ANTIMICROBIAL RESISTANCE PATTERN OF BACTERIA ASSOCIATED WITH RESPIRATORY TRACT INFECTION IN GOATS

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Session: 2019-2020

A thesis submitted in the partial fulfilment of the requirements for the degree of Master of Science in Medicine

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JUNE 2021

Authorization

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June 2021

Acknowledgements

At the very beginning, I would like to express my ever gratefulness to **The Supreme God, Lord Buddha** for giving me such strength and opportunity to complete the research and thesis successfully for the degree of **Master of Science (MS)** in Medicine under the **Department of Medicine and Surgery**, Chattogram Veterinary and Animal Sciences University (**CVASU**), Bangladesh.

Secondly, I would like to express the first and foremost appreciation, deepest sense of gratitude and best regards to my respected supervisor **Prof. Dr. Mohammed Yousuf Elahi Chowdhury,** Department of Medicine and Surgery (DMS), CVASU. It was my pleasure and great experience to work with him. His cordial supervision and important suggestions helped me a lot complete this thesis.

I feel much pleasure to convey my profound thanks to my co-supervisor, **Prof. Dr. Mohammad Mahmudul Hassan**, Department of Physiology, Biochemistry and Pharmacology, CVASU for his valuable advice, scholastic guidance, and well thought suggestions during my study.

I humbly thank to **Dr. Shuvo Singha**, Phd fellow, Dept. of Veterinary and Animal Science, University of Milan, Italy, and **Md. Ashiqur Rahman**, Senior officer, BRAC Artificial Insemination Enterprise, for their suggestions, encouragement and support during research work.

I would like to express my deep sense of gratitude to **Poultry Research and Training Centre**, CVASU, and **Ministry of Science and Technology**, People's Republic of Bangladesh for providing necessary research fund.

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

Finally, I am ever indebted to my respected mother, siblings, husband, seniors and juniors for all of their immense sacrifices, blessings and impulsive encouragement.

June 2021

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List of abbreviations

Abbreviation and symbols	Elaboration
AMR	Antimicrobial Resistance
AMC	Amoxicillin-Clavulanic acid
BHIB	Brain Heart Infusion Broth
MDR	Multidrug-resistant
RTI	Respiratory Tract Infection
%	Percent
>	Greater than
<	Less than
≥	Greater than equal
≤	Less than equal
=	Equal to
°C	Degree Celsius
MHA	Mannitol Salt Agar
MHB	Muller-Hinton Broth
EMB	Eosin Methylene Blue agar
Bp	Base Pair
BPW	Buffered Peptone Water
CFU	Colony Forming Unit
CI	Confidence Interval
CLSI	Clinical and Laboratory Standards Institute
CS	Culture Sensitivity
PRTC	Poultry Research and Training Center
SAQTVH	S. A. Quaderi Teaching Veterinary Hospital
CVASU	Chattogram Veterinary and Animal Sciences University
DAEC	Diffusely Adherent *E. coli*
DNA	De-oxy Ribonucleic Acid
S. aureus	*Staphylococcus aureus*
P. multocida	*Pasteurella multocida*

E.coli	*Escherichia coli*
InPEC	Intestinal pathogenic *E. coli*
ExPEC	Extra-intestinal pathogenic *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
EHEC	Enterohemorrhagic *E.coli*
EAEC	Enteroaggregative *E. coli*
EIEC	Enteroinvasive *E. coli*
AIEC	Adherent-invasive *E. coli*
DAEC	Diffusely adherent *E. coli*
UPEC	Uropathogenic *E. coli*
NMEC	Neonatal Meningitis *E. coli*
APEC	Avian pathogenic *E. coli*
PORT	Pneumonia Patient Outcome Research
CAP	Community acquired pneumonia
HP	Haemorrhagic pneumonia
EMB	Eosin Methylene Blue
ESBL	Extended Spectrum β-Lactamase
ETEC	Enterotoxigenic *E.coli*
CNF1	Cytotoxic necrotizing factor 1
PEGE	Pulse field agarose gel electrophoresis
MLST	Multilocus sequence typing
PLs	Phospholipids
LPs	Lipopolysaccharides
Kb	Kilo Base
µL	Microliter
mA	Milliampere
mL	Millilitre
Mm	Millimetre
Ng	Nanogram
Mm	Millimetre

MRSA	Methicillin Resistant *Staphylococcus aureus*
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
Rpm	Rotation Per Minute
STEC	Shiga Toxigenic *E. coli*
TAE	Tris Acetate EDTA
WHO	World Health Organization
w/v	Weight/Volume
CIP	Ciprofloxacin
TE	Tetracycline
CRO	Ceftriaxone
SXT	Sulfamethoxazole & Trimethoprim
CN	Gentamycin
ENR	Enrofloxacin
AMP	Ampicillin
AML	Amoxicillin
AZM	Azithromycin
P	Penicillin

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ABSTRACT

Antimicrobial resistance of bacteria causing respiratory tract infection (RTI) in ruminants especially in goats is a very common issue in today's world. In this study, a total number of 120 nasal swab samples were collected from goats having respiratory illness presented to S. A. Q. Teaching Veterinary Hospital (SAQTVH), CVASU. This study was designed to isolate and identify bacteria causing respiratory tract infections including *S. aureus*, *E. coli* and *P. multocida* in goats with their antibiotic resistance pattern. In the investigation, 16 (19.2%) isolates were confirmed as *S. aureus* whereas 8 (6.67%) isolates were confirmed as *E. coli* based on cultural, morphological and biochemical tests. Then the isolates were screened against 11 antimicrobial agents using the disc diffusion method. All the *S. aureus* isolates were resistant to ampicillin and highest number of isolates were equally resistant to amoxicillin and penicillin (93.75%, 95% CI 69.77 – 99.84) followed by amoxicillin-clavulanic acid (62.5%, CI 35.43 – 84.8). All the isolates of *E. coli* were resistant to amoxicillin and penicillin and most of the isolates were equally resistant to ampicillin and azithromycin (87.5%, CI 47.35 – 99.68). Moreover, higher resistance against enrofloxacin (62.5%, CI 24.49 – 91.48) and tetracycline (50%, CI 15.7 – 84.3) also found. Furthermore, highest number of *S. aureus* isolates (85.71%) showed multidrug resistance (MDR) against atleast 3 groups of antibiotics including the combination of beta-lactam, fluoroquilonone and macrolides (33.33%) and the combination of beta-lactam, fluoroquilonone, tetracycline (33.33%) groups. In case of *E. coli*, 40% isolates were found to be multidrug resistant against the combination of beta-lactam, fluoroquilonone, and macrolides. The infection rate of both bacteria were higher in cross breeds (*S. aureus*: 28.57%, CI 3.67 – 70.96 and *E. coli*: 14.29%, CI 3.67 – 70.96), goats that reared in farms (*S. aureus*: 16.67%, CI 4.73 – 37.38 and *E. coli*: 8.33%, CI 1.03 – 27), non-vaccinated goats (*S. aureus*: 14.42%, CI 8.3 – 22.67 and *E. coli*: 6.73%, CI 2.75 – 13.38), goats having poor BCS (*S. aureus*: 19.35%, CI 7.45 – 37.47 and *E. coli*: 9.68%, CI 2.04 – 25.75) and goats diagnosed with aspiration pneumonia (23.08%, CI 5.04 – 53.81). However, any of the association with risk factors was not significant according to the study.

This study identified pathogens in goats responsible for RTI with their AMR pattern in SAQTVH, Chattogram. Potential risk factors, measuring the strength of association of RTI caused by these pathogens were also determine.

CHAPTER-1

INTRODUCTION

Antimicrobial resistance (AMR) is considered one of the most prevalent global threats to both human and animal health (Vidovic et al., 2022). In the livestock sector, antimicrobials including antibiotics, antivirals, antifungals, and antiparasitics are commonly used for treatment, prophylaxis, and growth promotion to maintain health and improve productivity (Adekanye et al., 2020). Antimicrobials may become resistant to microbes because of irrational use, inherent capability of natural resistance of certain bacteria, genetic mutation, and acquired resistance from their surroundings (Bazzi et al., 2022 and Khan et al., 2020). The negative impacts of AMR can be treatment failure, scarcity of therapeutics resulting increased severity of illness, increased mortality, reduced productivity and increased production expenditure (Adekanye et al., 2020, Amin et al., 2020 and Khan et al., 2020). Very common and therapeutically significant microorganisms like *Escherichia coli*, *klebsiella pneumoniae*, *Salmonella* spp, *Enterococccus* spp, *Staphylococcus aureus* etc. are found to be bearing multi-drug resistant genes now a day's which is very alarming for animal and public health (Robinson et al., 2016).

Livestock is one of the most important sectors that contribute both poverty alleviation and the economy of agriculture-based country like Bangladesh. Small ruminants, in particular goats are very important in rural economies and nutrition that are widely used to reduce poverty here. There are about 26.6 million goats (*Capra hircus*) in this country (DLS, 2021). As such goat rearing is considered superior to the others in agricultural sector because of the assurance of return on investment in a relatively short period of time. Especially, poor people who are not able to buy and rear large ruminants, goats are ideally suitable for them. For this reason, goat rearing is becoming popular among poor women under scavenging system (Choudhury et al., 2013) while also contributing to the rural economy through women empowerment and income generation. Goats serve several activities and have a unique ability to adapt and maintain themselves in hard environmental conditions. That is why, the goat is described as "poor man's cow" (Clothier et al., 2012). According to the Food and Agriculture Organization (FAO), goat meat and skin account for 38% and 28% of all livestock meat and skin production in Bangladesh, respectively (Sarker and Islam, 2011). As goat rearing is less costly, less laborious, and more profitable, poor farmers

and distressed women rear goats for meat, milk and leather (Nath et al., 2014; Momin et al., 2011), thus play an important role in the national economy.

However, different infectious diseases such as pneumonia, enteritis etc. are considered as the major constraint of livestock development throughout the world that increases the production cost because of expensive treatment cost. There are multiple impacts of these diseases such as loss of farmers due to high mortality, reduced productivity, treatment and sanitation cost, poor quality livestock products, reduced market value etc. Among all diseases, respiratory tract infection (RTI) of all ages is considered as one of the most common illnesses in goats, which increase mortality and reduce the profit of farmers (Asaduzzaman et al., 2013; Momin et al., 2011). RTI's are generally caused by both physical stressors such as sudden weather changes, poor ventilation, high stocking density, transportation etc. and predisposing viral, bacterial and parasitic infection including *Peste des petits ruminants virus (PPRV), para influenza type 3, adenovirus, Pasteurella multocida (P. multocida), Mannheimia haemolytica, Mycoplasma* spp., *Pseudomonas* spp., *Bordetella pertussis, E. coli*, *Staphylococcus* spp., *Bacillus* spp., *Dictyocaulus filaria, Protostrongylus rufescens, Varestrongylus pneumonicus*, etc. (Berge et al., 2006; Asaduzzaman et al., 2013; Momin et al., 2014; Sharma, 1994). RTI is characterized by high fever (104-106^{0}C), mucopurulent nasal discharge, coughing, dyspnea, anorexia, and depression (Rawat et al., 2019), mostly caused by bacteria are commonly found in respiratory tract and frequently associated with outbreak of respiratory diseases and death of goats in all ages under immune suppressive condition.

Several antimicrobials such as sulfonamide, ceftriaxone, penicillin, gentamicin, ciprofloxacin, amoxicillin, erythromycin, sulfadimidine, gentamicin-sulfadiazine-trimethoprim combination, tylosin, nalidixic acid, norfloxacin etc. are commonly used to treat different respiratory tract infection (Chowdhury et al., 2021; Khan and Rahman, 2018; Asaduzzaman et al., 2013) in Bangladesh. However, due to indiscriminate use, antimicrobial agents are losing their ability to stop growing or killing bacteria. As a result, standard treatment becomes ineffective; infection persists and may spread to others (WHO, 2019). Since the past 2 decades, a terrible rise in antibiotic resistance has been reported in many countries including Bangladesh. Moreover, *Staphylococcus* spp. was found to be resistant against penicillin, nalidixic acid, erythromycin, tetracycline, ciprofloxacin, trimethoprim and metronidazole (Sultana, 2019; Asaduzzaman et al., 2013; Momin et al., 2011), *E. coli* against

ampicillin, amoxicillin-clavulanic acid, sulfamethoxazole-trimethoprim, tetracycline, streptomycin and gentamycin (Islam et al., 2016), and *P. multocida* against tetracycline, sulfamethoxazole, erythromycin and penicillin (Akter et al., 2018; Momin et al., 2011). Its consequences the prolongation of treatment and delayed the responses against diseases. Subsequently, resistant organisms are becoming more resistant and human get resistant to the antibiotics due to transmission of resistant organism in human body from environment and food animal. However, only a few studies conducted regarding organisms causing respiratory tract infection of goats in Bangladesh. No comprehensive research has been done yet based on the identification of organisms causing respiratory tract infection with their resistance pattern to the antibiotics in Chattogram even after occurrence of upper respiratory tract infection and pneumonia recorded as 8.74% and 5.61%, respectively in this area (Nath et al., 2014). Therefore, determining the specific causative agents producing respiratory tract illness an

d their status against antimicrobials commonly using to treat in goats was the goal of the current study.

The following were the precise objectives of the current study:

1. Isolation and identification of the common bacteria causing respiratory tract infection in goats.
2. Determination of antimicrobial resistance pattern of bacteria from infected goats to select appropriate antibiotic during treatment.

CHAPTER-2

REVIEW OF LITERATURE

Goats usually suffer from different infectious (bacterial, viral, parasitic etc.) and non-infectious diseases. Respiratory tract infection usually occur in goats and bacteria is one of the major causes of RTI in goats. Antibiotics commonly used to treat goats infected with different bacterial RTI. Antibiotic resistance is increasing day by day due to indiscriminate use of antimicrobial agents. Information on antimicrobials, antimicrobial resistance, bacteria causing respiratory tract infection has thoroughly reviewed in the following literature. This review focuses on the up-to-date scientific information based on the past studies and describe the present condition on antimicrobial resistance in RTI infected goats.

2.1 History and scope of antimicrobial resistance:

Resistance to drug is a natural phenomenon. The more susceptible organisms succumb after exposure to an antimicrobial, leaving behind those resistant to the antimicrobial and pass on their resistance to their offspring. Antimicrobial resistance has become an emerging global health threat due to indiscriminate use of antimicrobials both in human and animals. Now-a-days, most of the clinically important bacteria are multiple drug resistance and multidrug resistant genes commonly found in pathogens like *klebsiella pneumoniae, Salmonella* spp., *Enterococcus* spp. and *staphylococcus aureus* (Robinson et al., 2016). Antimicrobial drug resistance increased after use of antibiotics as growth promoter and therapeutic and prophylactic application (Roess et al., 2013). Within few years after invention, new antibiotics got resistance due to inappropriate use. Consequently, antibiotic resistant microorganisms are alarming high in human as well as animals. In 1928, the very first antibiotic discovered by Sir Alexander flaming (1881-1955) that started a revolution in the medicine. Ernst Chain and Howard Florey Purified Penicillin G in 1942 (Durand et al., 2019). Penicillin became very popular for treatment after invention. After that, several antibiotics have discovered as tetracycline, chloramphenicol, and gentamycin but unfortunately, *S. aureus* developed resistance against penicillin and was no longer susceptible (Davies and Davies, 2010).

In 1950-1960, first Multidrug resistance recorded in enteric bacteria such as *Escherichia coli* (*E. coli*) and *Salmonella* spp. (Spang et al., 2013). According to the surprising discovery of genetically antibiotic resistance in Japan in the mid-1950s, antibiotic resistance genes could be disseminated by bacterial conjugation among an

entire species of bacterial pathogens (Ezeamagu 2014; Munita and Arias, 2016). All classes of antimicrobials such as sulfonamides, penicillins, tetracyclines, ampicillins, aminoglycosides, and cephalosporin (cephalexin) used in human and veterinary medicine due to therapeutic purpose increases drug resistance as shown in Figure 2.1 (Collignon et al., 2016).

Some researchers and clinicians already predict a crisis stage of antibiotic and we may go to face some destructive diseases that will not be cured without antimicrobials (Lipsitch et al., 2002). Day by day resistance microorganisms are significantly increasing because of travelling worldwide frequently as well as increasing population rapidly both in developed and developing countries. Antimicrobial stewardship programs and government targets to reduce inappropriate prescribing of antibiotics often highlighted as an important way to tackle AMR. However, reducing the need for antibiotic therapy by reducing the demand for antibiotics is also key to driving down AMR acquisition. Government initiatives to prevent the incorrect prescription of antibiotics and antimicrobial stewardship programs are frequently mentioned as key strategies for combating AMR. To lower the spread of AMR, it is also important to reduce the need for antibiotic therapy by lowering drug demand.

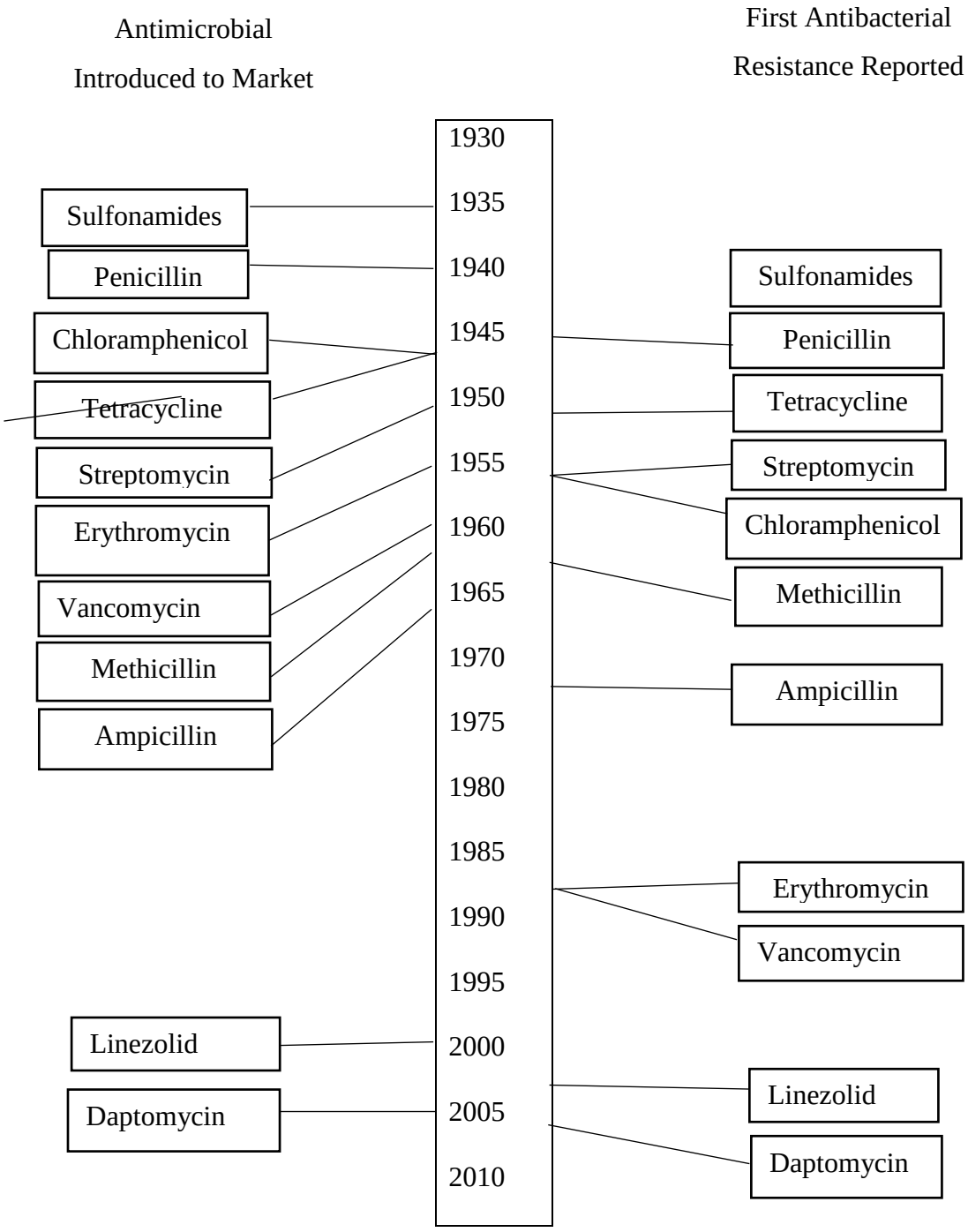

Figure 2.1: The timeline of introduction of anti-bacterial and resistance development (Holmes et al., 2016).

