clear indication of it. Staring from 1950 to 1970s a number of antibiotics were discovered and marked as the golden era of antibiotic discovery. Although unfortunately no single new class of antibiotics has been discovered since then but some modification of existed antibiotics have done to maintain the antibiotics sensitive (Nicolaou *et al*., 2018). After the invention of antibiotics the treatment of diseases was changed worldwide. The most important success was showed by the developed countries. For example in the US the major cause of death is non-communicable diseases such as cancer, stroke, and cardiac diseases (Banin *et al*., 2017). But the invention of antibiotics may not work up to the mark in every corner of the world due to poverty, inadequate public health, poor hygienic measure, improper sewage system and sanitation facility, lower vaccination coverage etc.

Table 2.1- Antibiotics according to their mode of action (Wanda., 2018)

Mechanism of Action	Antimicrobial Groups
Inhibit Cell Wall Synthesis	β-Lactams Carbapenems CephalosporinsMonoba ctams Penicillins

	Glycopeptides
Depolarize Cell Membrane	Lipopeptides
Inhibit Protein Synthesis	Bind to 30S Ribosomal Subunit- Aminoglycosides Tetracyclines Bind to 50S Ribosomal Subunit- Chloramphenicol Lincosamides Macrolides Oxazolidinones Streptogramins
Inhibit Nucleic Acid Synthesis	Quinolones Fluoroquinolones
Inhibit Metabolic Pathways	Sulfonamides Trimethoprim

Table 2.2. Bacterial targets of antibiotics in clinical use (Chellat *et al*., 2016)

Target	Type of Antibiotic
Cell-wall biosynthesis	Penicillins, cephalosporins, carbapenems, monobactams, cycloserine, fosfomycin, glycopeptides, lipoglycopeptides
Protein synthesis	Aminoglycosides, tetracyclines (*Subunit 30S*) Oxazolidinones, macrolides, thiopeptides, chloramphenicol, fusidic acid, clindamycin (*Subunit50S*)
DNA replication andrepair	Rifamycins, ansamycins, actinomycins, tiacumycins (*RNA polymerase*) Fluoroquinolones, aminocoumarins (*DNA gyrase*)
Folic acid metabolism	Sulfonamides, trimethoprim
Membrane structure	Lipopeptides, polymyxins

2.2 Resistance to antibiotics

The most important factor to resist the achievement of antibiotic era is termed as antibiotic resistance (Kapoor *et al*., 2017). It can be defined as the ability of the bacteria to resist against the antibiotic effectiveness which was initially sensitive to. Resistance can be grown through genetic mutations or section pressure etc. (Banin *et al*., 2017). Some resistant pathogen such as 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 done 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 is generally shared within specific

bacteria and independent of antibiotic exposure and not related to horizontal gene transfer. Reduced permeability of the outer membrane is the most common way of intrinsic resistance mechanism. Besides that they can also affect the cellular efflux pumps (Xie *et al*., 2018).

Table 2.3. Organism and resistance pattern

Organism	Intrinsic resistance
Bacteroides (anaerobes)	Aminoglycosides, many β-lactams, quinolones
All gram positives	Aztreonam
Enterococci	Aminoglycosides, cephalosporins, lincosamides
Listeria monocytogenes	Cephalosporins
All gram negatives	Glycopeptides, lipopeptides
Escherichia coli	Macrolides
Klebsiella spp.	Ampicillin
Serratia marcescens	Macrolides
Pseudomonas aeruginosa	Sulfonamides, ampicillin, 1st and 2nd generation cephalosporins, chloramphenicol, tetracycline
Stenotrophomonas maltophilia	Aminoglycosides, β-lactams, carbapenems, quinolones
Acinetobacter spp.	Ampicillin, glycopeptides

2.2.2 Acquired resistance

Way of getting resistant genetic materials by any of the three methods transformation, transposition and conjugation is called acquired resistance. Horizontal gene transfer is the key of this three mechanism along with mutation in the own chromosomal DNA. This resistance may be temporary or permanent. Most common route is termed as plasmid mediated and bacteriophage mediated transmission is rare. There are many ways of becoming resistant such as stressors (chemical or physical) or genetic mutations (substitution, deletion etc.). Average bacterial mutation occurred 1 for every 10^6 to 10^9 cell divisions and this mutation generally deleterious to the cells. Mutation aiding antimicrobial resistance occurred in specific genes such as drug targets or drug

transporters, regulator genes or antibiotic modifying enzymes gene etc. (Aanen*et al*., 2019).

2.2.3 Mechanism of Antibioticresistance

Mechanism of antimicrobial resistance mechanisms fall into four main groups:

- Limiting uptake of adrug
- Modifying a drugtarget
- Inactivating adrug
- Active drugefflux.

Bacteria may manifest resistance to antibacterial drugs through a variety of mechanisms. Some species of bacteria are innately resistant to 1 class of antimicrobial agents. In such cases, all strains of that bacterial species are likewise resistant to all the members of those antibacterial classes. Of greater concern are cases of acquired resistance, where initially susceptible populations of bacteria become resistant to an antibacterial agent and proliferate and spread under the selective pressure of use of that agent.

Several mechanisms of antimicrobial resistance are readily spread to a variety of bacterial genera. First, the organism may acquire genes encoding enzymes, such as lactamases, that destroy the antibacterial agent before it can have an effect. Second, bacteria may acquire efflux pumps that extrude the antibacterial agent from the cell before it can reach its target site and exert its effect. Third, bacteria may acquire several genes for a metabolic pathway which ultimately produces altered bacterial cell walls that no longer contain the binding site of the antimicrobial agent, or bacteria may acquire mutations that limit access of antimicrobial agents to the intracellular target site via down regulation of porin genes (McManus M.C., 1997).

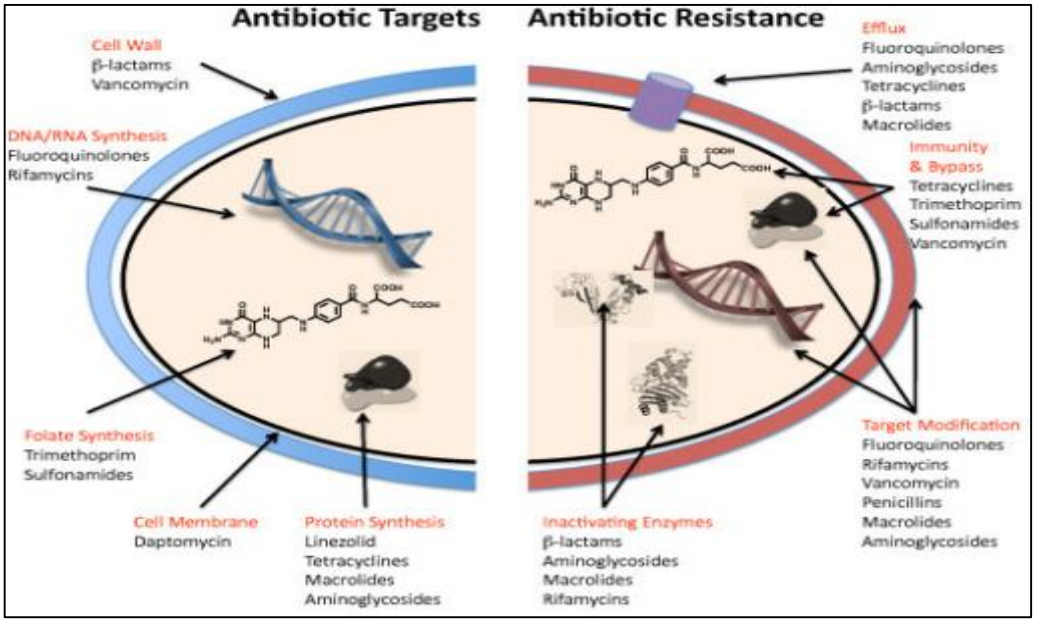

Figure 2.1: Antibiotics, effects and mechanisms of resistance

Thus, normally susceptible populations of bacteria may become resistant to antimicrobial agents through mutation and selection, or by acquiring from other bacteria the genetic information that encodes resistance. The last event may occur through 1 of several genetic mechanisms, including transformation, conjugation, or transduction. Through genetic exchange mechanisms, many bacteria have become resistant to multiple classes of antibacterial agents, and these bacteria with multidrug resistance (defined as resistance to 3 antibacterial drug classes) have become a cause for serious concern, particularly in hospitals and other healthcare institutions where they tend to occur most commonly. As noted above, susceptible bacteria can acquire resistance to an antimicrobial agent via new mutations. Such spontaneous mutations may cause resistance by (1) altering the target protein to which the antibacterial agent binds by modifying or eliminating the binding site (e.g., change in penicillin-binding protein 2b in pneumococci, which results in penicillin resistance), (2) upregulating the production of enzymes that inactivate the antimicrobial agent (e.g., erythromycin ribosomal methylase in **staphylococci**), (3) down regulating or altering an outer membrane protein channel that the drug requires for cell entry (e.g., OmpF in **E coli**), or(4)upregulatingpumpsthatexpelthedrugfromthecell(effluxoffluoroquinolones in S aureus). In all of these cases, strains of bacteria carrying resistance-conferring mutations are selected by antimicrobial use, which kills the susceptible strains but allows the newly resistant strains to survive and grow. Acquired resistance that

develops due to chromosomal mutation and selection is termed vertical evolution. Bacteria also develop resistance through the acquisition of new genetic material from other resistant organisms. This is termed horizontal evolution, and may occur between strains of the same species or between different bacterial species or genera. Mechanisms of genetic exchange include conjugation, transduction, and transformation. For each of these processes, transposons may facilitate the transfer and incorporation of the acquired resistance genes into the host's genome or into plasmids. During conjugation, a gram-negative bacterium transfers plasmid-containing resistance genes to an adjacent bacterium, often via an elongated proteinaceous structure termed a pilus, which joins the 2 organisms. Conjugation among gram-positive bacteria is usually initiated by production of sex pheromones by the mating pair, which facilitate the clumping of donor and recipient organisms, allowing the exchange of DNA. During transduction, resistance genes are transferred from 1 bacterium to another via bacteriophage (bacterial viruses). This is now thought to be a relatively rare event. Finally, transformation, i.e., the process whereby bacteria acquire and incorporate DNA segments from other bacteria that have released their DNA complement into the environment after cell lysis, can move resistance genes into previously susceptible strains. Mutation and selection, together with the mechanisms of genetic exchange, enable many bacterial species to adapt quickly to the introduction of antibacterial agents into their environment. Although a single mutation in a key bacterial gene may only slightly reduce the susceptibility of the host bacteria to that antibacterial agent, it may be just enough to allow its initial survival until it acquires additional mutations or additional genetic information resulting in fullfledged resistance to the antibacterial agent.18 However, in rare cases, a single mutation may be sufficient to confer high-level, clinically significant resistance upon an organism (e.g., high-level rifampin resistance in S aureus or high-level fluoroquinolone resistance in Campylobacter jejuni). The following case studies, which involve 3 different bacterial species, serve to illustrate several of the ways in which bacteria develop resistance to antibacterial drugs and how different resistance mechanisms may interact to increase the level or spectrum ofresistanceofanorganism.Resistancepatternsassociatedwiththesebacterial pathogens are discussed in greater detail in other articles in this supplement (Tenover*et al*., 2006).

2.3 Some Leading Resistant Pathogens

Many types of microorganisms cause infection in humans and animals, so disease prevention and treatment strategies must be adapted to reflect infection risk factors and available treatment options. Over the past decades, most pathogenic species have developed resistance to one or more antimicrobials. Some of the species in which resistance is of greatest public health concern are listedbelow.

- *Escherichicacoli*
- *Staphylococcus aureus*, including community-associated MRSA (Methicillin-Resistant *S. aureus*)
- *Mybocaterium tuberculosis* (cause oftuberculosis)
- *Neisseria gonorrhoeae* (cause ofgonorrhoea)
- *Salmonella*Typhi
- *Streptococcuspneumonia*
- Methicillin-resistant *Staphylococcus aureus* (MRSA) has become a focus of public health concern due to its increased virulence and resistance to an increasingly broad spectrum of antibiotics (Aliberti *et al*., 2012; Johnson *et al*., 2011).

2.4 AMR bacteria in livestock (Bacteria of concern)

Contemporaneous concerns about AMR spread from livestock to humans are highlighted on resistant bacteria known to enter the food chain, or otherwise transmit to humans, which have zoonotic potential and/or which harbor mobile genetic elements (principally plasmids) encoding AMR. Zoonotic bacteria of concern are: *Salmonella enterica*, *Campylobacter*, and livestock-associated methicillin-resistant ***S.aureus*** (LA-MRSA). Organisms with less zoonotic potential, but which pose a warning to susceptible individuals and may carry mobile resistance determinants, include**: *E. coli***, other members of the Enterobacteriaceae (particularly *Klebsiella* spp.) and *Enterococcus* spp.LA-MRSA can colonize humans and may go on to cause antimicrobial-resistant disease in some circumstances (Becker *et al*., 2017). Incidence of LA-MRSA human colonization appears variable, according to prevailinglocal livestock activity and MRSA colonization (Feingold et al., 2012). The danger of severe disease appears to be lower than with MRSA of typical human subtypes. In Germany, a single-hospital, admission screening at one hospital identified 25% of MRSA isolates to be of livestock-associated type, yet LA-MRSA amounted to only 7% of MRSA isolates from clinical sample submissions (Köck et al., 2011). The identification since

2005 of widespread LA-MRSA colonization of pig herds in many countries with intensive pig production (Lassok & Tenhagen, 2013), plus in retail pork and other meats (Fox et al., 2017), has led to a focus on characterization and control of this AMR organism, plus substantial interest in the media and among a general public familiar with MRSA from health care and community sources.

2.5 An overview of *E. coli* and *S. aureus*; most frequently noticed bacteria in livestock products

2.5.1 Structure of *E. coli* and *S.aureus*

E.coli is an onsporulating bacterium and cells are typically rod-shaped, and are about 2.0 μm long and 0.25–1.0 μm in diameter, with a cell volume of 0.6–0.7 μm^3. Cell wall is composed of a thin peptidoglycan layer and an outer membrane. Strains that possess flagella are motile. The flagella have a peritrichous arrangement (Scheutz, 2005). Italso attaches and effaces to the microvilli of the intestines via an adhesion molecule known as intimin. Pathogenic E. coli is divided into two major groups according to their infection sites namely extra intestinal pathogenic *E. coli* (ExPEC) and intestinal pathogenic *E. coli* (InPEC) (Leomil *et al*., 2005). ExPEC can cause diseases in urinary tract, meninges etc. but InPEC is subdivided into several categories such as enteropathogenic *E.coli* (EPEC), enterotoxigenic *E.coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and adherent-invasive *E. coli* (AIEC). That infection is occurred for both human and animals (Moriel *et al*., 2012).

On the other hand the cell wall of *S. aureus* consists of a very thick peptidoglycan layer. They are 0.5-1.5 μm in diameter and spherical in shape with the absence of flagella moreover sometimes divide in more than one plane to form grape-like clusters (Braga *et al*., 2004).

Both the bacterial scientific taxonomy is given in **Table 2.4.**

Table 2.4: Scientific taxonomy of *E.coli* and *S. aureus*

Taxonomy	
Escherichia coli	*Staphylococcus aureus*
Domain: Bacteria Phylum: Proteobacteria Class: Gammaproteobacteria Order: Enterobacteriales Family: Enterobacteriaceae Genus: *Escherichia* Species: *Escherichia coli*	Domain: Bacteria Phylum: Firmicutes Class: Bacilli Order: Lactobacillales Family: Staphylococcacea Genus: *Staphylococcus* Species: *Staphylococcus aureus*

2.5.2. Virulence factors

2.5.2.1 Virulence factors of *E. coli* (Raeispour *et al*.,2018)

- Fimbrial adhesions (F2- F6, F17, F18,F41)
- Heat-stable (STa, STb) and heat-labile (LTp/h, LT-IIa, LT-IIb)enterotoxins
- Attaching and effacing (AE) lesion; type 4BFP fimbriae by typical (t) EPEC of humans (dogs,cats)
- Vero toxins (VTx), afimbrial and fimbrialadhesion
- Small fimbrial adhesions (AAF/Hda); toxins (Pet, EAST1, ShET1) transcriptional activator gene (aggR) Adhesions of the A Fimbrial Adhesion (AFA)

Cytotoxic Necrotizing Factors (CNF) 1 or 2 and hemolysis (Hly) fimbrial (Pap/Prs, Sfa/F1C and /orF17) and/or afimbrial adhesions (AFAfamily); siderophores; resistance to complement.

2.5.2.2 Virulence factors of *S. aureus* (Lentz *et al*.,2018)

- Enzymes such as coagulase, hyaluronidase, deoxyribonuclease, lipase, staphylokinase, beta-lactamaseetc.
- Toxins such asTSST-1, and enterotoxin type B,Exfoliativetoxins
- Toxins that act on cell membranes include alpha toxin, beta toxin, delta toxin, and several bicomponenttoxins
- Panton-Valentine leucocidin (PVL) andbacteriophage

2.5.3 Method of detecting *S. aureus*

2.5.3.1 Cultural characteristics of *Staphylococcus aureus* (Murray *et al.*,2013)

1. Staphylococci grow readily on most bacteriologic media under aerobic or microaerophilicconditions.
2. Colonies on solid media are round, smooth, and raised, andglistening.
3. *S. aureus* usually forms gray to deep golden yellowcolonies.
4. **Mannitol Salt Agar:** circular, 2–3 mm in diameter, with a smooth, shiny surface; colonies appear opaque and are often pigmented goldenyellow.
5. **Tryptic Soy Agar**: circular, convex, and entiremargin.
6. **Blood Agar**: beta-hemolysis.
7. **Brain heart infusion agar:** Yellow pigmentedcolonies.

2.5.3.2 Biochemical characteristics of *Staphylococcus aureus* (Rusenova *et al*., 2017)

- Catalasepositive
- Oxidasenegative
- OF test –fermentative
- Coagulase positive: the presence of free and /or boundcoagulase
- Indolenegative
- Gasnegative
- Hydrogen sulfidenegative
- Methyl redpositive

- VP positive
- Nitrate reductionpositive
- Gelatin hydrolysispositive
- Beta hemolysis on Bloodagar
- Citrate positive and Ureasepositive
- Motilitynegative
- PYRnegative

2.5.3.3 Microscopic Feature

Microscopy is useful for pyogenic infections but not blood infections or toxin-mediated infections.A direct smear for Gram staining may be performed as soon as the specimen is collected.The Gram stain showing **typical Gram-positive** cocci that occur singly and in pairs, tetrads, short chains, and irregular **grape-like clusters** can be suspected to be
S. aureus.

2.5.3.4 Characteristics on growth medium (El-Jakee *et al.*,2008)

- The organism is isolated by streaking material from the clinical specimen (or from a blood culture) onto solid media such as **blood agar, tryptic soy agar,** or **heart infusionagar**.
- Specimens likely to be contaminated with other microorganisms can be plated on **mannitol salt agar** containing 7.5% sodium chloride, which allows the halo- tolerant staphylococci togrow.
- The inoculated plates should be incubated at 35°C to 37°C for 24 to 48hours.
- On **Blood agar**, growth occurs abundantly within 18 to 24 hours. Round, raised, opaque, yellow to golden yellow colonies of 1-2mm in diameter are seen with or without beta hemolysis.
- On **Mannitol Salt Agar (MSA)**, a selective media, S. *aureus* being a mannitol fermenting bacteria gives yellow or goldcolonies.
- An 18 h to 24 h culture can be used as the inoculum for additionaltests.

Isolates should be sub cultured at least once on a nonselective medium after initial isolation before being used in a diagnostic test that requires pure culture or

heavyinoculum.

2.5.3.5 Presumptivei dentification

- Large mannitol fermenting colonies on MSA
- Gram-positive cocci inclusters
- Catalase-positiveorganisms
- Coagulase-positiveorganisms

2.5.3.6 Confirmatory tests

Confirmatory tests include biochemical tests, PCR, or mass spectrometry.

