Screening and Molecular Characterization of Antibiotics Resistance Genes of *Staphylococcus* Species Isolated from the Nasal Cavity of Goats in Adama, Ethiopia

Leta Guta

A Thesis Submitted to the Department of Applied Biology, School of Applied Natural Science Presented in Partial Fulfillment of the Requirment for the Degree Master's in Biology Specialization in Biotechnology

Office of Graduate Studies
Adama Science and Technology University

March, 2022
Adama, Ethiopia

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Advisor: Seid Mohammed Ebu (PhD)
Co-advisor: Mr. Melaku Sombo

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DECLARATION

I hereby declare that this Master Thesis entitled **"Screening and Molecular Characterization of Antibiotics Resistance Genes of *Staphylococcus* Species Isolated from the Nasal Cavity of Goats in Adama, Ethiopia"** is my original work. That is, it has not been submitted for the award of any academic degree, diploma or certificate in any other university. All sources of materials that are used for this thesis have been duly acknowledged through citation

Name: Leta Guta **Signature** **Date:**

RECOMMENDATION

We, the advisors of this thesis, hereby certify that we have read the revised version of the thesis entitled **"Screening and Molecular Characterization of Antibiotics Resistance Genes of *Staphylococcus* Species Isolated from the Nasal Cavity of Goats in Adama, Ethiopia"** prepared under our guidance by **Leta Guta** submitted in partial fulfillment of the requirements for the degree of Mater's of Science in Biotechnology. Therefore, we recommend the submission of revised version of the thesis to the department following the applicable procedures.

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Mr. Melaku Sombo

Co-advisor: Signature Date

APPROVAL SHEET

We, the advisors of the thesis entitled **"Screening and Molecular Characterization of Antibiotics Resistance Genes of *Staphylococcus* Species Isolated from the Nasal Cavity of Goats in Adama, Ethiopia"** and developed by **Leta Guta**, hereby certify that the recommendation and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

Major Advisor: **Seid Mohammed (PhD)** Signature Date

Co-advisor: Mr. **Melaku Sombo** Signature Date

We, the undersigned, members of the Board of Examiners of the thesis by **Leta Guta** have read and evaluated the thesis entitled **"Screening and Molecular Characterization of Antibiotics Resistance Genes of *Staphylococcus* Species Isolated from the Nasal Cavity of Goats in Adama, Ethiopia"** and examined the candidate during open defense. This is, therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Master of Science in Biotechnology.

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ABBREVIATION AND ACRONYMS

ABC	ATP Binding Cassette
AME	Aminoglycoside Modifying Enzymes
AST	Antimicrobial Susceptibility Test
BLAST	Basic Local Alignment Search Tools
CDW	Cell Dry Weight
CLSI	Clinical and Laboratory Standards Institute
CoNS	Coagulase Negative *Staphylococcus*
CoPS	Coagulase Positive *Staphylococcus*
dNTPs	Deoxynucleotide Triphosphates
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FISH	Fluorescent *in situ* Hybridization
MALDI TOF MS	Matrix-assisted Laser Desorption Ionization Time Flight Mass-Spectroscopy
MATE	Multidrug and Toxic Compound Enterotoxins
MFS	Major Facilitator Superfamily
MRSA	Methicillin Resistant *Staphylococcus aureus*
MSA	Mannitol Salt Agar
MSA	Multiple Sequence Alignment
NCBI	National Center for Biotechnology Information
NCGs	Nasal Cavity of Goats
PBP	Penicillin Binding Protein
RNA	Resistance Nodulation Cell Division
SMR	Small Multidrug Resistance
TBE	Tris-Borate Ethylene Diamine Tetra Acetic Acid
TE	Tris-Ethylene Diamine Tetra Acetic Acid
TSB	Tryptophan Soy Broth
VIRSA	Vancomycin Intermediate Resistance *Staphylococcus aureus*
VRE	Vancomycin resistant *Enterococcus*

ABSTRACT

Staphylococci spp. are found everywhere in nature and they occur as a part of the normal microbial flora, asymptomatically colonizing the skin and mucous membranes in the nostrils of humans and animals. The aim of this study was to screen and molecularly characterize the antibiotic resistance of Staphylococcus species isolated from the nasal cavity of goats in Adama city. In this study, 40 samples of mucus were collected from the nasal cavity of goats (NCGs). Screening for possible Staphylococcus species isolate (PSSI) such as coagulase, DNase, growth on Mannitol salt agar (MSA) medium, gram staining, and morphological characterization were performed. Factors affecting S. aureus growth were determined and optimization was performed for PSSI-D8. Antimicrobial activities were determined against S. aureus. Molecular characterizations were performed to identify Staphylococcus species. Data were analyzed using Microsoft Excel, SPSS, and Origin Pro8.5. In this study, 34 isolates of PSSIs were obtained from NGCs. All are morphologically cocci. All PSSIs were positive results for catalase, gram staining, and urease test. Few isolates were found to be coagulase positive. These isolates might be S. aureus. In this study, PSSIs were identified as S. aureus, S. sciuri, S. simulans, and S. xylosus using MALDI-TOF MS analysis. Antibiotic resistance genes such as blaZ, mecA, and thermo-tolerant gene nuc were detected for PSSIs that isolated NCGs using PCR analysis. Based on 16S rRNA analysis, PSSI-D8 was confirmed to be Staphylococcus aureus with 99.47% sequence similarity. It was found that the isolated strains were (100%) susceptible to chloramphenicol and vancomycin antibiotics. The optimum condition for growth and biomass production for Staphylococcus aureus INCG8 was obtained at pH7, temperature (37^{o}C), and 1.28M of NaCl using glucose as the main carbon source. The Staphylococcus species isolated from the nasal cavity of goats are highly resistant to different antibiotics. This designates the need for further studies on mechanisms of antibiotic resistance

Keywords: Antibiotic resistance, goats, MALDI TOF MS, molecular characterization, nasal cavity, and *Staphylococcus*

1 INTRODUCTION

1.1 Background of the study

Staphylococcus species are members of gram-positive bacteria belonging to *micrococcaceae* family (Stapleton and Taylor, 2002). The name had been coined in 1880 by Scottish surgeon and bacteriologist Alexander Ogston. *Staphylococcus* contains more than 40 species. These species are cosmopolitan in nature and they occur as a part of the normal microbial flora, asymptomatically colonizing the skin and mucous membranes in the nostrils of humans and animals (Pekana & Green, 2018). *Staphylococcus* species are naturally facultative anaerobic microorganisms, these species usually occurred in a single group. The same species are able to form pairs and tetrads-like structures. These species have shown distinctive irregular grape-like structures, which is mainly a common characteristic feature of the *Staphylococcus* genus (Stapleton and Taylor, 2002). *Staphylococcus* species are among non-spore formers and most resistant to dehydration and have been killed at 60^{o}C within 30 minutes. However, these species are able to tolerate common disinfectants better than most other bacterial species (Coia & Cubie, 1995).

Staphylococci constitute the main groups of medically important pathogens. *Staphylococcal* infections range from trivial to rapidly fatal. They can be very difficult to treat, especially those contracted in hospitals, because of the remarkable ability of *Staphylococci* to become resistant to antibiotics (Richard *et al*., 2013). These species can also able to affect diverse host ranges in livestock. These species are broadly grouped into coagulase-negative *Staphylococcus* (CoNS) and coagulase-positive *Staphylococcus* (CoPS) (Beyene et al., 2017). Among coagulase-positive *S. aureus, S. intermedius, and S. hyicus* are associated in many diverse and specialized forms with healthy and diseases of farm animals. Among the CoPS species, *S. aureus* is more pathogenic than other common members of the genus and foodborne diseases. This species can be transmitted from contaminated animal food staff (Adugna *et al*., 2018).

It has been also stated that *methicillin*-resistant *S. aureus* strains (MRSA) are considered as major pathogens that infect and colonize both humans and animals (Mourabit et al., 2020). However, *Staphylococcus* species such as *S. delphini, S. epidermidis, S. hyicus,* and *S. pseudintermedius* are used to cause various infections, especially abscesses, mastitis, and pyoderma (Markey *et al*., 2013). The causation of lesions in the skin and mucous membranes by *Staphylococcus* depends on the number of factors such as a trauma, the immune status of

the animal, disturbance of the normal microflora and whether or not the particular *Staphylococcus* strain produce certain toxins and enzymes (https://www.anipedia.org). The same species are able to form toxin that leads to fever, sickness and in some cases death (Stapleton & Taylor, 2002; Ben Nejma *et al.*, 2006).

Different antibiotics have been used to treat *Staphylococcus* species for a long period of time. These of antibiotic used for treatments of *Staphylococcus* species are including such as vancomycin which targets the glycopeptide (Stapleton and Taylor, 2002) methicillin (Silva *et al*., 2020), clindamycin, ciprofloxacin, erythromycin, gentamicin, and trimethoprimm-sulfamethoxazole (Sadeghi & Mansouri, 2014). It has been also reported that clindamycin, cotrimoxazole (trimethoprim/sulfamethoxazole), linezolid and rifampin are tended to kill some species of *Staphylococcus* (Gittens *et al*., 2020).

However, nowadays these species able to develop resistance against these and other antibiotics which is a major threat to human and animals health (Nemeghaire *et al.*, 2014; Liu *et al.*, 2018; Zhao *et al.*, 2018; Verdú-Expósito *et al.*, 2020). For instance, hospital-acquired *S. aureus* strain (Nemeghaire et al., 2014) and clinical isolated *Staphylococcus argenteus* developed (Aung et al., 2019) resistant against methicillin, and ampicillin & tetracycline, respectively. Additionally, it was reported that *Staphylococcus aureus* strain isolated from goats has also shown resistance against antibiotics such as amoxicillin, furazolidone, lincomycin, roxithromycin and trimethoprim (Zhou et al., 2017).

More recently, molecular detection (PCR) based techniques have been used for the identification of antibiosis resistance strains of *Staphylococcus* species which are animals pathogens (Ventola, 2015). For instance, a multiplex polymerase chain reaction is used to detect *Staphylococcal* cassette chromosome *mec* (*SCCmec*) type IV and IVA (Ben Nejma et al., 2006). Seven enterotoxin and *mecA* gene profiles have been detected in the tested strains of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from bovine mastitis milk using polymerase chain reaction (Hoque *et al*., 2018). *Staphylococcus aureus* harboured various virulence genes encoding for a variety of virulence protein like haemolysins that encoded by; *cna, cla, hla*, *hlb*, *hlgA*, and *hlgB* Li et al (2019); Biofilm producing gene in *S. aureus* that isolated from bovine subclinical mastitis have been reported as *icaA, icaD* (Marques et al., 2021). It has been confirmed *Staphylococcus* surface components which is adhesive matrix molecules are encoded by *sdrC, sdrD, sdrE* gene (Sabat et al., 2006). The enterotoxin encoding gene was identified as *sea*, *sei*, and *seg* (Yu et al., 2012).

In the past decades, the *mecA* gene is commonly used to identify the resistance of CoPS but is nowadays harboured by many CoNS *mecA* encodes PBP2a (Williams et al., 2020). It has been confirmed that there is a plasmid-mediated gene which is similar the *mecA* gene which is chromosome-mediated in *S. aureus* (Okiki et al., 2020). A β-lactamase encoded *blaZ* gene has been isolated from Penicillin resistance in *S. aureus* using the molecular technique. The *S. aureus* produces thermonuclease enzyme encoded by *nuc* gene which is a calcium-dependent enzyme that catalyses the hydrolysis of both DNA and RNA at 5' position of the phosphodiester bond yielding 3'-mononucleotides and dinucleotides (Hynes & Fox, 1991).

1.2 Statement of the problem

Goats are becoming a very important component of livestock production in the Great Rift Valley including Adama and its area. Adama is one of the places where goats and other livestock market is taking place and distribute to the final destination for butchers, group consumers, supermarkets, and abattoirs. *Staphylococci* are the most prevalent bacteria isolated from bovine mammary secretions. They are not only originate from cases of intra-mammary infections, but also from teat canal, skin, nasal cavity, and other environmental sources (Wald *et al*., 2019). *S. aureus*, next to *Escherichia coli* and several *Streptococcal* species such as *Streptococcus uberis*, and *Streptococcus agalactiae*, is a major cause of mastitis in lactating cows, goats and sheep and incurs a significant economic loss to the dairy industry (Haag *et al*., 2019).

Antimicrobial resistance of pet origin, responsible for both direct and indirect threats on human health, regard principally Methicillin-Resistant *Staphylococcus aureus* (MRSA) (Palma *et al*., 2020). Some study has been performed on the isolation and characterization of *Staphylococcus* species particularly, *S. aureus* from milk (Abera *et al.*, 2010; Mekonnen *et al*., 2018), abattoirs, dairy farms and humans, and food of bovine in our country (Beyene et al., 2017). In human *S. aureus* nasal carriage has been far studied in patients and healthy individuals in different areas of our country (Shibabaw et al., 2013; Getenet et al., 2019).

The transfer of antibiotic resistance from animals/goats to humans via different mechanisms needs more attention to detect the antibiotic-resistant gene of *Staphylococcus* species. Now days, many other *Staphylococcus* species are the carriers of different antibiotic resistance genes (Rahimi et al., 2015). In small ruminants multiple microbial resistance of *Staphylococcus* species are a great concerns for their potential spread through the dairy food chains. In Ethiopia, there is a limited published and research work on identification and

molecular characterization of *Staphylococcus* species that act as an important reservoir of antimicrobial resistance gene. Optimization for *Staphylococcus aureus*, their growth conditions were also not briefly addressed in Ethiopia. Therefore, the current study was aimed at a molecular characterization of *Staphylococcus aureus* from nasal cavity of goats, optimization of their growth condition, suitable carbon source, identification of isolates using 16S rRNA for further confirmation and checking for antimicrobial susceptibility testing for this isolates.

1.3 Objective of the study

1.3.1 General objective

- To screen and molecularly characterize antibiotic resistance genes in *Staphylococcus* species isolated from the nasal cavity of goats.