2.2 Mechanisms of antimicrobial resistance:

Antibiotics can be classified on the basis of their mode of action. Any deviation in the process of mechanism of action of antibiotics may cause antibiotic resistance.

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

Mechanism of Action	Antimicrobial group
Inhibit Cell Wall Synthesis	β-Lactams Carbapenems Cephalosporins Monobactams 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

Antimicrobial drugs specifically target key bacterial cell wall functions, such as limiting cell wall synthesis, upsetting the composition and function of the cell membrane, obstructing the production of essential proteins, or hindering the production of genomic RNA or DNA (O'Connell et al., 2013). Among these agents, bactericidal are those that inhibit cell-wall construction leading to bacterial cell death. On the other hand, agents that inhibit protein synthesis and therefore simply prevent the growth of the bacteria are termed as bacteriostatic such as tetracycline. Some antibacterial agents referred as 'narrow-spectrum antibacterial agents' in which glycopeptides only show activity against Gram-positive organisms, however, other antibacterial such as β-lactams termed as 'broad-spectrum antibacterial agents' as they

target processes across different species (Luc, 2015). Some bacterial species have innate resistance to antibiotics, such as natural resistance, which results from a particular antibiotic defense mechanism or from a genetic feature with a different function that also confers resistance (Munita and Arias, 2016). Organisms showing intrinsic resistance (natural resistance) to different classes of antibiotics presented on Table 2.2 and mechanism of resistance to different classes of antimicrobials have summarized in Figure 2.2

Table 2.2: 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

Organization of these mechanisms can be done into four categories:

I. Reduction of entry or access to the target site of the antimicrobial drug has noticed in *Pseudomonas aeruginosa (P. aeruginosa).* This category includes organisms that have thickened cell wall to trap the drug before entry into the cell, e.g: *Klebsiella* spp. resist to β-lactam antibiotic and *S. aureus* intermediate resistant to vancomycin (Munita and Arias, 2016).

II. Expelling antimicrobial agents such as tetracycline, macrolides, lincosamide, and streptogramins from the bacterial cell by activation of efflux mechanism. There are variety of structurally different antimicrobials that possess the ability to expel multiple drugs (Sanchez et al., 2016). This phenomenon has been observed in *E. coli* and other members of Enterobacteriaceae family against tetracycline and chloramphenicol, in *S. aureus* and *Streptococcus pneumonia* against fluoroquinolones (Marr et al., 2006; Munita and Arias, 2016).

III. Degradation or modification of antimicrobials by using enzyme either inside or outside the bacterial cell for example hydrolytic degradation of the β-lactam ring in penicillin and cephalosporin by the bacterial β-lactamases (King et al., 2016).

Modification of the antimicrobial drug targets within the bacterial cell. This mechanism observed in methicillin resistant *S. aureus* (MRSA) through change or acquisition of different penicillin binding proteins and in vancomycin resistant enterococcus (Blair et al., 2015). Some other susceptible organisms go through genetic alteration within their genome either by mutation or by horizontal transfer of gene to acquire resistance (Von et al., 2016).

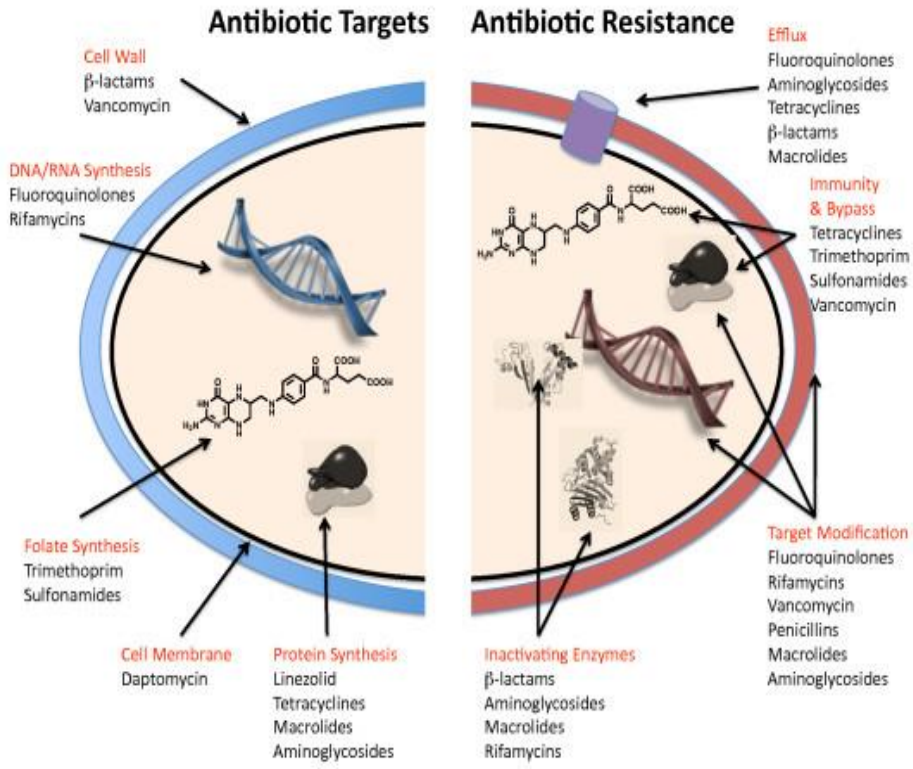

Figure 2.2: Antibiotics target and mechanism of resistance (Munita and Arias, 2016)

In general, resistance determinants transferred through a variety of techniques, such as bacteriophage transduction, plasmid conjugation, and transformation by integrating free DNA segments into the chromosome, the whole process shown in Figure 2.3.

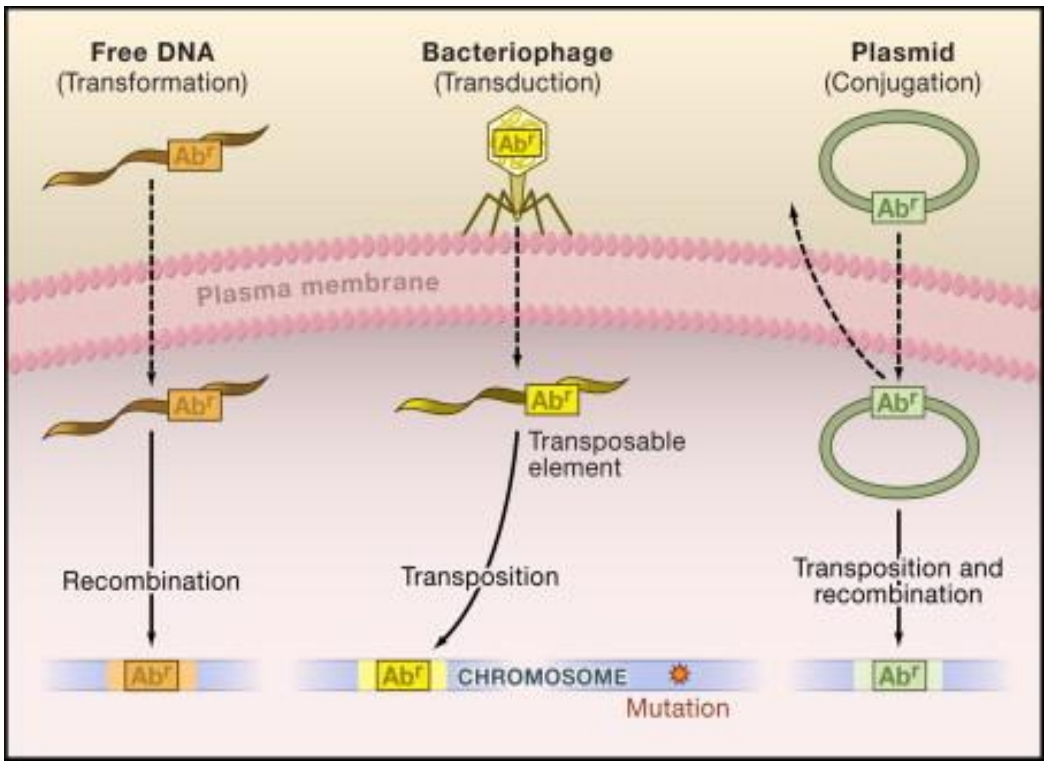

Figure 2.3: Antibiotic-resistant bacteria that result from a chromosomal mutation in the target gene (Arcilla et al., 2016).

Acquired resistance caused by chromosomal mutations found in *Mycobacterium tuberculosis* leads to rifampicin resistance, mutation in the drug's targets DNA gyrase and topoisomerase IV leads to fluoroquinolone resistance and horizontal acquisition of *mecA* in methicillin resistance (Bajaj et al., 2016). Intrinsic and acquired resistance can affect any of the four mentioned major resistance pathways.

2.3 Respiratory diseases of small ruminants:

Infectious respiratory illnesses in small ruminants can classified into the following groups depending on the etiological agent involved (Kumar et al., 2014; Zareh et al., 2021) shown in Table 2.3:

Table 2.3: Classification of disease according to etiological agent (Kumar et al., 2014; Zareh et al., 2021)

Etiological Agents	Diseases
Bacterial	Pasteurellosis, Ovine progressive pneumonia, mycoplasmosis, enzootic pneumonia, caseous lymphadenitis
Viral	PPR, parainfluenza, caprine arthritis encephalitis virus, blue tongue
Fungal	Fungal pneumonia
Parasitic	Nasal myiasis, verminous pneumonia
Others	Enzootic nasal tumors, ovine pulmonary adenomatosis

Secondary invaders frequently affect diseased individuals more severely due to environmental stress, immunosuppression, and poor managemental techniques; also, mixed infections with different aetiologies are a regular occurrence (Rajiv et al., 2000; Kumar et al., 2013). These conditions mostly affect the respiratory tract, with lesions restricted to either the upper or lower respiratory tract (Amit et al., 2012; Pavia, 2011). These diseases can grouped as followed (Rajiv et al., 2000; Kumar et al., 2013).

1. Upper respiratory tract diseases, namely nasal myiasis and enzootic nasal tumors that mostly affect the sinuses, nostrils, and nasal cavity. In the upper respiratory tract of sheep and goats various tumors like polyps (adenopapillomas), squamous cell carcinomas, adenocarcinomas, lymphosarcomas, and adenomas commonly found. However, the rate of occurrence is extremely low, with just rare examples reported.
2. Lower respiratory tract diseases, namely PPR, parainfluenza, Pasteurellosis, Ovine progressive pneumonia, mycoplasmosis, Caprine arthritis encephalitis virus, caseous lymphadenitis, verminous pneumonia, and many others involving lungs and lesions, observed in alveoli and bronchioles.

High morbidity and mortality can be observed in animals of various ages, depending on the severity of the infections and the physical condition of the diseased animals. These diseases, alone or in conjunction with other linked ailments, their onset may be acute or chronic, and they are a major cause of small animal industry losses. Thus,

based on the onset and duration of diseases, respiratory diseases can also classified into following mentioned groups:

1. Acute: Bluetongue, PPR, Pasteurellosis and parainfluenza
2. Chronic: Mycoplasmosis, verminous pneumonia, nasal myiasis and enzootic nasal tumors
3. Progressive: Ovine progressive pneumonia, Caprine arthritis encephalitis virus, caseous lymphadenitis and pulmonary adenomatosis.

2.4 Respiratory tract infection in goats:

Goat is the second most significant livestock in Bangladesh. They kept as a source of meat, milk, and fiber and are frequently referred to as the "poor man's cow" because they can thrive in environments where cows cannot. (Clothier et al., 2012). Respiratory tract infections are prevalent in farm animals especially in goats. Along with decreasing productivity in older animals, respiratory infections are the primary cause of death in kids. Most of respiratory tract infections are endogenous, meaning they caused by bacteria that are naturally present in the upper respiratory tract, although exogenous infections can also acquire by direct contact with ill animals or infected aerosol. Lung infection is caused by both infectious and non-infectious sources. Stress factors, bacteria, and viral infection all play role in respiratory disease, making complex respiratory affection. Bacterial pneumonia considered as one of the most common and major causes of death and economic loss connected with respiratory diseases (Andrawis, 2001). Pneumonia in goat is an infection of the lungs characterized by fever (40-41ºC), anorexia, painful coughing, dyspnea, mucopurulent nasal discharge, and depression. *Pasteurella maltocida*, *Streptococcus pneumoniae*, *Klebsiella pneumonia*, *Staphylococcus aureus*, and other infectious agents more frequently linked to an outbreak of acute pneumonia and the death of goats in all ages. (Falade, 2002). Most of these bacteria found in the upper respiratory tract of goats. Goats are more susceptible to respiratory tract infection mainly pneumonias due to inadequate management, transportation stress, overcrowding pens, unexpected environmental changes, poor living conditions, viral infection (e.g., parainfluenza-3 virus), lung parasites, and other stressful situations. All these conditions are responsible for high mortality, reduced live weight gain, delayed marketing, treatment cost and unthriftiness among the survivors (Daniel et al., 2006). The most common cause of goat deaths is pneumonia, which affects 24-51% of goats and results in economic losses due to deadly lung infections and poor animal performance (Hakim

et al., 2014). In order to take effective preventative and control actions against respiratory tract illness in goats, bacterial identification is crucial.

2.5 Overview of *S. aureus*:

Staphylococcus aureus is gram-positive, round-shaped bacterium that is a member of the Firmicutes, are frequently found in the upper respiratory tract and commonly associated with nosocomial infection. A Scottish surgeon Sir Alexander ogston discovered *S. aureus* in 1881. *Staphylococcus* clinically classified into two groups: coagulase-positive and coagulase-negative *staphylococcus*. In the coagulase-positive group, *Staphylococcus aureus* is the most important staphylococci. It commonly found in the nasal cavity and skin or mucous membrane of both human and animals. About 15% people persistently carry *S. aureus* in the anterior nares, and up to 50% of individuals with *S. aureus* colonization (Rasigade et al., 2014). However, *S. aureus* is associated with various life-threatening diseases including pneumonia, osteomyelitis, endocarditis, septicemia, meningitis etc. (Loir et al., 2003).

2.5.1 Morphology of *S. aureus*:

S. aureus is 0.5-1.5 µm in diameter and spherical in shape without any flagella. Moreover, sometimes it divides itself to form clusters like grapes. The cell wall of the organism consists of a very thick peptidoglycan layer.

2.5.2 Taxonomy of *S. aureus*:

Domain: Bacteria

Phylum: Firmicutes

Class: Bacilli

Order: Lactobacillales

Family: Staphylococcacea

Genus: *Staphylococcus*

Species: *Staphylococcus aureus*

2.5.3 Virulence and pathogenicity of *S. aureus*:

Although *S. aureus* is not always pathogenic (can be found as a commensal), however, pathogenic strains often involve with infection by producing virulence factors such as potent protein toxins and the expression of a cell-surface protein that binds and inactivate antibodies (Klimešová et al., 2017). Staphylococcal virulence factors can be

classified based on their mechanism of action and pathogenicity as presented in the following table:

Table 2.4: Virulence factors of S. aureus and their function (Gnanamani et al., 2017)

Factors	Functions
• Microbial Surface Components Recognizing adhesive matrix molecules (MSCRAMM)	Helping attachment to host tissues
• Polysaccharide microcapsule • Protein A • Panton-Valentine Leukocidin (PVL) • Alpha-toxin (Alpha hemolusin) • Chemotaxis-inhibitory protein of S. aureus (CHIPS)	Breaking /evading the host immunity
• Extracellular adherence protein (Eap) • Proteases, lipases, nucleases, hyaluronatelyase, phospholipase C, metalloproteases (elastase), & Staphylokinase	Tissue invasion
• Enterotoxins • Toxic shock syndrome toxin-1 (TSST-1) • Exfoliative toxins A and B	Induces toxinosis

S. aureus's pathogenicity is primarily influenced by a trifecta of toxin-mediated virulence, invasiveness, and antibiotic resistance.

The organism can cause sepsis by entering the blood and spreading in different organs. Diseases such as endocarditis, osteomyelitis, renal carbuncle, septic arthritis, and epidural abscess may occurred due to this hematogenous spread. Specific syndromes such as toxic shock syndrome, scalded skin syndrome and food borne gastroenteritis can also occur due to extra cellular toxins without a blood stream infection.

The main *S. aureus* toxin (α toxin) acts by two mechanisms. Each mechanism requires ADAM10 receptor that contains metalloprotease and disintegrin domains. First

mechanism includes pore formation in a series of target cells by α toxin via formation of a heptameric pore. Secondly, epithelial, and endothelial breach caused by it via breaking adherens junctions and compromising the cytoskeleton (Figure 2.4).

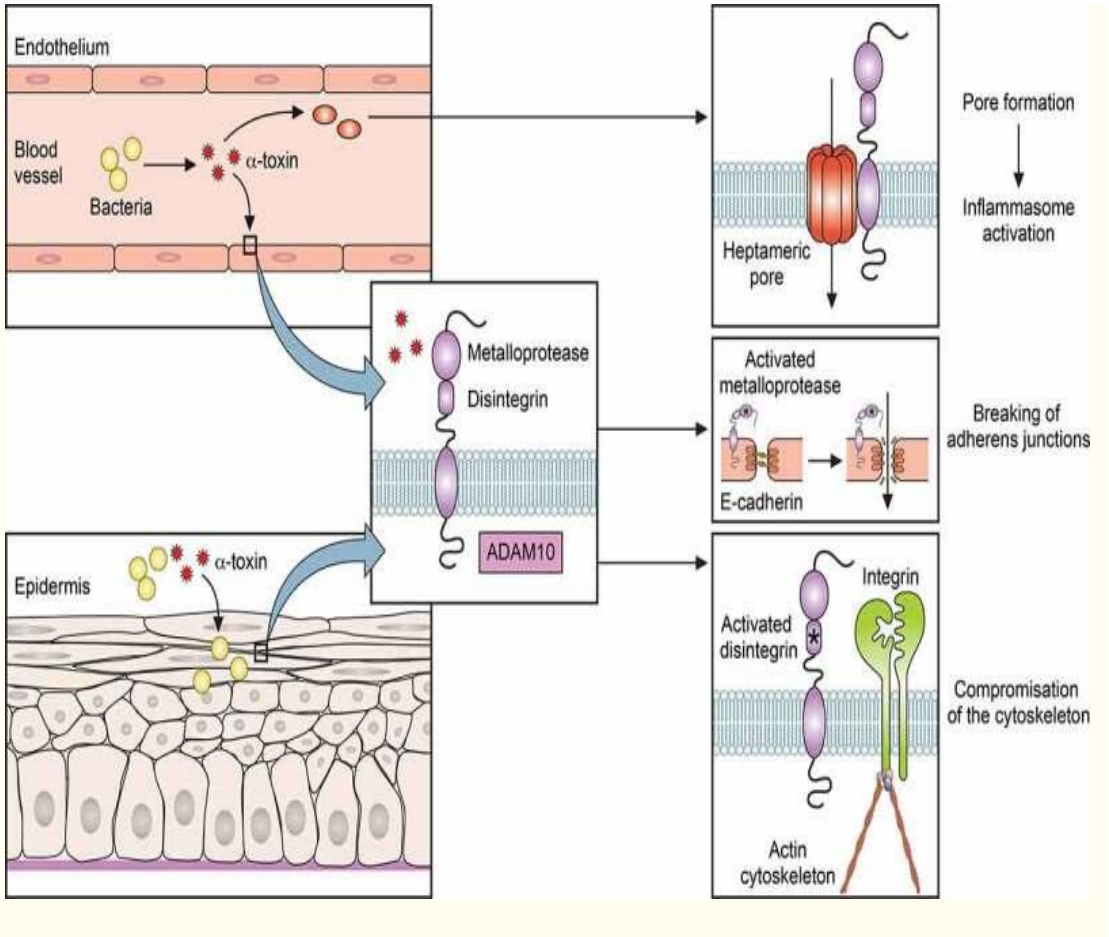

Figure 2.4: Importance of α-toxin in *S. aureus* infection (cheung et al., 2021)

Moreover, *S. aureus* also act as an opportunistic pathogen where primary harm done by other pathogens or predisposing factors. For example, secondary infection by *S. aureus* commonly the ultimate reason for death in lung infection that have begun by a viral infection such as the flu (McCullers, 2014 and Morens et al., 2008). Furthermore, the organism may inoculated into the skin from a site of carriage which results in different clinical manifestations of localized infections including carbuncle, cellulitis, impetigo bullosa or wound infection

2.5.4 Detection method of *S. aureus*:

2.5.4.1 Cultural characteristics of *S. aureus* (El-Jakee et al., 2008):

For cultural characterization, the first step is isolating the organism by streaking samples from clinical specimens (or from blood cultures) onto solid media like blood agar, tryptic soy agar, or brain heart infusion agar. To promote the growth of

halotolerant staphylococci, specimens that are likely to be contaminated with other microorganisms can be plated on specific media mannitol salt agar with 7.5% sodium chloride. After inoculation, the plates should incubate at 37°C for 24 to 48 hours.