2.5.3.7 Identification of toxins (Berube *et al*.,2013)

- This is important for more severe cases like toxic shock syndrome and food poisoning.
- Toxins produced by *S. aureus*, such as enterotoxins A to D and TSST-1 may be identified using agglutinationtests.
- The tests are determined by the clumping of the latex particles by the toxins present in thesamples.
- Commercial latex agglutination tests are available for thispurpose

2.5.3.8 Nucleic acid amplification tests (Kateete *et al*.,2010)

- Commercial nucleic acid amplification tests are available for the direct detection and identification of *S. aureus* in clinicalspecimens.
- Whereas the earlier versions of these tests required manual extraction of bacterial DNA and testing multiple specimens in large batches, integrated processing of specimens (extraction, gene amplification, and target detection) is now performed on highly automated platforms with disposable reagent strips or cartridges.
- They are useful for screening patients for carriage of methicillin-sensitive S. aureus (MSSA) andMRSA.

2.5.4 Different methods for detection of *E.coli*

2.5.4.1 Cultural characteristics of E. coli (Collee *et al*.,1996)

Nutrient Agar (NA)

- They appear large, circular, low convex, grayish,

white, moist, smooth, andopaque.

- They are of 2 forms: Smooth (S) form and Rough (R) form. Smooth forms are emulsifiable insaline.
- Due to repeated subculture, there is smooth to rough variation (S-R variation).

Blood Agar (BA)

- Colonies are big, circular, gray andmoist.
- Beta (β) hemolytic colonies areformed.

MacConkey Agar (MAC)

- Colonies are circular, moist, smooth and of entiremargin.
- Colonies appear flat and pink.
- They are lactose fermentingcolonies.

Mueller Hinton Agar (MHA)

- Colonies are pale strawcolored.

Eosin Methylene Blue (EMB) Agar

- Green Metallic sheen colonies areformed.

m-ENDO Agar

- Colonies are green metallicsheen.
- Metabolise lactose with the production of aldehyde andacid.

Violet Red Bile Agar (VRBA)

- Red colonies (pink to red) areformed.
- Bluish fluorescence around are seen around colonies underUV.

Cysteine Lactose Electrolyte-Deficient (CLED) Agar

- They give lactose positive yellowcolonies.

Characteristics on Liquid Media

- They show homogenous turbid growth within 12-18hours.
- R form agglutinates spontaneously, forming sediment

on the bottom of the test tubes.

- After prolonged incubation (>72 hrs), pellicles are formed on the surface of liquidmedia.
- Heavy deposits are formed which disperses onshaking.

2.5.5 Other methods (Lindstedt et al., 2003; Frydendahl et al., 2002)

- Polymerase Chain Reaction(PCR)
- Biochemicalprofiling
- Bacteriophagetyping
- DNA-fingerprintingmethods
- Pulsed field gelelectrophoresis
- TargetedRFLP
- Typing of virulence factor encodinggenes
- Multilocus variable-number tandem repeatanalysis

2.5.6 Diseases caused by *S. aureus*

2.5.6.1 Human diseases caused by *S.aureus*

- Staphylococcal skin infections Skin infections are the most common form of staphylococcal disease. Superficial infections may be diffuse, with vesicular pustules and crusting (impetigo) or sometimes cellulitis, or focal with nodular abscesses (furuncles and carbuncles) (Kwiatkowski *et al*,2017).
- Staphylococcal bacteremiarelated to intravascular catheters or other foreign bodies. It may also occur without any obvious primary site (Holland *et al*., 2018)
- Staphylococcal neonatal infections: Neonatal infections usually appear within 6 weeks after birth and include skin lesions with or without exfoliation, bacteremia,meningitis, pneumonia. On the other handsecondary pneumonia can be occurred with other virus infected patients with immune suppression. Along with that patients treated with corticosteroid sometimes more prone to those respiratory infections (Cailes *et al.*, 2018).

- *S. aureus* endocarditis is an acute highly febrile illness often accompanied by visceral abscesses, embolic phenomena, pericarditis, subungual petechial, subconjunctival hemorrhage, purpuric lesions, heart murmurs, perivalvular abscess, conduction defects, and heart failure secondary to cardiac valve damage (Liesenborghs *et al.*, 2019).
- Staphylococcal toxic shock syndrome may result from use of vaginal tampons or complicate any type of *S. aureus* infection (eg, postoperative wound infection, infection of a burn, skin infection). Although most cases have been due to methicillin-susceptible *S. aureus* (MSSA), cases due to MRSA are becoming more frequent (Krogman *et al*., 2017).
- Staphylococcal osteomyelitisoccurs more commonly in children, causing chills, fever, and pain over the involved bone. Subsequently, the overlying soft tissue becomes red and swollen. Articular infection may occur; it frequently results in effusion, suggesting septic arthritis rather than osteomyelitis (Kavanagh *et al*., 2018).

2.5.6.2 Diseases caused by *S. aureus* in Cattle

S. aureus, next to *E. coli* and several streptococcal species such as *Streptococcus uberis* and *Streptococcus agalactiae*, is a major cause of mastitis in dairy cows and incurs a significant economic loss to the dairy industry. Mastitis leads to the influx of leukocytes into the udder, and various thresholds for leukocyte numbers have been established for categorizing good milk quality. Taking cow milk as an example, milk with more than 200,000 leukocytes per ml is considered to be infected, and in the European Union when more than 400,000 cells per ml are found, the milk is deemed un-fit for human consumption. Thus, contamination of bulk milk can lead to food poisoning from fermented raw milk products (Martins *et al.*, 2019). Animal microbiota provides a reservoir of antibiotic resistance genes that can be acquired from their eco- logical niches and selected for by the use of antibiotics in agriculture (Sheppard *et al.*, 2018). The ability of some animal-adapted *S. aureus* strains to colonize and infect humans can give rise to the development of new epidemic clones with hitherto uncharacterized virulence capacity (Anjum *et al*., 2019). Moreover, an increased number of bovine-to-human transmissions have been reported in recent years. A closer analysis revealed that at least twoCC97 subclades for human infection had emerged that originated in bovine-

to-human host jumps and had there after spread through the human population (Haag *et al*., 2019). This provided further evidence that animals can provide a reservoir for the development of new *S. aureus* clones that can rapidly spread from animal to human and then through the population. This provided further evidence that animals can provide a reservoir for the development of new *S. aureus* clones that can rapidly spread from animal to human and then through the population ((Turner *et al*., 2019).

2.5.7 Diseases caused by *E. coli*

2.5.7.1 Human diseases caused by *E.coli*

- Enterohemorrhagic: These strains (including serotype O157:H7 and others) produce several cytotoxins, neurotoxins, and enterotoxins, including Shiga toxin (verotoxin), and cause bloody diarrhea (Ahsan *et al*., 2020), hemolytic-uremic syndrome develops in 2 to 7% of cases (Loos *et al*., 2017). Such strains have most often been acquired from undercooked ground beef but may also be acquired from infected people by the fecal-oral route when hygiene is inadequate.
- Enterotoxigenic: These strains can cause watery diarrhea, particularly in infants and travelers (traveler's diarrhea) (Mirhoseini *et al*., 2018).
- Enteroinvasive: These strains can cause inflammatory diarrhea (Farajzadeh *et al.*, 2020).
- Enteropathogenic: These strains can cause watery diarrhea, particularly in infants (Moxley *et al*., 2010).
- Enteroaggregative: Some strains are emerging as potentially important causes of persistent diarrhea in patients with AIDS and in children in tropical areas (Kaur *et al*., 2010).

Urinary tract infection which usually represent ascending infection (i.e., from the perineum via the urethra). *E. coli* may also cause prostatitis and pelvic inflammatory disease (PID) (Forsyth*et al*., 2020).

- 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 *et al*.,2000)

2.5.7.2 Cattle diseases caused by *E.coli*

In cases of diarrhea due to enterotoxigenic *E. coli* (ETEC), extensive fecal soiling of the perineum and dehydration and generalized muscle wasting may be observed on post-mortem (Das *et al*., 2013). The small and large intestine is distended with fluid and gas and the intestinal mucosa may be shiny to the naked eye. The intestinal mucosa usually appears normal on histopathological investigation. Lesions due to verocytotoxin-producing *Escherichia coli* (VTEC) are often severe and are most common in the colon, extending to the small intestine in severe cases. Lesions include edema, ulceration, and erosions in the large intestinal mucosa and subsequently localized and diffused hemorrhages can be observed in the intestinal lumen. There is extensive multifocal bacterial colonization of the surface epithelium by a thin layer of dark-stained coccobacilli, often oriented in a palisade pattern. Electron microscopy can show typical intimate attachment of bacteria to intestinal epithelial cells and effacement of microvilli. Petechial hemorrhages on the epicardium and serosal surfaces and there may be enlargement of the spleen and pulmonary edema and hemorrhage (Fairbrother *et al*., 2006). In mastitis of cows, lesions are often difficult to precisely localize because of the color and heat of the skin, and the subcutaneous fatedema.

2.5.8 Methicillin resistant *S.aureus*

Methicillin resistant Staphylococcus aureus (MRSA) is defined by the presence o f a large mobile genetic element called staphylococcal cassette chromosome, mec (SCCmec). It carries the mecA gene that codes for an alternative penicillin binding protein, PBP2a, with low binding affinity to all P-lactams (Ito *et al*., 1999). MRSA strains were first described in hospital settings, after the introduction of p-lactamase-insensitive penicillins into medical practice, and they continue to be a serious problem in health care due to their ability to acquire multidrug resistance determinants. Although outbreaks of diseases in a hospital may also be caused by methicillin sensitive S. aureus (MSSA) (Kurlenda et a l, 2009), MRSA infections are especially easily spread throughout a hospital and. without implementation o f a special surveillance program with control procedures, a risk of an epidemic in such a hospital is high (Kurlenda et a l.2007).

2.5.8.1 Disc diffusion test for MRSA

Disc diffusion test is employed by incubating S', aureus on Muller Hilton agar (MHA) impregnated with Oxacillin or Methicillin (1 or 5^g) and Cefoxitin (30^ig) discs. MRSA is identified by assessing zone of inhibitions with oxacillin < 14 mm and/or cefoxitin < 21 mm (CLSI, 2007). Cefoxitin disc diffusion test is considered superior to oxacillin disc diffusion test due to its ease of reading and higher sensitivity. Cefoxitin induces mecA gene of MRSA and its results have been found in concordance to PCR (Broekema *et al*., 2009; Rao *et al*., 2011). Thus, Cefoxitin disc duffusion test can be alternative to PCR for the detection of MRSA in resource constraintsettings.

2.5.8.2 Oxacillin M1C test

Gradient plates of MHA containing 2% NaCI with doubling dilutions from 0.25 ng/ml to 256 ng/ml of oxacillin are prepared. *S. aureus* inoculum is prepared by diluting 0.5 9 McFarland equivalent suspension of a strain with sterile normal saline to the concentration of l04CFU/ml. The plates arc spot inoculated and incubated at 35°C for 24 h. An oxacillin MIC of less than or equal to 2 ig/ml is indicative of susceptibility and that of > 2 Mg/ml resistance (CLSI, 2007).

2.5.8.3 Chromogenic Media

These are selective and differential media used for direct detection Of MRSA. This type of media contains specific chromogenic substrate and antibiotics like ccfoxitin. MRSA will grow in the presence of antibiotics producing colored colonies due to hydrolysis of chromogenicsubstances.

2.5.8.4 PCR

Polymerase chain reaction (PCR) is used for detection of mccA gene of S. aureus. This can be done by using mecA gene specific primers (Bhanderi and Jhala, 2 0 1 1). But, use of PCR method is limited only to sophisticated laboratories. (Garcia-Alvaraz *et al*., 2 0 1 1) found isolates resistant to penicillin but negative for mecA gene which has led scientists think about possible mechanism rendering S. aureus resistant to beta lactamse other than presence of mecA gene.

2.5.8.5 Typing of MRSA Strains

The S. aureus population, including MRSA, consists of different clonal lineages, also

called clonal complexes. Clones or strains of MRSA are differentiated using genetic typing tests, such as spa typing. Multi Locus Sequence Typing, Pulsed-Field Gel Electrophoresis, SCCmec typing and other tests (Leonard and Markey, 2008; Catry *et al*., 2010). These techniques are mainly useful for epidemiological M RSA typing systems are important for investigation of outbreaks of infection, aid the clinical treatment of patient, to discriminate between successive and recurrent infections, understanding epidemiology of infections. MRSA strains can be idcntitied/typed using phenotypic and molecular methods.

2.6 Importance of bovine 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 considerable amount of antibody in comparison of the normal lactation secretion. Bovine milk is the main dairy product having major portion of dairy industry. From the milk there are different by products are made and have large impact for the livestock industry. Milk contains both protein and carbohydrate along with other necessary ingredients (O'Connor *et al*., 2018). 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 countries, up to 5% of food borne infections were related to theconsumptionofmilkanddairyproducts (Ahmed*etal*.,2014).Thecasescenario could be worse for developing countries where high rates ofmilk contamination associated with unhygienic milk production and lack of efficient preservation (Garedew et al., 2012). *E. coli*pathogens are often used as indicator of fecal contamination of milk and may impose presence of pathogenic serotypes for humans (Ahmed*et al*., 2014; Garedew et al., 2012). Whereas,*S aureus*contamination of milk either associated with milkers or milk handlers, especially those with poor hygienic habits as coughing or sneezing during milking or milk handling (Kadariya*et al*., 2014), or with infected cows as reservoirs of S. aureusinfection (Abebe*et al*., 2016). In addition, *E. coli*and*S. aureus* are major causes of subclinical and clinical mastitis (Abebe*et al*., 2016; Hinthong*et al*., 2017). In subclinical mastitis, *E. coli* and *S. aureus* are shed in milk without abnormalities in milk consistency or udder shape; hence, humans may impacttheir health by consuming or processing milk from these cases

Table 2.5 Worldwide Prevalence of AMR gene in *S. aureus* isolated from bovine milk

Antibiotic	Resistant gene	Prevalenceand study area	Reference
Methicillin	*mecA*	44% of bulk tank milk samples contained mecA gene in South eastern Sicily, Italy	Antoci *et al.*, 2013
	mecA	England and Wales prevalence2.15%	Paterson *et al*., 2014
	mecA	34% prevalence of MRSA in overall bovine milk from district Faisalabad, Pakistan with 30% and 38% prevalence in cattle and buffalo,respectively	Aqib *et al*., 2017
	mecA	Herd prevalence of methicillin-susceptible MSSA was 84%, while MRSA herd prevalencewas	Haran *et al*., 2012
		4% in Minnesota	
	mecA	0.3% of cows in Northern Ethiopia	
Tetracycline	*tetM*	76.7% resistant in Tehran Province, Iran	Jamali *et al.*, 2014
	-	32–35% resistant in Northern Ethiopia	Kalayu *et al*., 2020
	tetK	85.71% in Urmia, Iran	Bahraminia *et al.* 2017
Erythromycin	*erm*(A), *erm*(B), *erm*(C), *msr*(A), *msr*(B)	32–35% resistant in China 32% *erm*(B), 20.4% *erm*(C) in Urmia, Iran	Liu *et al.*, 2017 Bahraminia *et al.* 2017
Penicillin	*blaZ*	Resistance against penicillin G (86%) in Tehran Province,Iran	Jamali *et al.*, 2014
	-	> 90% resistant Northern Ethiopia	Kalayu *et al*., 2020
	-	97.6% resistant in Oromia Region, Ethiopia	Regasa *et al.*, 2019

Table 2.6 Worldwide Prevalence of AMR gene in *E.coli* isolated from bovine milk

Antibiotic	Resistant gene	Prevalence area and study	Reference
Ceftriaxone	*bla*CTX-M	22.6% UK in Nottingham,	Ibrahim *et al*., 2016
		54.54% in West Bengal, India.	Batabyal *et al*., 2018
		66.7% Malaysia in Putra,	Kamaruzzaman *et al*. 2015
Ampicillin	*blaTEM*	7.5% UK in Nottingham,	Ibrahim *et al*., 2016
		83.1% Northern, SouthernChina	Yu *et al.,* 2020
Sulphur drug	*sul1*	83% in West Bengal, India.	Mahanti *et al*. 2020
		15.7%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 *et al*., 2020
	tetB	16% in West Bengal, India.	Das *et al*., 2017
	tetC	57% Irbid, Jordan	Ismail *et al*., 2020
	tetD	71% Irbid, Jordan	Ismail *et al*., 2020
Colistin	*mcr-1*	2.0% resistant in Beijing,China	Liu *et al*., 2020
	mcr3	2.6% in Lyon, France	Haenni et al., 2018

2.6 Transmission of antimicrobial resistance via livestock

Microorganisms containing AMR genes spread throughout the environment and animals incurring diseases (Baker *at 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 spreading of resistance from animals to humans, some pathogens follow direct. Environment and fauna become a reservoir of antibiotic resistance and also serve as the source of proliferation of antibiotic-resistant bacteria and their spread among humans and animals. It is because of the fact that 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 similar to those used in humans and can select 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 blaTEM, sul2, sul3, aadA, tetA, tetB, etc. These results provide evidence that livestock production farms are important reservoirs of antimicrobial resistant genes (ARGs) (Adelowo *et al*., 2014).

2.6.1 Animal-to-human antibiotic resistance transmission pathways.

- **Direct exposure**

Farmworkers, veterinarians, slaughterhouse workers and animal food handlers who are under the direct exposure with animals and their biological substances, are at high risk of being colonized with antibiotic-resistant bacteria from animals and animal farms, which subsequently may provide an opportunity for the entry of ARGs/ARBs in local communities and health care settings (Marshall & Levy, 2011). Reynaga *et al*. reported that the high prevalence (81/140, 57.9%) of pig farmworkers had been colonized or infected with livestock-associated clonal lineage ST398 of MRSA

(Reynaga *et al*.,2016). It has been found that *E. coli* strains isolated from turkey and broiler farmers from the Netherlands were nearly resistant to all tested antibiotics. These livestock- associated ARGs/ARBs could further transmit from farmers to their families and the local community by human-to-human transmissions, resulting in increasing cause of colonization and infections in people with/without livestock contact (Larsen, 2015).

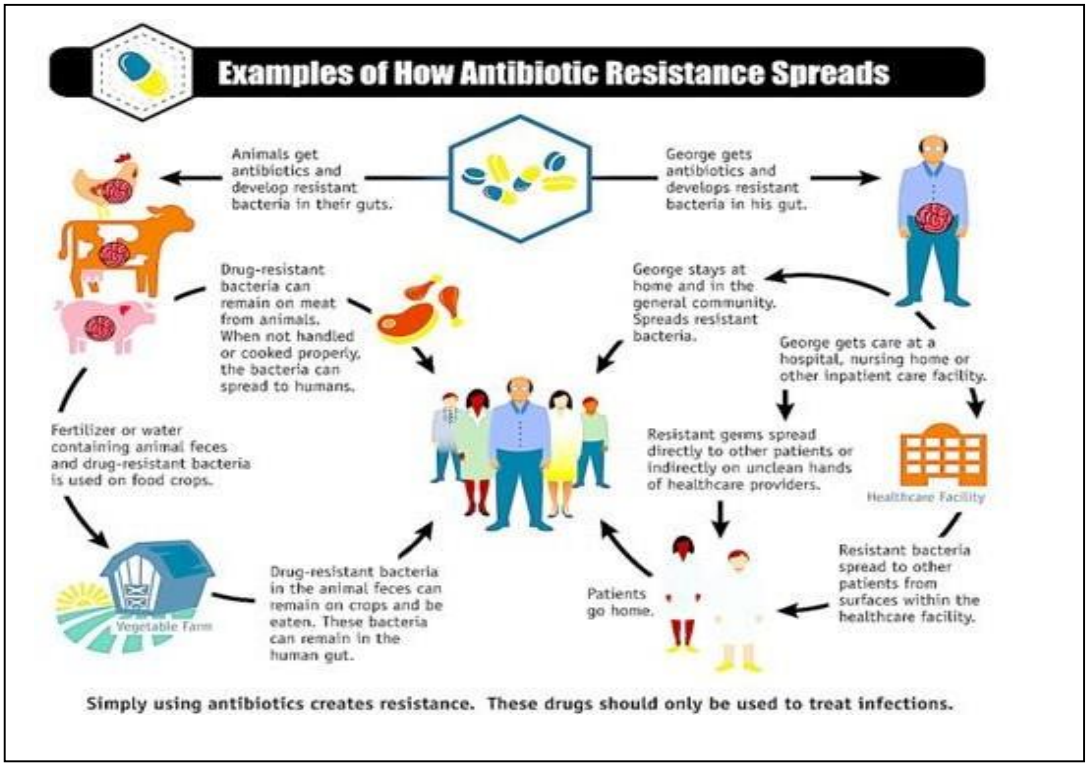

Figure 2.2: The U.S. Centres for Disease Control and Prevention; Transmission of antimicrobial resistance.