1.3.2 Specific objectives

- To isolate and characterize *Staphylococcus* species from the nasal cavity of the goats
- To conduct optimization and culture condition for newly isolated *Staphylococcus* isolates that obtained from nasal cavity of the goats
- To identify the phenotypic antibiotic resistance of *Staphylococcus* species isolated from the nasal cavity of goats using disk diffusion method
- To detect the occurrences of the antibiotics-resistant gene in the test organisms using PCR techniques
- To identify the isolates using 16S rRNA genes sequence

1.4 Significance of the study

The study was provided information on the presence of *Staphylococcus* species in the nasal cavity of goats. This study was also used to identify the most common antimicrobial-resistant *Staphylococcus* species isolated from goats and their effective drug for its treatment strategy. In this study, genes responsible for antibiotic resistance were also predicted using molecular techniques. This is a great importance to decide which antibiotic/drugs should be administered as well as monitoring the spread of multiple resistant strains on dairy goats. In this study, the isolated species of *Staphylococcus* was sequenced using 16S rRNA for conformation.

2 LITERATURE REVIEW

2.1 Classification and characteristics of *Staphylococcus*

Staphylococcus bacteria identified as the cause of various pyogenic infections in man are Gram-positive characterized by irregular clusters. Based on the ability to produce the extracellular enzyme coagulase the species of *Staphylococcus* is divided into coagulase-positive *Staphylococci* (CoPS) which are pathogenic and the coagulase-negative *Staphylococci* (CoNS) which are commensal on the skin (Nizet & Bradley, 2011). As the name suggests *Staphylococcus* bacteria have a spherical shape (cocci). Unlike other cocci bacteria that may exist as single cells or in pairs etc. The cell wall is composed of peptidoglycan and teichoic acid in *Staphylococcus*. It has been reported that peptidoglycan type L-Lys-GLY5-6 is found in several *Staphylococci* such as (*Staphylococcus aureus, Staphylococcus hyicus* and *Staphylococcus cohnii* and others).

2.2 Biochemical identification of *Staphylococcus* species

Biochemical tests are used for microbial identification based on difference lye in their biochemical activities exhibited by different types of bacteria (Shoaib et al., 2020). Biochemical test can reveal the vital information necessary for accurately identifying the genera of various bacteria within a sample. In their nature bacteria can produce large volume of enzyme and that enzyme allow for their identification through biochemical methods. Biochemical reaction used in biochemical tests depend on the presence of such bacteria (Aslanzadeh, 2006). Several biochemical tests available for bacterial identification, among them some commonly used in the identification of *Staphylococcus* species are mentioned below.

2.2.1 Various enzyme reported for *Staphylococcus*

Catalase, an enzyme within the cytochrome enzyme system, is responsible for the decomposition of hydrogen peroxide (H_2O_2) formed during aerobic respiration. All organisms using the cytochrome system of respiration will give a positive catalase reaction when tested. Those organisms using a different system will not produce catalase and will yield a negative reaction. The mechanism of action is as follows:

$$2H_2O_2 + \text{catalase} = 2H_2O + O_2$$

The possession of catalase test is primarily used to distinguish among Gram-positive cocci. Members of the genus *Staphylococcus* are catalase-positive, and members of the genera

Streptococcus and *Enterococcus* are catalase-negative (Kompaníková et al., 2016). A positive test is a bubbling reaction caused by the release of O_2 from the H_2O_2 in the presence of catalase. A negative test is the absence of any bubbling reaction. The test is performed either using tube or slide method by mixing the colony of bacteria with few drops of 3% H_2O_2 on slide or to the test tube and looking for bubble formation within 10 seconds (Facklam & Elliott, 1995).

Coagulase test is used to recognize the bacteria having the ability to produce the coagulase enzyme. Mostly, help to identify the *Staphylococcus aureus* which is positive bacteria for coagulase test and catalase test. Among the virulence of factors of *S. aureus*, coagulase is one of them. Coagulase enzyme will coagulate the blood plasma during reaction process. This test is performed by mixing the blood plasma with colony of bacteria. Bacteria will produce the coagulase enzyme that will coagulate the blood plasma indicative of positive reaction (Shoaib et al., 2020).

Most strains of *S. aureus* produce one or two types of coagulase; free coagulase and bound coagulase. Bound coagulase is localized on the surface of the cell wall and reacts with α- and β-chains of the plasma fibrinogens to form a coagulation. Free coagulase is an enzyme that is secreted extracellularly and bound coagulase is a cell wall associated protein. Free coagulase can be detected in tube coagulase test and bound coagulase can be detected in slide coagulase test. A tube coagulase may be used for further confirmation. The coagulase test is used to distinguish between pathogenic and nonpathogenic members of the genus *Staphylococcus*. All the pathogenic strains of *S. aureus* are coagulase positive whereas the nonpathogenic species (*S. epidermidis*) are coagulase negative (Kompaníková et al., 2016).

The DNA hydrolysis test detects the presence of enzyme DNase in an organism. The presence of DNase is used in clinical laboratories for the presumptive characterization of *Staphylococcus aureus,* either as well as or instead of coagulase for presumptive pathogenicity (Cowan and Steel's, 1993). Using DNase medium, DNase-positive *Staphylococci* are differentiated from other *Staphylococcus* spp. The media contains either toluidine blue or methyl green, which upon hydrolysis of the incorporated DNA turns colorless. The media is inoculated with the organism and incubated overnight at 35^{o}C. The plate is examined for evidence of growth and loss of color (positive reaction). No color change indicates a negative reaction (Murray et al., 2003).

2.3 Antimicrobial resistance in Gram-positive bacteria

Gram-positive bacteria are among the most common bacterial causes of clinical infections. Although recent global attention has been focused on multidrug resistant of Gram-positive bacteria are also a serious public health concern. Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VRE) are leading causes of healthcare-associated and community-acquired infections (Munita & Arias, 2016). MRSA and VRE have the potential for zoonotic transfer between humans and companion animals. *Enterococcus faecium* and *Staphylococcus aureus* are commensal bacteria living on the skin and in the intestinal tracts of both humans and animals (Rendle & Page, 2018).

Gram-positive bacteria are covered by a thick layer of peptidoglycan, consisting of short glycan chains of N-acetylmuramic acid and β-1-4-N-acetylglucoseamine residues. Penta-glycine cross-bridges are cross-linked in the cytoplasmic membrane through trans-peptidization which is catalyzed by the PBP. The β-lactam class of drugs inhibits the trans peptidase activity by acting as an analogue for the terminal D-alanyl bonds of the stem peptides. In methicillin-resistant staphylococci, β-lactams have a weak affinity towards PBP2a, an altered form of PBP2. This is due to the structural change to the serine active site resulting in decreased accessibility of β-lactams to its binding site (Stapleton and Taylor, 2002).

2.4 Mechanisms of acquiring resistance

The resistance to antibiotics in bacteria often develops as a result of unnecessary and inappropriate use of antibiotics. Through the intense use of antibiotics, resistant microorganisms have emerged over the years, and problems were started to be experienced for the treatment of these infections emerged with these resistant microorganisms. Today, on the one hand trying to develop new drugs, on the other hand, there are difficulties in treatment as a result of the development of resistance to these drugs rapidly. The development of resistance to antibiotics is a major public health problem all over the world (Cesur & Demiröz, 2013).

Antimicrobials act by targeting important bacterial functions such as cell wall synthesis (beta-lactams and glycopeptides), protein synthesis (aminoglycosides, tetracycline's, macrolides, lincosamides, chloramphenicol, mupirocin and fusidic acid), nucleic acid synthesis (quinolones), RNA synthesis (rifampin), and metabolic pathways such as folic acid metabolism (sulphonamides and trimethoprim) (Akanbi *et al*., 2017). The development of

resistance to antibiotics may be grouped into natural (Intrinsic) and acquired resistance (Cesur & Demiröz, 2013).

2.4.1 Natural (intrinsic) resistance

Depends on a specific characteristic of the bacterium and the biology of the microorganism bacterial resistance to antibiotics will be intrinsic or innate (Agnė et al., 2011). This kind of resistance is always expressed in the species and the genes are naturally occurring in the bacteria (Reygaert, 2018; Admassie, 2018). The innate ability of bacterial species caused by the structural characteristics of bacteria and is not associated with the use of antibiotics (Cesur & Demiröz, 2013). It has no hereditary property. It develops as a result of the natural resistance, or the microorganisms not including the structure of the target antibiotic, or antibiotics not reaching to its target due to their characteristics (Hasan & Al-harmoosh, 2020). Such natural insensitiveness can be due to lack of affinity of the drug for the specific bacterial, unreachability of the drug into the bacterial cell, extrusion of the drug by chromosomally encoded active exporters, and innate production of enzymes that inactivate the drug (Figure 1). Intrinsic resistance is that the innate ability of a bacterial species to resist the activity of a specific antimicrobial agent through its inherent structural or functional characteristics, which permit a tolerance of a particular drug or antimicrobial class (http://amrls.cvm.msu.edu.).

Bacterial cells are surrounded by a cell wall made of peptidoglycan, which consists of long sugar polymers (Senka, 2008; Kapoor et al., 2017). The Gram-positive bacteria consists of a cytoplasmic membrane surrounded by a tough and rigid mesh cell wall and Gram-negative bacteria consist of a thin cell wall which is surrounded by a second lipid membrane called an outer membrane (OM). The OM is another protective layer in Gram-negative bacteria and prevents from several substances entering into the bacterium. The OM is the major barrier for antibiotics penetrate through porins or by passive diffusion through the OM phospholipids bilayer (Kapoor et al., 2017). Membrane proteins export antibiotics from the cell and maintain their low intracellular concentration are called efflux pumps. Efflux systems in Gram-positive bacteria always comprise a single polypeptide located in the cytoplasmic membrane. When antibiotics are entering the cell through efflux mechanisms bacterial are pumping them out before they reach their target (Senka, 2008; Kapoor et al., 2017). Efflux pumps can be specific to antibiotics. The cell envelope of Gram-negative bacteria is a major barrier for antibiotics (Admassie, 2018).

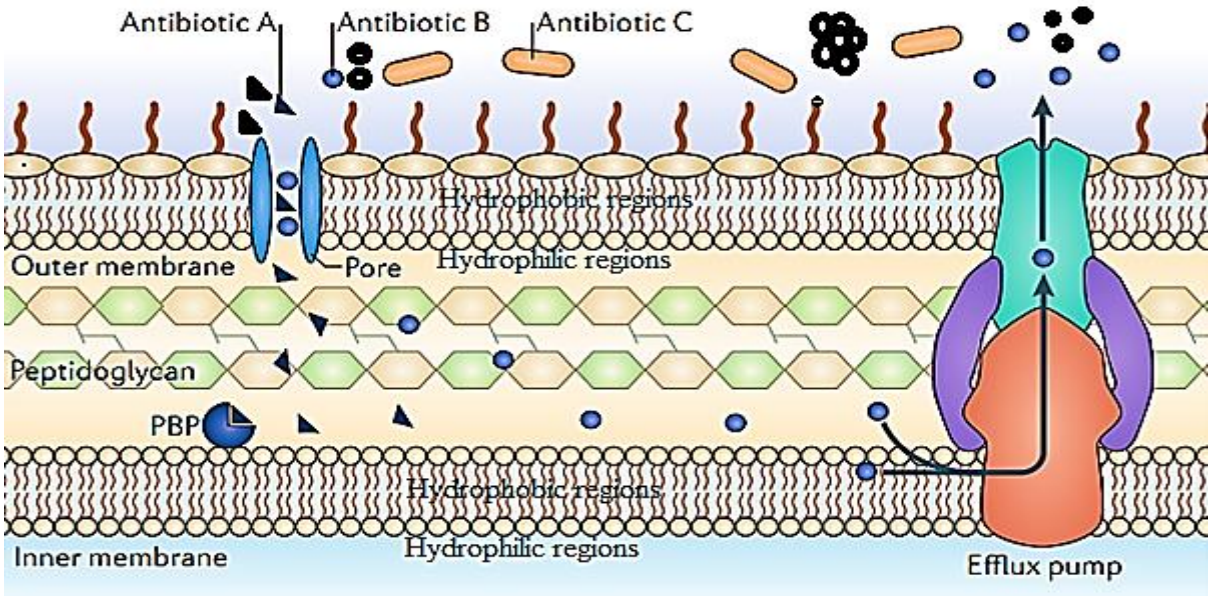

Figure 1: Intrinsic mechanism of antibiotic resistance. Picture obtained from (Blair et al, 2015) and modified

The figure shows an overview of intrinsic resistance mechanisms. The example shown is of β-lactam antibiotics targeting a penicillin-binding protein (PBP). Antibiotic A can enter the cell via a membrane-spanning porin protein, reach its target and inhibit peptidoglycan synthesis. Antibiotic B can also enter the cell via a porin, but unlike Antibiotic A, it is efficiently removed by efflux. Antibiotic C cannot cross the outer membrane and so is unable to access the target PBP.

2.4.2 Acquired resistance

Changing the bacterial genetic materials due to mutation leads to a change in the nature of proteins expression levels in bacterial strain such as *Staphylococcus aureus* (Admassie, 2018). This type of resistance commonly occurred when a previously responsive strain had been exposed to variety of antibiotics. This antibiotics resistance are also occurred due to structures of a chromosome or extrachromosomal (plasmid, transposon, etc.) (Nikaido, 2009; Hasan & Al-harmoosh, 2020).

Chromosomal resistance arises from mutations in developing in the spontaneous bacterial chromosome (spontaneous) (Cesur & Demiröz, 2013). A spontaneous alteration within the DNA sequence that may lead to a change in the trait which it codes for any change in a single nucleotide may result in corresponding modification of one or more amino acids that it codes, which can then change the enzyme or cell structure that consequently changes the affinity or effective activity of the targeted antimicrobials (Admassie, 2018). Such mutations

may occur according to some physical (starvation, ultraviolet, chemical, etc.) on the bacteria are common causes of genetic mutations (substitutions, deletions, etc.) (Reygaert, 2018). This can be a result of structural changes in bacterial cells. The result may be reduced permeability of bacterial drug or changes of the target of the drug may be in the cell. Antibiotics like Streptomycin, aminoglycosides, erythromycin, lincomycin can develop resistance against these natures (Cesur & Demiröz, 2013).