On blood agar, *S. aureus* forms round, smooth, raised, opaque, yellow to golden yellow colonies of 1-2 mm in diameter with characteristics β-hemolysis. On mannitol salt agar (MSA), it ferments the mannitol and forms circular, 2-3mm in diameter, pigmented golden yellow colonies with smooth and shiny surface. On tryptic Soy agar, it forms circular, convex colonies. After initial isolation, isolates should sub-cultured on a nonselective medium at least once before employed in a diagnostic test that needs pure culture or heavy inoculum.

2.5.4.2 Microscopic characteristics *S. aureus*:

On gram's staining, *S. aureus* shows typical gram-positive, spherical shaped cocci that occurs singly or in pairs, short chain or irregular grapes-like clusters ranges from 0.5-1.0 μm in diameter. The name 'Staphylococcus' derived from Greek word 'staphyle' which means bunch of grapes and 'kokkos' means berry (Licitra, 2013). On the transmission electron microscopy, the cells show thick cell wall, distinctive cytoplasmic membrane, and amorphous cytoplasm (Touhami et al., 2004).

2.5.4.3 Biochemical characteristics of *S. aureus*:

Following biochemical tests are done for confirmation of *S. aureus*-

Coagulase test:

For the coagulase test, whole blood from a horse was drawn into commercially available lavender tops that had been treated with EDTA. Then using a refrigerated centrifuge device, the blood centrifuged at 2600 rpm for 10 minutes. The follow-on supernatant, the plasma instantly transferred to a sterile 1.5 ml eppendorf tube using sterile micropipette and tips and kept at -20ºC for further use.

Tube coagulase test:

For coagulase test, Brain Heart Infusion Broth (BHIB) prepared according to the instructions of manufacturer (Oxoid, Basingstoke, Hampshire, UK). Positive colonies transferred to a 10 ml test tube containing 5 ml of BHIB and incubated at 37ºC for 6 hours. Then, 50μL BHIB containing cultivated sample transferred to the sterile tubes containing 50μL of horse plasma and incubated at 37ºC for 6 hours. Because of the massive, ordered coagulation and coagulation of all the contents of the tube that do not

fall off when inverted, the presence of coagulates was justified (Graham et al., 2006). For validation of the result, a control tube without plasma also placed.

Slide coagulase test:

Horse plasma is dropped onto a spotless, grease-free glass slide for the slide coagulase test. A loopful of suspected colony taken and mixed with the plasma to check for agglutination. A positive test result showed by proper agglutination and thus confirmed as *S. aureus*.

Slide Catalase test:

A fresh, clean and grease free slide was taken where small amount of colony placed. One drop of 3% H2O2 poured on the colony and a cover slip placed. A positive test result indicated by bubble formation.

2.4.4.4 Identification of toxins (Berube et al., 2013):

Toxin identification is important in severe cases such as food poisoning and toxic shock syndrome. *S. aureus* produces different toxins such as enterotoxins A to D and TSST-1 that may be identified by using agglutination tests. The toxins present in the samples clumps the latex particles and determines the test result. For this purpose, commercial latex agglutination tests are available.

2.5.4.5 Nucleic acid amplification tests (Kateete et al., 2010):

For the direct detection and identification of *S. aureus* in clinical specimen commercial nucleic acid amplification tests are available. Integrated specimen processing (extraction, gene amplification, and target detection) now performed on highly automated platforms with disposable reagent strips or cartridges, whereas earlier versions of these tests required manual extraction of bacterial DNA and testing multiple specimens in large batches. These tests are useful for screening the patients for carriage of methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA).

2.5.5 Antibiotic-Resistance pattern of *S. aureus*:

Staphylococci are resistant to many antimicrobials and according to the history, AMR in staphylococci started at the beginning of the antibiotic era. Resistance to different antibiotics described below.

2.5.5.1 Beta-lactam resistance:

2.5.5.1.1 Penicillin resistance:

In 1928, Alexander Fleming discovered the first beta-lactam antibiotic penicillin G that used in human as chemotherapeutic agent in 1941 (Fletcher, 1984). The antibiotic was powerful against Gram-positive pathogens and a strong weapon against Staphylococcal infection. After a year of its clinical use, first report of *S. aureus* strains resistant to penicillin appeared. Penicillin-resistant isolates carry penicillinase enzyme that is responsible for cleaving the beta-lactam ring of penicillin and thus inactivate antibiotic. The first wave of resistance refers to the establishment and spread of penicillinase-mediated resistance in *S. aureus*. In the 1960's, the situation became pandemic after alarming spread. By the late 1960's, around 80% of both community and hospital acquired *S. aureus* isolates became resistant to penicillin (Chambers and Deleo, 2009). By the early 2000s, more than 90% of Staphylococcal isolates expressed penicillinase enzyme, regardless of whether they came from the community or a hospital (Lowy, 2003).

2.5.5.1.2 Methicillin resistance:

The discovery of methicillin, a penicillinase-stable semisynthetic penicillin used to counter the penicillinase resistance in *S. aureus*. From 1961, people started using the drug into clinics and in the same year, methicillin resistance (MRSA) was reported. MRSA clones spread quickly over the world after the initial revelation, although only in nosocomial settings. In *S. aureus* infection, this referred as second wave of beta-lactam resistance (Enright et al., 2002). Presence of mecA gene was responsible for methicillin resistance. Increased MRSA infection rate in hospitals resulted in high morbidity and mortality, as well as raised the expense of health treatment (Klein et al., 2007 and Köck et al., 2010).

In the early 1990s, reports of MRSA infections in the community triggered the third wave of beta-lactam resistance in *S. aureus*. Community MRSA strains have infiltrated hospital settings in the recent decade, blurring the line between HA and CA MRSA (Mediavilla et al., 2012).

2.5.5.2 Quinolone resistance:

Quinolones work as antibacterial agent by blocking bacterial topoisomerases (topoisomerase IV and DNA Gyrase), which are required for de-supercoiling DNA and separating concatenated DNA strands. *S. aureus* develops resistance to quinolones

in a stepwise way, owing to point mutations in the GrlA subunit of topoisolmerase IV and the GyrA subunit of Gyrase. The expression of NorA efflux pumps is another way through which *S. aureus* becomes resistant to quinolones (Hooper, 2000).

Quinolone resistance commonly related with methicillin resistance in *S. aureus*, even though the mechanism of resistance and encoding genes are completely distinct. Fluoroquinolone resistance was 70.3% among MRSA isolates implicated in acute bacterial skin and skin structure infections (ABSSSIs) in hospitals in 2008. Even use third- a fourth-generation quinolones have ruled out for treatment of MRSA in the hospitals settings due to high level of quinolone resistance (Jones et al., 2010). Though CA-MRSA infections were previously amenable to non-beta-lactam antibiotics such as quinolones, the situation has altered in recent years, with an increase in the number of CA-MRSA infections that were multi-drug resistant (Dalhoff, 2012).

2.5.5.3 Vancomycin resistance:

In 1952, a glycopeptide antibiotic named Vancomycin discovered from a microbial source (*Streptomyces orientalis*). Vancomycin was approved for clinical use in 1958, but it was quickly surpassed by methicillin and other anti-staphylococcal penicillins that were less toxic than vancomycin but similarly effective against penicillin-resistant Staphylococci (Levie, 2006). Vancomycin's clinical efficacy in treating MRSA infections has well proven over the period of 1980s, thus the antibiotic has emerged as 'workhouse anti-MRSA' drug (Rodyold and McKoneghy, 2014). First report of a *S. aureus* strain showing vancomycin MIC of >128mg/L was published in 2002. The strain was methicillin-resistant and had the *VanA* gene, which conferred high-level vancomycin resistance (Sievert et al., 2002). This followed by rare reports of *S. aureus* strains resistant to vancomycin being isolated (Sievert et al., 2008). These strains all shown to have a high vancomycin MIC (> 8 mg/L) and known as vancomycin-resistant *S. aureus* (VRSA)

A transposon maned Tn1546, which obtained from vancomycin-resistant *Enterococcus faecialis* found in VRSA strains. The VanA-type resistance mediated by the transposon encodes a dehydrogenase (VanH) that converts pyruvate to D-Lac, as well as VanA Ligase that catalyzes the creation of an ester link between D-Ala and D-La.

2.5.5.4 Resistance to other antibiotics:

Because HA_MRSA strains are frequently MDR phenotypic, drugs including sulfonamides, tetracyclines, aminoglycosides, chloramphenicol and clindamycin ruled out due to inactivity, leaving vancomycin as the backbone of treatment. Resistance to sulfonamides and trimethoprim (Then et al., 1992), tretracycline (Schmitz et al., 2001), aminoglycosides (Schmitz et al., 1999), chloramphenicol (Fayyaz et al., 2013), and clindamycin (Frank et al., 2002) has been commonly observed in *S. aureus*, particularly in MRSA.

2.6 Overview of *E. coli*:

Escherichia coli is a gram-negative, non-spore forming, facultative anaerobic bacteria with which are commonly found in living human and animal digestion as normal microflora. In 1885, a German bacteriologist Theodor Escherich first discovered *E. coli*. According to the infection sites, pathogenic *E. coli* classified into two major groups: intestinal pathogenic *E. coli* (InPEC) and extra-intestinal pathogenic *E. coli* (ExPEC). Pathogenic strains of *E. coli* recognized as causing foodborne diarrhoea. On the other hand, extra-intestinal pathogenic strains related to diseases outside the gastrointestinal tract including urinary tract, respiratory system, central nervous system, and circulatory system (Russo and Johnson, 2003). Again, InPEC subdivided into several categories including- 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). These causes infection to both human and animals (Moriel et al., 2012). More than two decades ago, the Pneumonia Patient Outcome Research (PORT) found that *Escherichia coli* was the fourth most prevalent causal pathogen and the second most common cause of "bacteremic" Community acquired pneumonia (CAP) (Marrie et al., 1998).

2.6.1 Morphology of *E. coli*:

E. coli are short rods measuring 2.4 x 0.4-0.7 μm with a cell volume of 0.6-0.7 μm3, composed of a thin peptidoglycan layer and an outer membrane. It possesses flagella in petritichous manner. It also has an adhesive molecule namely intimin which helps to attach and efface to the microvilli of the intestines. Based on the differences in antigenic structure on the surface, the bacteria can be characterized by a method of serotyping. The serotyping includes bacterium's O-antigen (Ohne), a polysaccharide domain in the bacterium's lipopolysaccharide (LPS) in the outer membrane, and the

H-antigen (Hauch) consisting of flagella protein. Serotyping may also consist of the K-antigen (Kapsel) and the F-antigen (Fimbriae).

2.6.2 Taxonomy of *E. coli*:

Domain: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Enterobacteriales

Family: Enterobacteriaceae

Genus: *Escherichia*

Species: *Escherichia coli*

2.6.3 Virulence factors and pathogenicity of *E. coli*:

E. coli mostly remain as harmless commensal in the gastrointestinal tract of warm-blooded animals. However, some pathogenic subtypes possess virulence attributes that make them capable of causing a variety of illnesses in healthy human and animals (Kaper et al., 2004).

Table 2.5: *E. coli* pathogenic types with their disease occurrence based on virulence factors (Kaper et al., 2004):

Pathotype	**Virulence factors**	**Diseases**	**symptoms**	**References**
Enteric *E. coli*				
Enteropathogenic *E. coli* (EPEC)	Bfp, Intimin, LEE	Diarrhoea in children	Watery diarrhoea and vomiting	Kaper et al., 2004
Enterohaemorrhagic *E. coli* (EHEC)	Shiga toxins, Intimin, Bfp	Haemorrhagic colitis, HUS	Bloody diarrhoea	Kaper et al., 2004 Bilinski et al., 2012
Enterotoxigenic *E. coli* (ETEC)	Heat-labile and heat-stable	Traveller's diarrhoea	Watery diarrhoea and vomiting	Qadri et al., 2005

	toxins, CFAs			
Enteroaggregative *E. coli* (EAEC)	AAFs, cytotoxins	Diarrhoea in children	Diarrhoea with mucous and vomiting	Weintraub, 2007
Diffusely Adherent *E. coli* (DAEC)	Daa, AIDA	Acute diarrhoea in children	Watery diarrhoea, recurring UTI	Servin, 2005
Enteroinvasive *E. coli* (EIEC)	Shiga toxin, hemolysin, Cellular invasion, Ipa	Shigellosis – like	Watery diarrhoea, dysentery	Kaper et al., 2004
Adherent Invasive *E. coli* (AIEC)	Type 1 fimbriae, Cellular invasion	Associate with crohn disease	Persistent intestinal inflammation	Negroni et al., 2012
Extraintestinal *E. coli* (ExPEC)				
Uropathogenic *E. coli* (UPEC)	Type 1 and P fimbriae, AAFs, hemolysin	Lower UTI and systemic infections	Cystitis, pyelonephritis	Kaper et al., 2004
Neonatal Meningitis *E. coli* (NMEC)	S fimbriae, K1 capsule	Neonatal meningitis	Acute meningitis, sepsis	Pouillot et al., 2012
Avian Pathogenic *E. coli* (APEC)	Type 1 and P fimbriae, K1 capsule	Probable source of food-borne disease		Rodriguez-Siek et al., 2005

The extra-intestinal pathogenic *E. coli* (ExPEC) causes mostly urinary tract infection and bacteremia. However, it rarely causes dramatic lung illness characterized by fatal necrotizing hemorrhagic pneumonia (HP) with multi-organ involvement and

overwhelming sepsis. All the reports to date noted that all *E. coli* HP isolates contain the virulence gene cnf1 (cytotoxic necrotizing factor 1 [CNF1]). The regulatory Pho, Rac, and Cdc42 GTPases in eukaryotic cells permanently activated by deamination of a glutamine residue, therefore new activities in cells promoted, including gene transcription and cell proliferation, and thus the survival of the bacteria also promoted (Fabbri et al., 2010).

2.6.4. Detection method of *E. coli*:

2.6.4.1 Cultural characteristics of *E. coli* (Aryal, 2020):

On Nutrient Agar (NA):

- *E. coli* forms large, circular, low convex, grayish, white, moist, smooth and opaque colonies
- They found in 2 forms: Smooth (S) form and Rough (R) form
- Smooth forms are emulsifiable in saline
- There is smooth to rough variation (S-R variation) due to repeated subculture.

On Blood Agar (BA):

- Colonies are big, circular, gray, and moist.
- There may be found Beta-hemolytic or non-hemolytic (gamma-hemolysis) colonies.

On MacConkey Agar (MAC):

- Colonies are circular, moist, smooth, and of entire margin.
- Colonies appear flat and pink.
- They are lactose-fermenting colonies.

On Muller Hinton Agar:

- Colonies are pale straw colored.

On Eosin Methylene Blue Agar (EMB):

- Colonies forms Green metallic sheen.

On m-ENDO Agar:

- Colonies form green metallic sheen.
- Metabolise lactose with the production of aldehyde and acid.

On Violet Red Bile Agar (VRBA):

- There is formed red colonies (pink to red).

- Bluish fluorescence around colonies seen under UV.

On Cystine Lactose Electrolyte-Deficient (CLED) Agar:

- They form lactose-positive yellow colonies.

***E. coli* on liquid media:**

- On liquid media, there is found homogenous turbid growth within 12-18 hours.
- R form agglutinate spontaneously, forming sediment on the bottom of the test tubes.
- Pellicles formed on the surface of the liquid media after prolonged incubation (> 72 hours).
- Heavy deposits are formed which disperse on shanking.

2.6.4.2 Microscopic Characteristics of *E. coli* (Zinnah et al., 2007):

E. coli forms gram-negative, pink colored, rod shaped appearance and arranged in single or in pair.

2.6.4.3 Biochemical characteristics of *E. coli* (Zinnah et al., 2007)

Motility test:

- Motility test performed by hanging drop method to differentiate motile and non-motile bacteria.
- *E. coli* is positive to motility test.

Reaction in TSI agar slant:

- Test organism cultured into TSI agar slant by stab or streak method.
- *E. coli* indicated by yellow slant, yellow butt, forming gas bubbles and absence of black precipitation in the butt (due to production of H2S).

Carbohydrate fermentation test:

- Inoculate 0.2 ml of nutrient broth culture of the isolated organism into tubes containing 5 basic sugars including dextrose, maltose, lactose, sucrose and mannitol
- Incubate at 37ºc for 24 hours
- Color change from red to yellow indicates acid production
- Accumulation of gas bubbles in the inverted Durham's tube indicates gas production

Catalase test:

- 3 ml catalase reagent (3% H_2O_2) taken in test tube
- Single colony from pure culture merged in the reagent
- Formation of bubble indicates positive test reaction.

Methyl Red test:

- Single colony from pure culture inoculated in 5 ml of sterile MR-VP broth
- After 5 days, incubate at 37ºc and add 5 drops of methyl red solution
- Development of red or yellow color indicates positive or negative result, respectively.

Voges-Proskauer (VP) test:

- Test organism grown in 3 ml sterile MR-VP broth at 37 ºc for 48 hours
- Add 0.6 ml of 5% alphanapthol and 0.2 ml of 40% potassium hydroxide containing 0.3% creatine added on per ml of broth culture.
- Shake and stand for 5-10 min to observe color formation
- Positive result indicated by development of pink red color

Indole test:

- Test organism cultured in 3 ml peptone water containing tryptophan at 37ºc for 48 hours
- Add 1 ml diethyl ether, shaken and allowed to stand until the ether rises to the top
- 0.5 ml Kovacs reagent gently run down to the side of the test tube to form a ring between the medium and the ether.
- Positive test result indicated by formation of brilliant red colored ring.