- **Food chain and food trade**

Food chains are far-reaching and complicated pathways for the AMR transmission pathways from animals to humans. Numerous studies have demonstrated that the high abundance of ARGs/ARBs in animal foods, including pork, chicken, beef and fish (Founou *et al*., 2016). For instance, in a study on MRSA in meat from the retail trade, the contamination rate was found to be the highest in turkey (35%), chicken (16%), veal (15%), pork (10%) and beef (10%), and lower in final products (3%) comparing to the meat at processing (4.2%) (de Boer *et al*., 2009). Another example is the *mcr-1* gene, which spread from animals to humans via food chains as evidenced by the higher detection rate in animal samples (21%) and raw meat (15%) as compared to its low prevalence in clinical samples (1%) in China (Liu *et al*.,2016).

2.6.2 Animal-to-human transmission via environmental compartments

The environment plays an essential role in the global transport of ARGs from food animals to humans (Graham *et al*., 2019). Mainly two processes are involved in this environmental dissemination from animal farms: the selection by antibiotic residues and the dispersal of ARGs/ARBs. Antibiotics and resistant bacteria have been detected in the farm dust, air current inside the feeding operations, as well as in the groundwater under the influence of animal husbandry and feedlot areas in general (McEachran *et al*., 2015). Exposure scenarios with contaminations in the farm environments also provide pathways of the ARGs transmission to humans (Marshall and Levy, 2011). It has been shown that proximity to animal feeding operations or areas with fecal contaminations can increase the risk of MRSA (Casey *et al*., 2013). However, transmissions of animal sourced ARGs are not occur only in the animal farming environments. To unfold the complexities of AMR across animals, environments and humans, we here propose the animal-to-human AMR transmission pathways by focusing on the environmental transmission pathways in a One Healthperspective.

- **Via manured farml and soils**

Manure, as a fertilizer with rich nutrients and organic matters, is commonly used worldwide in agriculture for improving the soil properties and enhancing the crop growth. However, manure has become an important reservoir of antibiotic compounds, ARBs, ARGs, and MGEs. ARGs in manure-impacted soils can potentially enter the food chain, and the consumption of organically produced vegetables and fruits from manured-soils is likely another route for the ARGs dissemination to humans (Berger *et al*., 2010; Zhang *et al*., 2019). Soil microbes have a profound impact on the development of bacterial communities of below- and above-ground parts of plants, shown by a sizeable functional overlap between leaf- and root-derived bacteria (Bai *et al*., 2015). A strong association exists between endophytic and rhizosphere bacteria, and many facultative endophytic bacteria can also survive in the rhizosphere (van der Lelie *et al*., 2019). Besides, the rhizosphere is known as a hotspot for the horizontal gene transfer (Elsas *et al*., 2003). A previous studies demonstrated that soil bacteria could survive in the interior of the root and become root endophytes (Bulgarelli *et al*., 2012). As a consequence, the bacterial community of plants growing on manure-amended soil can be influenced by soil bacterial communities. Manure has been shown to stimulate the horizontal transfer of ARGs in soils (Heuer *et al*., 2011; Zhu *et al*., 2013) and from soils to vegetable microbiome (Zhang *et al*., 2019). Previous studies have detected various ARGs in vegetables and fruits (including root endophytes, leaf

endophytes and phyllosphere) after growing in the manure-amended soil (Berger *et al*., 2010; Marti *et al*., 2013; Ruimy *et al*., 2010; Wang *et al*.,2015; Zhu *et al*.,2017). A total of 134 ARGs were detected in the conventionally and organically produced lettuce using high-throughput quantitative PCR (HT-qPCR), and the phyllosphere of organic produce had a higher abundance of ARGs than the phyllosphere of conventionally produced lettuce (Zhu *et al*., 2017). Vegetables and fruits from manured soils, especially those are eaten raw, could therefore represent an important vehicle for the antibiotic resistance transmission into humans (Berger *et al*., 2010). In this situation, a better understanding of ARGs in the soil-plant system under manure fertilization, is crucial in helping control ARGspread.

The influence of manure application on soil ARGs also depends on agricultural via aquatic environments practices.

A Finish study revealed the only temporary increase in ARGs after manure fertilization under the restricted antibiotic use policy in Finland, implying that the negative influence of manure application on ARG contamination in soils might be mitigated under the restricted policy on antibiotic usage in animals (Muurinen *et al*., 2017). However, it could also be attributed to the limited manure application rates and volumes (Muurinen *et al*., 2017).

Aquatic environments are considered an ideal setting for the acquisition and dissemination of ARGs (Marti *et al*., 2014). Genetic exchange and recombination can frequently occur in aquatic environments in order to shape the evolution of aquatic microbial community (Watts, 2017). The genetic plasticity of aquatic microbes enables the quick movement of ARGs in aquatic bacterial population and community to combat the potential pressure from antibiotic pollutants and/or to enhance the competitiveness. Moreover, once microbes acquired ARGs (or MGEs carrying the ARGs), the acquired ARGs tend to be more persistent in aquatic environments compared to terrestrial environments, held even with absence of the selection pressure (Manu, 2010). Aquaculture is a gateway to development and globalization of AMR in aquatic environments (Cabello *et al*., 2016). Large amounts of selective agents and fecal contaminations containing ARGs/ARBs from aquaculture enter our water bodies, stimulating the bacterial mutation, recombination and horizontal gene transfer, thereby increasing the level of ARGs in natural aquatic environments and risk of spreading ARGs from aquaculture to humans (Watts, 2017).

The dissemination pattern of ARGs from aquaculture varied among different aquaculture systems. In open systems, a high proportion (70%–80%) of used antibiotics

end up as residues, persist and select for ARBs in aquatic ecosystems, together with ARGs excreted by fishes and other seafood animals leaving a legacy effect on aquatic microbial communities (Watts, 2017). In closed systems, farming systems are usually separated from the general environments. In near zero-discharge recirculating aquaculture closed systems (RAS), limited exchange happens between aquaculture water systems and the surrounding environments, but antibiotics accumulate in the systems promoting the ARBs on the RAS biofilter (Li, Zhang, *et al*., 2017). In integrated aquaculture systems, fish farming is usually connected with livestock farming or/and crops for better resource utilization. However, they also represent a high degree of ARG pollution and gene transfer in the soil-water-plant systems, providing an elevated risk of resistome transmissions to humans (Klase *et al*., 2019). In other regular closed systems, antibiotics and ARGs can be reduced by waste treatments and discharge controls before reaching the environments. The wastewater containing antibiotic residues and ARGs usually either flow into aquatic environments after the wastewater treatment or being treated to produce aquaculture sludge as organic fertilizers. In some cases, the treated aquaculture effluents are used in irrigation for crops or urban parks, together with the land application of aquaculture sludge as organic fertilizer, therefore provides a pathway for ARGs from animals to soils and crops, which potentially impact downstream occupational workers, crop consumers and urban residents (Chen *et al*., 2016; Fahrenfeld *et al*., 2013; Wang *et al*., 2014). However, its hould benoted that the great majority of the developing countries has no or minimal waste treatments in aquaculture systems or lacks regulations, thus untreated aquaculture wastewater may directly flow into the surrounding water bodies, potentially promoting the risk of spreading ARGs from aquaculture to humans via aquatic environments (U.S. Centers for Disease Control & Prevention,2018).

2.6.3 The horizontal gene transfer promotes the transmission

The horizontal transfer of ARGs potentially promotes the flow of ARGs from animals to humans (Soucy *et al*., 2015; von Wintersdorff *et al*., 2016). As many environmental microbes especially aquatic bacteria from aquaculture share a large number of MGEs, e.g., plasmids, integrative conjugative elements, integrons and transposons, significant genetic exchange and recombination can occur for various purposes (Elsas *et al*., 2003; Marti *et al*., 2014; von Wintersdorff *et al*., 2016). Furthermore, the strong association between antibiotic resistome in soil environments and human clinical pathogens has been demonstrated showing that MGE-mediated HGT assembles tandem arrays of distinct ARGs into integrons, transposons and plasmids and then makes them mobile

(Forsberg *et al*., 2012). These mobile ARGs and microbes can disseminate into surrounding environments and migrate into our food chains (Zhu *et al*., 2017; Zhu *et al*., 2018). ARGs transfer to human pathogens by the transduction, bacterial conjugation and with the bacterial uptake of "free" DNA can also happen (Zhu et *al*., 2017; Zhu *et al*., 2018). Therefore the Class 1 integrons, which are often physically linked to multiple resistant determinants for antibiotics, are proposed to be the most critical and widespread agents of ARGs and a useful proxy for ARGs with anthropogenic origins including the animal-food producing industry (Gaze *et al*., 2011; Gillings, 2018; Gillings *et al*., 2015). Conjugation is the transfer of DNA from a contributing cell to a recipient cell via bacterial pili or adhesins, and it has also been recognized to have a great influence on the dissemination of ARGs among the bacterial population, compared to transformation and transduction (von Wintersdorff *et al*., 2016). In livestock systems especially the aquaculture, ARGs are commonly associated with conjugative plasmids, integrons or transposons (Watts, 2017). Once ARG exchange events have occurred in environmental bacteria, the ARGs can be further disseminated among local bacterial populations including human pathogens and then spread globally through the international transport of food products as well as global travelers (Cabello *et al*., 2016; Zhu *et al*., 2017; Zhu *et al*., 2018). Several studies have revealed that livestock environments might have contributed tot he emergence of the plasmid-encoded *qnrA* gene conferring low-level resistance to quinolones, and the *qnrA* gene were associated with waterborne species *Shewanella* spp, which are widely disseminated in marine and freshwater environments (Poirel *et al*., 2005; Yan *et al*., 2017). Yang *et al*. investigated the resistome in sediment samples from a marine fish farm using high-throughput sequencing and observed that several contigs containing resistance genes (e.g., *strAB*, *qnrA* and *tetL*) and transposons or plasmids, were highly identical (>90%) to those from human pathogens (Yang *et al*., 2013). There is another interesting example that plasmid-borne *mcr* genes may have primarily originated from the aquatic systems as a result of aquaculture activities that transport *mcr* genes from aquatic bacteria to terrestrial bacteria (Cabello *et al*., 2017). One evidence is that amino acid sequences of *mcr-3* and *mcr-4* were significantly identical to phosphoethanolamine transferases found in fish pathogens *Aeromona Salmonicida* (84%) (Yin *et al*., 2017) and *Shewanella frigidimarina* (99%) (Carattoli *et al*., 2017). Furthermore, a recent study showed that aquaculture is a significant reservoir of *mcr-1* gene, observed that geographical zones with low aquaculture activity have significantly lower odds of *mcr- 1* positivity (odds ratio = 0.5, 95% confidence interval: 0.3–0.7)

than those with high aquaculture activity (Shen *et al*., 2018). So far, *mcr* genes have been globally identified in animal farms, animal food products, vegetables, imported reptiles, environments (sewage and soils), and humans (Liu *et al*., 2016; Shen *et al*., 2018; Wang *et al*., 2017, 2018). Further public health risk arose from this gene family has emerged with subsequent global dissemination (Wang *et al*., 2018). This scenario is worrying, as colistin remains as a last-line antibiotic against the global emergence of MDR Gram- negative bacteria in clinicalsettings.

2.7 Public Health Importance of Antimicrobial Resistance

Infectious diseases were the primary cause of mortality in mankind prior to the discovery and use of antimicrobials. In much of the developing world without access to good quality medicines, infections continue to be the major killers, and in all countries healthcare-associated infections with resistant microorganisms are a major cause of death (Jinadal *et al*., 2015; Ferri *et al.*, 2017). Depending on the scenario, it is estimated that failing to tackle AMR will mean that the world population by 2050 will be between 11 million and 444 million lower than it would otherwise be in the absence of AMR. The lower bound is a result of a scenario where resistance rates have been successfully kept at a relatively low rate while the upper bound reflects a scenario for a world with no effective antimicrobial drugs (Taylor*et al*., 2014).

2.7.1 Antimicrobial resistance is an unusual public health threat

- Antimicrobial resistance is not a "disease". Typically, there is no difference in the severity of disease caused by susceptible strains and resistant ones. Resistance is generally not a problem of disease pathology but one of limited therapyoptions.
- The core issue is our dependence on antimicrobials for treating infections. If there were alternate methods of treating infections, antimicrobial resistance would persist in the world but would no longer be relevant as a public healthconcern.
- Antimicrobial resistance is a public health threat driven by healthcare practices, most notably the overuse of antimicrobials in conditions for which they provide nobenefit.

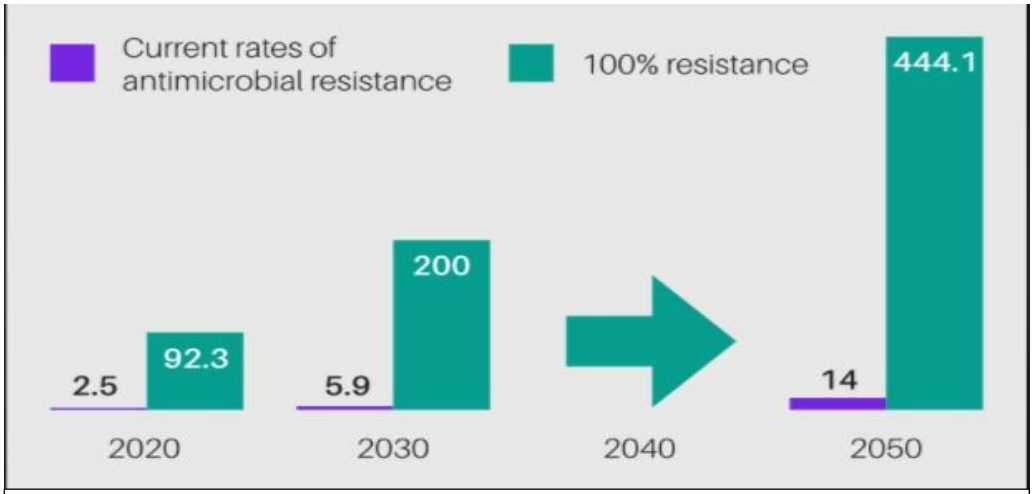

Figure 2.3: Global AMR consequence in near future

- 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, J.,2016)
- Resistance is a characteristic of many pathogens causing different diseases. Containment strategies thus must be adapted to the needs of specific disease control and treatment programs (Jinadal *et al*., 2015; Ferri *et al.*,2017).

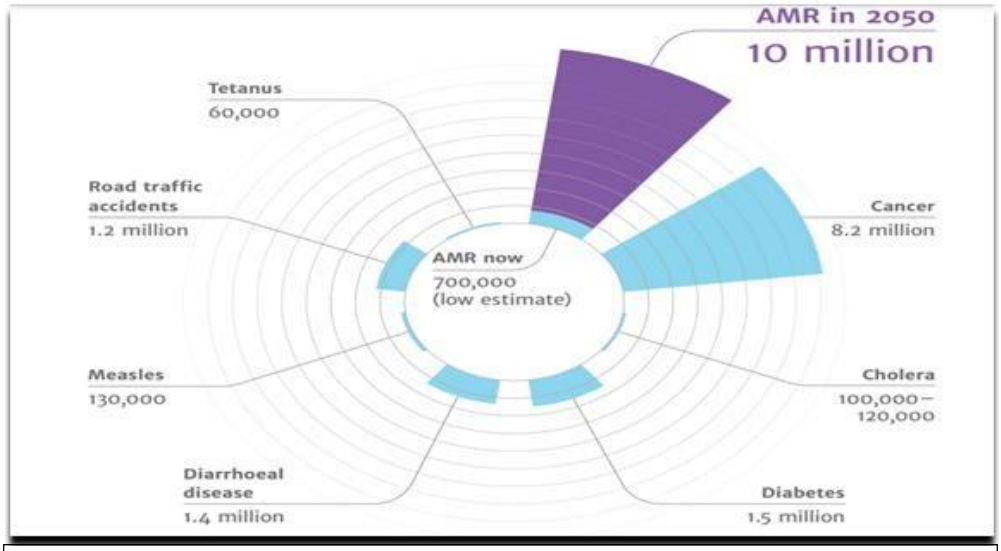

Figure 2.4: Worldwide economic loss due to AMR

2.8 Managing resistance in farm animals

i) Surveillance

An important step towards assessing any threat to public health from AMR in farm animals is to determine levels of resistance in those populations. National level reporting of AMR in farm animals typically relies on passive surveillance. For AMR in humans, alternatives to passive surveillance have been considered. In principle, these kinds of approaches could be extended to farm animals, as has been suggested in the context of emerging zoonotic diseases in general (Keusch *et al*., 2009)

ii) Reducing antimicrobial usage in farm animals

Reducing the levels of antimicrobial consumption in farm animals has not proved straightforward, as the experience of the EU-wide ban on growth promoters. Outside Europe, the adoption of voluntary codes and the development of guidelines for drug use, while welcome in themselves, seem unlikely to reduce consumption dramatically. There may be some potential for more effective use of antimicrobials in farm animals, particularly if this generated tangible benefits in terms of reduced costs or improved productivity. These include the same approaches that have been proposed for human medicine, such as overkill strategies, combination therapies and drug reuse and recycling (Imamovic*et al.,* 2013). Again as for humans, there would be obvious advantagesofrapiddiagnosisofbacterialinfectionsandreal-timeprofilingof resistance determinants using whole genome sequence data (Gordon *et al*., 2014) to determine treatment strategies more quickly and accurately.

A complete ban on the use of antimicrobials in farm animals would inevitably have serious repercussions for animal health, welfare and productivity, and consequently on food prices. However, reduced antimicrobial consumption in farm animals could form part of a coordinated strategy across the different sectors (Davies *et al.,* 2013). Any adverse effects of this on the agricultural industry would be at least partially alleviated if viable alternatives to antimicrobials wereavailable.

iii) Alternatives to antimicrobials for farm animals

There are currently a number of prebiotics and probiotics available, though their efficacy is unclear and likely variable. Mixing the two has also been proposed, so-called ‘synbiotics’. Phage therapy can be effective, for example against *Salmonella* Typhimurium in poultry and pigs, although this requires rapid selection and administration of the phage and high bacterial loads (Allen *et al.,* 2013). It may be possible to use purified phage lysins directly rather than the phage itself, thus precluding unintended transfer of genetic material from the phage. However, none of

these possibilities is close to being available for commercial use on a global scale against the full spectrum of microbial disease in farmsanimals.

A more immediately practical proposition may be to expand the range of vaccines available for veterinary use. Although vaccines are already available against many of the major viral diseases of livestock, there is currently limited routine use of vaccines that protect against bacterial infection and disease. Even when it is available, a vaccine is not automatically adopted by producers: for example, one trial of a live oral *Lawsonia* vaccine in pigs resulted in both 80% lower consumption of oxytetracycline and increased productivity (Bak *et al.,* 2009), but the vaccine is not widely used. As long as antibiotics are still available and effective, there is arguably little commercial incentive either to use existing or to develop new antibacterial vaccines for farmanimals.