Acquired resistance phenomena occurred by extrachromosomal elements such as integrons, plasmids that independently replicated, and transposons (Agnė et al., 2011). Plasmid carried gene fragment that able to deactivate various antibiotics. These, resistance gene can be transferred from one bacterial cells such as *Staphylococcus aureus* to other different strain through conjugation transduction, and transformation, a Horizontal Gene Transfer (HGT) methods (Figure 2) (Agnė et al., 2011; Reygaert, 2018; Peterson & Kaur, 2018)

2.4.2.1 Transformation

This is the process whereby bacteria uptake short fragments of DNA and incorporate them into their genome. Briefly, DNA is released into the environment from dead bacterial cells. Competent bacteria take up this free DNA from the environment and through recombination events, the acquired DNA is expressed by the recipient cell (Munita et al., 2016). Early studies to identify if *S. aureus* could take up free DNA from the environment revealed very low transfer efficiency. This transfer was dependent on the presence of prophage or lytic phage, and subsequently it was shown that this transfer required phage proteins, specifically tail proteins. Transformation pathways may be responsible for the rare transfer of very large pieces of DNA that have moved into some isolates creating unique *S. aureus* hybrid strains. Certain bacteria such as *Acinetobacter* species are naturally competent, and therefore capable of acquiring genetic material directly from the outside environment (Reygaert, 2018).

2.4.2.2 Transduction

Transduction is the simply the transfer of DNA from one cell to another by bacteriophage (a virus that infects bacteria). During the replication process, bacteriophages inadvertently take up bacterial host genomic DNA (Figure 2). Once, the bacteriophage lyses a host cell it is released into the environment. Lysis of the bacterial cell releases infectious phage particles which bind specifically to other *S. aureus* cells. The receptor for phage binding has recently been discovered to be glycosylated wall teichoic acid. These bacteriophages then infect a new bacterial host and transfer the acquired DNA. Phage particles inject their DNA into the

recipient cell where it either integrates into the chromosome as a prophage (lysogenic pathway), or goes on to generate more infectious phage particles and kill the recipient host (lytic pathway). These lysogenic, double-stranded DNA phage are common in *S. aureus*, with most naturally occurring isolates carrying between one and four different prophage types This DNA is then incorporated and expressed in the bacterial genome (Munita & Arias, 2016).

2.4.2.3 Conjugation

This is the transfer of genetic material between bacteria directly via a connecting tube pilus or pore. Conjugation requires a direct cell to cell contact between the donor and recipient bacterial cells (Figure 2), creating a mating pair (Hiroshi, 2010). In *S. aureus* pili are not seen, and it is presumed that pores are formed between neighboring cells in close proximity. Plasmids and transposons are the main mobile genetic elements transferred during conjugation, but integrons can also be frequently transferred (Peterson & Kaur, 2018).

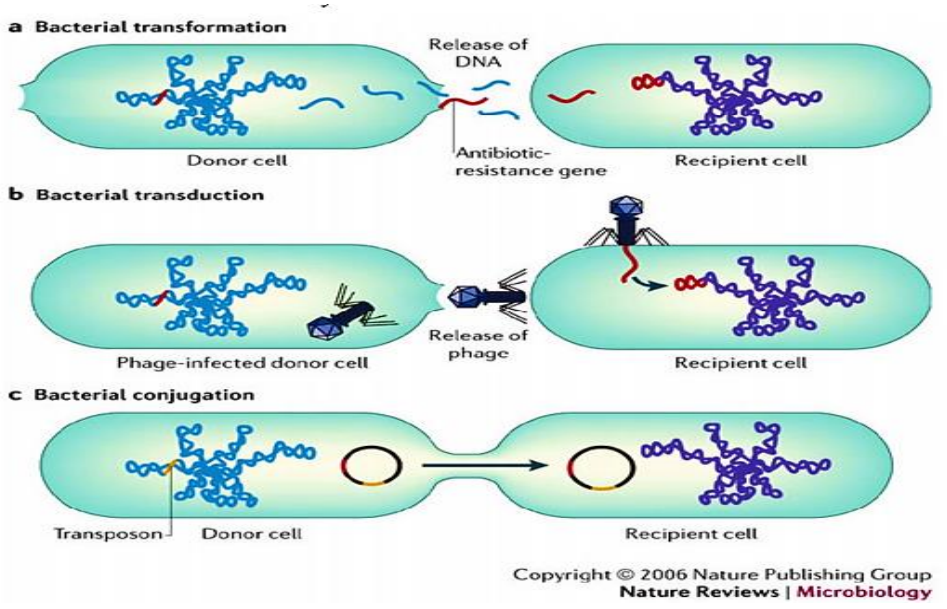

Figure 2: Means of transferring of resistance gene

2.4.3 Cross-resistance

Some microorganisms are resistant to a certain drug, that acts with the same or similar mechanism and also resistant to other drugs. This condition is usually observed in antibiotics whose structures are similar: such as resistance between erythromycin, neomycin-kanamycin, or resistance between cephalosporin's and penicillin's. However, sometimes it

can also be seen in completely unrelated drug groups. There is an example of cross-resistance between erythromycin-lincomycin. This may be chromosomal or extrachromosomal origin.

2.4.4 Multi-drug resistance

Multidrug-resistant organisms are usually bacteria that have become resistant to the antibiotics used to treat them. This means that a particular drug is no longer able to kill or control the bacteria. Inappropriate use of antibiotics for therapy resulted in the selection of pathogenic bacteria resistant to multiple drugs. Multidrug resistance in bacteria can be occurred by one of two mechanisms. First, these bacteria may accumulate multiple genes, each coding for resistance to a single drug. This type of resistance occurs typically on-resistance (R) plasmids. The second type of resistance, namely multidrug resistance may also occur by the increased expression of genes that code for multidrug efflux pumps, enzymatic inactivation, changes in the structure of the target, etc. If the bacterial strains resistant to three or more classes of antimicrobials, it is considered as multi-drug resistant (Hiroshi, 2010).

2.5 Mechanisms of resistance to antibiotics in *Staphylococcus* species

Understanding the mechanisms of microbial resistance to antibiotics has become a significant controversial issue since long years (Senka, 2008). This phenomenon has been confirmed for some isolates *Staphylococcus aureus* strains in which this isolate develop resistance against drug such as ampicillin Orrett & Land (2006), ceftriaxone Bissong & Ateba (2020), Methicillin Rijnders et al (2009), oxacillin Thapaliya et al (2017), penicillin, tetracycline Orrett & Land (2006), vancomycin Bissong & Ateba (2020). However, *Staphylococcus aureus* strains such as K1 and K1M200 had been found to be fully susceptible to erythromycin kanamycin, teicoplanin, tetracycline, and vancomycin (Wu et al., 2001). Furthermore, it has been confirmed that *Staphylococcus aureus* strains is shown sensitivity to antibiotic such as ciprofloxacin, clindamycin, erythromycin, gentamicin and trimethoprim-sulfamethoxazole (Sadeghi & Mansouri, 2014).

The alterations found in the receptor that connected to the drug and the region of the connection ‘Connection of the antibiotics’ target areas is different. Resistance associated with alterations in the ribosomal target is mostly observed in macrolide antibiotics. Mutations in PBPs (beta-lactamase enzymes) in *Staphylococcus aureus, Streptococcus pneumoniae, Neisseria meningitides,* and *Enterococcus faecium* strains can develop resistance to penicillin. Changes in the structure of the target, beta-lactam, quinolones,

glycopeptides, macrolides, tetracycline, and rifampicin resistance is an important mechanism in the development (Hiroshi, 2010). The major resistance mechanisms like; enzymatic inactivation of antibiotics, decreased drug uptake/reduction of inner and OM permeability, efflux pumps, and alternative metabolic pathways described below.

2.5.1 Enzymatic inactivation of antibiotics

In bacteria, defense mechanisms within the category of antibiotic inactivation include the production of enzymes that degrade or modify the drug itself (Senka, 2008). Most Gram-positive and Gram-negative bacteria synthesize enzymes that degrade/inactivate antibiotics. In *S. aureus* there are two main ways to inactivate antibiotics using enzymes; by actual degradation of the drug by chemical group transfer and inactivation by hydrolysis (Reygaert, 2018).

Antibiotic inactivation by chemical group transfer: The most diverse family of resistant enzymes is Phosphoryl transferases, nucleotidyl transferases, acetyltransferases, and glycosyltransferase (Kapoor et al., 2017). These enzymes inactivate antibiotics like aminoglycosides, chloramphenicol, streptogramin, macrolides, or rifampicin) by chemical substitution (phosphoryl, nucleotidyl, acetyl, and glycosyl groups are added to the periphery of the antibiotic molecule) (Senka, 2008). These modifications are achieved in the process of transport across the cytoplasmic membrane (co-substrate ATP, acetyl-CoA, NAD^+, UDP-glucose, or glutathione) (Reygaert, 2018). Aminoglycoside Modifying Enzymes (AME) reduces the affinity of a modified molecule, inhibit binding to the 30s ribosomal subunit, and provide extended-spectrum resistance to AG's and FQ. AMEs are identified in *S. aureus* (Kapoor et al., 2017).

β-lactamases (inactivation by hydrolysis): β-lactamases hydrolyze nearly all β-lactams that have ester and amide bonds, e.g., penicillin, cephalosporin, monobactams, and carbapenems (Senka, 2008; Kapoor et al., 2017). Several enzymes are known to destroy antibiotic activity by targeting and cleaving these bonds. These enzymes can often be excreted by the Gram-positive and Gram-negative bacteria, inactivating antibiotics before they reach their target within the bacteria (Senka, 2008). In *Staphylococcus aureus*, β-lactam drugs are the most widely used group of inactivation by hydrolysis of antimicrobial agents. The members of this drug group all share a specific core structure which consists of a four-sided β-lactam ring (Blair et al., 2015). Resistance to the β-lactam drugs in *Staphylococcus aureus* occurs through three general mechanisms: (1) preventing the interaction between the target PBP and the drug, usually by modifying the ability of the drug to bind to the PBP (this

is mediated by alterations to existing PBPs, (2) the presence of efflux pumps that can extrude β-lactam drugs, and (3) hydrolysis of the drug by β-lactamase enzymes (Reygaert, 2018).

2.5.2 Reduction of the inner and outer membrane permeability

Antimicrobial compounds almost always require access into the bacterial cell to reach their target site where they can interfere with the normal function of the bacterial organism. Porin channels are the passageways by which these antibiotics would normally cross the bacterial outer membrane. Some bacteria protect themselves by prohibiting these antimicrobial compounds from entering past their cell walls (Darai & Sonntag, 2009; Olowe, 2012). Gram-negative bacteria are intrinsically less permeable to many antibiotics than Gram-positive bacteria as their outer membrane forms a permeability barrier (Blair et al., 2015). One mechanism that results in reduce drug permeability in bacteria is by modifying the cell membrane porin channel frequency, size, and selectivity. The resistance due to alters in the internal and external membrane permeability decreases the drug uptake into the cell or quickly ejected through the active pump systems. Prohibiting entry in this manner will prevent these antimicrobials from reaching their intended targets that, for aminoglycosides and beta lactams, are the ribosomes and the penicillin-binding proteins (PBPs), respectively (Darai & Sonntag, 2009). This strategy have been observed in vancomycin intermediate-resistant *S. aureus* or VIRSA strains with thickened cell wall trapping vancomycin (Peterson & Kaur, 2018).

2.5.3 Active efflux Pump System

One of the most common drug resistance mechanisms is the active efflux of antibiotics/drugs from the inside of bacterial cells (Varela & Kumar, 2014). Some bacteria possess membrane proteins that act as an export or efflux pump for certain antimicrobials, extruding the antibiotic out of the cell as fast as it can enter. Some efflux pumps selectively extrude specific antibiotics such as macrolides, lincosamides, streptogramins and tetracyclines, whereas others (referred to as multiple drug resistance pumps) expel a variety of structurally diverse anti-infective with different modes of action (Darai & Sonntag, 2009; Admassie, 2018).

Based on the structure and energy source there are five main families of efflux pumps in bacteria (Figure 3); the multidrug and toxic compound extrusion (MATE) family, the small multidrug resistance (SMR) family, the major facilitator superfamily (MFS), the resistance-nodulation-cell division (RND) family, and the ATP-binding cassette (ABC) family (Reygaert, 2018). The transporters of the first four families are secondary transporters that

use the proton motive force to derive the extrusion of their substrates by an antiport H^+ drug mechanism with the exception of the MATE family which can also use the sodium membrane gradient as the source of energy. The transporters of the ABC family transporters that use ATP to drive the extrusion of their substrates (McMurry., 1981). In *Staphylococcus aureus* three families (MATE, SMR, and MFS) and more than ten multidrug efflux pumps encoded in the chromosome or in plasmid are observed (Costa et al., 2013).