2.6.4.4 Confirmatory diagnostic procedures:

Latex agglutination test (Duffy et al., 2000):

This test based on the latex bead coated with an antibody specific to *E. coli*. The beads adhere to the cells in the presence of the specific antigen that causes clumping of latex particles.

Immunological procedure (Beutin et al., 2002):

Immunological techniques based on the reaction between an antibody and antigen specific to VTEC. Some of these assays use surface antigens to detect VTEC cells belonging to specific serogroups, while others rely on ELISA to detect the toxins produced by VTEC. In conjunction with direct microscope detection, a fluorescent-labeled monoclonal antibody test for *E. coli* O157:H7 has developed. This method applied to detect pathogen in fecal samples.

Enzyme Linked Immunosorbent Assay (ELISA) (Beutin et al., 2002):

There are many commercial ELISA kits formats. However, most used format is sandwich assay. These assays have similar sensitivity to standard culture procedures, but they have advantages in terms of speed, labor cost savings, and large volume throughput.

Pulse field agarose gel electrophoresis (PEGE) (Goering, 2010):

In typing bacterial isolates, pulse field agarose gel electrophoresis considered as the gold standard method. It entails extracting DNA from a plug and then restricting digestion using appropriate restriction enzyme (eg: Xbal and NotI). Restriction enzyme digestion is separated on agarose gel by alternating field direction (current) which results in production of large DNA fragments (30-50 Kb). This causes the small fragments to move quicker on the agarose gel than the large fragments, resulting in a DNA pattern unique to each clone. PFGE is highly reproducible method that is easy to interpret and compare within and across different laboratories. Different studies have demonstrated that PFGE has very strong discriminatory capabilities in compared to other typing methodologies including MLST and RAPD. However, PFGE is labor expensive, time consuming, and requires specialized equipment and expertise as compared to other genotyping procedures.

Multilocus sequence typing (MLST) (Cooper and Feil, 2004; Gordon, 2010):

Multilocus sequence typing is a well-established method for characterization of bacteria. This method used to understand the clonal groups and phylogenetic relatedness. Seven housekeeping genes from the core genome were chosen for MLST, and a 300-700 bp section of each sequenced. These sequences referred to allele and each unique combination of alleles corresponds to a certain sequence type (ST). To determine the ancestry and relatedness of each strain, these sequence profile can used.

However, based on the core genes selection, MLST can be biased and cannot applied to all *E. coli* strains with the same set of seven genes.

Clermont phylogenetic grouping (Gall et al., 2007):

Clermont phylogenetic grouping is a quick and easy method for identifying *E. coli* phylogenetic groups. This method examines the presence and/or absence of two genes (*chuA* and *yjaA*) and a DNA fragment using a triplex PCR to determine the phylogenetic group (TSPE4.C2). *E. coli* is grouped into four main phylogenetic groups by this method including A, B1, B2 and D. The method's dependability has demonstrated in studies, and it is currently widely used in the molecular characterization of *E. coli*.

2.6.5 Mechanism of resistance on *E. coli*:

E. coli belongs to the gram-negative bacteria group. Gram-negative bacteria are more resistant to antibiotics than Gram-positive bacteria because they have an outer membrane that the Gram-positive bacteria lack. Thus, this outer membrane protects gram-negative bacteria from antibiotics. In the outer structure, this outer membrane contains inner structure that is composed of phospholipids (PLs) and lipopolysaccharides (LPs) (Kallau et al., 2018). As PLs and LPs formed of saturated chains and so are hydrophobic, thus they operate as bacterial membrane defences (Blair et al., 2015). The negative charge of the molecules that make up PLs and LPs allows for intermolecular linking connections through the binding of divalent cations (84) and the presence of porin as ion selective channels to limit antibiotic absorption (Henderson et al., 2016). *Escherichia coli* has the ability to modify antibiotic targets such as against the aminoglycoside class of antibiotic by modifying the ribosomal subunit through the acquisition of plasmids carrying 16S rRNA methyltransferases (Delcour, 2009), against the fluoroquinolone group by mutations in the GyrA subunit, which is chromosomally coded from gyrase (*gyrA* gene) or the ParC subunit of topoisomerase IV (*parC* gene) (Mingeot-Leclercq et al., 2009), and against penicillin through modification of penicillin-binding protein (PBP) (Schultsz and Geerlings. 2012). The bacteriolytic mechanism of action against bacteria mediated by β-lactam antibiotics, which have three carbon rings and one nitrogen ring. B-lactam acts by preventing the production of peptidoglycan, a key component of bacterial cell walls. By generating β-lactamase enzymes, *E. coli* can become resistant to β-lactam antibiotic. The Ambler structural classification is the most generally used classification for the β-lactamase enzyme which is based on sequence similarity and divides this

protein into four groups: serine-β-lactamase classes A, C, and D, and metalo-β-lactamase class B (Sauvage et al., 2008). Broad-spectrum β-lactamase (ESBL), AmpC β-lactamase (AmpC) and carbapenemase are three enzymes produced by *E. coli* that have variable hydrolytic activity against β-lactam antibiotics (Bush and Jacoby, 2010). In the ambler classification, ESBL is in the class A group predominantly and Penicillins, first, second, and third generation cephalosporin, and monobactams (for example, aztreonam) are all resistant to ESBL, but it cannot hydrolyze cephamycins (cefoxitin) or carbapenems (imipenem, meropenem), and it can be inhibited by β-lactamase inhibitors such as clavulanic acid, tazobactam, and sulbactam (Psichogiou et al., 2008). When a strain of *E. coli* possesses both AmpC and ESBL, the mechanism of action of antibiotics becomes more convoluted, and resistance increases (Kallau et al., 2018). Carbapenems bind to penicillin-binding proteins, causing the production of spheroplasts and cell lysis. The carbapenemase-producing *E. coli* strain can hydrolyze the antibiotic carpabenem (Nordmann et al., 2011). The ATP binding cassette (ABC) family, the multidrug and toxic compound extrusion (MATE) family, the major facilitator superfamily (MFS), and the resistance-nodulation cell division (RND) family are all efflux pumps found in *E. coli*. *E.coli* has an ABC group efflux pump known as the MacAB transporter that provides resistance to several macrolides (Lubelski et al., 2007). Mate that is capable of transporting fluoroquinolones (Kuroda and Tsuchiya, 2009), MFS that is capable of transporting macrolides (MefB and MdfA pumps), fluoroquinolones (QepA2, EmrAB-Tolc and MdfA pumps), tetracyclines (EmrAB-Tolc and MdfA pumps), trimethoprim (Fsr pumps), and Cholramphenicol (MdfA pumps) (Kumar et al., 2013).

2.7 Overview of *P. multocida*:

Pasteurella multocida is a gram-negative, spherical, or rod-shaped, non-motile, facultative anaerobic, penicillin-sensitive coccobacillus of the family pasteurellaceae that is responsible for pasteurellosis and severe economic loss in cattle, sheep, goat and poultry. In 1878, *Pasteurella multocida* first discovered in cholera-infected birds and in 1878, Louis Pasteur isolated it. Based on capsular antigen, *P. multocida* divided into five serotypes namely A, B, D, E, and F. Moreover, the bacteria divided into 16 somatic serovars based on lipopolysaccharide (LPS) antigens including serotypes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16.

The bacteria are commonly pathogenic to ruminants and poultry. *P. multocida* causes fowl cholera in poultry, Septicemia Epizootica (SE)/ Hemorrhagic Septicemia (HS)

and Pasteurellosis Septicemia in cattle and buffaloes, Pneumonia and Pasteurellosis Septicemia in goats and sheep, and Pneumonia, atropic rhinitis and septicemia in pigs (Wilkie et al., 2012). Among the respiratory tract infections, pneumonic pasterellosis or respiratory manheimiosis is most common with a wide range of prevalence in ruminants. Due to exposure to physical stress or unfavourable environmental condition, small ruminants mainly goats are fairely susceptible and contract the disease (Mohamed and abdelsalam, 2008). It is one of the most frequent respiratory problems in goats all around the world (Marru et al., 2013) and clinically characterized by anorexia, fever (40-41^{o} C), painful coughing, dyspnes, mucopurulent nasal discharge, and depression. For attachment and penetration in the host cells, as well as survival in a hostile environment, the *P. multocida* toxin possesses surface adhesins and iron acquisition proteins.

2.7.1 Morphology of *P. multocida*:

Pasteurella are short (0.5-1.5, 0.25-0.5mm) coccovoids with rounded ends. The genome of *P. multocid*a reveals 129 lipoproteins that secreted and found in the outer membrane. Protein H shown to be the most abundant polypeptide in the *P. multocida* outer membrane. *P. multocida* has a capsule that helps to avoid phagocytosis. Moreover, the bacteria have lipopolysaccharides that are important for survival of the bacteria into the host (Harper et al., 2006).

2.7.2 Taxonomy of *P. multocida*:

Domain: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Pasteurellales

Family: Pasteurellaceae

Genus: *Pasteurella*

Species: *Pasteurella multocida*

2.7.3 Virulence (Harper et al., 2006) and pathogenicity of *P. multocida* (Wilkie et al., 2012):

Capsule:

By serological methods, *P. multocida* can be classified into five capsule groups such as A, B, D, E and F.

Lipopolysaccharides:

Lipopolysaccharides plays a critical role in the pathogenesis of the disease of *P. multocida*. It is considered as a protective antigen and stimulates humoral immunity.

Fimbriae and adhesions:

Many genes in the *P. multocida* genome, such as ptfA, fimA, flp1, flp2, hsf 1 and hsf 2, encode proteins that are similar to fimbriae or fibrils in other bacteria. Fimbriae play important role in the surface adhesion. They have been seen on the surface of those *P. multocida* serotype A strains that were able to adhere to mucosal epithelium, but not on the surface of those strains that were unable to adhere.

Toxins:

Most strains of *P. multocida* that cause poultry cholera, hemorrhagic septicemia, or pneumonia known to lack toxins. The dermonecrotic toxin, PMT, is the only toxin found to date and is responsible for the clinical and pathological indications of atrophic rhinitis. It mostly expressed by serogroup D strains.

Iron regulated and iron acquisition proteins:

Iron is an essential element that bacteria must obtain to survive. The amount of free iron available in vivo is extremely limited due to its inherent toxicity, hence *P. multocida*, like other bacterial species, has evolved several systems for iron uptake. *P. multocida* PM70 sequence analysis found that a significant proportion of the genome (over 2.5%) encodes 53 proteins that are comparable to proteins involved in iron absorption or acquisition.

Sialic acid metabolism:

Some bacterial species manufacture sialidases, which extract sialic acid from host glycosylated proteins and lipids for use as a carbon source. These enzymes may also increase bacterial virulence by exposing important host receptors and/or decreasing the efficiency of host defences like mucin. Sialidase produced by the majority of *P. multocida* strains and both cell-bound and extracellular sialidases have found in *P. multocida.*

Hyaluronidase:

Even though the significance of hyaluronidase in pathogenesis is unknown, it found in many of the *P. multocida* serotype B strains that cause bovine haemorrhagic septicaemia.

Outer membrane proteins:

OmpH is one of *P. multocida's* key outer membrane proteins. Antibodies produced against this protein offer some protection against diseases. *P. multocida* has been demonstrated to adhere to fibronectin and collagen type IX in recent research exploring its potentiality to bind host extracellular matrix proteins. OmpA, Oma87, Pm1069, and the iron-related proteins Tbp (transferrin binding protein) and the potential TonB receptor HgbA all have discovered as possible adhesins. OmpA, an ion channel protein with a β-barrel, has been discovered as playing a direct function in adhesion. In *E. coli*, *Haemophilus influenza* and other bacteria, homologogs to this protein are significant adhesions. Recombinant *P. multocida* OmpA binds to bovine kidney cells and interacts with heparin and fibronectin, two extracellular matrix components found in the host.

Given the extensive range of diseases produced by *P. multocida* and their global economic significance, it may come as a surprise that little known about the cellular and molecular pathogenesis of pasteurellosis. The main reason for this lack of information that effective genetic modification of *P. multocida* has just achieved in the last decade or so. Prior to the genomic era, most research was descriptive; genes or proteins responsible for virulence-related rarely found or described.

2.7.4 Detection methods of *P. multocida*:

2.7.4.1 Cultural Characteristics of *P. multocida* (Kalhoro et al., 2015):

On blood agar:

- On blood agar, colonies of the organism found moist, mucoid and shiny appearance without any hemolysis.

On MacConkey agar:

- No growth appeared on MacConkey agar

On Muller Hinton Agar (MHA):

- Colonies are light white colored

On Brain Heart Infusion (BHI) broth:

- Colonies are white colored

On broth:

- Medium granular deposits form on the bottom of the tube.

2.7.4.2 Microscopic characteristics of *P. multocida* (Kalhoro et al., 2015):

On grams' staining, the organism found gram-negative, arranged in single, small rods or coccobacillary, bipolar and non-motile.

2.7.4.3 Biochemical characteristics of *P. multocida* (Jabeen et al., 2013):

Oxidase test:

- Oxidase reagent poured on filter paper
- *P. multocida* isolates spread over it with help of loop
- *P. multocida* is oxidase positive as the enzyme oxidase oxidizes phenylenediamine.

Catalase test:

- In test tube some amount of H2O2 taken
- *P. multocida* colony picked up with the help of wire loop and dipped into H2O2
- Bubbling appeared in the tube due to production of H2S gas indicates positive test result

Indole test:

- Colony of *P. multocida* was picked up by using a sterilized loop and dipped into the indole contained test tube and mix up
- Then incubate for 24 hours
- Positive test result indicated by appearance of oily ring on the surface of the media after adding Kovacs reagent

Triple Sugar Iron Test (TSI):

- Take TSI agar slant
- Pick up colony with the help of wire loop and streak over the slant
- Incubate for 24 hours
- Positive test result indicated by bubble formation in the tube

Glucose test:

- Transfer colony into a glucose test tube with the help of a loop

- Rotate inside the test tube for proper mixing
- Incubate for 24 hours
- Observe glucose test positive as it carried out fermentation.

Sugar test:

- Carry the test using 2% sorbitol, xylose, and maltose
- 1 ml each added to glucose media and incubate for 24 hours
- Sugar test result indicated negative for sorbitol and positive for xylose

Nitrate test:

- Pick up colony with help of a loop and mix into nitrate reagent
- Incubate for 24 hours
- Add 1ml of reagent 1(sulphonic acid) and then again add 1 ml of reagent 2 (alpha-naphthalene)
- Appearance of red color indicate positive test result

Motility test:

- Motility checked on peptone water agar
- Pass straight wire loop over the flame and pick up a colony
- Then dip in peptone water agar
- Negative test result indicate *P. multocida* is non-motile

2.7.5 Antibiotic-Resistance pattern of *P multocida*:

2.7.5.1 Resistance to Tetracycline:

At least nine tetracycline resistance genes (*tet* genes) have been found in bacteria from the genera *Pasteurella*, representing two resistance mechanisms (tetracycline exporters and ribosome protecting proteins). *Tet(*A), *tet(*B), *tet*(C), *tet*(G), *tet*(H), *tet*(L), and *tet*(K) are among the *tet* genes coding for membrane-associated proteins of the major facilitator superfamily that specifically export tetracyclines from the bacterial cell, however *tet*(K) is only typically found on tiny plasmids in human pathogens. On *P. multocida* isolates, the corresponding transpon, Tn5706 identified in 1998 which is a 4,378-bp non-conjugative composite transposon that is the first known resistance-mediating transposon discovered among *Pasteurella* species (Hunt et al., 2000). The sul2 sulfonamide resistance gene, as well as the *str*A and *str*B streptomycin resistance genes, flank the *tet*R-*tet*(H) gene area of the *P. multocida* plasmid pVM111, forming a new resistance gene cluster (Kehrenberg et al., 2003). The *Tet*(G) discovered on the

chromosome of six epidemiologically similar *M. haemolytica* isolates from cattle, as well as on the plasmid pJR1 from avian *P. multocida* (Wu et al., 2003). Surprisingly, the *tet*(G) structural gene was discovered in plasmid pJR1 without a corresponding *tet*R repressor gene, which is thought to be required for tetracycline-inducible expression of *tet*(G). It should note that the resistance genes identified on plasmid pJR1 have not introduced into susceptible recipient strains for phenotypic confirmation of their activity (Wu et al., 2003). The appropriate regulatory region, on the other hand, was missing from plasmid pCCK3259, although the upstream sequence had all the components required for constitutive expression of the *tet*(L) gene (Kehrenberg et al., 2003).

2.7.5.2 Resistance to β-Lactam antibiotics:

Generally, Pasteurellaceae resistance to β-lactam antibiotics based on the development of a β-lactamase enzyme or presence of penicillin-binding proteins with low affinity to β-lactams (Kehrenberg et al., 2006 and Schwarz 2008). In Pasteurellaceae, mechanisms such as reduced outer membrane permeability or multidrug efflux systems capable of efficiently exporting β-lactams from the bacterial cell have identified only rarely (Kehrenberg et al., 2006 and Schwarz 2008). Pasteurellaceae has so far found five -lactamase (*bla*) genes: *bla*ROB-1(San Millan et al., 2007), *bla*TEM-1 (Naas et al., 2001), *bla*PSE-1 (Wu et al., 2003), *bla*CMY-2 (Chander et al., 2011), and *bla*OXA-2 (Michael et al., 2012). Interestingly, the complete *bla*oxa-2 gene identified as part of ICEPmu1, was found functional in *E. coli* but non-functional in *P. multocida* (Michael et al., 2012). The ROB-1 and TEM enzymes classified as Ambler class A because of their structure, and Bush class 2b due of their substrate profile, according to existing β-lactamase categorization schemes. Members of this family have a narrow spectrum of activity and can hydrolyze penicillins and first-generation cephalosporins, but they are sensitive to inhibition by β-lactamase inhibitors such clavulanic acid. The PSE-1 β-lactamase belongs to the Amber class A but not to the Bush class 2c. This enzyme, also known as CARB-2-lactamase, can hydrolyze carbenicillin and inactivated by clavulanic acid. Only one study revealed the discovery of a TEM-1 β-lactamase in a *P. multocida* strain from a human dog bite wound (Naas et al., 2001). Similarly, PSE-1-lactamase has also discovered in a single avian *P. multocida* isolate (Wu et al., 2003). The TEM-1-lactamase validated in these studies by isoelectric focusing and sequencing analysis of a portion of the *bla*TEM-1 gene, whereas the *bla*PSE-1 gene sequenced entirely. According to Chander et al., (2011),

the *bla*CMY-2 gene only discovered by PCR in an apparently ceftiofur-resistant *P. multocida* strain from a pig. The role of *bla*CMY-2in ceftiofur resistance *P. multocida* remains questionable as the *bla*CMY-2 gene also detected in ceftiofur-susceptible isolates in the same study and no functional analysis performed.

2.7.5.3 Resistance to Aminoglycosides and Aminocyclitols:

Resistance to aminoglycoside and aminocyclitol antibiotics is mainly mediated by enzymes that adenylate, acetylate, or phosphorylate the medicines, rendering them inactive. Furthermore, mutations in chromosomal genes have discovered to mediate resistance to specific antimicrobial drugs in these classes (Schwarz et al., 2006).