A longer term vision for reducing antimicrobial usage in farm animals might include the use of livestock that are genetically resistant to infection or disease, likely through the use of genetic modification technologies. One example of early progress in this direction comes from the development of transgenic chickens that do not transmit avian influenza (Lyall *et al.*,2011)

Overall, however, it is clear that there would need to be considerable investment in research and development before any of the above approaches to disease control in farm animals become effective replacements forantimicrobials.

2.9 One-Health approaches to check the AMR issue

The complex epidemiology of AMR together with the socio-economical drivers make this topic the quintessential One-Health issue. Transectoral and transdisciplinary approaches are a “must-do” to tackle AMR appropriately. Reducing the dissemination and transmission of resistant bacteria within and between animal and human populations is central when aiming to fight AMR. The ability of bacteria to disseminate from one setting to another, sometimes over large geographic distances and among the different populations, makes it difficult to explain with certainty the origin of resistant bacteria strains. Therefore, the reservoirs and the transmission pathways of antimicrobial-resistant bacteria merit further investigation, ideally through a One- Healthapproach.

It is, therefore, important to improve our knowledge on how animal contacts and trade (direct transmission), farm management, and the wider farm environment (indirect transmission) drive the dissemination of AMR and to identify potential interventions to counteract this phenomenon.

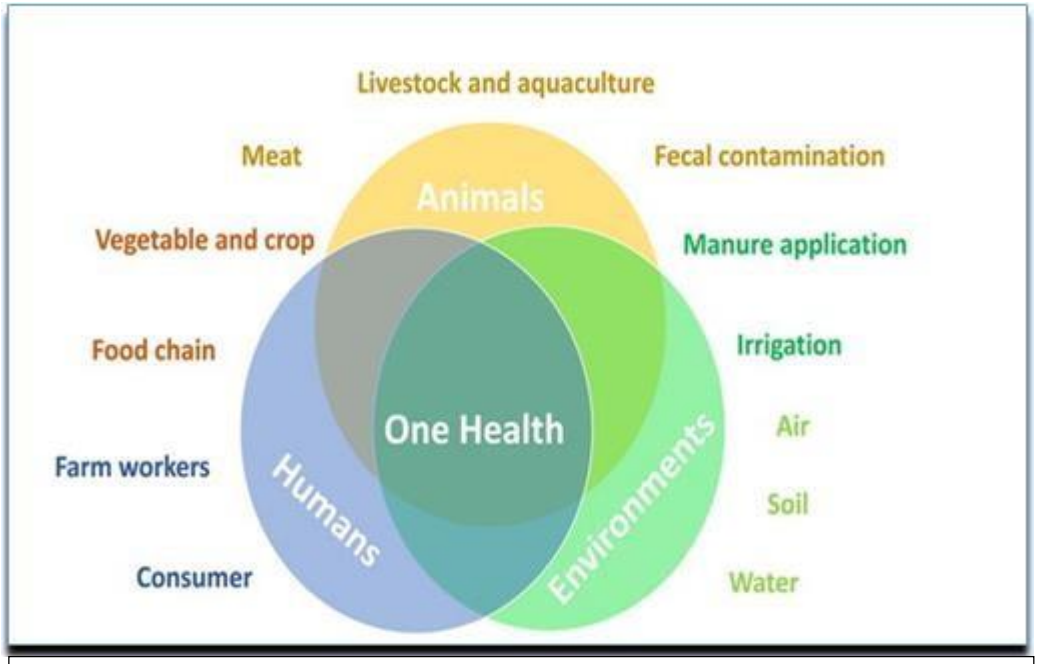

Figure 2.5: One Heath concept

Farm management studies could include all those practices that potentially facilitate spread of resistant bacteria within and between farms and from farms to the environment, such as farm hygiene and biosecurity, animal waste management, structure (and construction material) of holdings as well as animal production intensity. One-Health approaches should always be backed with molecular epidemiological data, which can provide information about links between resistance genes observed in different samples, such as from animals of different origin. Resistance genes should be studied not only in animal samples but also in the wider farm environment, such as farmers, other livestock species, farm pets, wildlife, manure, and water. These ecological data can provide the molecular link to characterize reservoirs of resistant bacteria and could support studies on transmission pathways between animal populations but also from animals to humans and *vice versa*. Source attribution can be of help to shed light on the contribution of AMR originating from livestock to the public health resistance burden. Moreover, it can also be an important piece of evidence when developing targeted interventions against AMR. Genomic data might also provide some additional information on potential evolutionary processes in bacteria during transmission within the studied populations. Furthermore, molecular epidemiology data can shed some light on how much of the resistance reservoir is attributed to the spread of resistant bacteria or *de novo* emergence due to AMU selection pressure in the studied farms.

CHAPTER-III

MATERIALS AND METHODS

3.1 Study area

Ten dairy farms of eight locations under Chittagong Metropolitan Area (CMA), Bangladesh (Bandar Thana, Kotwali Thana, Chandgaon, Patenga Thana, Shikalbaha, Jalalabad Market, Halishahar, Karnaphuli area) were selected randomly for sample collection.

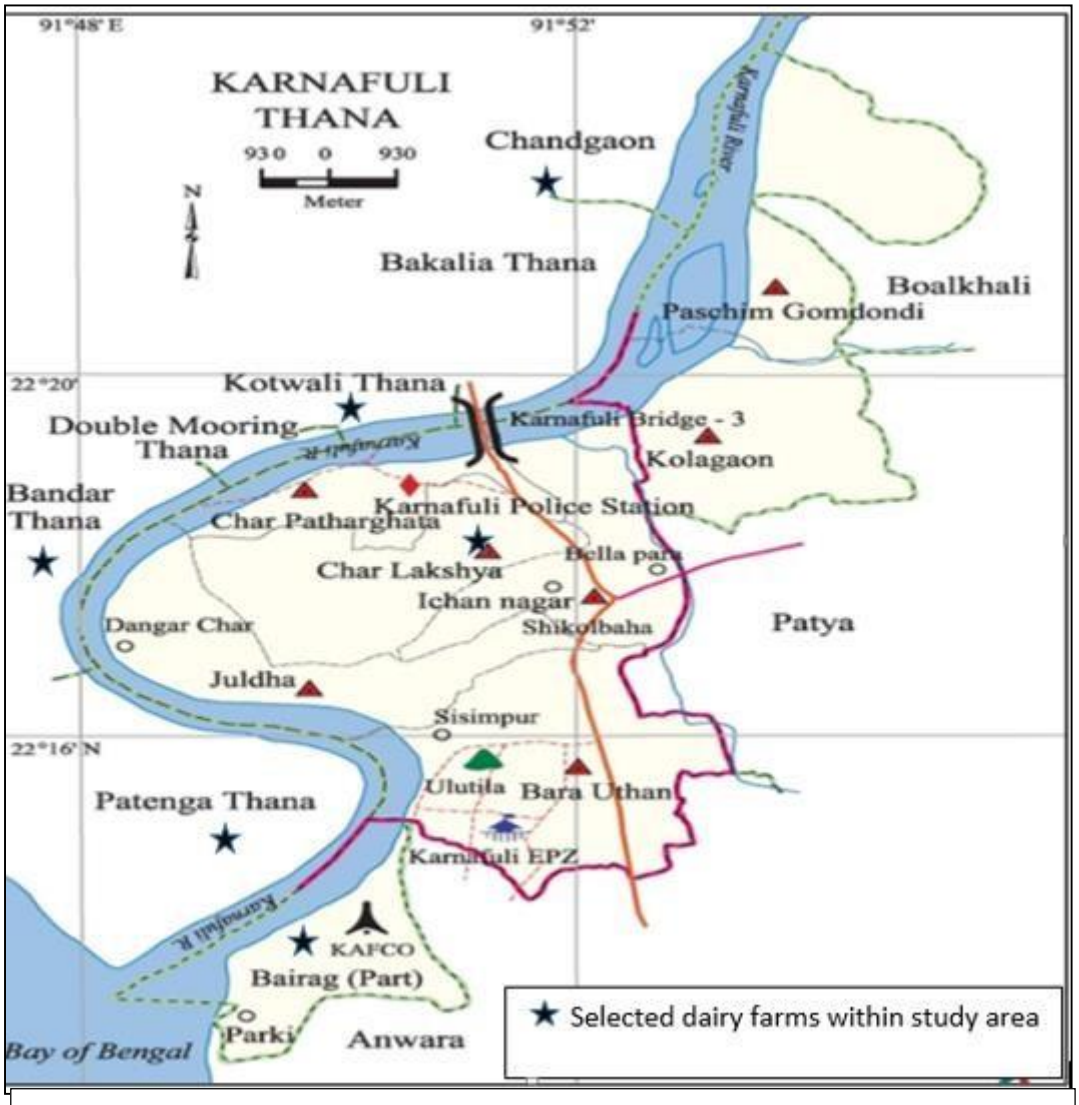

Figure 3.1: Geographical locations of the sites of different kinds of samples collected for the study

3.2 Sample collection duration

The samples were collected spanning the time between September 2019 and January 2020.

3.3 Study population

A total of 175 bovine raw milk samples were collected from different geographical locations. All the ten dairy farms were clustered based on farm size:

i) Large farm (farms having more than 50cows)

ii) Medium farm (farms having 20 to 50cows)

iii) Small farm (farms having less than 20cows)

Then the total samples were collected randomly from each cluster based on proportion.

3.4 Sample collection procedure

The samples were collected aseptically in clean sterile 15 ml labelled falcon tubes from the individual bucket full of milk used for individual cow. California Mastitis Test (CMT) was done for each cow. CMT positive and negative both samples were collected. Soon after collection, samples were kept into a cool box with ice for ceasing the growth and activity of acid producing organisms. The samples were then shipped to the clinical pathology laboratory of Chattogram Veterinary and Animal Sciences University (CVASU), where they were kept at 0°C until investigation but not exceeding 6 hours.

3.5 Questionnaire used for sample collection

To collect the data a structured questionnaire was followed during the study period. A detailed literature review was done before constructing the questionnaire to identify potential factors responsible for AMR. The questionnaire contained information about farm address, farm size, housing system, rearing system, type of breed, body condition score, milk yield, lactation number, udder cleanliness, floor cleanliness, stage of lactation, history of any disease, type of drug used. Data collection was done following a mixed (open and close ended) questionnaire. The sets of questionnaires used can be seen in Appendix-1.

3.6 Bacteriological Investigation

3.6.1 Isolation of *S. saureus*

Selective enrichment of milk samples were performed in Muller Hinton Broth (Oxoid, Basingstoke, Hampshire, UK) with 6.5% NaCl at 37°C overnight incubation and then inoculated onto Mannitol salt agar (Oxoid, Basingstoke, Hampshire, UK), where S.

aureus produced bright yellow coloured colonies after incubation of 24 hrs at 37°C.The presumptive positive colonies were identified based on the colony characteristics on MSA. The presumptive positive colonies (bright yellow color) were then sub cultured onto blood agar and incubated at 37ºC for 24 hours to detect characteristics appearance on blood agar and the haemolytic properties of organism (Rana et al.,2020).Suspected colonies were biochemically confirmed by catalase and coagulase test.

3.6.1.1 Coagulase test

To conduct the coagulase test, whole blood from horse was collected into commercially available EDTA -treated lavender tops. Then blood was centrifuged at 2600 rpm for 10 minutes using a refrigerated centrifuge. The resulting supernatant, the plasma was then immediately transferred to a sterile 1.5 ml eppendorf tube using a sterile micropipette. The plasma was then stored at - 20°C for future use.

3.6.1.2 Tube coagulase test

All the positive samples were subjected to coagulase tests for biochemical confirmation of *Staphylococcus spp.* as previously described (Monica, 1991). For this, few colonies were picked up and transferred to a 10 ml test tube containing 5 ml of BHIB which was prepared according to the instructions of manufacturer (Oxoid ltd, Basingstoke, Hampshire, UK), incubated at 37 °C for 6 h. On the other hand, whole blood from horse was collected into commercially available sterile tubes containing Ethylene Diamine Tetra Acetic Acid (EDTA). Blood was then centrifuged at 2600 rpm for 10 minutes at 4°C. Resulting supernatant, the plasma was immediately transferred to the sterile 1.5 ml eppendorf tube using sterile tip and stored at -20ºC for furtheranalysis.

Fifty micro liters of cultivated samples containing BHIB was transferred to the sterile tubes containing 50 µL of horse plasma and incubated at 37ºC for 6 hour. The presence of coagulates were considered when large organized coagulation of all the contents of the tube occurred which do not come off when inverted (Brasil, 2003). A control tube without horse plasma also is placed to validate the result.

3.6.2 Isolation of *Escherichia coli*

For screening of *E. coli* sample was selectively enriched in MacConkey broth (Oxoid, Basingstoke, Hampshire, UK) at 37°C overnight. After enrichment, milk sample was inoculated onto MacConkey agar (Oxoid, Basingstoke, Hampshire, UK), where *E. coli* produces large pink colour colony after incubation of 24 hrs at 37°C.The suspected large colour colony was inoculated onto Eosin Methelene Blue (EMB) (Oxoid ltd,

Basingstoke, Hampshire, UK) agar and incubated for 24 hours at 37ºC to verify whether such population produced colonies with metallic sheen, a diagnostic criterionfor*E.coli*(DyesEosinYandMethyleneBluereactwithproductsreleasedby *E. coli* from lactose or sucrose as carbon and energy source, forming metallic green sheen).Typical metallic sheen colony was sub-cultured onto Blood agar and finally tested for standard biochemical tests for *E. coli*, e.g Catalase test. Indole, Methyl red, Voges- Proskauer test, Nitrate reduction, Urease production, Simmon's citrate agar, and various sugar fermentation tests (Table 3.1)

Table 3.1: Typical biochemical reactions shown by any isolate belonging to *E. coli*

Biochemical Test	**Reaction**
Lactose fermentation	+ve
Catalase	+ve
Simmon's Citrate	-ve
Indole Production	+ve
Nitrate Reduction	+ve
Methyl Red	+ve
Voges- Proskauer	-ve
Urease	-ve
Acid from Sugar	
Glucose	+ve
Mannitol	+ve
Lactose	-ve
Salicin& Sucrose	+ve

3.7 Preservation of isolates

All positive isolates of *E coli* and *S. aureus* were inoculated in Brain Heart infusion

(BHI) broth (Oxoid ltd, Basingstoke, Hampshire, UK) and incubated overnight at 37°C. Then, 700µl BHI broth culture and 300µl of 50% glycerol were added in a 1.5ml eppendorf tube for each isolate. Finally, the tubes were properly labeled and stored at - 80°C for furtherinvestigation.

3.8 Molecular detection of *Escherichia coli* and *Staphylococcusaureus*

Polymerase chain reaction was performed for molecular detection of ***E. coli***and ***S. aureus*** using 16s rRNA and Spa gene respectively as described earlier (Dashti et al., 2009; Khal *et al*., 2005)

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 polymerase chain reaction (PCR).

3.8.2 DNA extraction from the isolates

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

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

ii. Then the tubes were boiled at 99°C for 15 minutes in water bath. Immediately after boilingthetubeswereplacedintotheicepackfor5minutes.Theprocessofhigh temperature boiling and immediate cooling allowed the cell wall to break down to release DNA from the bacterial cell.

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

3.8.3 PCR reactions

All the molecular investigation of the isolates were conducted in Clinical

Pathology Lab, CVASU. The primer sequences used for the PCR are shown in

Table 3.2. Table 3.2: Oligonucleotide primer sequence of 16s rRNA and Spa genes

Gene	Primer Sequence (5′-3′)	Annealing temperature	Amplicon size (bp)	Reference
16s rRNA	GACCTCGGTTTAGTTCACAGA(F) CACACGCTGACGCTGACCA(R)	58°C	585	Dashti *et al.*,2009
spa	TAAAGACGATCCTTCGGTGAGC (F)	59ºC	variable	Kahl *et al*.,2005

Table 3.3: Cycling conditions used during PCR for detection 16s rRNA of *E. coli*

Serial No	Steps	Temperature and Time
1	Initial denaturation	95°C for 5 minutes
2	Final denaturation (35 cycles)	94°C for 1 minute
3	Annealing	58°C for 1 minute
4	Initial Extension	72°C for 1 minute
5	Final Extension	72°C for 7 minutes
6	Final Holding	4°C

Table 3.4: Cycling conditions used during PCR for detection spa gene of *S. aureus*

Serial No	Steps	Temperature and Time
1	Initial denaturation	94°C for 15 minutes
2	Final denaturation (25 cycles)	94°C for 30 seconds
3	Annealing	59°C for 1 minute
4	Initial Extension	72°C for 1 minute
5	Final Extension	72°C for 10 minutes
6	Final Holding	4°C

3.9 Screening of antimicrobial resistance pattern of *E. coli* and *S. aureus*

E. coli and *S. aureus* isolates were screened for antimicrobial susceptibility against a panel of antimicrobials using Kirby-Bauer disc diffusion method. Seven antimicrobials of six different groups of drugs having public health significance were selected for the cultural susceptibility (CS) testing. We screened the isolates against 6 groups of unrelated antimicrobials namely: β-lactam antibiotics, tetracyclines, polymyxins, aminoglycosides, quinolones and sulfonamides. The following anti-microbial agents (with respective disc potencies) were used: CAZ: Cefatazidime(30µg), E: Erythromycin(15µg), S: Streptomycin (10µg), DO: Doxycycline (30µg),CRO: Ceftriaxone (30µg), AMC: Amoxicillin+Clavulinic acid (10µg), TE: Tetracycline (30µg), CN: Gentamycin (10µg), AMP: Ampicillin (10µg), CTX: Cefotaxime (30µg), OT: Oxytetracycline (30µg), SXT: Sulfamethoxazole- trimethoprim (23.75µg+1.25µg), CIP: Ciprofloxacin (5µg), MEM: Meropenem(10µg),OX: Oxacillin (1µg). To interpret the result of CS test the CLSI standards are given in **Table 3.5.**

Table 3.5: Concentrations and diffusion zone breakpoints for resistance against antimicrobials standard for *Escherichia coli* and *Staphylococcus aureus* isolates (CLSI, 2011)

Antimicrobial Agent	**Disc Content**	**Diffusion Zone Breakpoint (diameter in mm)**					
		Staphylococcus aureus			***Escherichia coli***		
		R	**I**	**S**	**R**	**I**	**S**
Cefatazidime (CAZ)	30µg	≤14	15-17	≥18	≤17	18-20	≥21
Erythromycin (E)	15µg	≤13	14-22	≥23	≤13	14-22	≥23
Streptomycin (S)	10µg	-	-	-	≤11	12-14	≥15
Doxycycline (DO)	30µg	≤12	13-15	≥16	≤10	11-13	≥14
Ceftriaxone (CRO)	30µg	≤13	14-20	≥21	≤19	20-22	≥23
Amoxicillin+Clavulinic acid (AMC)	10µg	≤19	-	≥20	≤13	14-17	≥18
Gentamycin (CN)	10µg	≤12	13-14	≥15	≤12	13-14	≥15
Cefotaxime (CTX)	30µg	≤14	15-22	≥23	≤22	23-25	≥26
Oxytetracilin (OT)	30µg	≤14	15-18	≥19	≤11	12-14	≥15
Ciprofloxacin (CIP)	15µg	≤15	16-20	≥21	≤15	16-20	≥21
Meropenem (MEM)	10µg	≤13	14-15	≥16	≤19	20-22	≥23
Trimethoprim/Sulfamethoxazole (SXT)	25µg	≤10	11-15	≥16	≤10	11-15	≥16
Imepenem (IMP)	10µg	≤13	14-15	≥16	≤19	20-22	≥23
Tetracycline (TE)	30µg	≤14	15-18	≥19	≤11	12-14	≥15
Oxacillin (OX)	1µg	≤10	11-12	≥13	-	-	-
Ampicillin (AMP)	10µg	≤28	-	≥29	≤13	14-16	≤17

3.9.1 Procedure of cultural sensitivity test (CS) test:-

At first sub-culturing of the preserved organism was done on blood agar and incubated at 37° for 24 hours to obtain a pure growth. Using sterile inoculating loop 3 or 4 individual colonies from the blood agar were transferred into a tube containing 3ml of sterile phosphate buffer saline solution (0.85% w/v NaCl solution). Emulsification of the inoculums was done to avoid clumping of the cells inside test tube using vortex machine. Then the bacterial suspension was adjusted to the turbidity of 0.5 McFarland standard (equivalent to growth of 1-2×108CFU/ml). Within 15 minutes of preparing the inoculums, a pre-sterile cotton swab was dipped into the inoculums and rotated against the side of the tube with firm pressure to remove excess fluid. Then the swab was streaked over the entire dry surface of Mueller Hinton agar for three times rotating the plate approximately at 60 degrees. After 15 minutes of inoculation the discs were placed on the agar surface using a sterile forceps. After dispensing all the discs the agar plates were incubated at 37°C for 18 hours. After incubation the size of zone of inhibition (in mm) around a disc including the diameter of the disc was measured using a ruler and the result was interpreted according to CLSI, 2011.