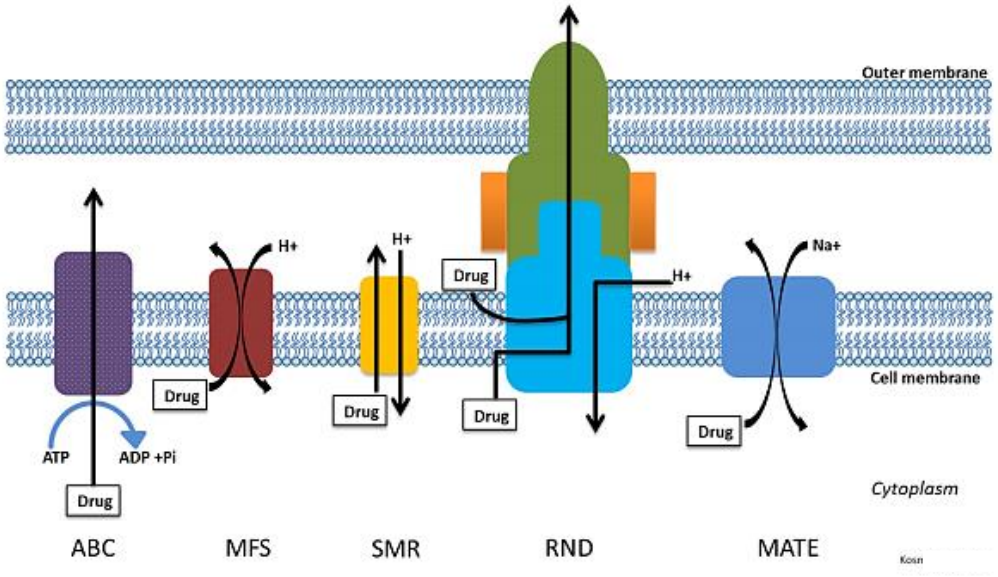

Figure 3: Structure of main efflux pump families (Reygaert, 2018)

2.5.3.1 MATE transporter family

The MATE efflux family uses a Na^+ gradient as the energy source, and efflux cationic dyes, and most efflux fluoroquinolone drugs. Very few of these have been characterized in Gram-positive bacteria, and most are found in Gram-negative organisms (Piddock, 2006). The efflux pump *mepA* was identified in studies with *S. aureus norA* disrupted mutants. The *mepA* is encoded by the chromosomal gene *mepA* and it was the first multidrug transporter from the MATE family to be described in *S. aureus*. This 451 amino acid protein has 12 transmembrane segments and presents 26% and 21% identity to the MATE transporters *cdeA* from *Clostridium difficile* and *norM* from chromosomal DNA in *Vibrio parahaemolyticus* (Kuroda, 2009).

2.5.3.2 SMR transporter family

The SMR efflux families are energized by the proton motive force (H^+), are hydrophobic, and efflux mainly lipophilic cations. SMR most commonly confer resistance to β-lactams and some aminoglycosides (Kumar, 2005). SMR pumps were first discovered on

Staphylococcus aureus (the SMR pump which transports ampicillin, erythromycin, and tetracycline (Yerushalmi et al., 1995). The efflux pump gene *smr* was identified by different groups in several plasmids conferring antiseptic and ethidium bromide resistance. This gene can be found in small non-conjugative plasmid such as *pSK89* and large conjugative plasmid like *pSK41* in both *S. aureus* and coagulative negative staphylococci and encodes the efflux pump *Smr*. Sequence analysis of the *smr* gene found in several plasmids showed that it is highly conserved with only one variant *smr* which shows a single nucleotide alteration that producing the *smr* protein. Other *S. aureus* plasmid borne efflux pumps conferring resistance to antiseptic and disinfectant include *qacG*, *qacH*, and *qacJ* (Costa et al., 2013).

2.5.3.3 MFS transporter family

The MFS efflux family catalyzes transport via solute/cation (H^+ or Na^+) symport or solute/H^+ antiport. They are involved in the transport of anions, drugs (e.g. macrolides and tetracycline), and metabolites (e.g. bile salts), and sugars (Blair et al., 2014). *S. aureus* strains that were resistant to multiple cationic bactericides and were causing hospital-acquired infections showed, however, that these strains contained plasmids coding for a multidrug efflux transporter *qacA* (or *qacB*), belonging to the MFS transporter family (Hiroshi, 2010). The chromosomally coded *NorA* of *S. aureus* pumps with a slightly broader substrate specificity which transports fluoroquinolones and chloramphenicol and *LmrS* pump which transports linezolid, erythromycin, chloramphenicol, and trimethoprim (Kumar et al., 2013). The *S. aureus* chromosome contains at least two other homologs of *norA*, *norB* and *norC*, which produce a similar phenotype. Thus, in about one-half of *S. aureus* strains isolated from the bloodstream of patients, efflux made a strong contribution to resistance, and more than half of these strains overexpressed the three chromosomally coded pumps just mentioned (Hiroshi, 2010; Costa et al., 2013).

2.5.4 Using an alternative metabolic pathway

Bacteria can create different ways to change the molecular targets of antimicrobial agents. Altered targets may include, mutated DNA gyrase, a targeted to FQ antibiotic resistance, Quinolones bind to DNA gyraseA subunit. The mechanism of resistance involves the modification of two enzymes: DNA gyrase coded by genes *gyrA* and *gyrB* and topoisomerase IV (coded by genes *parC* and *parE*) (Kim et al., 2002; Robicsek et al., 2006). Mutations in genes *gyrA* and *parC* leads to replication failure and as a result, FQ cannot bind (Kapoor et al., 2017). RNA polymerase, a target of rifampin Drapeau et al (2010), the prokaryotic ribosome, a target of tetracycline and other protein synthesis inhibitors Stapleton

& Taylor (2002), and targets of a chemical that inhibit the use of metabolite drugs, such as the sulfonamides and related drugs. *Staphylococcus aureus* becomes resistant to penicillin by any one of the several mechanisms such as a mutation in PBP or acquisition of new PBP with reduced affinity to penicillin, overexpression of PBP, etc. (Fisher et al., 2005). Another example of an altered cell wall precursor's synthesis in Gram-positive bacteria can be inhibited by glycopeptides, e.g., vancomycin or teicoplanin, by their binding to D-alanyl-D-alanine residues of peptidoglycan precursors. D-alanyl-alanine is changed to D-alanyl-lactate as a result of which glycopeptides do not cross-link with them, hence resistance to them develops (Grundmann et al., 2006; Senka, 2008).

2.6 Phenotypic detection of antimicrobial resistance

Phenotypic detection of antimicrobial resistance has been widely used in clinical and diagnostic microbiology laboratories (Markey *et al*., 2013). The principle of phenotypic testing is to compare observable or measurable traits to a known set of criteria. These methods have been well studied and standardized. They have the advantages of being low cost, easy to perform (automated systems), and interpretation criteria readily available for commonly encountered organisms. These assays also are essential for new resistance discovery (Chao et al, 2006).

2.6.1 Antimicrobial susceptibility testing

The principle of antimicrobial susceptibility testing is to determine the effectiveness of an antibiotic at inhibiting the growth of a bacterial isolate in a laboratory setting, also known as *in vitro* testing. Antimicrobial susceptibility testing follows a highly standardized protocol to ensure reproducible and reliable results (Balouiri *et al*., 2016). There are two main international standards committees recognized: The Clinical and Laboratory Standards Institute (CLSI), and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). These committees provide updated protocols for testing, and guideline criteria for interpretation of results. The standardization of antimicrobial susceptibility testing is important for generating accurate results and involves every step of the protocol, including, growth media, antibiotic concentration, inoculum concentration, incubation time and temperature, and the use of control bacterial strains. There have been several methods developed for drug susceptibility testing for bacterial strains like; agar dilution method, broth dilution method, disc diffusion method, and E-test method. Dilution based techniques can

be performed using either agar plates or broth media (Coia & Cubie, 1995; Abmm *et al*., 2020).

2.6.1.1 Disc diffusion method

The disc diffusion test is an antibiotic susceptibility test. It uses antibiotic disc to test the extent to which bacteria are affected by those antibiotics (Bauer et al., 1966). It entails the placing of discs impregnated with antimicrobial agents into plate seeded with the bacterium to be tested. The antimicrobial agents diffuses into the agar creating a zone saturated with the agent in which an organisms susceptible to that agent will not grow enough. This is called zone of inhibition. The size of zone of inhibition depends on many factors including; the bacterial concentration of the inoculum, the test medium, the depth of the agar, the pH of the medium, the antimicrobial agent and its concentration in the disk, incubation condition, and the test bacterium (Coia & Cubie, 1995).

Diffusion based techniques include the disk diffusion and gradient strip tests. Both tests use the same standardized protocol. To perform these assays, a fresh bacterial suspension in saline or deionized water is prepared. The inoculum is prepared to a density of a McFarland 0.5 standard, which corresponds to approximately 1.5 x 10^8CFU/ml. The inoculum is streaked out onto the surface of a 4mm thick Mueller Hinton Agar (MHA) plate, creating an even lawn of bacteria. After the plate is inoculated, antibiotic disks or antibiotic strips are placed on the plate and incubated at 35°C for 16-18 hours. Results are interpreted using the CLSI criteria (CLSI, 2014; Abmm *et al.*, 2020).

2.6.1.2 Automated antimicrobial susceptibility testing systems

The development of automatic susceptibility testing system consists of electronic devices; such as plate reader, video capture board, and software which automatically measure and interpret the susceptibility results from inhibition zone around the antimicrobial disc (Larsen et al., 2017). The measuring and interpreting processes are based on image processing principle such as image smoothening, edge enhancing, edge detection and area detection (Sriboonsong & Boonchoo, 2007).

2.7 Molecular identification of antibiotic resistance

Molecular methods involve the detection of resistance genes and can have advantages over conventional methods, particularly when the organism is slow-growing or difficult to isolate. Molecular identification of antibiotic resistance includes hybridization based techniques such as fluorescent in situ hybridization (FISH), amplification methods (PCR, qPCR and

RT-PCR), and DNA microarray and whole genome sequencing (WGS) (Ferone et al., 2020). The most molecular methods in current use for the detection of antibiotic resistance gene are based on either conventional or real time PCR (Coia & Cubie, 1995).

2.7.1 Polymerase chain reaction

Polymerase chain reaction (PCR) was first described by Kary Mullis in 1983 (Mullis et al., 1992). It is a technique that exploits the basic biochemistry of DNA replication, allowing for the exponential amplification of specific segments of DNA. The portion of DNA amplified contains diagnostically useful information such as bacterial resistance genes. This method produces millions of copies of a target sequence within a short timeframe by using a thermostable DNA polymerase enzyme, known as *Taq* polymerase, to mimic the natural process of DNA replication. It uses existing single-stranded DNA as a template and attaches deoxynucleotide triphosphates (dNTPs) into the replicating strand to make a complimentary copy. Conventional PCR consists of a cycle of three distinct temperature phases repeated 30 to 40 times: denaturation, annealing and extension (Figure 4) (Viljoen *et al*., 2005). This technique can be utilized to identify specific bacteria species and strains, single or multiple resistance genes, integrons, gene cassettes and plasmids.

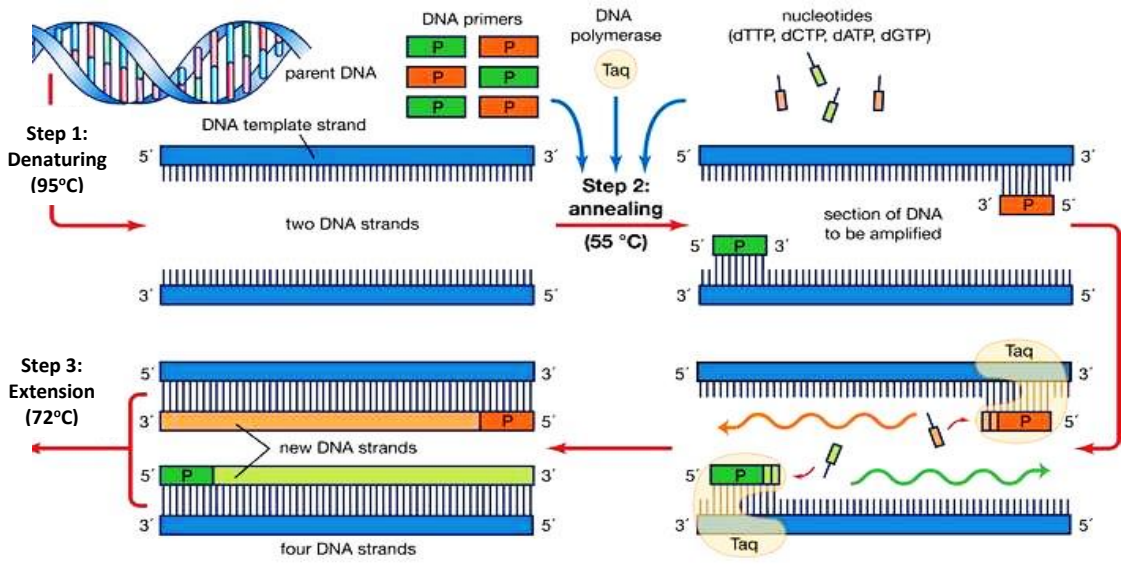

Figure 4: The three steps of PCR. Source: Encyclopedia Britannica, Inc.

2.7.1.1 DNA sequencing

Post-amplification analysis of PCR amplicons by DNA sequencing is commonly used to identify single nucleotide polymorphisms and for identifying specific alleles for strain typing. Amplicon sequencing is based on the dideoxy method also known as sequencing by termination or Sanger sequencing. Traditionally, this method substitutes dideoxynucleotide

triphosphates (ddNTP) for deoxynucleotides into a growing strand of DNA during polymerization resulting in the termination of DNA synthesis in a base-specific manner. Four separate sequencing reactions are performed where one radiolabeled-ddNTP analog in addition to the four deoxynucleotides (dATP, dGTP, dCTP and dTTP) are included to terminate at A, G, C and T residues. Each reaction creates specific fragments which could be resolved by gel electrophoresis (Sanger *et al*, 1977). Improvements to the original Sanger sequencing method increased the speed and efficiency of DNA sequencing by replacing radiolabelling with fluorometric based detection (allowing for one instead of four reactions) and the use of capillary based electrophoresis (Heather and Chain, 2016).

3 MATERIALS AND METHODS

3.1 Description of the study area

The study was conducted in Adama city in central Oromia region of Ethiopia located at about 92.2 km southeast of Addis Ababa. Adama is geographically located at 8.54ºN 39.27ºE at an elevation of 1712 meters. The city sits between the base of an escarpment to the west, and the Great Rift Valley to the east. Its annual rainfall ranges from 400-800 mm and annual temperature is 22ºC. The city has the total area of 9,616,399.5m^2 (OWWDS, 2012).