Resistance to aminoglycosides and aminocyclitols by enzymatic inactivation, Streptomycin and/or spectinomycin resistance mediated by enzymatic inactivation:

Streptomycin resistance genes were the first aminoglycoside resistance genes discovered in *Pasteurella* and *Mannheimia*. Streptomycin resistance in *P. multocida* is frequently associated with small non-conjugative plasmids of less than 15 kb (Millan et al., 2009). Additional resistant genes such as Sulfonamide resistance gene *sul2*, kanamycin/neomycin resistance gene *aphA1*, chloramphenicol resistance gene *catA3* and/or ampicillin resistance gene *bla*ROB-1 are carried by many of these plasmids. *StrA* is the most common streptomycin resistance gene found in *Pasteurella*, *Mannheimia*, and *Actinobacillus* bacteria. Its codes for a 269 amino acid aminoglycoside-3″-phosphotransferase and sometimes found combined with the *strB* gene, which codes for a 278 amino acid aminoglycoside-6-phosphotransferase. *StrA* is frequently intact in streptomycin resistant *Pasteurella*, *Mannheimia*, and *Actinobacillus* isolates, but several truncated *strB* genes have identified (Kehrenberg and Schwarz, 2001). On the 5.2-kb plasmid pJR2 from avian *P. multocida*, an *aadA1* gene coding for an aminoglycoside-3″-adenyltransferase that promotes resistance to both the aminoglycoside streptomycin and the aminocyclitol spectinomycin has discovered (Wu et al., 2003). On a short 5.2-kb plasmid from a bovine *P. multocida* capsular type F isolate from Belgium, a novel streptomycin-spectinomycin resistance gene, named *aadA14*, was identified (Kehrenberg et al., 2005). In addition to other resistance genes, the 92-kb ICEMh1 comprises *strA* and *strB*, which confer streptomycin resistance (MIC, 256 mg/liter), and *aphA1*, which confers kanamycin/neomycin resistance (MICs, 512 mg/liter and 64 mg/liter, respectively) (Eidam et al., 2015). In *P. multocida* strain 36950, the 82-kb ICEPmu1 includes 12

resistance genes, including *strA* and *strB*, *aadA25*, *aadB*, and *aphA1*, with the latter three genes providing resistance to streptomycin (MIC, 256 mg/liter) and spectinomycin (MIC, 512 mg/liter), gentamicin (MIC, 128 mg/liter), and kanamycin/neomycin (MICs, 128 mg/liter and ≥32 mg/liter) (Michael et al., 2012).

2.7.5.4 Resistance to Macrolides:

Because of permeability barriers or multidrug efflux pumps, many Gram-negative bacteria thought to be intrinsically resistant to macrolides. However, Mutations in ribosomal proteins or chemical alteration of the ribosomal target site by rRNA methylases have also described (Schwarz et al., 2006). The rRNA methylase genes *erm*(A) and *erm*(C) in *A. pleuropneumoniae* were discovered after studies of the existence of macrolide resistance genes in bacteria of the genus *Actinobacillus*. PCR investigation of bovine *P. multocida* and *M. haemolytica* with erythromycin MICs of less than 16 mg/liter revealed no evidence of the three genes *erm*(A), *erm*(B), or *erm*(C). In the chromosome of *M. haemolytica* and *P. multocida* isolates a novel monomethyltransferase gene, *erm*, has been discovered with high levels of resistance to various macrolides (Desmolaize et al., 2011). The monomethyltransferase gene's sequence discovered to be distinct from previously described *erm* genes, and it only detected through whole-genome sequencing of resistant isolates. Mutations were found in one of two locations (either A2058G or A2059G) in all six copies of the 23S rRNA in isolates of *P. multocida* and *M. haemolytica* reported to be highly resistant (MICs >64 mg/liter) to multiple macrolides including erythromycin, tilmicosin, tildipirosin, tulathromycin, and gamithromycin (Olsen et al., 2015).

2.7.5.5 Resistance to Phenicols:

Resistance to nonfluorinated phenicols may mediated by phenicol specific exporters, whereas resistance to fluorinated and/or nonfluorinated phenicols may mediated by enzymatic inactivation by chloramphenicol acetyltransferases (Schwarz et al., 2006). Other mechanisms, such as permeability barriers, have found in Pasteurellaceae only infrequently.

Chloramphenicol resistance mediated by enzymatic inactivation:

Chloramphenicol resistance mainly caused by enzymatic inactivation of the drug by chloramphenicol acetyltransferases. There are now two varieties of chloramphenicol acetyltransferases, A and B, which defined by several distinct *catA* and *catB* genes (Schwarz et al., 2004). Normally, the *catA* and *catB* genes located on plasmids,

transposons, or gene cassettes. In porcaine *P. multocida* isolates, bovine *P. multocida* isolates, and *M. haemolytica* isolates, plasmids mediating chloramphenicol resistance have been identified. On plasmid pJR1 from avian *P. multocida* (Wu et al., 2003), a *catB2* gene discovered, which codes for a different type of chloramphenicol acetyltransferase than the *catA* genes (Schwarz et al., 2004). Because the *catB2* gene is part of a gene cassette, its expression is dependent on the integron-associated promotor. If a suitable promotor is available, gene cassettes placed at secondary locations outside of integrons can expressed (Ojo et al., 2002). However, no such promotor has discovered in pJR (Wu et al., 2003).

2.7.5.6 Resistance to (Fluoro) quinolones:

Quinolones are antimicrobials with a broad spectrum of activity that inhibit bacterial DNA gyrase and topoisomerase IV. Resistance often caused by mutations in the genes that code for the various subunits of both enzymes, although it can also cause active efflux or protection of the enzymes by Qnr proteins (Schwarz et al., 2006).

(Fluoro) quinolone resistance mediated by target site mutations:

Resistance to (fluoro) quinolones in *Pasteurella*, *Mannheimia*, *Actinobacillus*, and *Haemophilus* poorly understood. *Pasteurella* spp. from humans and animals (Kaspar et al., 2007) isolates shown to be extremely susceptible to the fluoroquinolones examined in almost all cases. The first study of the quinolone resistance determining region (QRDR) of the proteins encoded by the genes *gyrA* and *parC* in *P. multocida* isolates found a Ser83Ile change in *GyrA* in an isolate with a nalidixic acid MIC of 256 mg/liter, while Asp87Gly changes were found in isolates with nalidixic acid MICs of 4 and 12 mg/liter (Cárdenas et al., 2001). Whole-genome sequencing of the multi-resistant bovine *P. multocida* strain 36950, which had an enrofloxacin MIC of 2 mg/ liter, revealed two base pair exchanges in the QRDR of *gyrA*, resulting in the amino acid modifications GGT AGT (Gly75Ser) and AGC AGA (Ser83Arg). Aditionally, a single base pair exchange in parC's QRDR, TCA TTA, that resulted in a Ser80Leu exchange, was also seen (Michael et al., 2012). More recently, fluoroquinolone-resistant clinical isolates of *P. multocida* shown to exhibit Asp87Asn or Ala84Pro mutations in *GyrA*, with MICs of 0.5 mg/liter for both enrofloxacin and ciprofloxacin (Kong et al., 2014).

2.8 Problems associated with antimicrobial resistance:

It is an alarming issue and major concern about the problem of antimicrobial resistance in the whole world. World Health Organization (WHO) is much concern about the AMR and increased anxiety about the role of antimicrobials used in animal husbandry. Many meetings and conferences occurred to prevent and control the emergence and spread of antimicrobial resistant microorganisms. Now it is impossible to return the pre-antibiotic era, so we must concern about the antimicrobial resistance.

AMR is a global threat to both human and animals and day by day it is increasingly growing and poses a huge health risk to the human, animals, and environment. Antimicrobial resistance has the direct and indirect effects on the health. When the levels of antimicrobials are high, then it can be toxic to the human or animals. Most of the antibiotics have the direct effect as for example Penicillin causes hypersensitivity reactions and produces allergy. In USA, self-reported penicillin allergy was reported about 80% to 90% of the individuals. The report also suggested that unnecessarily exposed to broader-spectrum antibiotics leads to developing of antimicrobial resistant microorganisms (Pongdee and Li, 2018). Some antimicrobials cause endocrine disruption such as oxytetracycline, tetracycline and sulfamethoxazole and some causes nervous effects (cefuroxime, neomycin) (Lee et al., 2001).

The main problem of AMR is growing the resistance to the specific antibiotics that would not work further. Improper and inappropriate use of antibiotics leads to develop the resistance. Most antibiotics used in two disciplines: treatment of humans and growth promotion and prophylaxis in animals. In recent years increasing of broad-spectrum agents to the patients and crowd of animals in hospitals are other major causes of transferring resistant microorganisms. The cost of the treatment increased due to the resistant pathogens and in most of the cases commercially available drugs didn't work for patients. So they had to buy uncommon antibiotics at a high price.

CHAPTER-3

MATERIALS AND METHOD

3.1 Study design and sampling:

The study was conducted at S. A. Q. Teaching Veterinary Hospital (SAQTVH) in Chattogram Veterinary and Animal Sciences University (CVASU) during the period of August 2019 to June 2021. Nasal Swab samples were collected from 120 goats having different respiratory signs such as nasal discharge, rhinitis, coughing, sneezing, pneumonia etc. (Excluding PPR cases). Samples were collected by inserting sterile cotton buds into the nostrils of clinically sick goats. All samples were collected prior to give antibiotics. The collected samples were inoculated into falcon tube containing 5 ml buffer peptone water (Oxoid, UK). Then the samples were transferred into the laboratory of the Department of Medicine and Surgery, CVASU using ice box to keep at 4°C until further investigation. Both sample collection and laboratory tests were performed simultaneously. Rest of the laboratory tests such as PCR and culture sensitivity test by disc diffusion method were done up to August 2021. A structured questionnaire was developed to note down the data related to the occurrence of respiratory tract infection in goats. The data were collected by direct interviewing the goat owners and by direct inspection and examination of the goats. Parameters including age, sex, breed, BCS, rearing system, health status, temperature, respiratory sound were considered for the study.

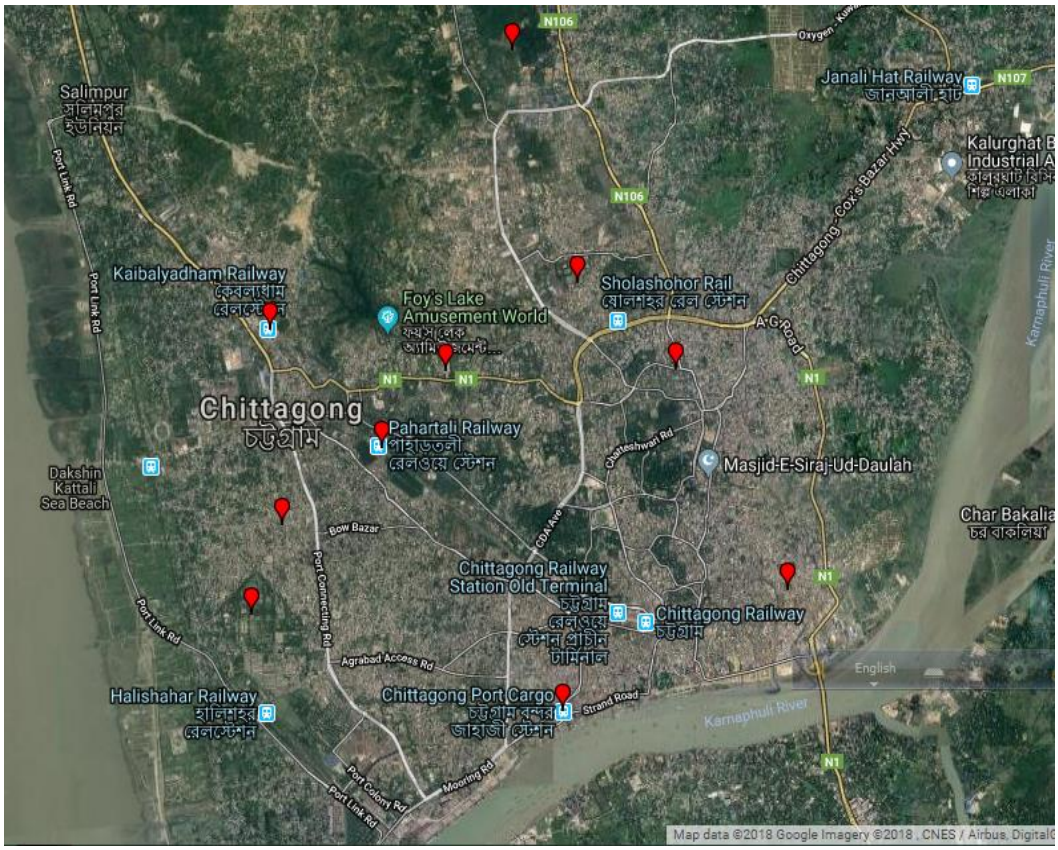

Figure 3.1: Geographical position of sampling area

3.2 Experimental design:

The study was divided into 4 major steps: Firstly, Collection and transportation of the sample to the laboratory. Secondly, bacterial isolation and identification based on cultural characteristics and grams staining. Thirdly, confirmation of isolated bacteria through different biochemical test and polymerase chain reaction (PCR). Lastly, culture sensitivity test to detect antimicrobial resistance pattern of isolates.

3.3 Laboratory investigation:

3.3.1 Isolation and identification of *S. aureus*:

Nasal swab samples were enriched into Muller-Hinton (MH) Broth (Oxoid, Basingstoke, Hampshire, UK) with 6.5% NaCl and incubated at 37ºC for 24 hours. Then the inoculum given into Mannitol salt agar (MSA) which was prepared according to the instructions of the manufacturer. Additionally, blood agar was prepared by adding 5% citrated- bovine blood in the blood agar base (Oxoid, Basingstoke, Hampshire, UK). The presumptive positive colonies were sub-cultured onto blood agar and incubated at 37ºC for 24 hours to identify the organisms hemolytic properties and appearance on blood agar (Rana et al., 2020). Grams' staining was also performed to

determine the morphology of the bacteria. Biochemical tests including catalase and coagulase tests were done to confirm suspected colonies.

Coagulase test:

Whole blood from a horse was collected into commercially supplied EDTA-treated lavender tops for the coagulase test. Then using a refrigerated centrifuge device, the blood centrifuged at 2600 rpm for 10 minutes. The follow-on supernatant, the plasma instantly transferred to a sterile 1.5 ml eppendorf tube using sterile micropipette and tips and kept at -20ºC for further use.

Tube coagulase test:

For coagulase test, Brain Heart Infusion Broth (BHIB) was prepared according to the instructions of manufacturer (Oxoid, Basingstoke, Hampshire, UK). Positive colonies transferred to a 10 ml test tube containing 5 ml of BHIB and incubated at 37ºC for 6 hours. Then, 50µL BHIB containing cultivated sample transferred to the sterile tubes containing 50µL of horse plasma and incubated at 37ºC for 6 hours. Because of the massive, ordered coagulation and coagulation of all the contents of the tube that do not fall off when inverted, the presence of coagulates was justified (Graham et al., 2006). For validation of the result, a control tube without plasma also placed.

Slide coagulase test:

For slide coagulase test, a drop of horse plasma placed on a clean grease free glass slide. A loopful of suspected colony taken and mixed with the plasma to check for agglutination. A positive test result showed by proper agglutination and thus confirmed as *S. aureus*.

Slide Catalase test:

A fresh, clean and grease free slide was taken where small amount of colony was placed. One drop of 3% H2O2 poured on the colony and a cover slip was placed. Positive test results indicated by bubble formation.

3.3.2 Isolation and identification of *E. coli*:

For the isolation and identification of *E. coli*, samples were selectively enriched on MacConkey broth (Oxoid, Basingstoke, Hampshire, UK) at 37ºC for 24 hours. After enrichment, samples were inoculated onto MacConkey agar which was prepared according to the instructions of the manufacturer (Oxoid, Basingstoke, Hampshire,

UK) and incubated at 37ºC overnight. Suspected colonies from MacConkey agar were further inoculated onto Eosin Methylene Blue (EMB) agar (Oxoid, Basingstoke, Hampshire, UK) and incubated at 37ºC for 24 hours. Colonies with typical green metallic sheen in EMB agar were sub-cultured again onto Blood agar and incubated at 37ºC overnight to identify the organism's appearance on blood agar. An isolated colony from blood agar taken to prepare smear on clean grease free microscopic glass slide and stained with grams staining method for morphological identification of the bacteria. Finally, standard biochemical tests including catalase test, indole, Methyl red, Voges-Proskaoer test, Simmons' citrate and various sugar fermentation tests were done to confirm *E. coli*.

Carbohydrate fermentation test:

Nutrient broth culture (0.2 ml) of the isolated organism inoculated into tubes containing five basic sugars including dextrose, maltose, lactose, sucrose, and mannitol. It was incubated at 37ºC for 24 hours.

Catalase test:

In a test tube, 3 ml catalase reagent (3% H2O2) was taken. Then, single colony from pure culture merged into the reagent.

Simmons' citrate test:

Small amount of isolate was taken and streaked into Simmons' citrate medium containing in a tube. It was incubated at 37ºC for 24-48 hours.

Indole test:

Test organism cultured in 3 ml peptone water containing tryptophan at 37 ºC for 48 hours. One ml diethyl ether was added, shaken, and allowed to stand until the ether rises to the top. Then, 0.5 ml Kovacs reagent gently ran down to the side of the test tube to form a ring between the medium and the ether.

Methyl Red test:

Single colony from pure culture was inoculated in 5 ml of sterile MR-VP broth. After 5 days, incubated at 37 ºC and 5 drops of methyl red solution was added.

Voges-Proskauer (VP) test:

Test organism grown in 3 ml sterile MR-VP broth at 37ºC for 48 hours. Five percent of alphanapthol (0.6 ml) and Forty percent of potassium hydroxide (0.2 ml) containing 0.3% creatine were added on each ml of broth culture. It was shaken and stand for 5-10 min to observe colour formation.

Motility test:

A straight needle was touched to a colony of isolates. It was stab once on SIM media to a depth of only 1/3 to 1/2 inch in the middle of the tube. It was then incubated at 37ºC and observed for a slightly opaque diffused zone of growth flaring out from the line of inoculation.

Reaction in TSI agar slant:

Test organism was cultured into TSI agar slant by stab or streak method.

3.3.3 Isolation and identification of *P. multocida*:

Nasal swab samples were directly inoculated onto blood agar base produced by the instructions of the manufacturer (Oxoid, Basingstoke, Hampshire, UK) and supplemented with 5% defibrinated cattle blood. All the agar plates were incubated for 24-48 hours at 37ºC. The plates were examined for bacterial growth and colonial characteristics. The suspected colonies from blood agar were inoculated onto MacConkey agar (Oxoid, Basingstoke, Hampshire, UK) and incubated at 37ºC overnight. Grams' staining was done by placing a pure isolated colony from blood agar on clean, grease free microscopic glass slide and morphological identification of the bacteria was done. Polymerase chain reaction (PCR) was done to confirm the suspected bacteria.

3.4 Grams' staining technique:

All suspected positive colonies were picked up from blood agar and smear was prepared on clean, grease free fresh microscopic glass slide which was followed by staining procedure of grams staining. It is a method of differentiation of two large groups of bacterial species (gram-positive and gram-negative) based on physical and chemical properties on their cell walls.

For Grams' staining of bacteria, smear was prepared on microscopic slide with distinct bacterial colony. The smear was properly flooded with crystal violet and waited for 1 minute. Then, the crystal violet was rinsed off gently with tap water followed by

flooding of grams iodine and waited for 1 minute. After 1 minute, the iodine was rinsed off gently with tap water again the smear was flooded with acetone and rinsed off the acetone with tap water after 5 seconds. The smear was then flooded with safranin and rinsed off the safranin gently with tap water after waiting for 30 seconds. The slide was then dried and examined with cover slip under 100X microscopic vision using emulsion oil.

3.5 Preservation of culture:

All positive samples were inoculated onto Brain Heart Infusion Broth (BHIB) (Oxoid, Basingstoke, Hampshire, UK) and incubated at 37ºC for 24 hours. For each isolate, 700µl of BHIB culture was placed in a 1.5ml Eppendorf tube in which 300µl of 50% glycerol were added. Lastly, the tubes properly labelled with sample details and stored at -80ºC for further use.