3.10 Polymerase chain reaction (PCR) to test for the presence resistantgenes

3.10.1 PCR for resistant genes in *E.coli*

To identify tetracycline resistant genes all positive *E. coli* isolates were tested for *tet*A, *tet*B, tetC genes, phenotypically those were resistant to ampicillin and cefotaxime were tested for bla_{TEM} and $bla_{\text{CTX-M}}$ genes, those were resistant to ciprofloxacin were tested for gyrA, gyrB and parC, and those were resistant to sulfamethoxazole/trimethoprim were tested for *Sul-I* and *Sul-II* genes respectively. All the positive *E coli* isolates were tested for *mcr-1, mcr-2, mcr-3 and mcr-4* genes to detect colistin resistance by PCR using the set of specific primers used for each gene described in **Table 3.6**.

Table 3.6: Oligonucleotide primer sequences used to detect the selected antimicrobial genes in the *E. coli* isolates

Antibiotic	Gene	Primer Name	Primer sequence (5´- 3´)	Amplicon size (bp)	Reference
Tetracycline	*tet*A	*tet*A-F	GGCGGTCTTCTTCATCATGC	502	(Lanz et al., 2003)
		*tet*A-R	CGGCAGGCAGAGCAAGTAGA		
	*tet*B	*tet*B-F	CATTAATAGGCGCATCGCTG	964	(Lanz et al., 2003)
		*tet*B-R	TGAAGGTCATCGATAGCAGG		
	tetC	*tet*C-F	GCTGTAGGCATAGGCTTGGT	888	(Lanz et al., 2003)
		*tet*C-R	GCCGGAAGCGAGAAGAATCA		
Sulfamethoxazole/ trimethoprime	*Sul*-I	*Sul*I-F	CGG CGT GGG CTA CCT GAA CG	779	(Lanz et al., 2003)
		*Sul*I- R	GCC GAT CGC GTG AAG TTC CG		
	Sul-II	Sul-II-F	CCTGTTTCGTCCGACACAGA	721	(Lanz et al., 2003)
		Sul-II-R	GAAGCGCAGCCGCAATTCAT		
Ampicillin	*bla*TEM	*bla*TEM F	TACGATACGGGAGGGCTTAC	964	(Hasman et al., 2005)
		*bla*TEM R	TTCCTGTTTTTGCTCACCCA		
Ceftriaxone	*bla*CTXM	CTXMF	ACGCTGTTGTTAGGAAGTG	557	(Feizabadi et al., 2010)
		CTXMR	TTGAGGCTGGGTGAAGT		
Ciprofloxacin	*gyrA*	GyrA-L	TACCGTCATAGTTATCCACGA	312	Wiluff et al.,2000
		GyrA-R	GTACTTTACGCCATGAACGT		
	gyrB	GyrB-L	GTCCGAACTGTACCTGGTGG	281	Wiluff et al.,2000
		GyrB-R	AACAGCAGCGTACGAATGTG		
	ParC	ParC-L	TGGGATCCAAACCTGTTCAGC GCCGCATT	261	Wiluff et al.,2000
		ParC-R	CGGAATTCGTGGTGCCGTTAA GCAAA		
Colistin Sulfate	*mcr-1*	CLR F	CGGTCAGTCCGTTTGTTC	309	Liu et al.,2016
		CLR R	CTTGGTCGGTCTGTAGGG		
	mcr-2	MCR2 IF	TGTTGCTTGTGCCGATTGGA	567	Xavier et al., 2016
		MCR2IR	AGATGGTATTGTTGGTTGCTG		
	mcr-3	mcr3F	TTGGCACTGTATTTTGCATTT	542	Yin et al., 2017
		mcr3R	TTAACGAAATTGGCTGGAAC A		
	mcr-4	mcr4F	ATTGGGATAGTCGCCTTTTT	487	Carattoli et al., 2017
		mcr4R	TTACAGCCAGAATCATTATCA		

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

Serial No	Name	Manufacturer
1	Master Mix	Thermo Scientific
2	Molecular marker	Thermo Scientific O‟ GeneRuler 100bp plus
3	Ethidium bromide solution (1%)	Fermantas
4	Electrophoresis Buffer 50X TAE	Fermantas
5	Agarose powder	Seakem® Le agarose-Lonza
6	Nuclease Free Water	Thermo Scientific

PCR reactions were conducted with a final volume of 25 µl. Proportions of different reagents used for PCR for two different resistance genes are given in Table 3.8.

Table 3.8: Contents of each reaction mixture of PCR assay

Serial No	Name of the Contents	Amount
1	Thermo Scientific Dream Taq PCR Master Mix (2x) ready to use	12.5µl
2	Forward primer	1µl
3	Reverse primer	1µl
4	DNA template	1µl
5	Nuclease free water	9.5µl
Total		25µl

PCR was run on a thermocycler (Applied Biosystem, 2720 thermal cycler, Singapore) following the cycling conditions mentioned in **Table 3.9.**

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

Gene name	***bla*TEM**	***bla*CTXM**	***tet*A, *tet*B**	***Sul*-I**	***Sul*-II**	***gyrA, gyrB, parC***	***mecA***	***mcr-1, mcr-2***	***mcr-3, mcr-4***
Initial denaturatio n	94°C for 3 mins	94°C for 3 mins	95°C for 4 mins	95°C for 5 mins	94°C for 4 mins	94°C for 3 mins	94°C for 4mins	94°C for 3 mins	95°C for 5mins
Cyclic denaturatio n	94°C for 1 min	94°C for 1 min	95°C for 1 min	95°C for 1 min	94°C for 1 min	94°C for 1 min	94°C for 30 secs	94°C for 30 secs	95°C for 30 secs
Cyclic annealing	50°C for 1 min	58°C for 30 secs	64°C for 1 min	68°C for 1 min	66°C for 1 min	60°C for 1 min	53°C for 30 secs	58°C for 90 secs	50°C for 90 secs
Cyclic extension	72°C for 1	72°C for 1 min	72°C for 1 min	72°C for 1 min	72°C for 1 min	72°C for 1 min	72°C for 1min	72°C for 30 secs	72°C for 45 secs
Final extension	72°C for 10 min	72°C for 10 min	72°C for 7 min	72°C for 10 min	72°C for 7 min	72°C for 10 min	72°C for 4min	72°C for 12 min	72°C for 12 min
Holding	4°C	4°C	4°C	4°C	4°C	4°C	4°C	4°C	4°C
Cycle number	25	36	35	35	35	30	30	25	30
References	Hasman et al., 2005	Feizabadi et al., 2010	Lanz et al., 2003	Lanz et al., 2003	Lanz et al., 2003	Wiluff et al.,2000	Liveira et al.,2002	Liu et al.,2016, Xavier et al., 2016	Yin et al., 2017, Caratto li et al., 2017

3.10.1 Visualization of PCR Product

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

i. 0.75 gm of agarose powder and 50 ml of 1X TAE buffer was mixed thoroughly in a conical flask and boiled in a microwave oven until agarosedissolved.

ii. Then the agarose mixture was cooled at 50°C in a water bath and one drop of ethidium bromide was added to themixture.

iii. Gel casting tray was assembled by sealing the ends of gel chamber with tape and placed appropriate number of combs in geltray.

iv. The agarose-TAE buffer mixture was poured into the gel tray and kept for 20 minutes at room temperature for solidification then combs were removed and the gel was shifted into an electrophoresis tank filled with 1X TAE buffer and kept until the gel is drownedcompletely.

v. An amount of 5 µl of PCR product for a gene was loaded into a gelhole.

vi. Then 3 µl of 100 bp plus DNA marker (O‟ GeneRular 100bp plus) was used to compare the amplicons size of a gene product and the electrophoresis was run at 110 volts and 80 mA for 30minutes.

vii. Finally the gel was examined by using a UV transilluminator for image acquisition andanalysis.

3.10.2 PCR for resistant genes in *S.aureus*

A total of 12 phenotypically oxacillin resistant isolates were selected for PCR assays to identify methicillin resistant gene, namely *mecA* gene. Phenotypically a total of 18 ampicillin resistant, 10 erythromycin, and 13 tetracycline resistant isolates were selected for PCR assays to detect *blaZ* gene, two erythromycin resistant genes, namely *ermB* and *ermC*, and two tetracycline resistant genes, namely *tetM*, and *tetK*, respectively.

For the multiplex PCR, system l (containing*nuc*, *ermC* and *tet M* genes) and strain system ll (containing *blaZ, erm B,* and *tet K* genes) were chosen. Using the multiplex PCR assay, 26 isolates were used for detection of all possible resistance gene patterns. Each single-gene PCR experiment contained 1 µl of template DNA, 1 µl of each primer pair (10 µM), 7 µl of double distilled (dd)H2 O, and 10 µl of 2 × Taqmix.a Initial denaturation, which occurred at 95°C for 5 min, was followed by 30 cycles of amplification using the following parameters: 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 30 sec, and a final extension step at 72°C for 5 min. The PCR products were analyzed by electrophoresis on a 2% agarose gel. Of the 2 multiplex PCR systems, System I wasdesignedtotestfor*nuc, tetM*, and*ermC,* and contained 1.5 µl of each of the nuc and tet M primer pairs, 0.5 µl of the *erm C* primer pair, 5 µl of template DNA, 3µl of ddH2O and 15µl of 2×Taqmix. and SystemII contained 1.5µl of each of the *erm B* and *blaZ* primer pairs, and 0.5 µl of the *tet K* primer pair. Other materials used in System II were similar to those of System I. A pre-PCR step at 95°C

for 5 min was applied. A total of 38 cycles were run at the following conditions: denaturation at 95°C for 30 sec, annealing at 50°C for 35 sec, and extension at 65°C for

1.5 min. After the final cycle, the preparation was kept at 65°C for 10 min to complete the reaction. The products were analyzed by electrophoresis on a 3% agarose gel following method described by Gao *et al.* (2011). Other than multiplex PCR, singleplex PCR aslo done to identify methicillin-resistant gene (mecA) and the procedure was followed by the other singleplex PCR used in case of *E. coli*.

Table 3.10. Primers used for the multiplex polymerase chain reaction results systems for the identification of *Staphylococcus aureus* and related antibiotic resistance genes.

Target gene	**Primer (5′–3′)**	**Annealing temperature (°C)**	**Product size (bp)**	**Accession no. or reference**
nuc	1- ATATGTATGGCAATCGTTTCAAT	56	395	AP009324
	2-GTAAATGCACTTGCTTCAGGAC			
blaZ	1-AAGAGATTTGCCTATGCTTC	55	517	Sawant et al., 2009
	2-GCTTGACCACTTTTATCAGC			
erm (B)	1-ACGACGAAACTGGCTAA	53	409	U00453
	2-TGGTATGGCGGGTAA			
erm (C)	1-CTTGTTGATCACGATAATTTCC	55	190	Martineau et al., 2000
	2-ATCTTTTAGCAAACCCGTATTC			
tet (K)	1-TCGATAGGAACAGCAGTA	55	169	Garofalo et al., 2007
	2-CAGCAGATCCTACTCCTT			
tet (M)	1-CCGCACCCTCTACTACAA	56	351	X56353
	2-CATTCCACTTCCCAACG			
mecA	1-TCCAGATTACAACTTCACCAGG	53	162	Liveiraet al.,2002

3.11 Statistical analysis

Epidemiological data were entered into a spread sheet program (Microsoft Office Excel 2010) and transferred to STATA-13 software for data summary and analysis.Any factor having p value ≤ 0.20 in univariable analysis was selected for multivariable logistic regression analysis to see the independent association of a risk factor with the isolation frequency of AMR *E. coli* and *S. aureus*. A p value less than or equal 0.05 was considered statistically significant in the final multivariable model.

CHAPTER-IV

RESULTS

4.1 *S. aureus* isolated from bovine milk

A total of 175 bovine milk samples were collected from 10 dairy farms of Chattagram Metropolitan area for the present study. Among those, 26 (14.86%; 95% CI 10% - 21%) isolates were confirmed as *S. aureus* based on the PCR assay Characteristic growth of *S. aureus* strain on a Mannitol Salt agar plate and β hemolysis due to growth of *S. aureus* on Blood agar plate are shown in **Figure 4.1** and **Figure 4.2**, respectively, and the result of Catalase and Coagulase test as well as Gram"s staining property of it are displayed in **Figure 4.3, Figure 4.4** and **Figure 4.5,** respectively. The results of PCR assay of some of the isolates after gel electrophoresis for the detection of the *spa* gene in these isolates are displayed in **Figure 4.6.**

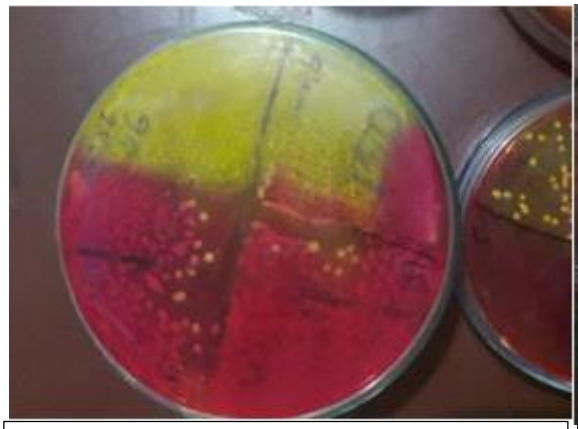

Figure 4.1: *Staphylococcus aureus* on Mannitol salt agar plates

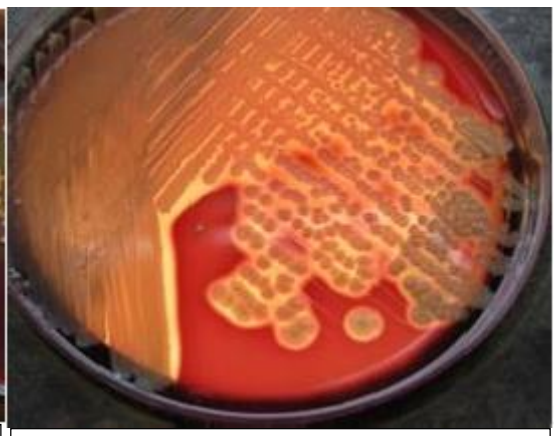

Figure 4.2: *Staphylococcus aureus* on blood agar plates

Figure 4.3: Catalase positive test

Figure 4.4: Coagulase positive test

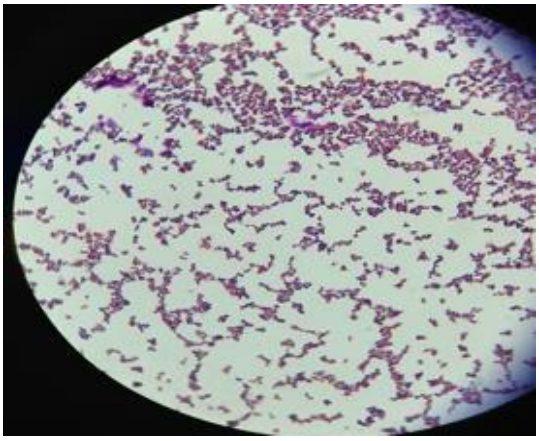

Figure 4.5: Gram's staining properties of *Staphylococcus aureus*

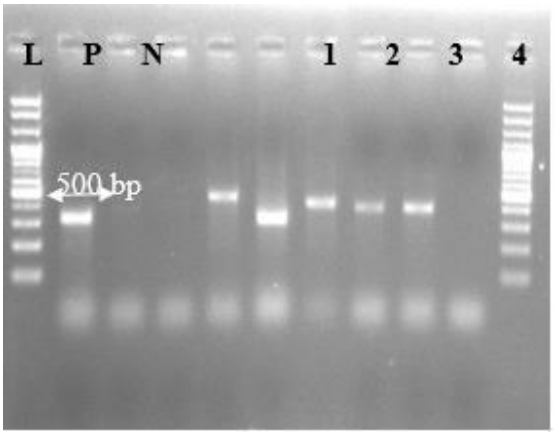

Figure 4.6: Result of PCR assay for the detection of the *spa* gene (variable bp)

4.2 *E. coli* isolated from bovine milk

A total of 20 (11.43 %; 95% CI 7.12% - 17.10%) isolates were confirmed as *E. coli* out of 175 bovine milk samples by PCR. Characteristic growth of *E. coli* strain on MacConkey agar plates and on EMB agar plate are shown in **Figure 4.7** And **Figure 4.8**, respectively, and the result of indole test and Gram‟s staining property of it are displayed in **Figure 4.9** and **Figure 4.10**, respectively. PCR assay of some of the isolates after gel electrophoresis for the detection of 16s rRNA gene in those are displayed in **Figure 4.11.**

Figure 4.7: *E. coli* producing large pink colour growth on McConkey

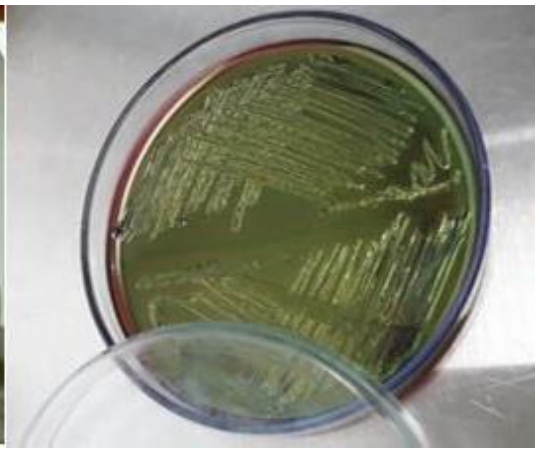

Figure 4.8: Metalic green sheen on EMB agar

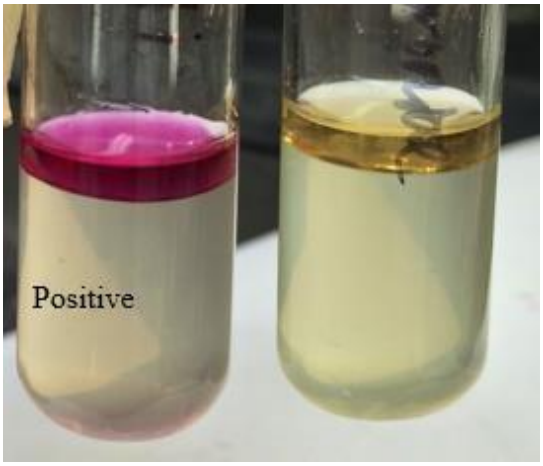

Figure 4.9: *E. coli*; Indole positive

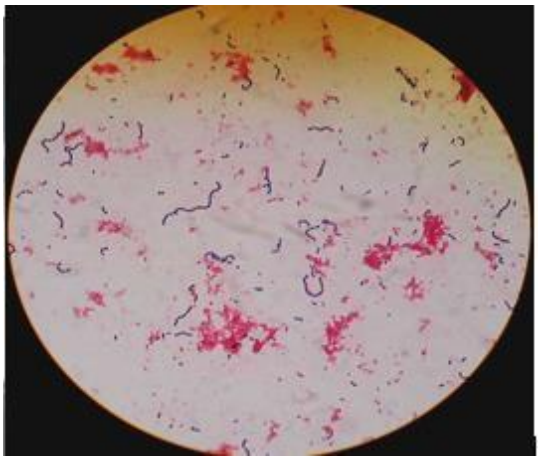

Figure 4.10: Gram's staining property of *E. coli*

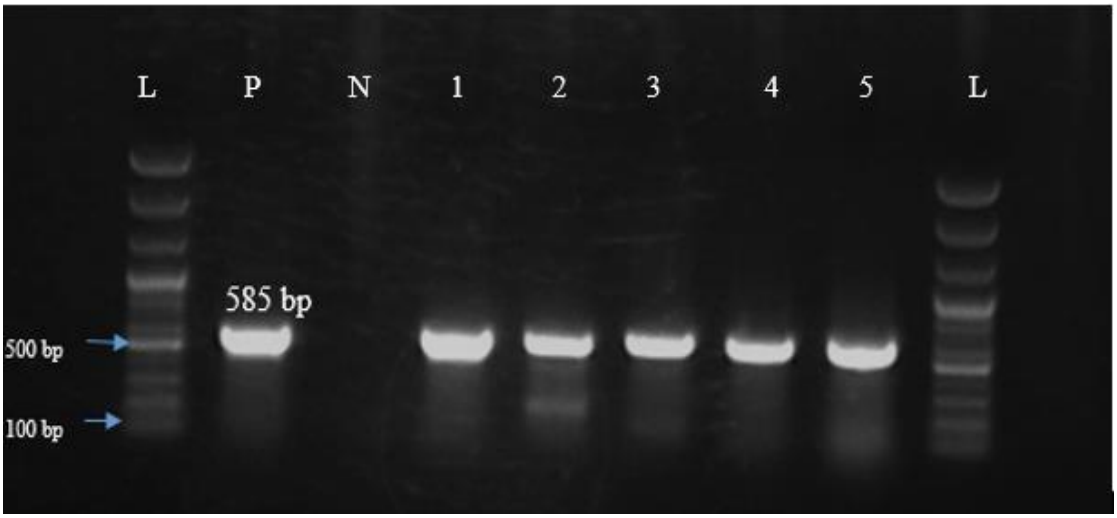

Figure 4.11: PCR assay for the detection of the 16s rRNA gene; Lane L: 1kb plus DNA ladder; Lane P: Positive control; Lane N: Negative control; Lane 1-5: gene-16s rRNA sized (585 bp) amplicon.