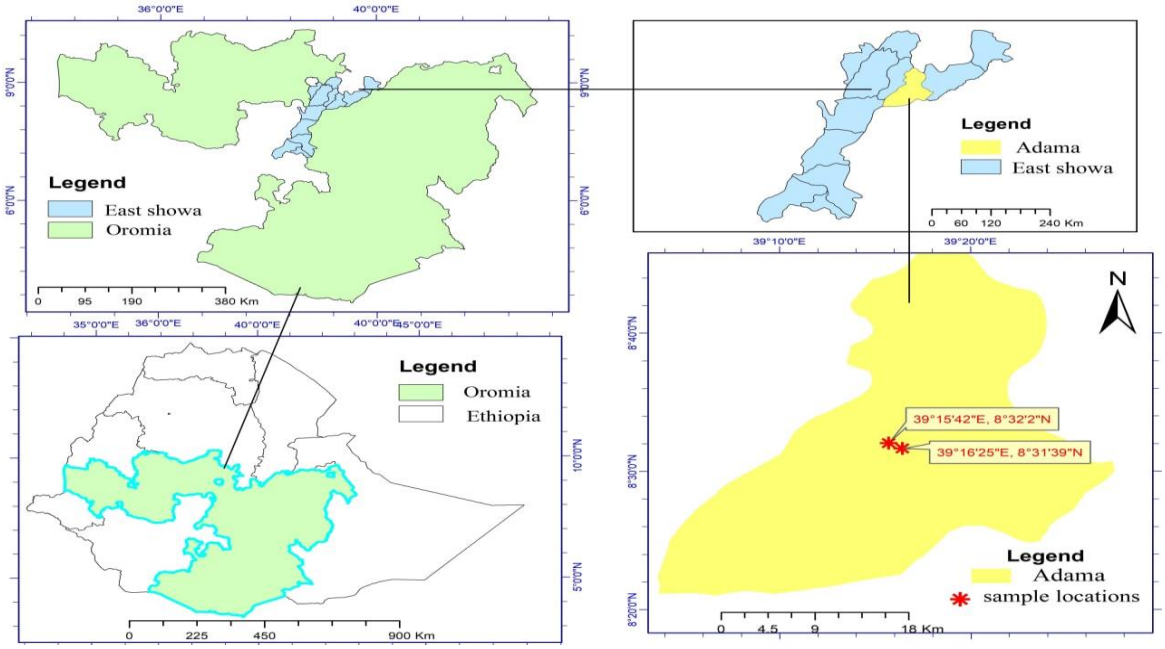

Figure 5: Location of the study area, coordinate system by Adindan UTM zone 37

3.2 Study design and sample collection

The study populations included healthy goats brought from different areas of the region and sold for the community for the slaughtering purposes. A total of 40 nasal swab samples were taken from nostrils of goats. The samples were selected using purposive sampling techniques from four herds of goat in the city for this study. The cotton swabs were inserted into the anterior nares of both left and right nostrils and softly rolled against the inner walls. One swab was used to take a sample from both nostrils then stored in Tryptone soy broth (Pancreatic digest of casein 17g/l; enzymatic digest of soybean 3g/l; NaCl 5g/l; K_2HPO_4 2.5g/l and Glucose, 2.5g/l at 25ºC and pH 6.8±0.2). The samples cultures were then transported to the applied biology microbiology laboratory using ice box. Finally, the samples were incubated at 37°C for 24 hours until isolation and screening was performed.

3.3 Screening of possible isolated *Staphylococcus* species

Preliminary screening such as catalase, coagulase, and DNase test were performed for PSSIs.

3.3.1 Growth on mannitol salt agar medium

Isolation of *Staphylococcus* species was done according to Singh and Prakash (2008) with slight modifications. A loop full of sample was aseptically spread on freshly prepared Mannitol Salt Agar (MSA) medium (Protease peptone 10g/l; NaCl 75g/l; D-mannitol 10g/l; Beef extract 1g/l; Phenol red, 0.025g/l; and agar, 15g/l) (Oxoid, UK). These all plates were incubated aerobically at 37^{o}C for 24-48 hrs. The cultures were examined for colony morphology, pigmentation and characteristics of mannitol fermentation. The possible colonies of *S. aureus* were further cultured onto mannitol salt agar (MSA) and repeatedly sub-cultured to get pure culture. The obtained pure cultures of the isolates were preserved for further bacterial identification.

3.3.2 Smear preparation and gram staining

Gram staining was performed according to the method of (Castenholz et al., 2001). Briefly, cleaned microscopic slides were taken. One drop of sterile distilled water was placed in the center of microscopic slide using a micropipette. Using an aseptic technique a very small part of a single colony was taken and transferred from solid medium to distilled water drop on a microscopic slide using sterilized loop. A thin smear was carried out and allowed to air dry at room temperature. The bacterial cells were fixed by passing the slides through the Bunsen burner flame for two-three times. The heat-fixed smears were placed on the staining rack and covered with crystal violet and left for one minute. The slide was washed gently with tap water and covered with Gram's iodine for one minute and the iodine was washed off by tilting the slide. The smear was decolorized with 95% of ethyl alcohol for about fifteen seconds and immediately washed with water. The smear was covered with safranin for one minute and washed with tap water. Finally, the smear was blotted and dried in the air to examine under the oil immersion objective.

3.3.3 Catalase test

Catalase test was performed for these newly isolated PSSIs. It was performed following the methods of (Taylor & Achanzar, 1972; Castenholz et al., 2001) with modification. Briefly, bacterial isolates were inoculated into LB agar. It was incubated at 37^{o}C for 24hrs. From 24hrs old culture, colony was taken and placed on microscopic slide. A 1-2 drops H_2O_2 were

added to bacterial isolates placed onto microscopic slide. Bubble gas was formed and considered as positive for catalase enzymes.

3.3.4 Coagulase test

Coagulase test was used to differentiate coagulase-positive *Staphylococcus* species from coagulase-negative *Staphylococcus* species. The coagulase test was performed according to the procedure of (Kateete et al., 2010). A 0.5 mL of human plasma was taken from student clinic and transferred into a sterile small test tube (one drop plasma plus nine drops of normal saline). A small number of colonies from the growth cultures were emulsified to the plasma tube and incubated at 35^{o}C for 4 hrs. Clot formation within four hours was interpreted as a positive test for cell-free coagulase (Aslanzadeh, 2006).

3.3.5 DNase test

DNase test was performed to differentiate and identify *S. aureus* form other *Staphylococcus* species. The DNase agar (Pancreatic digest of casein, 10g/l; yeast extract, 10g/l; deoxyribonucleic acid, 2g/l; NaCl, 5g/l; agar, 15g/l; methyl green, 0.5g/l and l liter distilled water) for characterization of *Staphylococcus* species. The test organism was taken from 18hrs old cultures and inoculated on the prepared DNase agar using a sterile inoculating loop. The plates were incubated at 35-37°C for 24 hrs. The incubated agar plates were flooded with a 1N HCl solution, and the excess acid is tipped off. The plates were observed for a clear zone around the colonies within 5 min (Kateete et al., 2010).

3.4 Identification of *Staphylococcus* species using MALDI-TOF MS

All isolates initially screened using biochemical tests and shown characteristics feature of *Staphylococcus* species were subjected to identification using Matrix-assisted laser desorption ionization-time flight mass-spectroscopy (MALDI-TOF MS). All the isolates classifications were carried out using the direct transfer method (MALDI Biotyper 3.1. User Manual, Bruker Daltonics Inc.).The representative single colonies of the possible *Staphylococci* isolated were smeared as a thin film directly into a spot on MALDI targeted plate using a tooth applicator. The MALDI target plate was overlaid with 1µL of 70% (v/v) of formic acid and allowed to dry at a room temperature. Immediately the spot was overlaid with 1µL of matrix solution α-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile (HCCA) solution and allowed to dry at room temperature. Plates were run on a Microflex LT/SH mass spectrometer (Bruker Daltonics). The resulting spectra were compared with reference spectra by using the Biotyper 3.1 software (Bruker MALDI Biotyper, UK). The

identification score cutoff values were applied to each measurement according to the manufacturer's instructions. Isolates with a score of ≥2.00-3.00 for a given species were considered as high confidence identification, ≥1.70-1.99 low confidence identification and the range value between 0.00-1.69 no organisms identification possible (Tomazi et al., 2014). *E. coli* was used as a standard for calibration and quality control.

3.5 Factors affecting the growth of newly isolated PSSI-D8

3.5.1 Effect of temperature on PSSI-D8

A 1L of Luria Bertani's broth (g/l) with the compositions of (glucose2g/l, NaCl5g/l, Tryptone 10g/l, and yeast extract 10g/l) was prepared. A 250ml of broth medium was poured into four conical flask (500 ml) and the media was then sterilized at 121^{o}C for 15 minutes. After sterilization 2 ml of the isolated *Staphylococcus aureus* strain was aseptically inoculated into the LB broth medium. The culture was incubated at different temperatures (25, 30, 37, and 40 ^{o}C) on a rotary shaker at 150 rpm for 48 hrs. Finally, the optical density of the culture was read in triplicate by UV-spectrophotometer at 600 *nm* and the cell dry weight was calculated.

3.5.2 Effect of pH on PSSI-D8

Effects of various pH value of the medium were prepared to study of the optimum pH for the isolated *Staphylococcus aureus* strain. Three 500 ml conical flasks each containing 250 ml of LB broth (g/L) glucose 2g/l, NaCl 5g/l, Tryptone 10g/l, and yeast extract 10g/l was set to various pH values (5, 7, and 10) using 1N HCl (v/v) and 1N NaOH (w/v) and autoclaved at 121 ^{o}C for 15 min. After sterilization, then 2 ml of the isolated *Staphylococcus aureus* suspensions was inoculated into each adjusted pH and incubated at 37^{o}C on a rotary shaker at 150 rpm for 48 hrs. The OD600 *nm* of culture and CDW (g/mL) measured at 600 *nm* wavelength using UV-spectrophotometer (SM-1600) and digital electronic balance (ES52200-4) respectively. Finally, the mean value of OD600*nm* of culture and CDW (g/ml) were calculated for the triplicate data and plotted to identify the optimum pH value for *Staphylococcus aureus*.

3.5.3 Effect of salt concentration on PSSI-D8

LB broth was prepared according to the manufacturer's instruction manual. A 250 ml of each of the three media were prepared using different NaCl (w/v) concentrations (1.28M, 1.70M, and 2.13M). Then it was dispensed into three conical flasks and corked tightly. The flasks were labeled and sterilized at 121^{o}C for 15 min. After sterilization, the isolated

bacterial suspension was inoculated into the three flasks with different NaCl concentrations. The inoculated LB broth was then incubated at 37^{o}C on a rotary shaker at 150 rpm for 48 hrs. At the end of incubation, the CDW (g/mL) and absorbance of the culture (OD at 600*nm*) were recorded in triplicate by using digital electronic balance (ES5200-4) and UV-spectrophotometer (SM-1600) respectively.

3.5.4 Effects of carbon source on isolates of PSSI-D8

The effects of different types of carbon sources on the growth of *Staphylococcus aureus* were also studied. Minimal salt medium (g/l) (K_2HPO_4, 1.73g/l, KH_2PO_4, 0.68g/l; $MgSO_4.7H_2O$, 0.1g/l; NaCl, 4g/l; $FeSO_4.7H_2O$, 0.03g/l; NH_4NO_3, 1g/l; and $CaCl_2$, 0.02g/l at 37^{o}C and 7.0 pH). Prior to sterilization, the initial pH of the medium was adjusted to pH 7. The minimal salt medium was sterilized at 121^{o}C for 15 min. A 2% (w/v) of different types of carbon sources such as fructose, glucose, mannitol, and sucrose were sterilized separately before being mixed with minimal salt media. Each culture medium with different carbon sources were inoculated with newly isolated *Staphylococcus aureus* and incubated at 37^{o}C in a rotary shaker at 150 rpm for 48 hrs. After the incubation time, the absorbance of the culture and biomass was recorded in triplicate using a UV-spectrophotometer (SM-1600) and digital electronic balance (ES5200-4) respectively.

3.6 Antimicrobial susceptibility testing

The isolated *Staphylococcus* species were screened for *in vitro* antimicrobial susceptibility using the disk diffusion method according to the procedure given by (Bauer et al., 1966). The bacterial suspension was prepared in 5ml of Mueller Hinton broth (g/l) (acid hydrolysate casein 17.5 g/l, beef infusion from buffalo 2 g/l, starch 1.5 g/l) and the turbidity was adjusted to meet 0.5 McFarland turbidity standards (~1.5x10^{8}CFU/ml). The isolates were inoculated on Muller Hinton agar medium (g/l) acid hydrolysate casein 17.5g/l, agar 17g/l, beef infusion from buffalo 300g/l, starch 1.5g/l and tested against impregnated of antibiotics disk such as ampicillin (10µg), amoxicillin (2µg), chloramphenicol (Himedia, India) (30µg), kanamycin (30µg) (Oxoid Company, Hampshire, England), nalidixic acid (30µg), oxacillin (1µg), penicillin (10µg), streptomycin (Himedia, India) (25µg), and vancomycin (30µg). This impregnated antibiotics disk symmetrically placed onto the medium by using sterilized tweezers. *Staphylococcus aureus* (ATCC 25923) was used as a positive control. All antimicrobial susceptibility testing assays were triplicated. After 24hrs of incubation, the clear zones of bacterial growth around the antibiotic disc diameter for individual

antimicrobial agents were measured by (digital antibiotic zone reader, M-10676, India) and then translated into sensitive (S) and resistant (R) categories according to the interpretation table of the (CLSI, 2014) and VET01-S2 (CLSI, 2015).