3.6 Molecular detection of *P. multocida*:

PCR was performed for molecular detection of *P. multocida* using *KMT1* gene and *Kmt1* gene. All the molecular investigations carried out in Poultry Research and Training Center (PRTC), CVASU.

3.6.1 Sub-culturing on blood agar:

The stored cultures thawed at room temperature after removal from -80ºC temperature. The isolates sub-cultured onto blood agar and incubated at 37 ºC for 24 hours. The colonies grew on blood agar were used for DNA extraction for further use in PCR.

3.6.2 DNA extraction procedure:

Boiling method was used for DNA extraction. Colonies from blood agar (approximately 3-4) were taken by inoculating loop and transferred to 1.5 ml eppendorf tube containing 100µl de-ionized water. After that, the tubes were vortexed to create a uniform cell suspension. There was a ventilation hole made on the lid of each tube. The tubes were then boiled in water bath at 99ºC for 15 minutes. After boiling, the tubes were immediately transferred at -20ºC for 5 minutes for cooling. The procedure of boiling at a high temperature and then cooling quickly causes the bacterial cell wall to break down, allowing DNA to be released from bacterial cell. The tubes containing suspension were centrifuged at 15000 rpm for 5 minutes. Finally, 50µl supernatant from each tube containing bacterial DNA was collected in sterile Eppendorf tubes and stored at -20ºC for further processing in PCR.

Primer sets: Two sets of published primers were used for molecular detection of *P. multocida* in this study (Verma et al., 2019; Rawat et al., 2019). The primer sequence used for PCR are shown in Table 3.1.

Table 3.1: Primer sequence of different genes:

Primer	Primer Sequence (5′-3′)	Specify	Amplicon size (bp)	Reference
KMT1SP6 F	GCTGTAAACGAACTCGCCAC	*Kmt1* gene	460	Verma et al., 2019
KMT1SP7 R	ATCCGCTATTACCCAGTGG			
KMT1 F	ATCCGCTATTTACCCAGTGG	*KMT1* gene	457	Rawat et al., 2019
KMT1 R	GCTGTAAACGAACTCGCCAC			

3.6.3 PCR amplification of *Kmt1* gene of *P. multocida*:

For PCR amplification, a 25µl reaction mixture was taken where 50ng DNA, 200 µM of dNTPs, 0.2 µM of each primer, 1.875 mM of Mgcl2 and 1.25 U of Taq DNA polymerase (Sigma-Aldrich, USA) in 1×PCR buffer were added. The amplification carried out with an initial DNA denaturation at 95ºC for 4 min, followed by 30 cycles of denaturation at 95ºC for 1 min, annealing at 55ºC for 1 min, initial extension at 72ºC for 1 min and final extension at 72ºC for 9 min.

Table 3.2: Cycling conditions used during PCR for detection Kmt1 gene of *P. multocida*

Serial No	Steps	Temperature and Time
1	Initial denaturation	95°C for 4 minutes
2	Denaturation (30 cycles)	95°C for 1 minute
3	Annealing	55°C for 1 minute
4	Initial Extension	72°C for 1 minute
5	Final Extension	72°C for 9 minutes

3.6.4 PCR amplification of *KMT1* gene of *P. multocida*:

A 25 µl reaction mixture containing 50ng DNA, 200 µM of dNTPs, 0.2 µM of each primer, 1.875 mM of Mgcl2 and 1.25 U of Taq DNA polymerase (Sigma-Aldrich, USA) in 1×PCR buffer was prepared. For performing amplification according to the

previous method an initial DNA denaturation at 95ºC for 3 min, followed by 30 cycles of denaturation at 95ºC for 1 min, annealing at 56ºC for 45 sec, initial extension at 72ºC for 1 min and final extension at 72ºC for 5 min was performed.

Table 3.3: Cycling conditions used during PCR for detection KMT1 gene of *P. multocida*

Serial No	Steps	Temperature and Time
1	Initial denaturation	95°C for 3 minutes
2	Denaturation (35 cycles)	95°C for 45 seconds
3	Annealing	56°C for 45 seconds
4	Initial Extension	72°C for 1 minute
5	Final Extension	72°C for 5 minutes

Table 3.4: Reagents used for PCR amplifications of the 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

Table 3.5: 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

3.6.5 Visualization of PCR Products:

To visualize the PCR product, 1.5% agarose gel (W/V) was used. In a conical flask, 0.75 gm of agarose power was mixed with 50 ml of 1X TAE and boiled in a microwave oven to dissolve the agarose powder. The agarose mixture was then cooled at 50ºC in a water bath. One drop ethidium bromide was added to the mixture. The ends of the gel chamber sealed with tape by assembling the gel casting tray and appropriate number of combs were placed in gel tray. The agarose mixture was poured into the gel tray and kept at room temperature for 20 minutes so that it could be solidified. After that, the combs were removed, and the gel was shifted into an electrophoresis tank filled with 1X TAE buffer and kept until the gel is drowned completely. In a gel hole, an amount of 5 µl of PCR product for a gene was loaded. To compare the amplicons size 3 µl of 100 bp DNA marker (O" gene Ruler 100bp plus) was used and the electrophoresis was run at 110 volts and 80 mA for 30 minutes. Lastly, a UV transilluminator was used to examine the gel for image acquisition and analysis.

3.7 Antimicrobial resistance testing of *Staphylococcus aureus* and *Escherichia coli:*

All positive bacterial isolates (*S. aureus* and *E. coli*) were examined for antimicrobial resistance testing to detect their diversity to antimicrobials. Kirby-Bauer disc diffusion procedure was used to carry out the screening of isolates against a panel of antimicrobials by disc diffusion method. Eleven molecules from six different groups of drugs including β-lactam, semi- synthetic β-lactam, tetracyclines, fluoroquinolone, sulfonamides, aminoglycosides were used for testing. The following anti-microbial agents (with respective disc potentials) were used: CRO: Ceftriaxone (30µg), CN: Gentamycin (10µg), CIP: Ciprofloxacin (5µg), ENR: Enrofloxacin (5µg), TE: Tetracycline (30µg), SXT: Sulfamethoxazole-trimethoprim (26 µg), AMC: Amoxicillin-Clavulanic acid (30µg), AMP: Ampicillin (10µg), AML: Amoxicillin (10µg), AZM: Azithromycin (15µg), P: Penicillin-G (10µg). For interpretation of the CS test result the CLSI standard given in Table 3.2

3.7.1 Culture Sensitivity (CS) test procedure:

To run CS test, the stored positive isolates were sub-cultured onto blood agar and incubated at 37ºC for 24 hours. Muller-Hinton agar was prepared according to the instructions of the manufacturer (Oxoid Ltd, Basingstoke, Hampshire, UK) and incubated at 37ºC overnight. Three ml of sterile phosphate buffer saline solution

(0.85% w/v NaCl solution) was taken in a test tube where 3-5 well-isolated individual colonies were transferred from blood agar using a sterile inoculating loop. To avoid clumping of the cells inside the test tube emulsification of the inoculums done using a vortex machine. The bacterial suspension was adjusted to the turbidity of 0.5 McFarland standard (equivalent to growth of 1-2×108 CFU/ml). A sterile cotton swab was dipped into the inoculum and rotated against the side wall of the tube with slide pressure so that excess fluid from the cotton swab removed. The surface of the Muller-Hinton agar plate inoculated by streaking the swab for 3 times rotating the plate approximately 60 degrees angle for even distribution of inoculum. Then the antibiotic discs were placed on the agar surface with a sterile forceps and slightly pressed down to ensure complete contact. The discs were placed not more than 24mm from each other and total 6 discs in 150 mm plate. The agar plates were incubated at37ºC for 18 hours after proper distribution of all discs. After incubation, the size of the zone of inhibition (in mm) including the diameter of the disc was measured and the result was interpreted considering as Resistant (R), Intermediate (I) and Sensitive (S) according to CLSI, 2021.

Table 3.6: Concentration and diffusion zone breakpoints for resistance against antimicrobials standard for Staphylococcus aureus and Escherichia coli (CLSI, 2021)

Antimicrobial Agent	**Disc Content**	**Diffusion Zone Breakpoint (Diameter in mm)**					
		S. aureus			***E. coli***		
		R	**I**	**S**	**R**	**I**	**S**
Ceftriaxone (CRO)	30µg	≤13	14-20	≥21	≤19	20-22	≥23
Amoxicillin-Clavulanic acid (AMC)	30µg	≤19	-	≥20	≤13	14-17	≥18
Gentamycin (CN)	10µg	≤12	13-14	≥15	≤12	13-14	≥15
Ciprofloxacin (CIP)	5µg	15	16-20	21	21	22-25	26
Trimethoprim/Sulfamethoxazole (SXT)	26µg	10	11-15	16	10	11-15	16
Enrofloxacin (ENR)	5µg	15	16-20	21	21	22-25	26
Tetracycline (TE)	30µg	14	15-18	19	≤11	12-14	≥15

Ampicillin (AMP)	10µg	≤28	-	≥29	≤13	14-16	≤17
Amoxicillin (AML)	10µg	≤28	-	≥29	≤28	-	≥29
Azithromycin (AZM)	15µg	≤13	14-17	≥18	≤12	-	≥13
Penicillin-G (P)	10µg	28	-	29	15	-	14

3.8 Statistical Analysis:

All field and laboratory data were sorted into Microsoft office excel 2013 and the spreadsheet was inserted into STATA-IC 13® (StataCorp. College Station, Texas, USA) software to perform statistical analysis.

3.9 Descriptive analysis:

Descriptive analysis was performed to calculate the percentage and 95% confidence interval (CI) of antimicrobial resistance pattern of isolated organisms. A chi-square test was performed to check the association between isolated organisms' status with the different demographic and management practices of studied animals.

CHAPTER-4

RESULTS

4.1 Confirmation of *S. aureus*:

Among 120 samples, 16 (19.2%) isolates were confirmed as *S. aureus* based on the cultural characteristics on mannitol salt agar plate and blood agar plate after incubation at 37ºC for 24 hours. Bright yellow colonies surrounded by yellow zones on MSA indicated positive for *S. aureus* due to the fermentation of mannitol (Kateete et al., 2010). On blood agar, *S. aureus* showed yellow to golden yellow colony with β-hemolysis. In catalase and coagulase test, bubbles and coagulates formation were found respectively, indicating *S. aureus* positive. In grams' staining, violet-colored cocci that were arranged in grapes like clusters revealed as Gram-positive *S. aureus*. All the cultural, biochemical, and microscopic characteristics of *S. aureus* shown in figure 4.1.

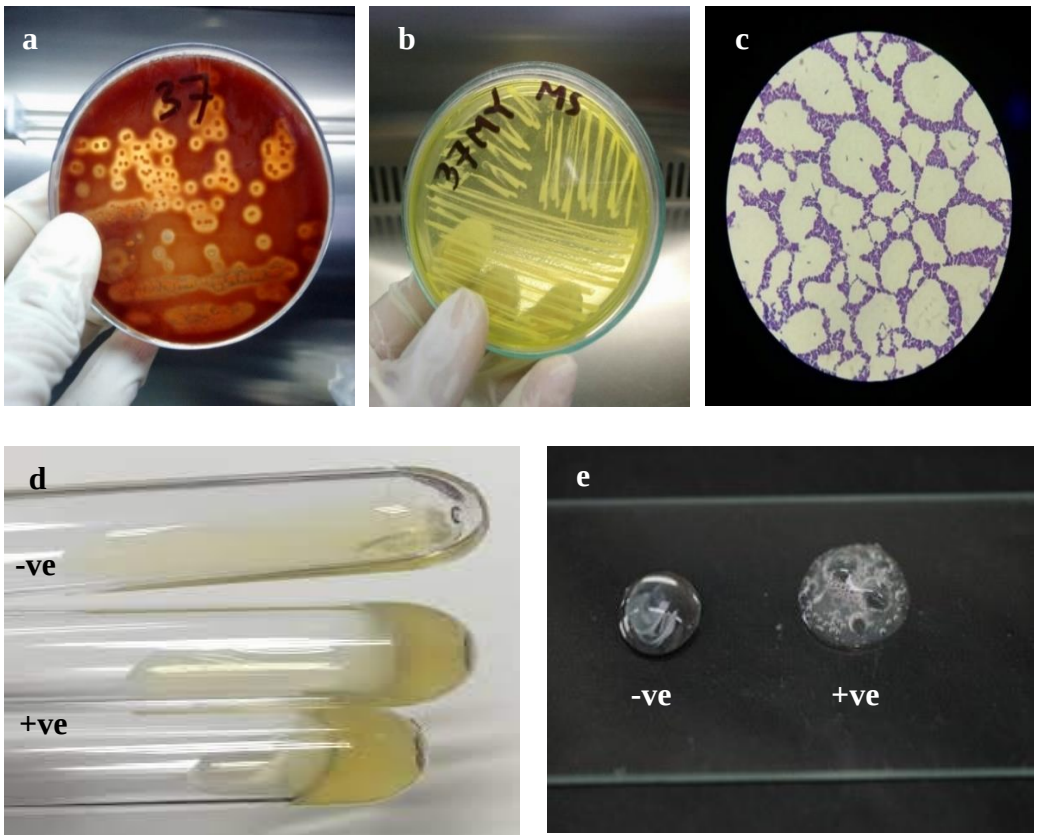

Figure 4.1: Confirmation of *S. aureus* on (a) blood agar indicating β-hemolysis (b) mannitol salt agar indicating bright yellow colonies (c) cluster of grapes like colony of *S. aureus* on grams' staining (d) tube coagulase test for *S. aureus* (e) slide catalase test for *S. aureus*

4.2 Confirmation of *E. coli*:

In MacConkey agar plate, Eosin methylene blue (EMB) agar plate, 8 (6.67%) isolates from 120 samples were confirmed as *E. coli* based on their cultural characteristics. On MacConkey agar, *E. coli* produced smooth pink colonies. On EMB agar, positive colonies identified as smooth, large, circular, blue-black colonies with green metallic sheen. In biochemical tests, *E. coli* was motile, lactose fermenting, indole positive, Methyl Red positive, Voges- Proskauer negative. In grams' staining, *E. coli* found Gram-negative, pink coloured, small rod-shaped bacillus arranged in single, pairs or short chain. All the cultural, biochemical, and microscopic characteristics of *E. coli* shown in figure 4.2.

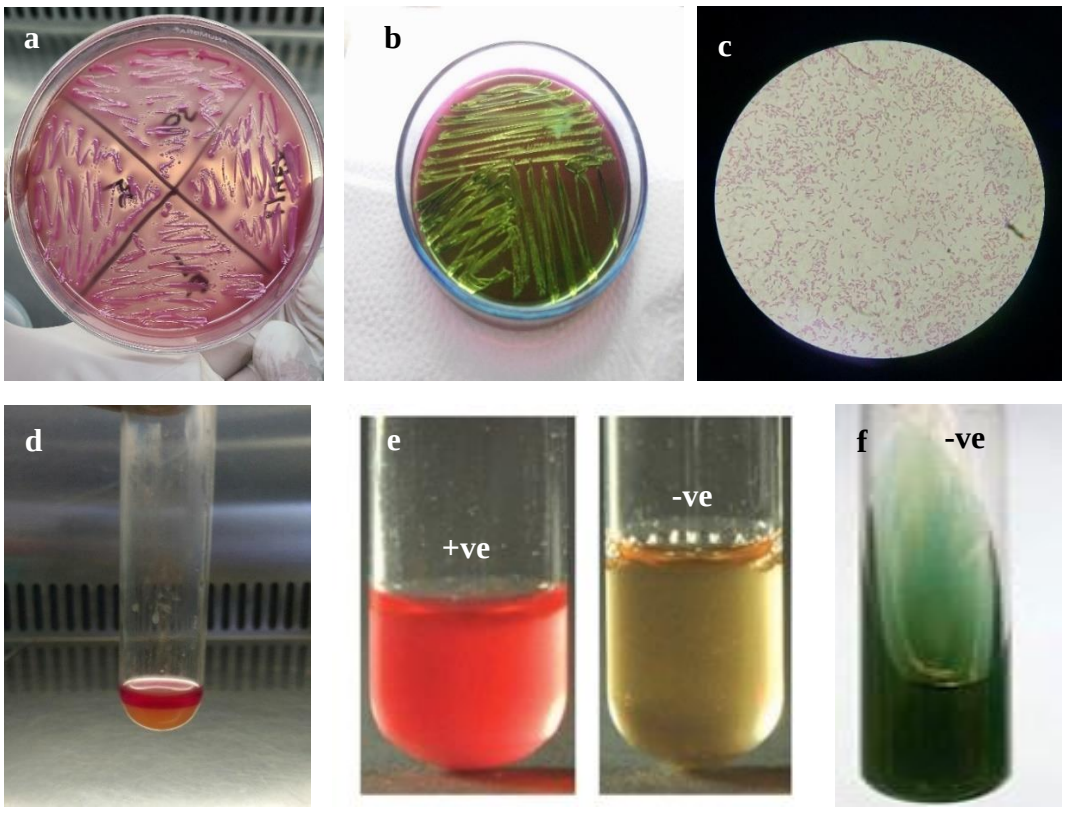

Figure 4.2: Confirmation of *E. coli* on (a) MacConkey agar indicating pink colonies, (b) EMB agar indication metallic sheen, (c) pink coloured rod shaped colonies of *E. coli* on grams' staining, (d) Indole positive (e) MR positive and VP negative (f) Simmons' citrate negative.

4.3 Confirmation of *P. multocida*:

4.3.1 Cultural and morphological detection of *P. multocida*:

Among 120 samples, 26 samples were suspected to be *P. multocida* based on their cultural and morphological characteristics. On blood agar, suspected colonies formed small, translucent, glistening, dewdrop like non-hemolytic colonies. Suspected colonies failed to grow on MacConkey agar. On grams' staining, gram-negative, small pink coloured, rod shaped organisms were suspected as *P. multocida.* All the cultural and microscopic characteristics of *P. multocida* shown in figure 4.3.

4.3.2 Detection of *P. multocida* by PCR method:

All the 26 suspected isolates were subjected to PCR assay for the confirmation of *P. multocida* organism through *KMT1* gene and *kmt1* gene. Unfortunately, none of isolates found to be positive for *P. multocida* in PCR assay.

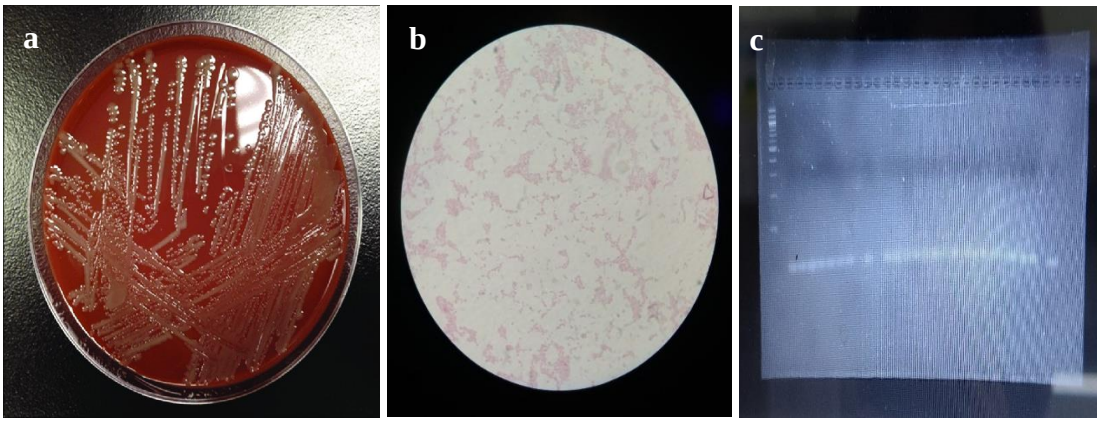

Figure 4.3: Suspected *P. multocida* colonies on (a) blood agar, (b) pink coloured rod shaped colonies on grams' staining, (c) no band on PCR assay.