4.3 Antimicrobial resistance profile of *S. aureus* and *E.coli*

All the *S. aureus* (26) and *E. coli* (20) isolates were found to be resistant to at least one type of selected antimicrobials phenotypically. Isolate showing sensitivity and resistance to different antimicrobials is shown in **Figure 4.12** and McFarland Standard in **4.13**, respectively. Antimicrobial susceptibility patterns of the isolates were interpreted following the guidelines of Clinical and Laboratory Standard Institute (CLSI). The susceptibility patterns of the isolates are shown in **Table 4.1.** We observed that, highest number of *S. aureus* (80.7%, 95% CI 60.6-93.4) isolates were resistant to streptomycinandallthe *S. aureus* (100%) weresensitivetomeropenem. Meanwhile,

all the *E. coli* isolates were resistant to ciprofloxacin whereas 95% (95% CI 75.1% - 99.9%) showed sensitivity to meropenem and 80% (95% CI 56.3%-94.2%) were sensitive to gentamicin.

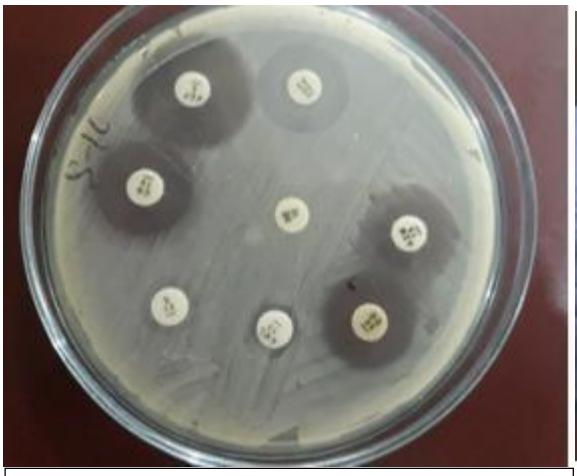

Figure 4.12: Bacterial zone of inhibition

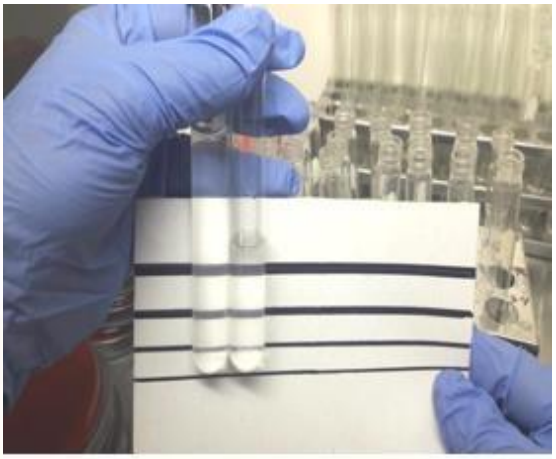

Figure 4.13: Comparing inoculum with McFarland Standard

Table 4.1: Antimicrobial resistance pattern of *S. aureus* & *E. coli* isolates:

Code of Antibiotics	Sensitive % (n) (95% CI)		Intermediate % (n) (95% CI)		Resistant % (n) (95% CI)	
	S. aureus	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*
AMP	30.7% (8) (14.3-51.8)	30%(6) (11.9-54.2)	-	10%(2) (1.2-31.6	69.3%(18) (48.2-85.7)	60%(12) (36.0-80.8)
E	34.6%(9) (17.2-55.2)	25%(5) (8.7-49.1)	26.9%(7) (11.5-47.7)	20%(4) (5.7-43.6)	38.5%(10) (20.2-59.4)	55%(11) (31.5-76.9)
S	15.4%(4) (4.3-34.8)	30%(6) (11.9-54.2)	3.8%(1) (0.09-19.6)	-	80.7%(21) (60.6-93.4)	70%(14) (45.7-88.1)
DO	53.8%(14) (33.3-73.4)	65%(13) (40.8-84.6)	11.5%(3) (2.4-30.1)	-	34.6%(9) (17.2-55.6)	35%(7) (15.4-59.2)
AMC	26.9%(7) (11.5-47.7)	55%(11) (31.5-76.9)	23.0%(6) (8.9-43.6)	20%(4) (5.7-43.6)	50%(13) (29.9-70.0)	25%(5) (8.7-49.1)

CN	38.5%(10) (20.2-59.4)	80%(16) (56.3-94.2)	3.8%(1) (0.09-19.6)	5%(1) (0.1-24.8)	57.6%(15) (36.9-76.6)	15%(3) (3.2-37.9)
OT	42.3%(11) (23.3-63.0)	60%(12) (36.0-80.8)	19.2%(5) (6.6-39.3)	-	38.5%(10) (20.2-59.4)	40%(8) (19.1-63.9)
CIP	38.5%(10) (20.2-59.4)	-	15.4%(4) (4.3-34.8)	-	46.1%(12) (26.5-66.6)	100% (20)
MEM	100% (26)	95%(19) (75.1-99.9)	-	5%(1) (0.1-24.8)	-	-
SXT	57.6%(15) (36.9-76.6)	20%(4) (5.7-43.6)	11.5%(3) (2.4-30.1)	10%(2) (1.2-31.6)	30.9% (8) (14.3-51.8)	70%(14) (45.7-88.1)
TE	26.9%(7) (11.6-47.8)	30%(6) (11.9-54.2)	-	-	73.1%(19) (52.2-88.4)	70% (14) (45.7-88.1)
CRO	50%(13) (29.9-70.0)	40%(8) (19.1-63.9)	-	15%(3) (3.2-37.9)	50%(13) (29.9-70.0)	45%(9) (23.0-68.5)
OX	46.1%(12) (26.5-66.6)	-	7.8%(2) (0.9-25.1)	-	46.1%(12) (26.5-66.6)	-
CAZ	-	50% (10) (27.2-72.8)	-	5%(1) (0.1-24.8)	-	45%(9) (23.0-68.5)
CTX	-	45%(9) (23.0-68.5)	-	-	-	55%(11) (31.5-76.9)

AMP: Ampicillin, E: Erythromycin, S: Streptomycin, DO: Doxycycline, AMC: Amoxicillin+Clavulinic Acid, CN: Gentamycin, CIP: Ciprofloxacin, MEM: Meropenem, SXT: Sulphamethoxazole-Trimethoprim, TE: Tetracycline, CRO: Ceftriaxone, OX: Oxacillin, CAZ: Cefatazidime, CTX: Cefotaxime

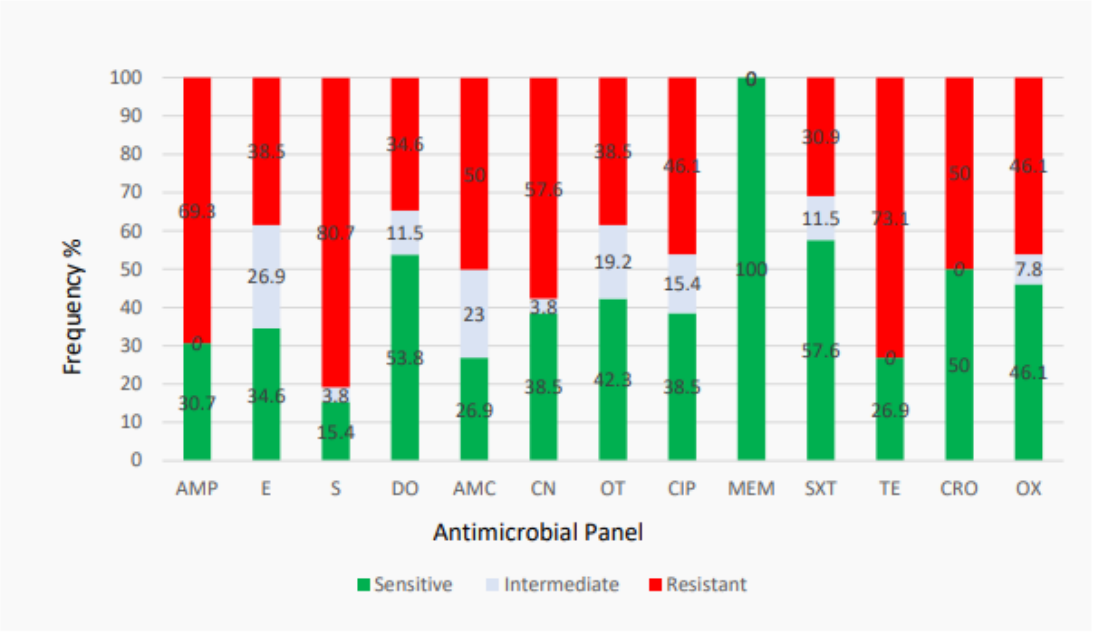

Figure 4.14.: Antibiogram profile of *S. aureus* isolates isolated from bovine milk

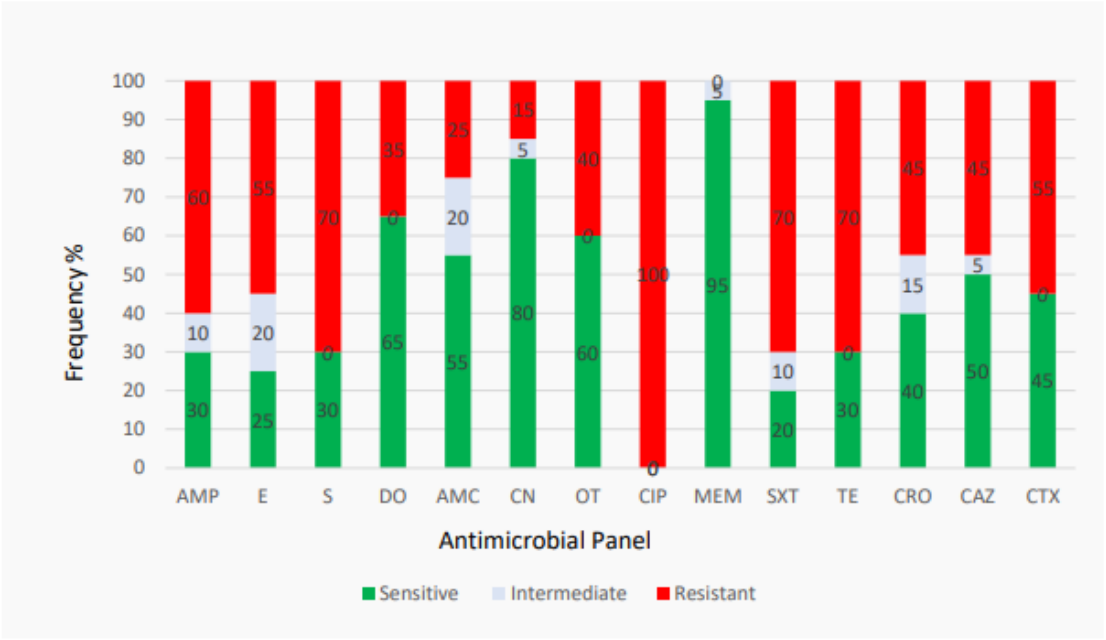

Figure 4.15.: Antibiogram profile of *E. coli* isolates isolated from bovine milk

4.4 Prevalence of Antimicrobial Resistant (AMR) Genes in *S.aureus*

All the phenotypically resistant isolates (n=26) were selected for PCR assays to detect antimicrobial resistant genes and among those 22 (84.6%; 95% CI 65.1%-95.6%) isolates were characterized with the presence of AMR genes. The results on the distribution of different genes detected in the isolates are in Table 4.2. Among the 26 *S. aureus*, 5 isolates (19.2%) were positive to *mecA* gene. *tetK* gene was identified among the 19 (73.1%) *S. aureus*isolates and belonged the highest proportion. On the other hand, *tetM* gene was found between the 2 isolates showing the least percentage. The overall prevalence of all antimicrobial resistant genes among the isolates tested are graphically displayed in **Figure 4.16.**

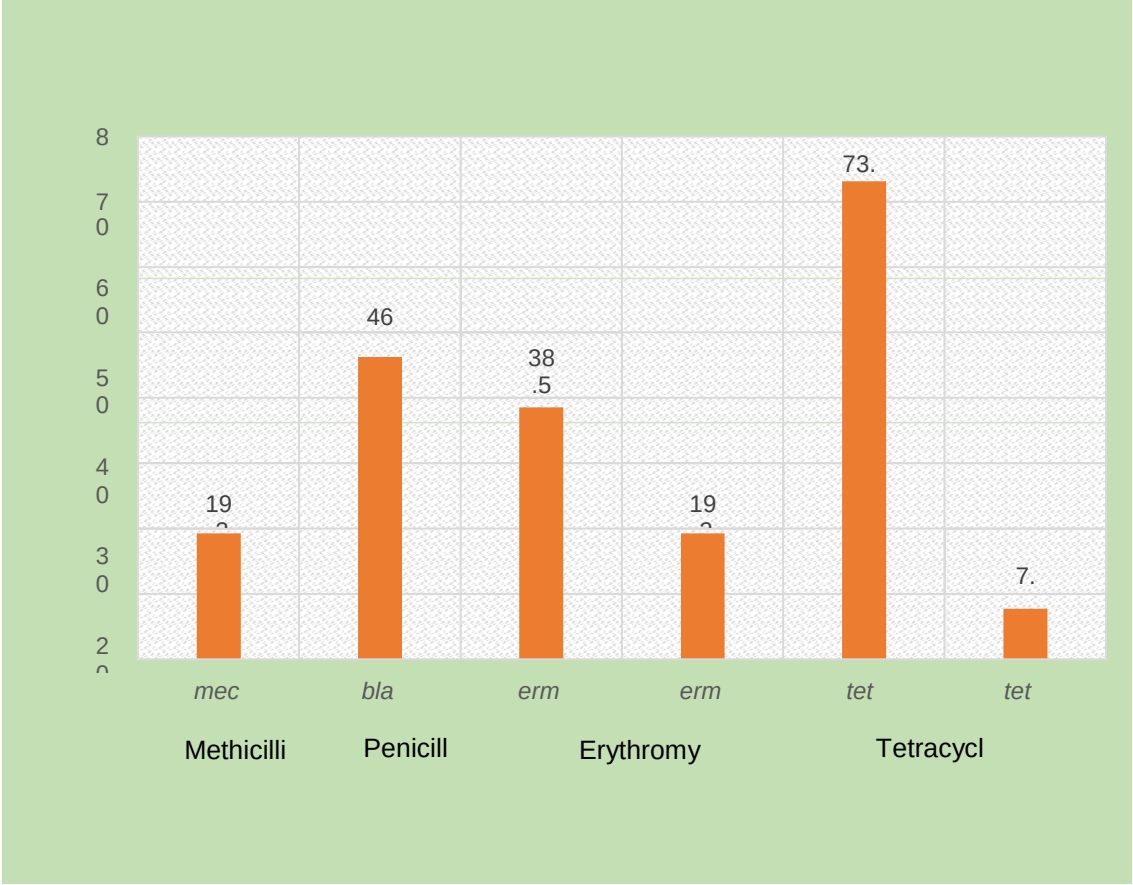

Figure 4.16.: Overall prevalence estimates of resistant genes from *S. aureus* isolates (n=26)

Table 4.2: Antibiogram profile of *S. aureus* isolates isolated from bovine milk (Phenotypically and genotypically found resistant isolates, n=26)

Code of Antibiotic	**Overall Prevalence; 95%CI,(n) [**Phenotypically resistant isolates]	**Overall Prevalence; 95%CI,(n)**[Genotypically resistant isolates]	**Resistant Gene, (n) %**
Ox	46.1% (26.6-66.6), (12)	19.2% (6.6-39.3) (5)	*mecA*, (5) 100%
AMP	69.2% (48.2-85.7), (18)	46.2% (26.6-66.6), (12)	*blaZ*, (12) 100%
E	38.5% (20.2-59.4), (10)	23.1% (8.9-43.6), (6)	*ermB*, (1) 16.7%
			ermC, (5) 83.3%
TE	73.1% (52.2-88.4),(19)	73.1% (52.2-88.4),(19)	*tetK*, (17) 89.5%
			Both *tetM* and *tetK*, (2) 10.5%

OX: Oxacillin, AMP: Ampicillin,E: Erythromycin,TE: Tetracycline

4.5 Prevalence of Antimicrobial Resistant (AMR) Genes in *E.coli*

Phenotypically resistant all isolates (n=20) were selected for PCR assays to detect antimicrobial resistant genes and among those 17 (85%; 95% CI 62.1%-96.8%) isolates were characterized with the presence of AMR genes. The results on the distribution of different genes detected in the isolates are in Table 4.3. Ciprofloxacin resistant gene, *gyrA*, was identified among the 19 (95%) *E. coli* isolates and it was the highest proportion. No colistin resistant gene was detected, whereas, 50% of the *E. coli* isolates represented sulphamethaxosole-trimethoprim resistant gene *sul1* and *sul2*. Similarly, 10 (50%) *E. coli*isolates were positive to ampicillin resistant gene bla_{TEM} and 30% were $bla_{CTX\text{-}M,}$ resistant to ceftriaxone.

The prevalence of all antimicrobial resistant genes among the isolates tested are graphically displayed in **Figure 4.17**.