3.7 DNA extraction

DNA extraction and PCR for the amplification of antibiotic resistance genes were conducted in National Animal Health Diagnosis and Investigation Center (NAHDIC) in Sebeta, Oromia. DNA extraction was performed using Qiagen DNeasy extraction kit protocol for bacterial culture (Thermo Scientific, Germany). Briefly, seventeen newly isolated *Staphylococcus* species isolates were grown overnight at 37ºC in brain heart infusion agar (g/l) (Himedia, India); HM infusion powder 12.5 g/l, BHI powder 5 g/l, Proteose peptone 10 g/l, Dextrose 2 g/l, NaCl 5 g/l, Na_2HPO_4 2.5 g/l and Agar, 15 g) and final pH (at 25ºC) 7.4±0.2. One-two loop full distinct colonies of bacterial isolates were taken and suspended in 600 µl of sterile PBS buffer. The suspended bacterial culture was centrifuged 20,0000xg for 5 min at 25ºC and the supernatant was discarded and the pellet was harvested. The pellet was suspended within 180 µl of enzymatic lysis buffer and vigorously vortexed for 10-20 sec and the cells were incubated at 37ºC for 30 min in the water bath. After incubation 25 µl of proteinase K and 200 µl of AL buffer were added respectively to the tube and briefly vortexed. The tubes were incubated at 56ºC for 30 min in the water bath. After incubation 200 µl of 100% (v/v) ethanol was added and vortexed. Using a micropipette the entire contents of the extracted sample were transferred to the labelled spin column tube and the column was centrifuged at 10,000x g for 1 min at 25ºC. The column was removed from the collection tube and placed in a new collection tube. 500 µl of AW1 buffer was added to the column and centrifuged at 10000xg for 1 min at 25ºC. The column was removed from the collection tube and the column was placed in a new collection tube. A 500 µl of AW2 buffer was then added to the column and centrifuged 20,000x g for 3 min at 25ºC. The tubes were carefully removed from the centrifuge. The column was then transferred to a 1.5 ml tube and 200 µl of AE buffer was added to the column. The column was stood at room temperature for 1 min. Finally, column was centrifuged 10,000x g for 1 min at 25ºC and pure DNA was collected and stored at -20 ºC until used for further analysis.

3.8 PCR for amplification of antibiotic resistance

Seventeen genomic DNA of *Staphylococcus* species isolates were subjected to PCR in order to amplify the resistance genes using PCR master kit with 25 µl mixtures including 2 µL of

each primer, 1.5 µl of 25 mM of $MgCl_2$, 5 µl of PCR buffer, 0.5 µl of dNTP, 0.5 µl of Taq polymerase enzyme, 11.5 µl of RNase free water and 2 µl of extracted DNA. Sterile water was added instead of nucleic acids for the negative control. *Staphylococcus aureus* (ATCC25923) strain was used as a positive control for all selected primers. The PCR tubes containing the mixture were tapped gently and the PCR tubes with all the components were transferred to (Flex cycler, Biometra Gmbh, Germany) thermal cycler. Three sets of primers were used for amplification of targeting antibiotic resistance genes. These genes are including such as; *mec*A, *nuc,* and *bla*Z gene. These primers are commercially synthesized by Eurofins genomics India Pvt. Ltd. Oligonucleotide primers *mecA*-F (gtagaaatgactgaacgtccgatga) and *mecA*-R (ccaattccacattgtttcggtctaa) was used to target oxacillin resistant genes (Hoque et al., 2018). The *mec*A PCR program included denaturation at 95°C for 5 min followed by (37) cycles at 95°C for 30 sec, 55°C for 30 sec, 72°C for 60 sec, and a final extension at 72°C for 10 min has completed the amplification (Salisbury *et al*, 1997). Oligonucleotide primer *nuc*-F (gcgattgatggtgatacggtt) and *nuc*-R (agccaagccttgacgaactaaagc) was used to detect the thermo-tolerant gene. The *nuc* amplification was performed under the following condition: One cycle for initial denaturation 95°C for 5 min, 37cycles of 95^{o}C for the 30 sec, 55^{o}C for 30 sec, and 72°C for 1 min, 1 cycle of 72^{o}C for 10 min. Oligonucleotide primer *blaZ* was used to detect penicillin resistance gene. Primers used for amplification of *blaZ* gene of *Staphylococcus* isolates *blaZ*-F (tacaactgtaatatcggaggg) *blaZ*-R (cattacactcttggcggtttc) (Okiki et al., 2020). The *blaZ* amplification program was performed under the following conditions: Initial denaturation 95°C for 5 min, 37 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min with a final extension of 72°C for 10 min.

3.9 Agarose gel electrophoresis and visualization

The PCR products were separated on 1.5% (w/v) agarose gel containing ethidium bromide (2µL) that intercalate into the DNA and used for visualization. A 8µl amplification product of each sample with 2 µl of loading dye was applied into the slots of agarose gel. DNA marker of 100 bp (Qiagen; Germany) was used to estimate the molecular weight of the DNA fragments. Electrophoresis (Apelex Midigel XL, France) was performed in 1X in Tris-TBE buffer at 100v for 1 hr. The products were visualized with UV illumination (Gel Doc^{TM} XR^{+}, BioRAD; Germany) and imaged with a gel documentation system.

3.10 Amplification for 16S rDNA gene for isolate identification

Amplification for 16S rDNA and sequencing of the purified PCR product were conducted in the laboratory of heredity life sciences Pvt Ltd, Odisha, India. One isolate that had a positive result for the selected primers were selected for 16S rDNA sequencing studies. The genomic DNA of the isolates was extracted using Quick-DNA™ Fungal/Bacterial Miniprep Kit Catalog No. D6005 from Zymo Research. About 10^9 (50–100 mg) wet weight of bacterial cells that re-suspended in up to 200 µl of isotonic buffer was added to a ZR BashingBead™ Lysis Tube (0.1 mm & 0.5 mm). A 750 µl BashingBead™ buffer was added to the cap tube that prevents leakage. Bead beater fitted with a 2 ml tube holder assembly was secured and centrifugation at maximum speed for ≥ 5 min was processed. The ZR BashingBead™ Lysis Tube (0.1 & 0.5 mm) in a micro centrifuge was centrifuged at 10,000xg for 1 min at 25ºC. Up to 400 µl of supernatant was transferred to a Zymo-Spin™ III-F filter in a collection tube and centrifuged at 8,000xg for 1 min at 25ºC. A 1,200 µl of genomic lysis buffer was added to the filtrate in the collection tube from step 4. A 800 µl of the mixture was transferred from Step 5 to a Zymo-Spin™ IICR column in a collection tube and centrifuged at 10,000xg for 1 min at 25ºC. The flow-through from the collection tube was discarded and Step 6 was repeated. 200 µl DNA pre-wash buffer was added to the Zymo-Spin™ IICR column in a new collection tube and centrifuged at 10,000xg for 1 min at 25ºC. A 500 µl gDNA wash buffer was added to the Zymo-Spin™ IICR column and centrifuge at 10,000xg for 1 min at 25ºC. The Zymo-Spin™ IICR Column was transferred to a clean 1.5 ml micro centrifuge tube and 50 µl DNA elution buffer was directly added to the column matrix and centrifuge at 10,000xg for 30 sec at 25ºC to elute the DNA.

The concentration of each DNA sample was measured by Nanodrop (Biotech Instruments, USA). DNA was stored at-80ºC for further use. The ratio of absorbance at 260*nm* and 280*nm* is used to assess the purity of DNA. A ratio of ~1.8 to 2.0 is generally accepted as pure for DNA analysis. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol, or other contaminants that absorb strongly at or near 280*nm*.

PCR was performed in a total volume of 25 µl containing 10 pmol each of forward (27F-agagtttgatcctggctcag) and reverse (1492R-tacggttaccttgttacgactt) primers, 2.5 mM of $MgCl_2$, 200 µM each of the four deoxy ribonucleotide triphosphates (dNTPs), 0.5U of Taq DNA polymerase, 1x concentration of PCR buffer (Invitrogen, Life Technologies, Brazil) and 50 to 100 ng of isolated bacterial genomic DNA. The template was denatured by heating at a pre-denaturation of 95 °C for 5 min. This was followed by 39 cycles of denaturation 30 sec

at 95 °C, 45 sec annealing, and 1 min elongation at 72°C, with a final extension of 7 min at 72°C. The amplicons were resolved in 1.5% agarose gel using 0.5x tris-acetate-EDTA (TAE) buffer at 50V for 30 to 45 min till DNA fragments are migrated across gel. The gel was photographed on a gel documentation system.

The sequencing of the purified PCR product was performed by the Sanger sequencer method (ABI 3730XL) genetic analyzer version. The generated sequence was submitted for a Basic Local Alignment Search Tool (BLAST) in the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/). The sequence with the highest homology with a maximum query coverage and the maximum score was used to ascertain the identity of the *Staphylococcus* species (Higgins *et al*., 2007).

3.11 Phylogenetic tree analysis

The 16S rDNA gene sequences were analyzed with Basic Local Alignment Search Tool (BLAST) program available on the National Center for Biotechnology Information (NCBI). The 16S rRNA gene sequences were extracted from the genomic DNA of strains these showing sequence similarity more than 99% with recent our isolate (PSSI-D8) after blasting it using tools that available at http://www.ncbi.nlm.nih.gov/blast. The 16S rDNA genes sequence of newly isolated bacterial species and other taxonomically related strains were also collected from GenBank database using EzTaxon server (https://www.ezbiocloud.net/identify). These strains were aligned by multiple sequence alignment program **(**Align by Muscle, codons) and phylogenetic tree was performed with the MEGA version 11 software package (Tamura et al., 2021). The related sequences for phylogenetic tree analysis were obtained from NCBI database using NCBI-BLAST tools. Phylogenetic tree was performed for aligned sequence and evolutionary distances were done by using Maximum likelihood statistical method and Kimura two parameter distance model at has invariant sites with the gap or missing data treatment as partial deletion (Tamura et al., 2011). Confidence values were estimated from bootstrap analyses of 1,000 times (Saitou & Nei, 1987). *Erwinia tasmaniensis* Et199 (T) (HG522775.1) was designated as an out-group for the phylogenetic tree to be constructed.

3.12 Data management and analysis

Data were computed into Microsoft excel to calculate mean, standard deviation, and standard error for isolate's biomass (CDW, g/ml) against different carbon source, physical parameter such as pH and temperatures. IMB SPSS 24 and OriginPro8.5 statistical software were used

for data analysis. All data were mean of experiments performed in triplicate. Descriptive statistics were used to summarizing quantitative data.

3.13 Ethical clearance

Sampling from animals were carried out according to the experimental practice and standards approved by the University Ethical Review Board (UERD) from Research Affairs Dean Office, Adama Science and Technology University (ASTU), with the verification number RECSoANS/BIO/03/2021, that is in accordance with the international guidelines for animal welfare (Appendix 3).

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4 RESULTS AND DISCUSSION

4.1 Screening of *Staphylococcus* species isolates

4.1.1 Growth on mannitol salt agar medium for PSSIs

In this study, 40 samples were obtained from NCGs. Out these PSSIs, 34 (85%) of them were able to grow on Mannitol salt agar (MSA). However, six (15%) of them were did not grow on MSA medium. As shown in (Figure 6a), the pure colonies of PSSIs were shown yellowish to golden color which is a characteristic feature of *Staphylococcus aureus.* In agreement with this study, it was confirmed that *S. aureus* able to ferment mannitol Kumar et al (2009) and forms golden yellow colonies on the MSA medium. Other possible *Staphylococcus* species PSSI-D16, PSSI-D20, PSSI-M23and PSSI-M31 isolates were pink or red colonies (Figure 6b). The color of the colonies and the medium is depending on the reactivity of phenol red indicator to the pH of the medium. Phenol red is red at pH 8.4 and phenol red is yellow at pH 6.8.

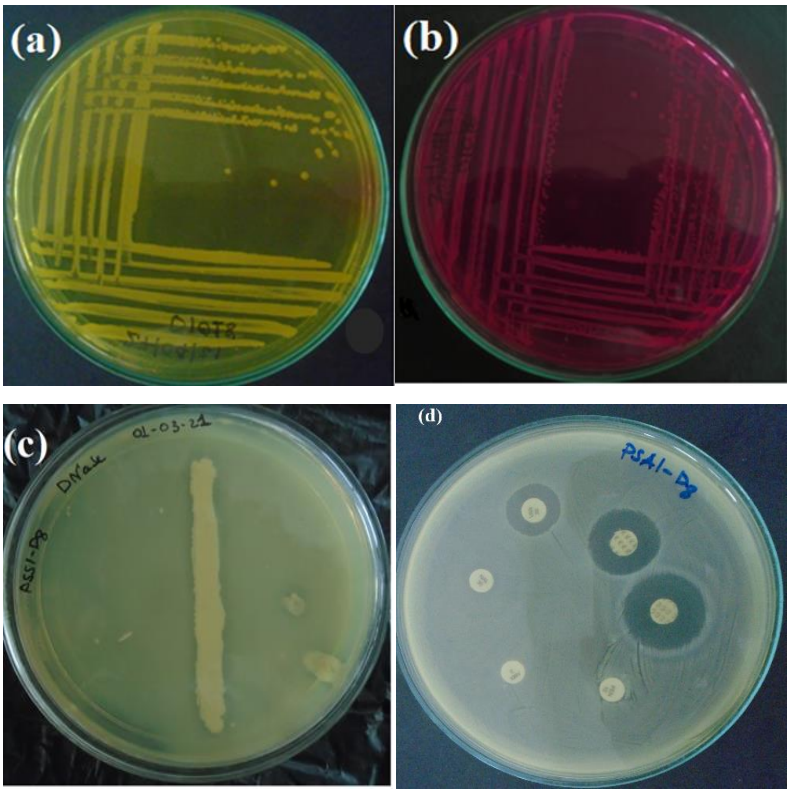

Figure 6: Pure colonies of PSSI-D8 these obtained from NCGs, and grown on MSA.

(a) Confirmed pure colonies of most likely *Staphylococcus aureus* (PSSI-D8) strain with a golden color. **(b)** Pure colony of *Staphylococcus simulans* that grown on MSA (PSSI-D9). **(c)** Pure colony of PSSI-D8 that grown on DNase agar medium with slight clear zone near

the growth of inoculum most likely due to hydrolytic enzyme. **(d)** The antibiotics susceptible test for various antibiotics disk

4.1.2 Gram staining and morphological characterization for PSSIs

From 34 isolates grew on mannitol salt agar, 28 isolates were found to be round shape (grape like clusters) in terms of their morphology. These cells were able to form clusters that resemble a bunch of grapes (Figure 7).