4.4 Antimicrobial resistance pattern of *S. aureus*:

To observe the antimicrobial resistance pattern, cultural sensitivity test was performed against 11 different antimicrobials. All *S. aureus* isolates (16) were found to be resistant to ampicillin (100%) (Figure 4.4). It was also found to be resistant against amoxicillin (93.75%, CI 69.77 – 99.84), amoxicillin-clavulanic acid (62.5%, 95% CI 35.43 – 84.8) and penicillin (93.75%, 95% CI 69.77 – 99.84) (Figure 4.4). On the other hand, highest number of *S. aureus* isolates were showed sensitivity against both sulfamethoxazole-trimethoprim (75%, CI 47.62 – 92.73) and gentamycin (75%, 47.62 – 92.73) followed by tetracycline (62.5%, CI 35.43 – 84.8), enrofloxacin (50%, CI 24.65 – 75.35). No isolates found sensitive to ampicillin. Sensitivity and resistance pattern of *S. aureus* shown in Table 4.1 and figure 4.6.

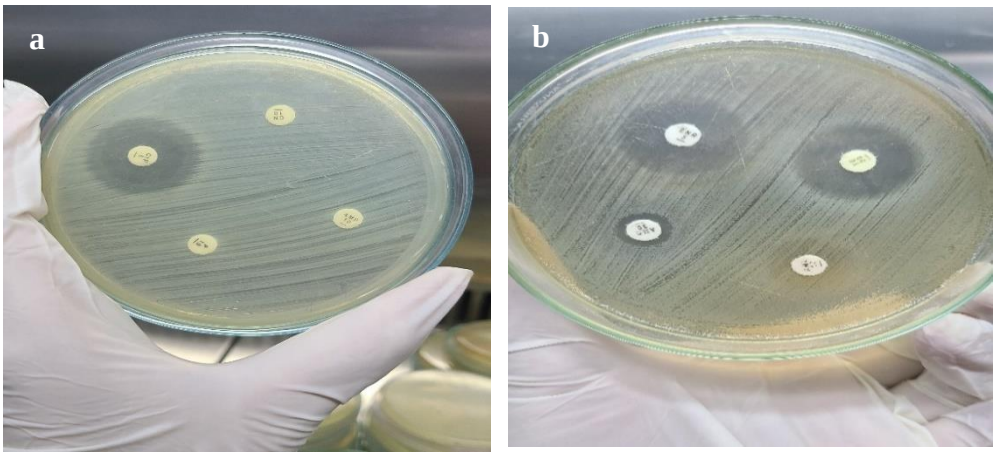

Figure 4.4: *S. aureus* resistance to (a) penicillin and ampicillin (b) amoxicillin and amoxicillin-clavulanic acid

4.5 Antimicrobial resistance pattern of *E. coli*:

The highest resistance in *E. coli* positive isolates (8) observed against amoxicillin (100%) and penicillin (100%) (Figure 4.5). After that, most of the isolates found resistant to ampicillin (87.5%, CI 47.35 – 99.68) (Figure 4.5), azithromycin (87.5%, CI 47.35 – 99.68). About 62.5% isolates displayed sensitivity against amoxicillin-clavulanic acid, ciprofloxacin, gentamycin, and ceftriaxone (62.5%, CI 24.49 – 91.48). Like *S. aureus*, sensitivity to ampicillin not found in any isolates of *E. coli*. Sensitivity and resistance pattern of *E. coli* shown in table 4.1 and figure 4.7.

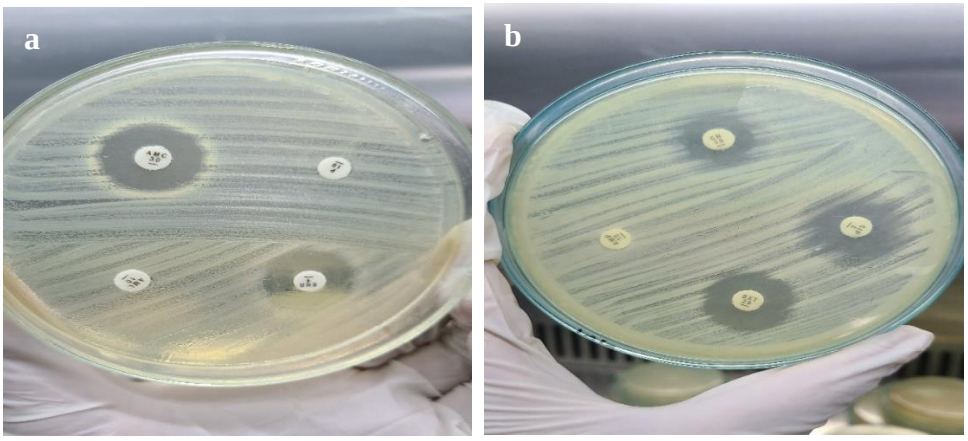

Figure 4.5: *E. coli* resistance to (a) penicillin and amoxicillin (b) ampicillin

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

Name of antibiotics	*S. aureus*				*E. coli*			
	S, n (%), 95% CI	I, n (%), 95% CI	R, n (%), 95% CI	I + R, n (%), 95% CI	S, n (%), 95% CI	I, n (%), 95% CI	R, n (%), 95% CI	I + R, n (%), 95% CI
AMP	0, 0 – 20.59	0, 0 – 20.59	16, (100%), 79.41 - 100	16, (100%), 79.41 - 100	0, 0 – 36.94	1, (12.5%), 0.32 – 52.65	7, (87.5%), 47.35 – 99.68	8, (100%), 63.06 - 100
AMC	6, (37.5%), 15.2 – 64.56	0, 0 – 20.59	10, (62.5%), 35.43 – 84.8	10, (62.5%), 35.43 – 84.8	5, (62.5%), 24.49 – 91.48	1, (12.5%), 0.32 – 52.65	2, (25%), 3.18 – 65.08	3, (37.5%), 8.52 – 75.51
AML	1, (6.25%), 0.16 – 30.23	0, 0 – 20.59	15, (93.75 %), 69.77 – 99.84	15, (93.75%), 69.77 – 99.84	0, 0 – 36.94	0, 0 – 36.94	8, (100%), 63.06 - 100	8, (100%), 63.06 - 100
CIP	7, (43.75 %), 19.75 – 70.12	4, (25%), 7.27 – 52.38	5, (31.25 %), 11.02 – 58.66	9, (56.25%), 29.88 – 80.25	5, (62.5%), 24.49 – 91.48	1, (12.5%), 0.32 – 52.65	2, (25%), 3.18 – 65.08	3, (37.5%), 8.52 – 75.51
ENR	8, (50%), 24.65 – 75.35	6, (37.5 %), 15.2 – 64.56	2, (12.5%), 1.55 – 38.35	8, (50%), 24.65 – 75.35	3, (37.5%), 8.52 – 75.51	0, 0 – 36.94	5, (62.5%), 24.49 – 91.48	5, (62.5%), 24.49 – 91.48
P	1, (6.25%), 0.16 – 30.23	0, 0 – 20.59	15, (93.75 %), 69.77 – 99.84	15, (93.75%), 69.77 – 99.84	0, 0 – 36.94	0, 0 – 36.94	8, (100%), 63.06 - 100	8, (100%), 63.06 - 100

AZM	1, (6.25%), 0.16 – 30.23	9, (56.25%), 29.88 – 80.25	6, (37.5%), 15.2 – 64.56	15, (93.75%), 69.77 – 99.84	1, (12.5%), 0.32 – 52.65	0, 0 – 36.94	7, (87.5%), 47.35 – 99.68	7, (87.5%), 47.35 – 99.68
CN	12, (75%), 47.62 – 92.73	3, (18.75%), 4.05 – 45.64	1, (6.25%), 0.16 – 30.23	4, (25%), 7.27 – 52.38	5, (62.5%), 24.49 – 91.48	1, (12.5%), 0.32 – 52.65	2, (25%), 3.18 – 65.08	3, (37.5%), 8.52 – 75.51
TE	10, (62.5%), 35.43 – 84.8	1, (6.25%), 0.16 – 30.23	5, (31.25%), 11.02 – 58.66	6, (37.5%), 15.2 – 64.56	4, (50%), 15.7 – 84.3	0, 0 – 36.94	4, (50%), 15.7 – 84.3	4, (50%), 15.7 – 84.3
CRO	7, (43.75%), 19.75 – 70.12	5, (31.25%), 11.02 – 58.66	4, (25%), 7.27 – 52.38	9, (56.25%), 29.88 – 80.25	5, (62.5%), 24.49 – 91.48	0, 0 – 36.94	3, (37.5%), 8.52 – 75.51	3, (37.5%), 8.52 – 75.51
SXT	12, (75%), 47.62 – 92.73	2, (12.5%), 1.55 – 38.35	2, (12.5%), 1.55 – 38.35	4, (25%), 7.27 – 52.38	4, (50%), 15.7 – 84.3	1, (12.5%), 0.32 – 52.65	3, (37.5%), 8.52 – 75.51	4, (50%), 15.7 – 84.3

AMP: Ampicillin, AMC: Amoxicillin-clavulinic acid, AML: Amoxicillin, CIP: Ciprofloxacin, ENR: Enrofloxacin, P: Penicillin, AZM: Azithromycin, CN: Gentamycin, TE: Tetracycline, CRO: Ceftriaxone, SXT: Sulfamethoxazole-Trimethoprim.

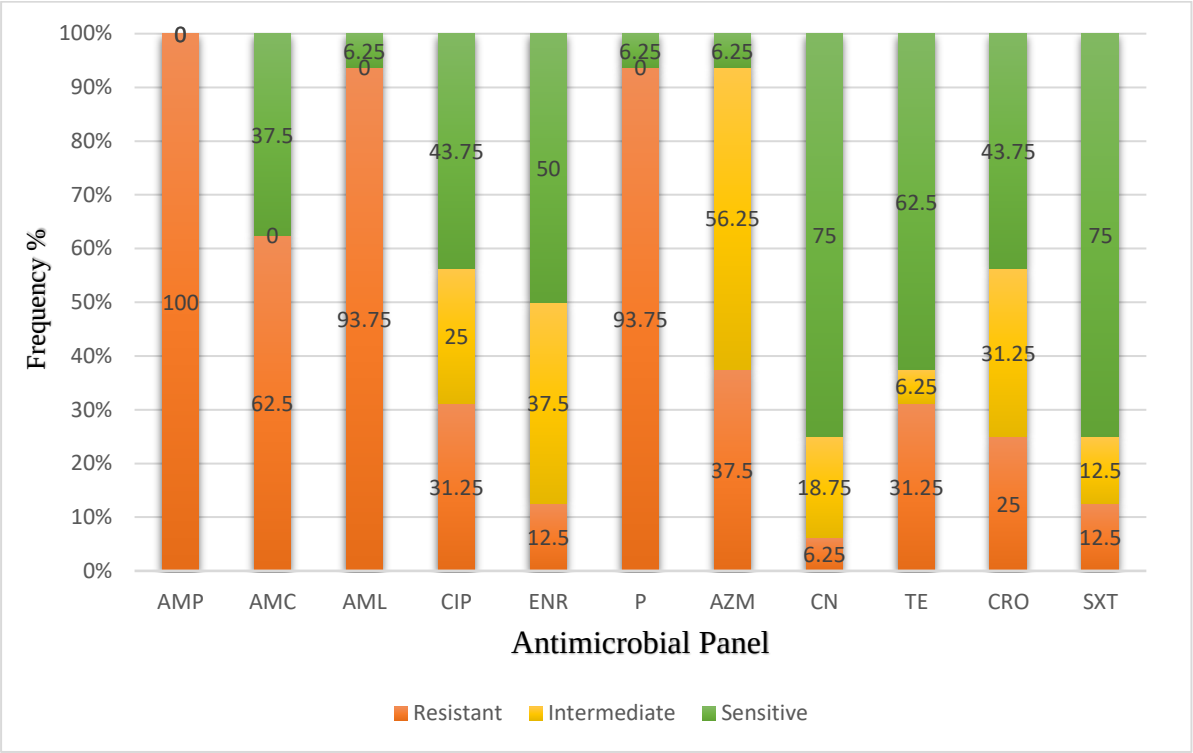

Figure 4.6: Antimicrobial panel of *S. aureus* isolated from infected goat

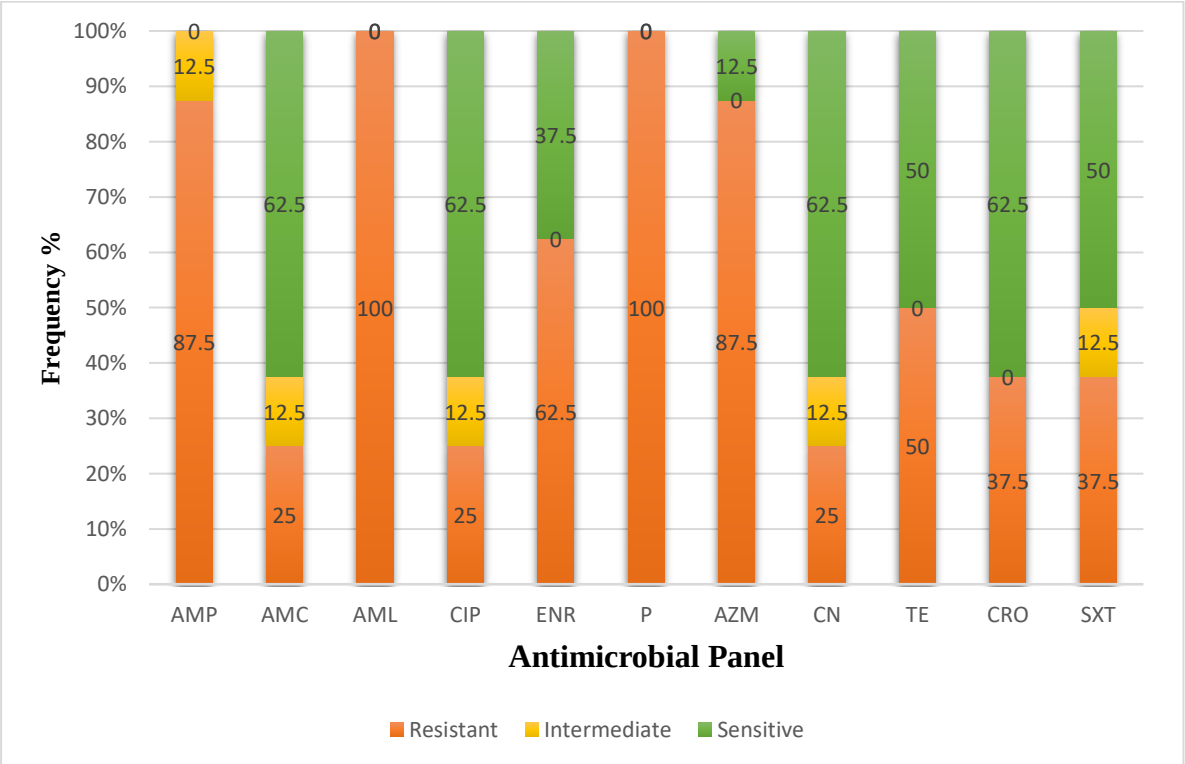

Figure 4.7: Antimicrobial panel of *E. coli* isolated from infected goat

4.6 Overall multidrug resistance pattern of *S. aureus*:

In this study, 16 isolates were positive to *S. aureus* and among those, 7 were identified as multidrug resistance. Highest number of isolates (85.71%) showed multidrug (MDR) resistance against atleast 3 groups of antibiotics and most of them were equally resistant against the combination of beta-lactam, fluoroquilonone and macrolides (33.33%) and the combination of beta-lactam, fluoroquilonone, tetracycline (33.33%) groups. Only 14.29% isolates found resistant against 5 groups of antibiotics that is beta-lactam, fluoroquilonone, macrolides, tetracycline and sulfonamides groups. No isolate was resistant against all the 6 groups of antibiotics. MDR pattern is highlighted in table 4.2 amd 4.3

4.7 Overall multidrug resistance pattern of *E. coli*:

Among all the multidrug resistant *E. coli*, around 40% isolates found resistant against atleast 3 groups of antibiotics and those isolates were corely resistant against the combination of beta- lactam, fluoroquilonone and macrolides (100%) groups. Similarly, 40% isolates displayed resistance against all the 6 groups of antibiotics separately and combindly also (100%) denoted against beta-lactam, fluoroquilonone, macrolides, aminoglycosides, tetracycline, sulfonamides groups. Moreover, 20% isolates showed resistance (100%) against the combiantion of 5 groups of antibiotics (beta-lactam, fluoroquilonone, macrolides, tetracycline, sulfonamides). MDR pattern is shown is table 4.2 amd 4.3.

Table 4.2: Overall pattern of multidrug resistance in *S. aureus* and *E. coli* isolated from animals

No of resistance antibiotic groups	*S. aureus*, n (%)	*E. coli*, n (%)
3	6, (85.71%)	2, (40%)
4	0, (0%)	0, (0%)
5	1, (14.29%)	1, (20%)
6	0, (0%)	2, (40%)

Table 4.3: Multidrug resistance patterns of *S. aureus* and *E. coli* isolated from animals

No of resistance antibiotic groups	Antibiotic groups	*S. aureus*, n (%)	*E. coli*, n (%)
3	Beta-lactam + Fluoroquinolone + Macrolides	2, (33.33%)	2 (100%)
	Beta-lactam + Macrolides + Tetracycline	1, (16.67%)	0
	Beta-lactam +Tetracycline + Sulfonamides	1, (16.67%)	0
	Beta-lactam + Fluoroquinolone + Tetracycline	2, (33.33%)	0
5	Beta-lactam + Fluoroquinolone + Macrolides +Tetracycline + Sulfonamides	1, (100%)	1, (100%)
6	Beta-lactam + Fluoroquinolone + Macrolides + Aminoglycoside + Tetracycline + Sulfonamides	0	2, (100%)

4.8 Prevalence of bacterial infection and association with different risk factors:

The rate of infection was higher in goats that were reared in farms than family for both bacterial infections (*S. aureus*: 16.67%, CI 4.73 – 37.38 and *E. coli*: 8.33%, CI 1.03 – 27). Occurrence of both *S. aureus* and *E. coli* infection were higher in cross breeds (*S. aureus*: 28.57%, CI 3.67 – 70.96 and *E. coli*: 14.29%, CI 3.67 – 70.96), non-vaccinated goats (*S. aureus*: 14.42%, CI 8.3 – 22.67 and *E. coli*: 6.73%, CI 2.75 – 13.38) and goats with poor BCS (*S. aureus*: 19.35%, CI 7.45 – 37.47 and *E. coli*: 9.68%, CI 2.04 – 25.75). Most of the infected goats were having pale mucous membrane in both bacterial infections (*S. aureus*: 14.71%, CI 4.95 – 31.06 and *E. coli*: 11.76%, CI 3.3 – 27.45). Different respiratory tract infections were diagnosed as aspiration pneumonia, bacterial pneumonia, rhinitis, upper respiratory tract infection (URTI), shipping fever etc. Among them, highest infection rate found in aspiration pneumonia (23.08%, CI 5.04 – 53.81) in both bacteria. Moreover, most used antibiotic group was Beta-lactam (*S. aureus*: 20.83%, CI 10.47 – 34.99 and *E. coli*: 8.33%, CI 2.32 – 19.98).