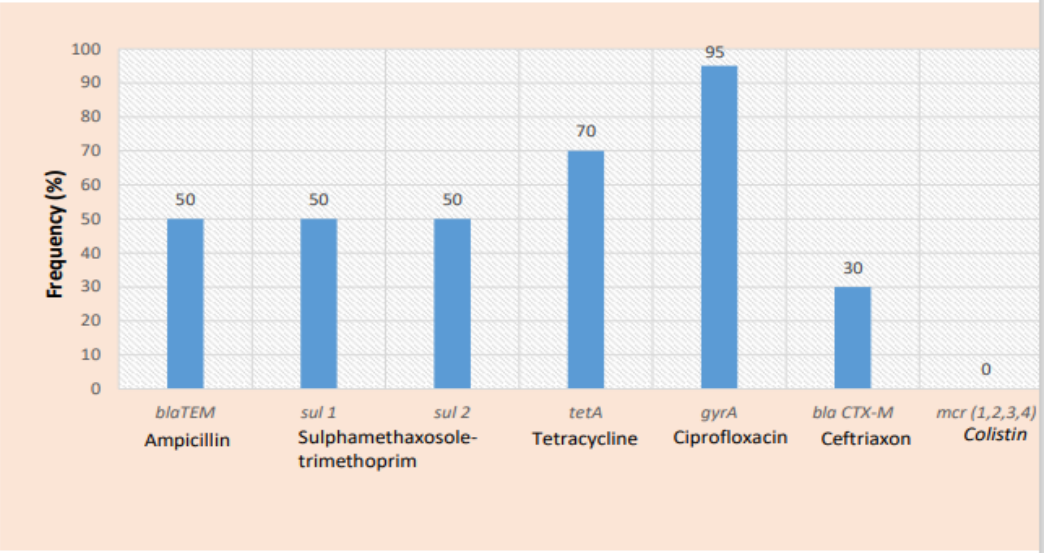

Figure 4.17: Overall prevalence estimates of resistant genes from *E. coli* isolates (n=20)

Table 4.3: Antibiogram profile of *E. coli* isolates isolated from bovine milk (Phenotypically and genotypically found resistant isolates, (n=20)

Code of Antibiotic	**Overall Prevalence; 95%CI,(n)** [Phenotypically resistant isolates]	**Overall Prevalence; 95%CI,(n)**[Genotypically resistant isolates]	**Resistant gene, (n) %**
AMP	60% (36.0-80.8), (12)	50% (27.2-72.8), (10)	bla_{TEM} ,(10) 100%
SXT	70% (45.7-88.1), (14)	65% (40.8-84.6),(13)	*sul 1*, (3) 23%
			sul 2, (3) 23%
			both, (7) 54%
TE	70% (45.7-88.1), (14)	70% (45.7-88.1), (14)	*tetA*, (14) 100%
			tetB, (0)
			tetC(0)
CIP	100% ,(20)	95% (75.8-99.9), (19)	*gyrA*, (19) 100%
			gyrB, (0)
			parC, (0)
CRO	45%,(9)	30% (11.9-54.2), (6)	bla_{CTX-M}, (6) 100%
CT	-	0%	*mcr-1*,(0)
			mcr-2,(0)
			mcr-4,(0)
			mcr-4,(0)

AMP: Ampicillin, SXT: Sulphamethoxazole-Trimethoprim, TE: Tetracycline, CIP: Ciprofloxacin, CRO: Ceftriaxone, CT: Colistin Sulphate

4.6 Overall Multidrug Resistance Pattern of *S. aureus* and *E.coli*

Among the all isolates, 60% *E. coli* isolates and 11.5 % *S. aureus* isolates showed multidrug (MDR) resistant pattern (≥ 3 groups of antimicrobial). Meanwhile, around 15% both *E. coli*and *S. aureus*isolates were not resistant to any kind of antimicrobial. MDR pattern is highlighted in the **Figure 4.18.**

Figure 4.19 is presented with amplicon of some of the *S. aureus*isolates tested positive for the system I (*nuc, ermC*and *tetM*gene), **Figure 4.20** with some*S. aureus* tested positive for the system II (*blaz, ermB* and *tetK*) and Figure 4.21 with some tested positive for the *mecA* gene. **Figure 4.22** with some tested positive for the bla_{TEM}gene in

E. coli. Amplicons showing the presence of the $bla_{CTX\text{-}M}$, *tetK, gyrA* and *sul1*of some of the *E. coli* isolates tested are in **Figures 4.23, 4.24**, **4.25,** and **4.26** respectively

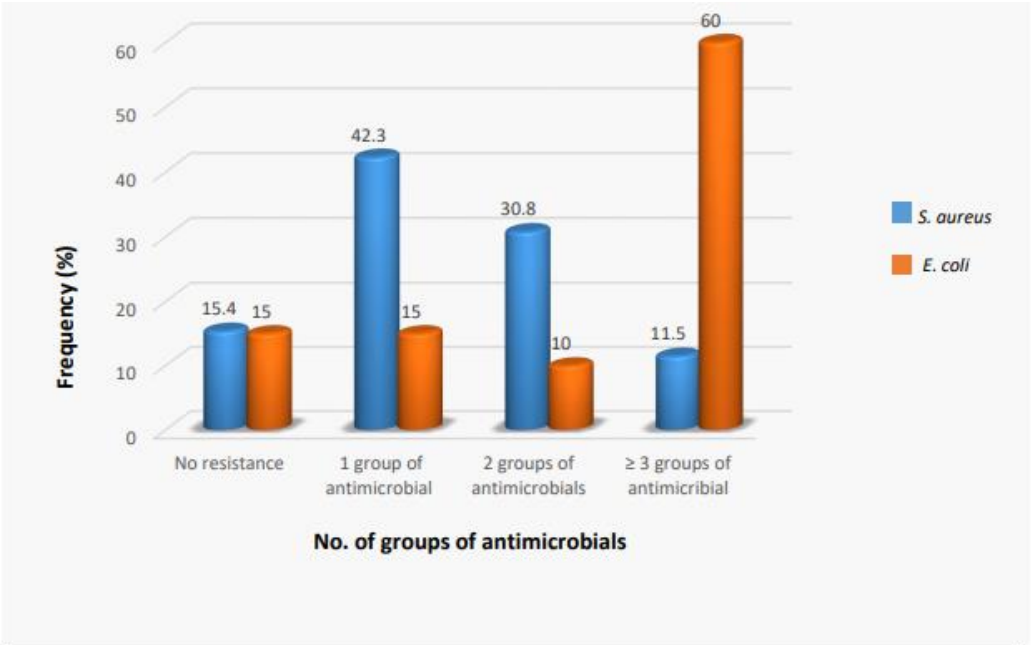

Figure 4.18: Multidrug Resistant Pattern of *S. aureus* and *E.coli*

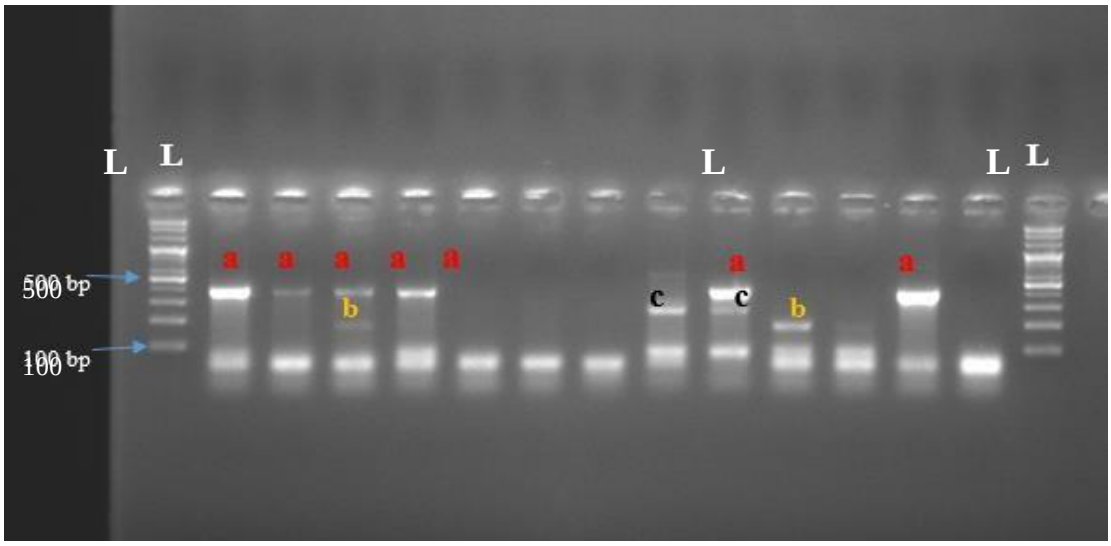

Figure 4.19: MultiplexPCR assay for the detection of the genes; Lane L: 1kb plus DNA ladder; Lane a: *nuc* gene (395 bp); Lane b: *ermC* (190 bp); Lane c: tetM (351 bp) amplicon.

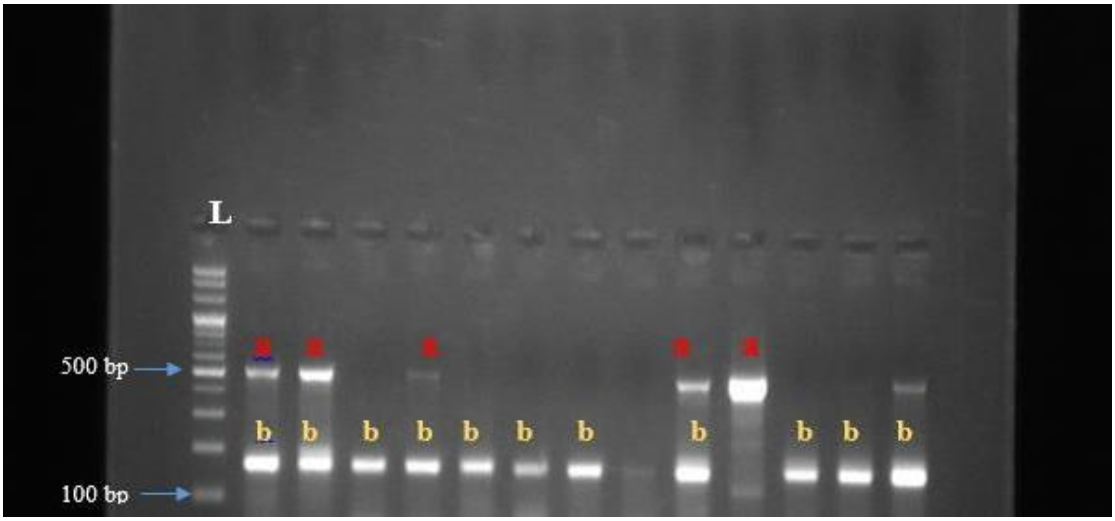

Figure 4.20: PCR assay for the detection of the genes; Lane L: 1kb plus DNA ladder; Lane a: *blaZ* gene (517bp); Lane b: *tetK* (169 bp) amplicon.

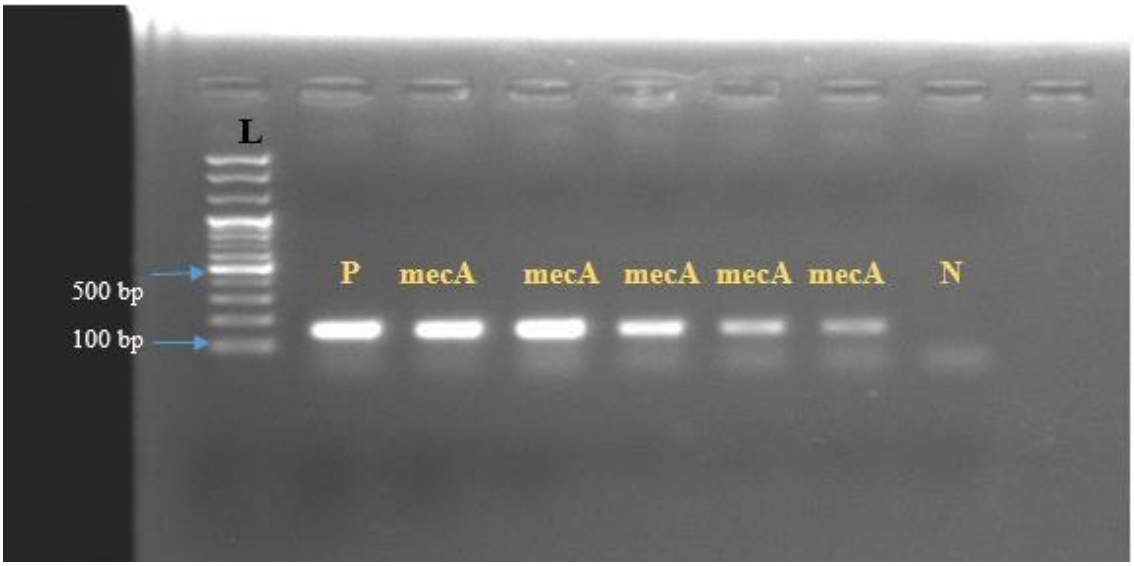

Figure 4.21: PCR assay for the detection of *mecA* gene

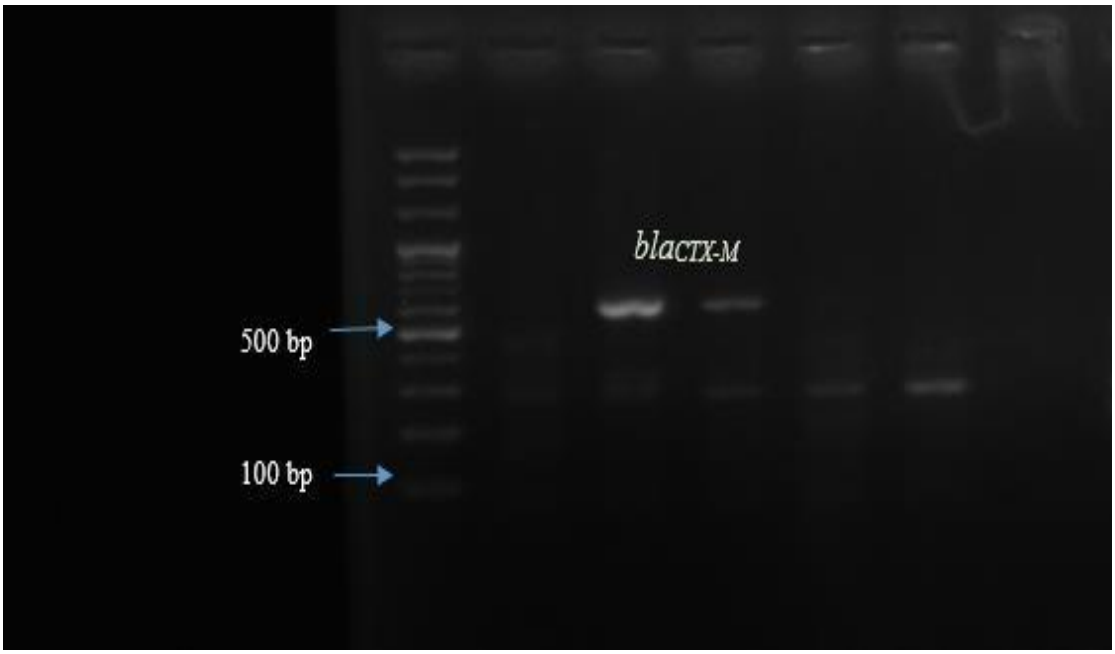

Figure 4.22: PCR assay for the detection of the *bla*$_{CTX-M}$gene (557 bp) in *E. coli*

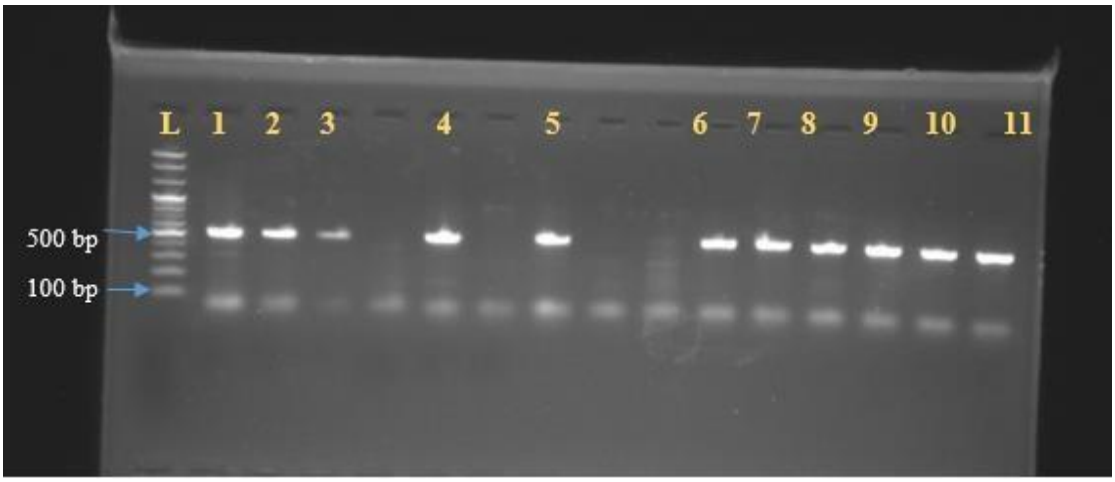

Figure 4.23: PCR assay for the detection of the *tetK* gene (502bp) in *E. coli*; Lane L: 1kb plus DNA ladder; Lane 1-11: *tetK* gene

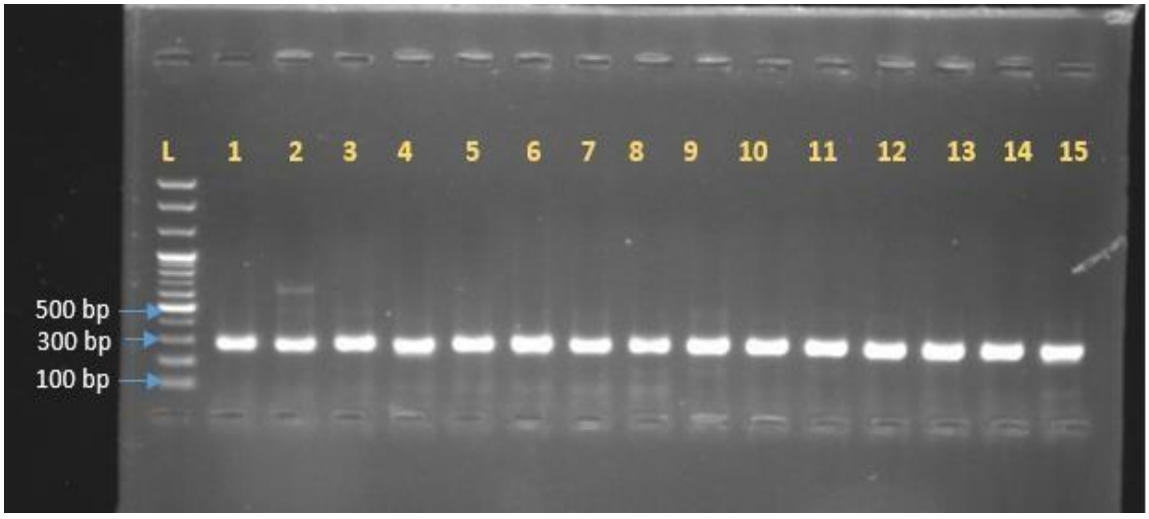

Figure 4.24: PCR assay for the detection of the *gyrA* gene (312bp) in *E. coli*; Lane L: 1kb plus DNA ladder; Lane 1-15: *gyrA* gene

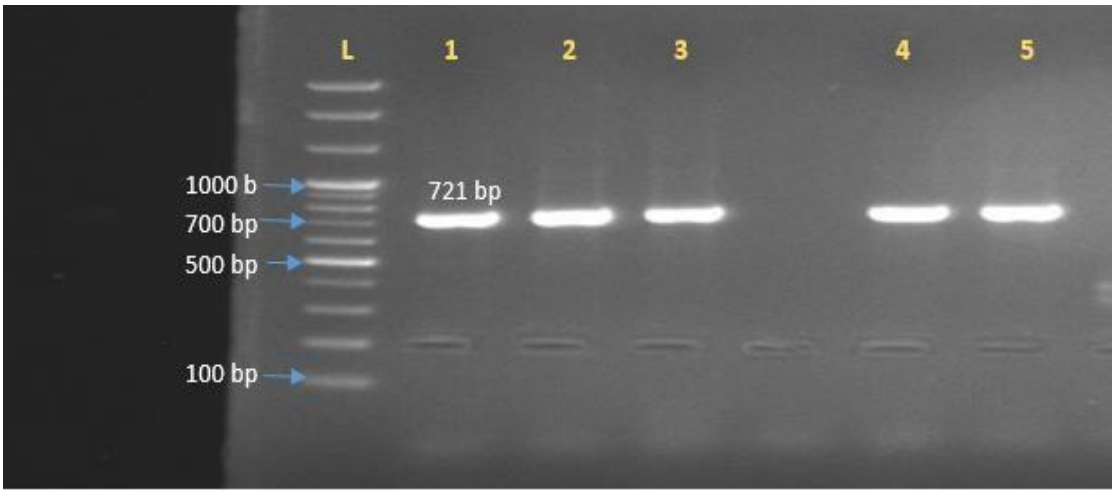

Figure 4.25: PCR assay for the detection of the *sul2* gene (721bp) in *E. coli*; Lane L: 1kb plus DNA ladder; Lane1-5:*sul2* gene

4.7. Risk factors associated with emergence of *S. aureus* and *E. coli* in bovine milk sample

After the univariable logistic regression analysis, the result showed that several factors such as CMT result, floor condition during milking**,** floor cleaning practice and udder cleanliness were remarkably contributing in the presence of *S. aureus* and *E. coli* in raw bovine milk sample **(Table 4.4 and Table 4.6)**. Then the factors were fitted for the multivariable logistic regression model to scrutinize whether those factors were significantly associated with having listed two organisms in sample **(Table 4.5 and Table 4.7)**.