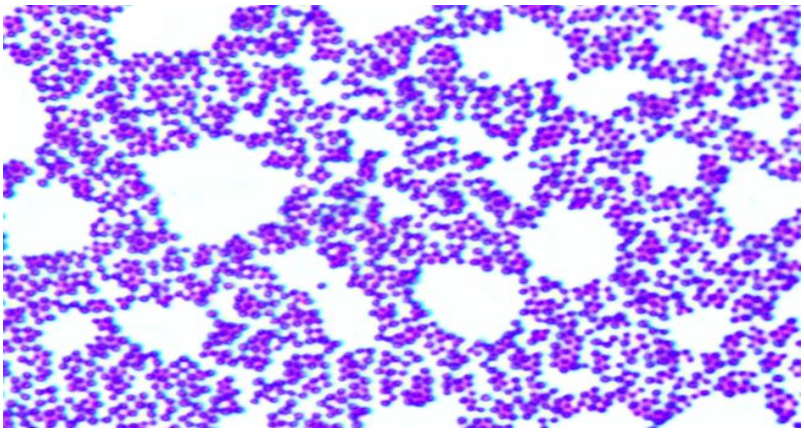

Figure 7: Gram staining for the isolates under the microscope. Gram staining for PSSI-D8 that obtained from NCGs, with cluster shaped cells.

All PSSIs were gram positive and cocci in morphology as indicated in above figure for sample collected from NCGs. This is a unique feature for *S. aureus. Staphylococci* are found to be non-sporulation, gram-positive cocci that are an average of >1 cells with grape-like clusters cocci Wollman et al (2016) when viewed under microscopy which is strongly in agreement with our result.

4.1.3 Catalase test for PSSIs

In the recent study, all the isolates of *Staphylococcus* showed catalase positive (Table 1). It was also suggested that these isolates harbored catalase enzyme that able to breakdown H_2O_2 into oxygen and water which resulted in formation of bubbles gas (Figure 8a). In agreement with this study, it was reported that there is a formation of bubble gas when H_2O_2 were applied on *Staphylococci* species (Jahan et al., 2015). These catalase test, thus found to be employed as a distinguishing methods of *Staphylococcus* from the *Streptococci* and anaerobes (Aslanzadeh, 2006).

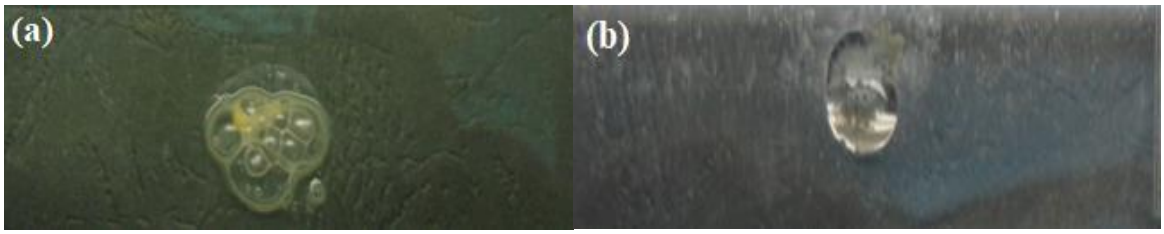

Figure 8: Catalase test

(a) Catalase positive isolate that obtained with bubble gas formation. **(b)** Catalase negative with no bubble formation.

4.1.4 Coagulase test for PSSIs

As indicated in Table 1, some species of *Staphylococcus* isolates were coagulase positive some were coagulase negative For instance, PSSI-D4, PSSI-D8, and PSSI-D18 isolates were found positive for coagulase. These isolates supposed to be *Staphylococcus* species. In line with is study, it was reported that majority of the *Staphylococcus aureus* strains are found to be coagulase-positive (Kateete et al., 2010). In line with this study, Lowy (1998) reported that *S. aureus* is distinguished from other species by its coagulase positive. As indicated in Table 1, most of these isolates did not show coagulase positive which have shown a characteristic features of coagulase-negative *Staphylococcus* species. Similarly it was reported that coagulase-negative *Staphylococci* species Zhao et al (2021) were among the most abundant species isolated from human nares and skin when these isolates had been identified by using molecular characterization. In line with this study, it was confirmed that *Staphylococcus aureus Ps55* Bannantine & Pattee (1996) contains coagulase gene known as *coa* that used to produce coagulase enzyme upon expression.

4.1.5 Deoxy ribonuclease (DNase) test for PSSIs

Staphylococcus species such as PSSI-D4, PSSI-D8 and PSSI-D18 showed positive results for DNase test in the present study (Figure 6c). However, the remaining isolates showed negative results for DNase. PSSI with positive DNase test was found to be the least prevalent (Table 1). The presence of DNase positive indicates that the isolates contain hydrolytic enzyme which is a DNase. From this result it is possible to reconfirm that isolates PSSI-D4, D8 and D18 isolates could be most likely *Staphylococcus aureus.* In agreement with this study it was reported that *Staphylococcus* species with DNase positive confirmed to be *Staphylococcus aureus* (Orrett & Land, 2006). It was further stated that DNase test is used to distinguish *Staphylococcus aureus* from other *Staphylococcus* species (Lowy, 1998).

Table 1: Biochemical tests for the isolated *Staphylococcus* species

S.N.	Samples	Gram staining	Shape	Catalase test	Coagulase test	DNase test	Mannitol fermentation
1	PSSI-D2	+	C	+	-	-	-
2	PSSI-D4	+	C	+	+	+	+
3	PSSI-D8	+	C	+	V	+	+
4	PSSI-D9	+	C	+	-	-	-
5	PSSI-D13	+	C	+	-	-	-
6	PSSI-D14	+	C	+	-	-	+
7	PSSI-D16	+	C	+	-	-	-
8	PSSI-D18	+	C	+	+	+	+
9	PSSI-D19	+	C	+	-	-	-
10	PSSI-D20	+	C	+	-	-	-
11	PSSI-M22	+	C	+	-	-	+
12	PSSI-M23	+	C	+	-	-	-
13	PSSI-M24	+	C	+	-	-	-
14	PSSI-M29	+	C	+	-	-	-
15	PSSI-M31	+	C	+		-	-
16	PSSI-M32	+	C	+	-	-	-
17	PSSI-M37	+	C	+	-	-	-
18	-Ve	-	-	-	-	-	-
19	*S. aureus* ATCC 25923^{T}	+	C	+	+	+	+

Key words: C, indicates that the isolates are cocci, **PSSIs**, indicates Possible *Staphylococcus* species isolates, **D** indicates Dambala, **M** indicates Mugar and **V** indicates variable. Both Dambala and Mugar are place where herds of goats from which the recent samples were collected for isolation of *Staphylococcus* species, Negative control (without inoculation isolates of pure NCGs), Positive control (*S. aureus*) ATCC 25923^{T}

4.2 Identification of *Staphylococcus* species by using MALDI-TOF MS techniques

Based on MALDI-TOF MS analysis, 17 (60.71%) isolates were identified as the *Staphylococcus* species, 9 (32.14%) were identified as *Enterococcus* species and 2 (7.14%) were identified as *Bacillus* species (Table 2). As indicated in Table 2, *Staphylococcus simulans* (52.94%) was found to be a dominant flora of NCGs and followed by *Staphylococcus aureus* and *S. xylosus* of which they are 17.64% each among identified *Staphylococci* species. This could be due to suitability of goat's nasal cavity for their growth. In agreement with the current study, *S. simulans* which is the opportunistic pathogen has been isolated from cows, goats, horses and other domesticated animals and associated with bovine mastitis (Gosselin et al., 2018; Moroz et al., 2020). Among the 17 *Staphylococcus* species analyzed by MALDI-TOF MS techniques, 3 (17.64%) isolates were found to be coagulase positive *Staphylococcus* species (CoPSS) and 14 (82.36%) was found to be coagulase negative *Staphylococcus* species (CoNSS).

In this study, four different species of *Staphylococcus* species were isolated. In confirmation with this study, *Staphylococcus* species were reported from nasal carriage of dairy goats in Canada and Poland (Moroz et al., 2020). In a clinical samples analysis and non-healthcare settings, *Staphylococcus epidermidis* were also found to be the most commonly reported *Staphylococcus* species (Xu et al., 2018; Shrestha et al, 2020).

Table 2: Type of isolate obtained from nasal cavity of goats

S.N	Genus level	Species level	Name of isolates	Numbers of isolation
1	*Enterococcus*	*E. faecium*	PSSI-D10, PSSI-M25, PSSI-M26, PSSI-M34, PSSI-M35 and PSSI-M39	6
		E.gallinarum	PSSI-D17, PSSI-M28 and PSSI-M33	3
2	*Staphylococcus*	*S.aureus*	PSSI-D4, PSSI-D8 and PSSI-D18	3
		S.xylosus	PSSI-D14, PSSI-M22 and PSSI-M23	3
		S.sciuri	PSSI-M24 and PSSI-M37	2
		S.simulans	PSSI-D2, PSSI-D9, PSSI-D13, PSSI-D16, PSSI-D19, PSSI-D20, PSSI-M29, PSSI-M31 and PSSI-M25	9
3	*Bacillus*	*B. cereus*	PSSI-M38 and PSSI-M40	2
Total	**3**			**28**

4.3 Antibiotic susceptibility testing against isolates of PSSIs

The susceptibility and resistant patterns were varied among all these *Staphylococcus* isolates (Table 3). In this study, antimicrobial resistant was observed for *Staphylococcus* species against antibiotics such as ampicillin (100%), Penicillin (88.25%), oxacillin (88.25%), amoxicillin (82.36%), and nalidixic acid (70.59%). It was confirmed that less number of resistance was observed in streptomycin (5.89%) and kanamycin (29.42%) respectively by *Staphylococcus* species. It was found that *Staphylococcus* species were susceptibility to vancomycin & chloramphenicol (100%), streptomycin (94.11%), & kanamycin (70.58%), respectively. Less susceptibility was observed for amoxicillin, oxacillin, nalidixic acid, and penicillin (Figure 6d and Table 3). From the current study, we noted that chloramphenicol, streptomycin and vancomycin were effective against *Staphylococcus species.* However, kanamycin is found to be moderately effective against to *Staphylococcus* species that isolated from the nasal cavity of goats which is in agreement with the finding of (Tefera et

al., 2019). In this study, *Staphylococcus* species are found to be 100% susceptible to chloramphenicol and vancomycin. In line with this study, MRSA was found to be more susceptible to bacitracin (89.3%), gentamicin (86.1%), sulfamethoxazole (94.3%), trimethoprim (88.5%), and vancomycin (Chang et al., 2015).

The findings showed that the isolated *Staphylococcus* species were found to be more susceptible to glycopeptides and aminoglycosides groups of antimicrobial agents than other groups of antibiotics. This result is in line with the reports of Abera et al (2010) in which the isolates were found to be 100% susceptible to Chloramphenicol, 88.9% to Kanamycin and 86.1% to Streptomycin. Zhou et al (2017) isolated *Staphylococcus aureus* from goats in China in which this isolate was susceptible to chloramphenicol (65.63%) streptomycin (87.5%), to kanamycin (81.25%), and vancomycin (87.5%). In this study, aminoglycosides and phenicols groups of antimicrobial agents were found to be effective against *Staphylococcus* species. These drugs are most likely used to inhibit protein and cell wall synthesis. This finding shows that all (100%) of the isolated *Staphylococcus* species were resistant to three or more classes of antibiotics (Table 3). From which 11.77, 41.17, 41.17 and 5.89% of the isolates were resistant to three, four, five and six antibiotics, respectively. The present study showed that all the isolates of *Staphylococcus* species are multidrug resistant. This could be due to the inappropriate use of antibiotics as means of treatment and thereby the development of antibiotic resistance.

In this study, the *Staphylococcus* isolates were shown resistant to penicillinase and *β*-lactams group of antimicrobial agents (Figure 6d). In addition, the isolated *Staphylococcus* strains were also displayed resistance to nalidixic acid which is a group of quinolones antimicrobial agent. As revealed in this study, *Staphylococcus* species shown resistance to various antimicrobial agents which is a similar finding to Girmay et al (2020) who reported that *Staphylococcus* strains developed resistant against ampicillin (100%), amoxicillin (85.71%) and nalidixic acid (100%). Abera et al (2010) have also reported that some *Staphylococcus* strains are able to develop resistant to ampicillin (94.4%) and to amoxicillin (36.1%). Weldeselassie et al (2020) have further confirmed that some of the *Staphylococcus* strains are able to show resistance to amoxicillin (96.9%) and penicillin (93.8%). Similarly, Jahan et al (2015) have demonstrated 100% resistance to penicillin and amoxicillin. Thaker et al., 2013, also reported that 100% resistance to penicillin, 40% to ampicillin and 20% to oxacillin. Beyene et al., (2017), have indicated 95.3% resistance to penicillin and 88.4% to nalidixic acid.

Table 3: Antibiotic resistance of *Staphylococcus* species isolates from goats against nine different antibiotics

Group of antimicrobial agent	**Antibiotic name**	**Percentage of resistance and susceptibility**	
		Susceptible	**Resistant**
	AMP	0 (0%)	17 (100%)
β-lactams	OX	2 (11.75%)	15 (88.25%)
	PEN	2 (11.75%)	15 (88.25%)
	AMX	3 (17.64%)	14 (82.36%)
Quinolones	NAL	1 (5.88%)	16 (94.12%)
Phenicoles	C	17 (100%)	0 (0%)
Glycopeptides	VAN	17 (100%)	0 (0%)
Aminoglycosides	ST	16 (94.11%)	1 (5.89%)
	K	12 (70.58%)	5 (29.42%)
Mean		**46.97%**	**53.03%**

AMX: Amoxicillin, AMP: Ampicillin, C: Chloramphenicol, K: Kanamycin, NAL: Nalidixic acid, OX: Oxacillin, PEN: Penicillin, ST: Streptomycin; VAN: Vancomycin

Certain ruminant's animals such as goats used to play significant role in transmission of zoonotic diseases. These animals are considered to be the sources of *Staphylococcus* species. There is a close contact between humans and goats during pastures and feeding or petting that provides the opportunity of transferring of *Staphylococcus* resistance genes between goats and humans and vice versa (Malik et al., 2006). Antibiotic resistance development among the bacteria poses a problem of concern. Effectiveness of the current treatments and ability to control infectious diseases in both animals and humans may become hazardous (Thaker et al., 2013). If the necessary action not taken against indiscriminate use of antibiotics, prevalence of antibiotic resistant *Staphylococcus* species may increase further to cause a serious health hazards to humans. The resistance of *Staphylococcus* species to penicillin and *β*-lactams groups of antibiotics closely related to the production of *β*-lactamase, an enzyme that inactivates penicillin and closely related antibiotics (Reygaert, 2018).