Apart from similarities, there were also some dissimilarities among the factors associated with *S. aureus* and *E. coli* infection. Prevalence of *S. aureus* infection was higher (13.43%, CI 6.33 – 23.97) in aged (> 12 month) than young goats (1-12 months, 7.55%, CI 2.09 – 18.21). Female goats were more prone to *S. aureus* infection (17.28%, CI 9.78 – 27.29) whereas in case of *E. coli* infection, male goats were more susceptible (10.26%, CI 2.87 – 24.22). Most of the goats reared in semi-intensive housing system affected with *S. aureus* infection (18.18%, CI 9.08 – 30.9). On the contrary, goats reared in intensive housing system were highly infected with *E. coli* infection (7.69%, CI 2.54 – 17.04). Prevalence of both bacterial infections associated with different risk factors shown in table 4.4.

Although, there are some similarities and dissimilarities in distribution of *S. aureus* and *E. coli* infection with different factors, their association were not significant according to the analysis of this study.

Table 4.4: Univariable analysis of factors associated with *S. aureus* and *E. coli* isolates from animals:

Factor	Category	*S. aureus*		*E. coli*		P
		n,(%)	95% CI	n, (%)	95% CI	
Source of animal	Farm (24)	4, (16.67)	4.73 – 37.38	2, (8.33)	1.03 - 27	0.791
	Family (96)	12, (12.5)	6.63 – 20.82	6, (6.25)	2.33 – 13.11	
Breed	Local (71)	8, (11.27)	4.99 - 21	6, (8.45)	3.16 – 17.49	0.393
	Jamnapari (42)	6, (14.29)	5.43 – 28.54	1, (2.38)	0.06 – 12.56	
	Cross (7)	2, (28.57)	3.67 – 70.96	1, (14.29)	0.36 – 57.87	
Age (month)	1-12 (53)	7, (13.21)	5.48 – 25.34	4, (7.55)	2.09 – 18.21	0.943
	>12 (67)	9, (13.43)	6.33 – 23.97	4, (5.97)	1.65 – 14.59	
Sex	Female (81)	14, (17.28)	9.78 – 27.29	4, (4.94)	1.36 – 12.16	0.123
	Male (39)	2, (5.13)	0.63 – 17.32	4, (10.26)	2.87 – 24.22	
BCS	Poor (31)	6, (19.35)	7.45 – 37.47	3, (9.68)	2.04 – 25.75	0.330
	Fair (54)	8, (14.81)	6.62 – 27.12	4, (7.41)	2.06 – 17.89	
	Good (35)	2, (5.71)	0.7 – 19.16	1, (2.86)	0.07 – 14.92	
Rearing system	Intensive (65)	6, (9.23)	3.46 – 19.02	5, (7.69)	2.54 – 17.04	0.336
	Semi-intensive (55)	10, (18.18)	9.08 – 30.9	3, (5.45)	1.14 – 15.12	
Vaccination	No (104)	15, (14.42)	8.3 – 22.67	7, (6.73)	2.75 – 13.38	0.661
	Yes (16)	1, (6.25)	0.16 – 30.23	1, (6.25)	0.16 – 30.23	
Feeding practice	Normal (18)	0	0 – 18.53	1, (5.56)	0.14 – 27.29	0.485
	Loss of appetite (72)	11, (15.28)	7.88 – 25.69	5, (6.94)	2.29 – 15.47	
	Off-feed (30)	5, (16.67)	5.64 – 34.72	2, (6.67)	0.82 – 22.07	

Mucous membrane	Pink (82)	11, (13.41)	6.89 – 22.74	4, (4.88)	1.34 – 12.02	0.562
	Pale (34)	5, (14.71)	4.95 – 31.06	4, (11.76)	3.3 – 27.45	
	Congested (4)	0	0 – 60.24	0	0 – 60.24	
Coughing	No (4)	1, (25)	0.63 – 80.59	0	0 – 60.24	0.701
	Yes (116)	15, (12.93)	7.42 – 20.43	8, (6.9)	3.02 – 13.14	
Diagnosis	Aspiration pneumonia (13)	3, (23.08)	5.04 – 53.81	3, (23.08)	5.04 – 53.81	0.498
	Bacterial pneumonia (8)	1, (12.5)	0.32 – 52.65	1, (12.5)	0.32 – 52.65	
	Pneumonia (22)	4, (18.18)	5.19 – 40.28	1, (4.55)	0.12 – 22.84	
	Pneumonia + myiasis (1)	0	0 – 97.5	0	0 – 97.5	
	RTI (31)	3, (9.68)	2.04 – 25.75	0	0 – 11.22	
	Rhinitis (13)	2, (15.38)	1.92 – 45.45	1, (7.69)	0.19 – 36.03	
	Shipping fever (6)	1, (16.67)	0.42 – 64.12	1, (16.67)	0.42 – 64.12	
	URTI (26)	2, (7.69)	0.94 – 25.13	2, (7.69)	0.94 – 25.13	
Antibiotics	Amoxicillin (61)	5, (8.2)	2.71 – 18.1	4, (6.56)	1.82 – 15.95	0.395
	Ampicillin (1)	1, (100)	2.5 - 100	0	0 – 97.5	
	Ceftriaxone (29)	7, (24.14)	10.3 – 43.54	3, (10.34)	2.19 – 27.35	
	Ceftiafur (18)	3, (16.67)	3.58 – 41.42	1, (5.56)	0.14 – 27.29	
	Oxytetracycline (8)	0	0 – 36.94	0	0 – 36.94	
	Sulphadimidine (1)	0	0 – 97.5	0	0 – 97.5	
	Gentamycine (1)	0	0 – 97.5	0	0 – 97.5	

	No antibiotic (1)	0	0 – 97.5	0	0 – 97.5	
Group of antibiotics	Semi synthetic beta-lactam (61)	6, (9.84)	3.7 – 20.19	4, (6.56)	1.81 – 15.95	0.803
	Beta-lactam (48)	10, (20.83)	10.47 – 34.99	4, (8.33)	2.32 – 19.98	
	Tetracycline (8)	0	0 – 36.94	0	0 – 36.94	
	Sulfonamodes (1)	0	0 – 97.5	0	0 – 97.5	
	Aminoglycosides (1)	0	0 – 97.5	0	0 – 97.5	
	N/A (1)	0	0 – 97.5	0	0 – 97.5	

CHAPTER-5

DISCUSSION

In this study 120 nasal swab samples were collected from goats showing the signs of respiratory tract infection and brought to the S. A. Q. Teaching Veterinary Hospital of CVASU for treatment. Samples were transferred to the laboratory for isolation, identification, and characterization of three organisms including *S. aureus*, *E. coli* and *P. multocida*. The samples were examined from which 19.2% isolates were positive for *S. aureus*, 6.67% were positive for *E. coli* and none of the isolates found to be positive for *P. multocida*.

Staphylococcus spp. is frequently isolated and considered as one of the most common causes of respiratory tract infection in goats (Islam et al., 2006). In this study, prevalence of *S. aureus* found 19.2% which is lower than the findings of Asaduzzaman et al., (2013) that depicted 30% prevalence of *S. aureus* from upper respiratory tract of Black Bengal goats. Also, Momin et al., (2011) found 26% prevalence of *S. aureus* in pneumonic goat. However, prevalence of *S. aureus* in this study was higher than Zaman et al., (2018) in which 14% prevalence noted. This type of variation in *Staphylococcus* spp. isolation could be due to geographic differences in the region where the samples obtained, mixed bacterial populations in animals, and differences in the procedures used by different laboratories to conduct the tests.

Prevalence of *E. coli* found 6.67% in this study. Akter et al., (2018) found 7.5% prevalence of *E. coli* from nasal swab samples of buffalo which is nearly like this study. However, Khalifa et al., (2021) depicted 47.4% prevalence of *E. coli* in sheep and goat showing respiratory signs in different private and government farms in Kafrelsheikh city, Egypt. Moreover, Asaduzzaman et al., (2013) recorded a higher prevalence (44%) from upper respiratory tract of Black Bengal goat. The variation in the prevalence of *E. coli* infection might be due to sampling variation, climatic and geographical diversity and mixed bacterial infection of the goats examined.

In this study, isolates of *S. aureus* and *E. coli* were identified and characterized by morphology, cultural properties, and biochemical tests. Selective media mannitol salt agar was used to identify colonial properties of *S. aureus*. *S. aureus* produced characteristics yellow color colony with change in the media color in mannitol salt agar. Cultural, morphological, and biochemical results of *S. aureus* in this study were like the findings of Saha et al., (2019), Jahan et al., (2014) and Das (2012). According

to their study, *S. aureus* produced yellowish colony on MS agar, cluster of grapes like arrangement of gram-positive cocci under microscope and catalase positive on biochemical test. In this study, 5% bovine blood was used to prepare blood agar due to limitations of sheep blood collection and all the *S. aureus* isolates produced β-hemolysis on blood agar which is supported by the study of Saha et al., (2019).

On the other hand, MacConkey agar and Eosin Methylene Blue (EMB) agar were used to find out cultural characteristics of *E. coli* in this experiment. On MacConkey agar, *E. coli* produced lactose fermented rose pink colored colony and on EMB agar it formed smooth, circular, black colored colonies with metallic sheen. After grams staining, *E. coli* revealed gram-negative, small rod shaped pink colored organisms arranged in single, pairs or short chain. On biochemical tests, isolates of *E. coli* showed complete fermentation of five basic sugars by producing both acid and gas. Additionally, isolates were found positive to catalase test, MR test and indole test but negative to VP test and Simmons' citrate test. All these findings agreed with the study of Akter et al., (2018) and Islam et al., (2016).

For the detection of *P. multocida* samples were tested for cultural, and morphological characteristics. For further confirmation DNA extraction and PCR was also conducted. On blood agar suspected colonies produced small, translucent, glistening, dewdrop like non-hemolytic colonies. Suspected colonies were inoculated onto MacConkey agar. On MacConkey agar there was no growth observed which was like Akter et al., (2018). Ara et al., (2016) suggested that *P. multocida* isolates showed gram-negative, small pink colored, rod shaped organism on gram's staining. Similar findings also recorded in this study. DNA was extracted from suspected isolates to use in PCR assay. For confirmation of *P. multocida,* PCR was done as described by Verma et al., (2019) and Rawat et al., (2019). Verma et al., (2019) used the primer pairs KMT1SP6 5′-GCTGTAAACGAACTCGCCAC-3′ and KMT1T7 5′- ATCCGCTATTACCCAGT GG-3′ for *Kmt1* gene and Rawat *et al*., (2019) used KMT1 F 5′-ATCCGCTATT TACCCAGTGG-3′ and KMT1 R 5′-GCTGTAAACGAACTCGCCAC-3 primer pairs for *KMT1* gene. Unfortunately, all samples screened for *P. multocida* gave negative result on PCR. This may be due to using only two primer sets to detect *P. multocida*.

The Culture sensitivity (CS) test for all 16 *S. aureus* positive isolates showed 100% resistance against ampicillin followed by penicillin (93.75%), amoxicillin (93.75%), amoxicillin-clavulanic acid (62.5%), azithromycin (37.5%), ciprofloxacin (31.25%),

tetracycline (31.25%), ceftriaxone (25%), enrofloxacin (12.5%), sulfamethoxazole-trimethoprim (12.5%) and gentamycin (6.25%).

According to Aziz and Lafta (2021), *S. aureus* showed 100% resistance against ampicillin which is similar this study. Furthermore, *S. aureus* revealed higher resistance against amoxicillin, penicillin, and amoxicillin-clavulanic acid in this study. These findings agree with Momin et al., (2011) where less sensitivity to amoxicillin and penicillin against *S. aureus* recorded. On the contrary, Akter et al., (2018) and Asaduzzaman et al., (2013) revealed higher sensitivity of *S. aureus* against ampicillin and amoxicillin.

The resistance to tetracycline was 31.25% in this study which is near to the resistance observed in the report of Aziz and Lafta (2021) and Vitale et al., (2019) where they have showed 33.3% resistance of tetracycline to *S. aureus*. Vitale et al., (2019) also revealed only 14% resistance against ceftriaxone and gentamycin that closely related to this study. However, Aziz et al., (2021) found higher (33.3%) resistance against gentamycin and no resistance against ciprofloxacin whereas 31.25% resistance against ciprofloxacin reported in this study.

In this experiment, *S. aureus* showed higher sensitivity to sulfamethoxazole-trimethoprim (75%). In contrast, Akter et al., (2018) and Asaduzzaman et al., (2013) reported resistance against sulfamethoxazole-trimethoprim in buffalo and black bengal goats in Mymensingh area, respectively. This difference may be due to higher usage of sulfamethoxazole-trimethoprim in Mymensingh area whereas lower usage in Chattogram area. A higher sensitivity to enrofloxacin (50%) in this study was similar to Momin et al., (2011).

The culture sensitivity test of *E. coli* isolates showed highest resistance against amoxicillin (100%) and penicillin (100%) followed by ampicillin (87.5%), azithromycin (87.5%), enrofloxacin (62.5%), tetracycline (50%), sulfamethoxazole-trimethoprim (37.5%), ceftriaxone (37.5%), amoxicillin-clavulanic acid (25%), ciprofloxacin (25%) and gentamycin (25%).

All the *E. coli* isolates were found to be resistant to amoxicillin and penicillin in this study. Gram-negative bacteria are frequently becoming resistant to penicillin especially β-lactam group of antibiotics which is terrifying (Islam et al., 2016). A higher resistance against amoxicillin reported by Singh et al., (2019) where 65.5%

isolates were resistant to amoxicillin. However, Akter et al., (2018) and Asaduzzaman et al., (2013) revealed intermediate and moderate sensitivity to amoxicillin.

A higher resistance against ampicillin, enrofloxacin and tetracycline were determined in this study. Similarly higher resistance showed by Islam et al., (2016) in which resistance to ampicillin and tetracycline were 65.38% and 51.28%. In another study Singh et al., (2019) showed similar higher (65.5%) resistance to enrofloxacin. But Akter et al., (2018) reported that *E. coli* was sensitive to ampicillin and tetracycline and Mandal et al., (2019) reported 82.50% sensitivity to enrofloxacin.

In current study, a relatively lower resistance of *E.coli* against ceftriaxone, sulfamethoxazole-trimethoprim, amoxicillin-clavulanic acid, ciprofloxacin, and gentamycin were reported. These findings of higher sensitivity supported by Akter et al., (2018). However, Islam et al., (2016) revealed higher resistance to amoxicillin-clavulanic acid (60.26%) and sulfamethoxazole-trimethoprim (52.56%) but lower resistance to gentamycin (37.18%) and ceftriaxone (21.79%). These similar findings of lower resistance to gentamycin and ceftriaxone in these studies could be due to lower usage of these two classes of antibiotics in respiratory tract infection.

The isolates that showed resistance against 3 or more groups of antimicrobials were considered as Multidrug- resistant (MDR). In this study, 85.71% isolates of *S. aureus* and 40% isolates of *E. coli* found resistant to 3 groups of antibiotics and all the combination of these 3 groups contains β-lactam group which group of antibiotics mostly used in respiratory diseases of goats. Moreover, resistance to the combination of β-lactam and fluoroquinolone group was found highest (*S. aureus* 33.3% and *E. coli* 100%). Similar findings recorded by Singh et al., (2019) where highest resistance rate to penicillin (65.5%) and fluoroquinolones was found from *E. coli* affected pneumonic and septicemic goats. He also described the occurrence of β-lactamase-producing extraintestinal *E. coli* associated with pneumonia in small ruminants. Resistance to multiple antimicrobials among both gram-positive and gram-negative bacteria indicates the emergence of multidrug resistance. High degree of MDR emergence to both *S. aureus* and *E. coli* depicts a critical condition where management of clinical conditions would be very difficult. MDR also threats the therapeutic use of antibiotics. Therefore, checking the emergence of AMR among both gram-positive and gram-negative bacteria on regular basis is important to select suitable and effective antimicrobials for therapeutic applications and to control the further spread of resistant pathogens.

CHAPTER-6

CONCLUSION

Antibiotics has been using in goats for treating, preventing, and controlling of diseases. Higher use of antibiotics increasing the emergence of antimicrobial resistance rapidly. AMR is accompanied by high mortality rates, it provokes hindrance of treatment of the diseases with the spreading of resistant pathogens, resulting in a persistent time of infection to the patient. All classes of antimicrobials including sulfonamides, penicillin, tetracycline, ampicillin, aminoglycosides, and cephalosporin used in veterinary and human medicine rises drug resistance. Respiratory tract infections are one of the most commonly found diseases in goats. Ruminants especially goats are susceptible to different respiratory diseases including bacterial pneumonia, rhinitis, upper respiratory tract infection, bronchopneumonia etc. that treated with antibiotics regularly. Thus, due to indiscriminate use antimicrobial agents are losing their ability to stop growing or killing bacteria day by day. This study revealed a significant infection rate of *S. aureus* (19.2%) and *E. coli* (6.67%) in goats affected with different respiratory tract infections. It is also a matter of great concern that both *S. aureus* and *E. coli* found resistant to commonly used antibiotics including penicillin, ampicillin, amoxicillin, amoxicillin-clavulanic acid, azithromycin etc. AMR is becoming a global hazard to both humans and animals, as microorganisms are developing resistance to certain antibiotics that will no longer be effective in the future. This increasing rate of antimicrobial resistance is affecting mostly the farmers and the farm owners as they must invest more on treatment and management cost. Therefore, it is the demand of time to assist guidelines to take proper treatment especially limiting the usage of antibiotics indiscriminately and taking preventive measures against respiratory tract infection and thus reduction of production, management, and treatment cost.

CHAPTER-7

LIMITATIONS

The study has following limitations:

- Although utmost sincerity and dedication was investigated to carry out the study, it could not go beyond limitations as the sample size was not large enough due to the Covid-19 pandemic situation.
- The study was conducted in small scale (only goats brought to SAQTVH hospital) due to time and resource limitations
- Failure to identify resistant genes of isolated organisms as laboratory resource and time was limited. However, willing to identify resistant genes of identified organisms in future.
- More primer sets could be chosen for detection of *P. multocida* molecularly as it remained undetected using only two primer sets.

CHAPTER-8

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APPENDIX

Questionnaire survey

Questionnaire number: **Date:**

Owner Details:

1. Name of the owner:
2. Phone no:
3. Address:

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4. Source: Farm animal/ Family Livestock/others
5. No. of goat rear:

Patient Details:

1. Breed:
2. Age:
3. Sex: M/F
4. Body weight:
5. BCS: Cachectic (1)/Poor (2)/Fair (3)/ Good (4)/ Obese (5)
6. Rearing System: Intensive/ Semi-intensive/ Extensive

Case Details:

1. Clinical History:

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2. Vaccination: Yes/No. If yes; name of vaccine:
3. Deworming: Yes/No. If yes; name of anthelmintics:
4. Clinical sign:
    - Temperature:
    - Feeding: Normal/Loss of Appetite/ Off-feed

- M/M: Pink/pale/congested
- Dehydration: Normal/Mild/Moderate/ Severe
- Weakness: Yes/ No
- Feces: Normal/ Diarrhea
- Nasal discharge: Present/ Absent
- Coughing: Yes/ No.
- Respiration: Normal/Abnormal; If abnormal, specify:
- Any abnormal respiratory sound:

Diagnosis:

Treatment:

1. Antibiotic:

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2. Others/ Supportive Drugs:

BIOGRAPHY

Shanta Barua passed Secondary School Certificate (SSC) examination from Dr. Khastagir Govt. Girls' High School in 2009 and then Higher Secondary School Certificate examination from Chittagong Govt. Girls' College in 2011. She completed Doctor of Veterinary Medicine (DVM) from Chattogram Veterinary and Animal Sciences University, Chattogram, Bangladesh in 2018 with CGPA 3.75 out of 4.00. She has been studying Masters in Medicine in the department of Medicine and Surgery at Chattogram Veterinary and Animal Sciences University, Chattogram, Bangladesh. She has great interest to work in antimicrobial resistance and zoonoses research.