Table 4.4: Output of univariable logistic regression analysis to identify the effect of different management and hygiene related factors on presence of *S. aureus* in bovine milk sample

Variable	Category (N)	No. of *S. aureus*	Prevalence (95%CI)	p-value (chi square)	OR (95% CI)	p value (univariable logistic regression)
Farm Size	≥100 (107)	15	14.02% (8.06-22.07)	0.696	Ref	
	≤100 (68)	11	13. 24% (6.2-23.6)		1.18(0.51-2.76)	0.696
CMT	Negative (105)	3	2.86% (0.6-8.1)	<0.0001	Ref	
	Positive (70)	23	32.9% (22.1-45.1)		16.6 (4.76-58.18)	<0.0001
Floor Condition	Clean (78)	3	3.8% (0.80-10.8)	0.001	-	

during milking	Moderately Clean (55)	11	20% (10.4-32.9)		6.25 (1.65-23.6)	0.007
	Dirty (42)	12	28.6% (15.7-44.6)		10 (2.63-38.0)	0.001
Floor Cleaning practice	Once daily with disinfectants (134)	15	11.2 % (6.4-17.8)	0.014		
	Once only with water and weekly disinfectants (41)	11	26.9% (14.2-43)		2.91 (1.21-7.0)	0.017
Udder Cleanliness	Clean (119)	5	4.20% (1.4-9.5)	<0.0001	-	
	Moderately clean (36)	13	36.1% (20.8-53.8)		12.8 (4.19-39.7)	<0.0001
	Contaminated with mud and cow dung (20)	8	40% (19.1-64)		15.2 (4.28-53.9)	<0.0001

Table 4.5: Multivariate logistic regression model of risk factors for the presence of *S. aureus* in bovine milk sample

Factor	**Category**	**OR**	**95% CI**	**p**
CMT result	negative	Ref	-	-
	positive	10.5	2.76-40.29	0.001

Table 4.6: Output of univariable logistic regression analysis to identify theof different management and hygiene related factors on the presence of *E.coli* in bovine milk sample:

Variable	Category (N)	No. of *E. coli*	Prevalence (95%CI)	p-value (chi square)	OR (95% CI)	p value (univariable logistic regression)
Farm Size	≥100 (107)	5	4.7% (1.5-10.6)	<0.0001	Ref	
	≤100 (68)	15	22.06% (12.9-33.8)		5.7 (1.99-16.8)	0.001
CMT	Negative (105)	2	1.9% (0.23-6.7)	<0.0001	-	
	Positive (70)	18	25.7% (16.0-37.6)		17.8 (4.0-79.8)	<0.0001
Floor Condition during milking	Clean (78)	1	1.28% (0.03-6.9)	<0.0001	-	
	Moderately Clean (53)	3	5.5% (1.1-15.1)		4.44 (0.45-43.9)	0.202
	Dirty (44)	16	36.3% (22.4-52.2)		47.4 (6.0-375.0)	<0.0001
Floor Cleanliness	once daily with disinfectants (134)	5	3.73% (1.2-8.5)	<0.0001		
	once only with water and weekly disinfectants (41)	15	36.59% (22.1-53.1)		14.9 (5.0-44.5)	<0.0001
Udder Cleanliness	Clean (119)	1	0.84% (0.02-4.6)	<0.0001	-	
	Moderately clean (36)	6	16.67% (6.4-32.8)		23.6 (2.73-203.53)	0.004
	Contaminated with mud and cow dung	13	65% (40.8-84.6)		219.1 (25.0-1923.5)	<0.0001

	(20)					

Table 4.7: Multivariate logistic regression model of risk factors for the presence of *E. coli* in bovine milk sample

Factor	Category	OR	95% CI	p
Floor condition during milking	clean	Ref	-	-
	Moderately clean	1.2	0.1-15.7	0.904
	contaminated with mud and cow dung	13.7	1.21-155.27	0.034
Udder cleanliness	clean	Ref	-	-
	Moderately clean	13	1.4-123.9	0.026
	dirty	142.3	14-1451.5	<0.0001

4.8 Risk factors associated with emergence of occurrence of AMR *S. aureus* and AMR *E. coli* in dairy farms

From the univariable logistic regression analysis it was evident that the odds of having AMR *S.aureus* was 2.41 (95% CI: 0.96-6.07) times higherin the farms where antibiotic course was not completed properly **(Table 4.8)**. Furthermore, the odds of having AMR *E.coli* in bovine raw milk was almost 23 (95% CI: 6.09-84.25) times higher in the dairy farms avoiding the practice of antibiotic course completion, 12 (95% CI: 3.31-44.12) times higher where withdrawal period was not followed, 18.38 (95% CI: 4.98-67.82) times attributed when the farms avoided proper dose maintenance and 7.62 (95% CI: 2.10-27.63) times greater in those dairy farms having the farm personnel without the knowledge of AMR**(Table 4.9)**.

Table 4.8: Output of univariable logistic regression analysis to identify the effect of different AMR related factors on occurrence of AMR *S. aureus* in dairy farms

Variable	Category (N)	No. of AMR resistant *S. aureus*	Prevalence (95%CI)	p-value (chi square)	OR (95% CI)	p value (univariable logistic regression)

Antibiotic Course Complete	yes (134)	14	10.45% (5.8-16.9)	0.056	Ref	
	no (41)	9	21.95% (10.56-37.61)		2.41 (0.96-6.07)	0.062
Withdrawal period maintain	yes (117)	13	11.11(6.1-18.2)	0.259	Ref	
	no (58)	10	17.24 (8.6-29.4)		1.67 (0.68-4.07)	0.262
Maintain Proper dose	yes (129)	14	10.85%(6.1-17.5)	0.133	Ref	
	no (46)	9	19.57% (9.4-33.9)		1.92 (0.8-4.9)	0.138
Farm personnel know about AMR	yes (101)	13	12.87% (7.03-21)	0.901	Ref	
	no (74)	10	13.51% (6.7-23.4)		1.06 (0.44-2.56)	0.901

Table 4.9: Output of univariable logistic regression analysis to identify the effect of different AMR related factors on the occurrence of AMR *E.coli* in dairy farms

Antibiotic Course Complete	yes (134)	3	2.24% (0.46-6.4)	<0.0001	Ref	
	no (41)	14	34.15% (20.1-50.6)		22.64 (6.09-84.25)	<0.0001
Withdrawal period maintain	yes (117)	3	2.56%(0.53-7.31)	<0.0001	Ref	
	no (58)	14	24.14% (13.9-37.1)		12.09 (3.31-44.12)	<0.0001
Maintain Proper dose	yes (129)	3	2.33% (0.48-6.64)	<0.0001	Ref	
	no (46)	14	30.43% (17.7-45.6)		18.38 (4.98-67.82)	<0.0001
Farm personnel know about AMR	yes (101)	3	2.97% (0.62-8.4)	<0.0001	Ref	
	no (74)	14	18.92% (10.7-29.7)		7.62 (2.10-27.63)	0.002

4.9 Multivariable analysis

After adjustment of the confounding effect of the factors in the multivariable logistic regression model, a potential risk factor (CMT result) was identified in this study. The odds ratio of presence of *S. aureus* in the CMT test 'positive' samples was 10.3 times (95% CI: 2.6-40.5) higher than the CMT test 'negative' samples **(Table 4.5)**.

In the case of *E.coli*, the multivariable analysis showed that the presence of *E.coli* increased by 13.7 times if the floor had been contaminated with mud and cow dung during the milking time and exclusively a hike odds found in case of dirty udder of cows, as well**(Table 4.7)**.

4.10 GenBank accession number for Methicillin Resistant *S. aureus* (mecAgene):

Among the 26 *S. aureus* isolates 12 showed resistance against Oxacillin (46.1%; 95% CI 26.6%–66.6%) phenotypically. All the 12 phenotypically oxacillin-

resistant isolates were tested for the presence of mecA gene by PCR. Of them 5 isolates were found positive (41.6%; 95% CI 15.1-72.3%) for the possession of mecA gene. The sequence data of four of those were selected for the direct submission to GenBank. The GenBank accession number for four submitted mecA gene sequences are MW439194, MW439195, MW439196, and MW439197.

CHAPTER-V

DISCUSSIONS

The overall prevalence of *S. aureus* and *E.coli* in bovine raw milk was assessed in the present study. The isolates showed antimicrobial-resistant attributes phenotypically were selected to identify antimicrobial resistant (AMR) genes, responsible for Multidrug-Resistant (MDR) features. Finally, the risk factors accountable for the AMR *S. aureus* and *E. coli* in association with the dairy farm's circumstances were evaluated. The overall prevalence of *S.aureus* in bovine raw milk was around 15% (26 out of 175 samples) in this study which is consistent with the finding of Patel *et al.* (2018) carried out in Gujrat, India; however a much higher prevalence in Beijing, China (46.2%) was reported by Wang *et al*. (2018). Nevertheless, the prevalence of Methicillin-resistant *S. aureus* (MRSA) in this study was approximately 3% showed similarity with the findings of Papadopoulos *et al*. (2018) who stated alike prevalence of MRSA (3%) in dairy products in north-western Greece. About 11.4% of the total milk samples screened were positive to *E. coli* isolates in this study which showed similarity to Batabyal *et al*. (2018), Elmonir *et al*. (2018) and Kamaruzzama *et al*. (2015), reported 12.1-13.7% *E. coli* positivity in the bovine milk samples in their study area; West Bengal, Egypt and Putra, Malaysia,respectively.

The cultural sensitivity test of 26 *S. aureus* isolates showed highest resistance against streptomycin (80.7%) followed by tetracycline (73.1%), ampicillin (69.3%),gentamicin (57.6%),ceftriaxone (50%), oxacillin(46.1%), ciprofloxacin (46.1%),oxytetracycline (38.5%), erythromycin (38.5%), doxycycline (34.6), and sulfamethoxazole-trimethoprim (30.9%). We observed that all the *S. aureus* isolates were resistant to at least one of the selected antimicrobials phenotypically. A similar elevated resistance to tetracycline, ampicillin and oxacillin was reported by Omwenga *et al.* (2020) where nearly 79% isolates were resistant to tetracycline. Another study reported that *S. aureus* isolates from bovine milk in Morogoro, Tanzania (Mohammed *et al*., 2018) exhibited 30.4% oxacillin resistance feature which is in harmony with this study. Relatively close research findings were published from different other studies (Jamali *et al*., 2014; Liu *et al*., 2017; Thaker *et al*.,2013).

Among the 20 *E. coli* isolates from bovine milk, all were associated with phenotypic Multidrug Resistant (MDR) traits. The cultural sensitivity test of *E.coli* isolates showed highest resistance against ciprofloxacin (100%) followed by streptomycin (70%),

sulfamethoxazole-trimethoprim (70%), tetracycline (70%), ampicillin (60%), erythromycin (55%), cefotaxime (55%), ceftriaxone (45%), cefatazidime (45%), oxytetracycline (40%), doxycycline (35%), amoxicillin+clavulanic Acid (25%), gentamicin (15%), and meropenem (5%). Different studies revealed a wide range of prevalence of MDR *E.coli* such as an escalated resistance against tetracycline (75%) (Thaker *et al*., 2017), ampicillin (51.85%) (Adzitey *et al.*, 2016), amoxicillin/clavulanic acid (83.33%), ceftazidime (91.67%) (Batabyal *et al*., 2018) were reported based on Culture Sensitivity test.

The emergence of methicillin-resistant *S. aureus* (MRSA) infection in dairy farms is of great concern for animal and public health worldwide. Recently, due to the excessive use of antibiotics in dairy industries and food animals, especially in case of dairy cows with subclinical and clinical mastitis, an MRSA strain with zoonotic potential emerged and became a pronounced risk to human health (Yi *et al*., 2018). In this study, A total of 26 (15%) *S. aureus* isolates were identified from bovine milk of which 5 (19.2%) possessed methicillin resistant (*mec*A) gene. A relatively similar result was found in the Aydin region in Turkey where 17.2% methicillin resistant gene *mecA* wasisolated from bovine milk samples (Türkyılmaz et al., 2010). On the contrary, a substantially high multidrug resistance rates were observed among the MRSA isolates reported in some recent studies, such as 25% methicillin (*mec*A) positive genes from bovine milk in India (Shah et al., 2019) and 52. 38% in Ilam province, Iran (Nemati, 2020) indicated an alarming scenario of antimicrobial practice worldwide in the dairy industry. Poor farm management and indiscriminate use of antibiotics may augment MRSA emergence in bovine milk (Joshi *et al*.,2014).

We observed that *S. aureus* showed resistance to most of the antibiotics which are frequently used for the treatment of bovine mastitis, especially penicillin. In this study, Penicillin resistant gene blaZ was detected in 46.2% (12/26) of bovine milk samples. Similar findings were reported from different household dairy farms of Lahore where genotypic characterization of isolates resulted in 40.0% (6/15) positive for bla Z (khan *etal*.,2020). Data from other countries also have exhibited a sharp high prevalenceof penicillin-resistant *S. aureus* ranging from 94.6 to 97% *blaZ* from the isolates (Yang *et al.*, 2015; Martini *et al*., 2017). Resistant genes *tetK* (73.1%), *tetM* (7.7%), *ermB* (3.8%) and *ermC* (19.2%) were detected among resistant isolates of *S. aureus* strain in this study, whereas Bahraminia *et al*. (2017) reported a very high proportion of resistant genes with 32% ermB and 20.40% ermC. Another study revealed that 85.71% isolates

harboured the *tetK* gene (Rahi *et al*., 2020).

Escherichia coli causes a broad range of infections in dairy cattle, as well as in humans. The prevalence of extended-spectrum beta-lactamase (ESBL)-producing *E. coli* is expanding in the world day by day (Batabyal *et al*., 2018). In *E. coli*, horizontal transfer of genes coding for ESBL is a common mechanism of dissemination of resistance to a broad range of β-lactams. Furthermore, ESBL-producing *E. coli* strains have a higher inclination to express multidrug resistance than non-ESBL-producing strains (Karkaba et *al.,* 2017), therefore complicating infection management. Approximately, 30% *E. coli* isolates were positive to $bla_{\text{CTX-M}}$ gene might be responsible for producing ESBL and 50% isolates showed bla_{TEM} through PCR essay in this study. The findings are supported by Ibrahim *et al*., 2016, where 22.6% $bla_{\text{CTX-M}}$ positive *E. coli* isolates were found with a low proportion of bla_{TEM} (7.5%). However, Batabyal *et al*. (2018) and Kamaruzzaman reported a high prevalence of ESBL-producing *E. coli* in milk showing 54.54% and 66.7% $bla_{\text{CTX-M}}$ gene in their isolates, respectively. Meanwhile, 65% *E.coli* isolates were genotypically resistant to sulfamethoxazole-trimethoprim with *sul1* (10, 50%) and *sul2* (10, 50%) genes supported by the study findings of Mahanti *et al*. (2020). Moreover, the most frequently identified resistant antimicrobial was ciprofloxacin, thus gyrA gene was detected in 95% of the *E.coli* isolates whereas the second highest identified resistant gene was tetA (70%) in this study. Tahar et al. (2020) reported 44.2% tetA positive *E.coli* isolates, much lower than the current study. Although 2% comistin resistant gene (*mcr-1*) was found in bovine mastitic milk in a previous study (Liu *et al*., 2020), we identified none among the *E.coli* isolates however from bovine non mastiticmilk.

Different studies revealed that the amount of antibiotic used in dairy farms contributed as a pivotal risk factor for antimicrobial resistant bacteria (Chantziaras *et al.,* 2014; Lam*etal.,* 2014), supported the current study. However, many of the effects of univariable analysis were found insignificant in multivariable models might be because low number of observations lowered the study power.

On the other hand, CMT positive samples showed almost 10 times higher association of having *S.aureus* in raw milk samples and it was confirmed by multivariable logistic regression analysis. Also, in case of *E.coli*, the multivariable analysis showed that the presence of *E.coli* increased by 13.7 times if the floor had been contaminated with mud and cow dung during the milking time and exclusively a hiked odds was found in case of dirty udders of cows, as well. Thus, the analysis highlighted the 'milking hygiene'

practice as a crucial fact for the increased prevalence of bacteria in raw milk and this circumstance was signified by the study findings of Schnitt *et al.* (2020) and Antoci *et al.*, (2013) where authors concluded that improper milking hygiene procedures may be a substantial risk factor for MRSA transmission within dairy herds.

CHAPTER-VI

CONCLUSION

Presence of *S. aureus* and *E. coli* in dairy products is the indication of breach in farm hygiene strategy. The study revealed a significant prevalence of *S. aureus (*14.86%) and *E. coli* (11.43%) in bovine raw milk sample within Chattogram Metropolitan Area. Moreover, 84.6% *S. aureus* and 85% *E. coli*were characterized with the presence of AMR genes indicating a complex phenomenon. Understanding the attitude and knowledge of farmers and veterinarians toward AMU and AMR is a crucial step for the design of strategies to combat this public health threat. The lack of detailed AMU data impacts our ability to interpret surveillance data on AMR and to design efficient interventions. Therefore, monitoring systems to fill this knowledge gap should be prioritized. Finally, the ecology of AMR should be addressed with a holistic, One-Health approach combining expertise from different disciplines.

CHAPTER-VII

LIMITATIONS

The study has following limitations:

- Due to time and resource limitation the study was conducted in small scale. In future, the study can be conducted involving a higher samplesize.
- Minimum inhibitory concentration (MIC) of any of the resistant isolates was not performed due to time and resourcelimitation.
- Sequencing of the described genes could have provided better understanding on their source of origin and spread.

CHAPTER-VIII

Appendix Questionnaire survey

1. Farm level baseline Information:

- ID no of farm:
- Date:
- Farm's and Farmer's contact address:
- Farm size:
- Types of breed:
- Housing system:
- Rearing system:
- Floor cleanliness:

2. Individual level information:

- Breed:
- BCS:
- Age:
- Body weight:
- Milk yield (daily):
- Duration of lactation:
- Lactation no:
- Date of occurrence:
- Udder cleanliness:
- Any history of disorder in & around calving: Yes /No
- Any history of lameness: Yes /No
- Floor cleanliness during milking:
- Udder cleanliness during milking:
- Floor cleaning frequency:

- **CMT Result:**

3. General information:

- Any knowledge about mastitis: Yes / No
- How do you manage problems of your animals: With the help of vet / VFA / Self
- What types of drug usually used?
- Any idea about CMT: Yes /No
- Materials used for cleaning:
- Do you complete the antibiotic course?
- Do you maintain withdrawal period?
- Do you maintain actual dose according to prescription?
- Do you have knowledge about AMR?

CHAPTER-IX

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CHAPTER-X

BIOGRAPHY

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