4.4 Physiological characterization and growth condition of PSSI-D8

4.4.1 Effect of carbon sources on newly isolated PSSI-D8

Optimization for carbon sources was performed and showed different growth patterns for PSSI-D8 (Figure 9a). The minimum biomass was obtained when sucrose (0.00213±000025g/ml) was provided as a sole carbon source. However, the maximum

biomass was obtained from glucose (0.0031±0.00026g/ml) followed by fructose (0.0026±0.00026g/ml). As indicated in (Figure 9a) the mean value (mean ± SD) of OD_{600nm} for the isolate *S. aureus* that obtained from the NCGs were 0.3333 ± 0.0058, 1.2267 ± 0.00577, and 1.55 ± 0.01 when sucrose, mannitol, fructose, and glucose had been supplied as a sole carbon source, respectively in increasing order. Significant variation (p>0.05) was observed among carbon source to support the growth of PSSI-D8 biomass.

In this study, we observed that variations in terms of detected for these isolates at a 95% confidence interval (p<0.05). As illustrated in (Figure 9a), statistically significant variations were observed among glucose and fructose (p=0.033), glucose and mannitol (p=0.009), fructose and sucrose (p=0.043), and glucose and sucrose (p=0.001), for CDW g/ml. However, there was no significant variations recorded for fructose and mannitol (p=0.416), and mannitol and sucrose (p=0.161) in terms of CDWg/ml for the isolate PSSI-D8 obtained from the nasal cavity of goats (Figure 9a). As it was witnessed from the figure in spite of variations detected in certain of these carbon sources, the isolate was able to produce more CDW (g/ml) with maximum OD_{600nm} (1.55±0.01) when grew on glucose as a carbon source. There is a significant variation (p<0.05) in the terms of OD_{600nm} for PSSI-D8 for these carbon sources having the same letter on their respective bars (Figure 9a).

The effects of carbon sources such as glucose, fructose, sucrose, and maltose were evaluated for the growth of PSSI-D8. The isolates were capable of growing on all selected carbon sources. Glucose is the only carbon source highly utilized by *S. aureus* next to fructose (Figure 9a). The highest cell dry weight (CDWg/ml) was also recorded when glucose employed as a sole carbon source and less CDW was recorded from sucrose that used as sole carbon source. In line with this study, Malairuang et al (2020) have found that glucose is a suitable carbon source for diverse microorganisms since glucose is easily metabolized and employed for microbial cellular growth. The speed at which the carbon source like glucose metabolized can be used to influence the formation of bacterial biomass and their metabolic activities. Our results are in agreement with the observation of Malairuang et al (2020) who indicated that glucose is very effective and can be used by many microorganisms as a carbon source because glucose supports fast growth as it is a single molecule and easy to consume and the first preferred carbon sources.

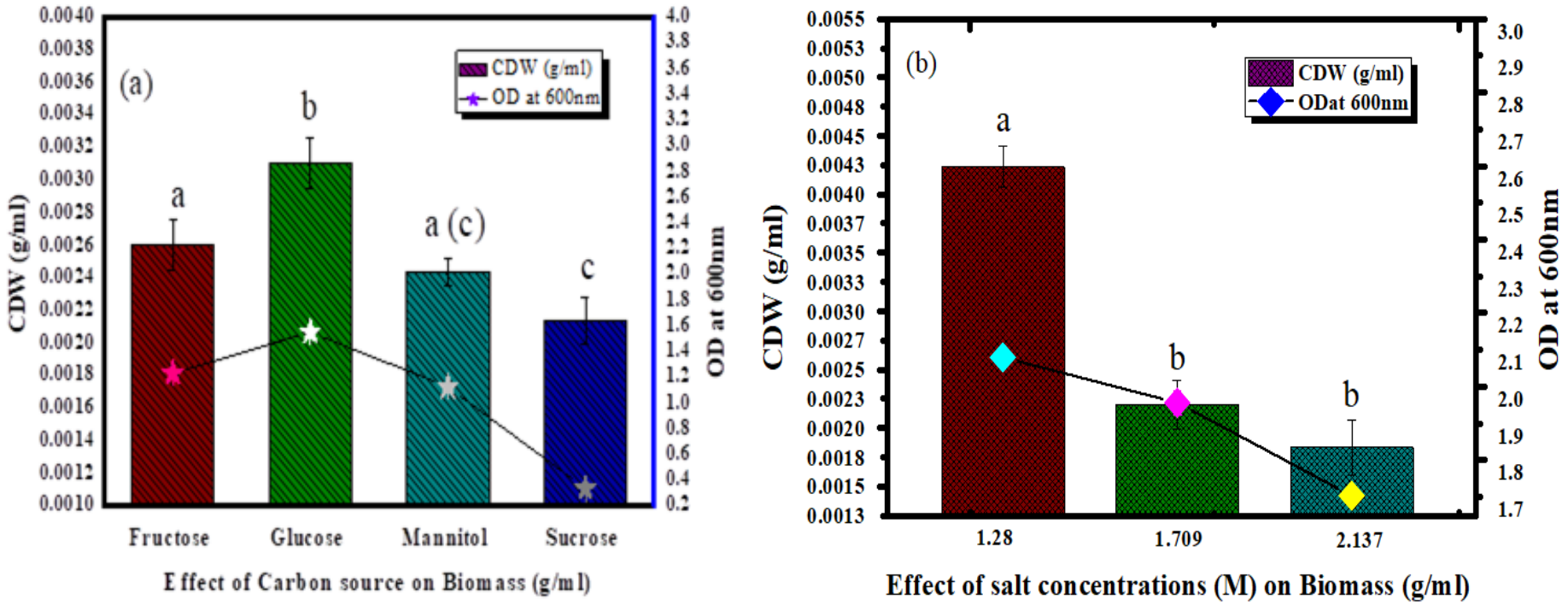

Figure 9: Effects of carbon sources and salt concentration on biomass and growth of PSSI-D8

(a) Effects of carbon sources on biomass and their respective OD_{600nm} of *S. aureus* **(b)** Effects of salt concentration on the growth of *Staphylococcus* species. Data represented mean ±SD of three replicate (n=3) of CDW (g/ml) and OD600nm for pH value and temperature. According to one way ANOVA there is no significant variation at 95% confidence interval by using LSD test (p>0.05) with the same latter on bar for carbon source and salt concentration.

4.4.2 Effects of salt concentration on PSSI-D8

We observed the growth of PSSI-D8 on the different salt concentrations of sodium chloride in Luria-Bertani's broth (Figure 9b). The isolate PSSI-D8 grew on LB broth containing 1.282M, 1.709M and 2.137M concentrations of sodium chloride. At optimum growth temperature, the result indicated that the optimum salt concentration for the growth of PSSI-D8 in the LB broth was 1.282M. In the present study, the isolated PSSI-D8 can grow up to a 2.13M concentration of sodium chloride. It was observed that in a flask containing a highly concentrated solution of sodium chloride, the growth was less prolific than in a flask of LB broth with less concentrated salt 1.282M. From this study, we observed that the isolate PSSI-D8 can grow in a high salt concentration of sodium chloride.

The salt concentrations were also evaluated against biomass production for PSSI-D8 isolates. As indicated in above (Figure 9b), the minimum CDW (0.0018±0.0004g/ml) (mean ± SD) was detected at a 2.13M concentration. However, the maximum CDW (0.0042±0.00031g/ml) was obtained from 1.282M salt concentration at optimum temperature 37^{o}C. In this study, great significant variations were observed at LSD Post Hoc Test between 1.282M and 1.709M (p=0.00) and 1.282M and 2.137M (p=0.00) salt concentration (Figure 9b) against biomass for *S. aureus*. However, no significant variation was detected for PSSI-D8 at 1.709M and 2.137M (p=0.258) salt concentration.

The PSSI-D8 is able to grow in the Mannitol Salt Agar (MSA) that contain high salt concentration (7.5%) (w/v) which is a similar result obtained by (Ribeiro de Souza da Cunha, 2018). These salt concentration found within MSA may assisted for inhibition of other possible bacterial contamination. It was also reported that (Medveov & Valk, 2012) the optimal growth condition for *S. aureus* was in the range from 35-37^{o}C in which these isolates are found to be grown up to 20% (w/v) of NaCl concentration. In line with this study, the recent PSSI-D8 is able to grow at higher salt concentration (2.137M) at 37^{o}C. The current study estimated *S. aureus* as a potential isolate that may contain osmo-protective systems in order to copy with such harsh environmental condition. These isolate may form biofilm that suggested resisting to high salt concentration. It was reported that intrinsic factor such as high salt concentration, water activities, pH-value and nutrient contents used to affect growth of *S. aureus* and its toxic production (Ebert, 2018). The same authors reported that *S. aureus* can existed in the food with high salt concentration due to its osmotolerant. It was

further stated that this high salt content in the food may be used to inhibit the growth of many other contaminant bacterial cells which is similar results to our estimation.

4.4.3 Effect of pH value on a biomass of a newly isolated PSSI-D8

The optimum pH value for growth of PSSI-D8 was observed at a pH7. In this study, 0.001±0.0002, 0.004167±0.0003055 and 0.003667±0.0004509 (mean ± SD) of CDW (g/ml) were obtained from PSSI-D8 at pH5, pH7, and pH10 at optimum temperature 37℃ respectively. Differences in pH tolerances are shown in (Figure 10a). The minimum CDW (0001±0.0002g/ml) was obtained from PSSI-D8 at pH5. However the maximum biomass (0.00417±0.0003g/mL) detected for PSSI-D8 at pH7 and followed by pH10 (0.0036±0.00045g/ml) at optimum temperature 37℃. Thus, the PSSI-D8 is active over a broad range of pH7 which is an optimum pH value. This pH value is lower than that of pH-value reported for *S. aureus* NCTC 8530 (SAL-1) and S. *aureus* PS54 (SAL-2) (Rosenstein, 2000).

As indicated in (Figure 10a) there is a significant variation ($p<0.005$) were observed between pH5 and pH7 ($p=0.00$) and pH5 and pH10 ($p=0.00$) in terms of CDWg/mL at optimum temperature 37℃. However, in this study there is no significant variation observed between pH7 and pH10 ($P=0.117$) at 37℃. In pH tolerance of an isolated PSSI-D8 there is statistically significant variation between pH5 & pH7, pH5 & pH10, and pH7 & pH10 with ($p=0.000$) at OD_{600nm}. In this study, at the optimum temperature, the maximum (2.0533±0.00031) and the minimum (0.0667±0.00577g/ml) biomasses were obtained at pH7 and pH5, respectively (Figure 10a).

It was found that the pH value used to affect the microbial growth. At optimum pH value, the maximum growth of PSSI-D8 was found to be occurred at pH7.0. This PSSI-D8 may be shown better metabolic activities at this pH value. Minimum growth of PSSI-D8 was also observed at pH5.0 and pH10.0. This indicated that the isolate may tolerate acidic and basic environment without cell division. In agreement with this study, the (James, 2005) it has been reported that *S. aureus* was able to grow over the range of 4.0 to 9.8 pH value. The same authors confirmed that the optimum pH range was found to be 6-7 pH value for PSSI-D8.

A complete inhibition of *S. aureus* was observed at pH5 Medveov & Valk (2012) which is a contrary finding to our recent finding. This indicated that the current our isolate can be *S. aureus* type strain. It was also found that an acidic stress and the drop of intracellular pH

alter the membrane structure and lead to a decrease in the activity of several enzymes which are pH-sensitive (Medveov & Valk, 2012). As indicated in the Figure 10a, cell biomass and OD_{600nm} showed direct relationship. For instance, at pH7.0 value both cell biomass (g/ml) and optical density (OD_{600nm}) at 600nm wavelength are found to be the maximum. This indicated that a maximum cell suspension can be occurred at this pH value.

4.4.4 Effect of temperature on newly isolated PSSI-D8

In this study the minimum (0.00193±0.00015g/ml) of CDW and the maximum (0.0045±0.00026g/ml) of CDW (mean ± SD) biomass were obtained at 30℃ and 37℃ respectively at optimum pH7. As indicated in (Figure 10b), larger biomass (0.0045±0.00026g/ml) CDW was obtained from PSSI-D8 at 37^{o}C with (2.056±0.01155) OD_{600nm}. Multiple comparison of One-way ANOVA at LSD Post Hoc Test indicated that statistically significant variations were detected among 30 & 35 ^{o}C (p=0.001), 30 & 40 ^{o}C (p=0.021), 30 & 37 ^{o}C, 35 & 37 ^{o}C, and 37 & 40 ^{o}C (p=0.000) at optimum pH value (pH7) for CDW (g/ml) (w/v) (Figure 11b). However, at optimum pH value there is no statistical significance variations detected in terms of isolate biomass (CDWg/ml) when this isolate was incubated at 35 and 40^{o}C (p=0.077). In terms of OD_{600nm} there is a significant variations between 30 & 35^{o}C, 30 & 37^{o}C, 30 & 40^{o}C, 35 & 37^{o}C, 35 & 40^{o}C, and 37 & 40^{o}C (p=0.000) at optimum pH7.0.

It has been stated that the optimum temperature growth for *S. aureus* is in the range from 37-40^{o}C and the minimal temperature for the growth is about 6.7^{o}C. Some strains of *S. aureus* is able to grow up to 50^{o}C (James, 2005; Sadeghi & Mansouri, 2014) which is the highest growth temperature when compared to the recent isolate of *Staphylococcus* species (PSSI-D8). Valero et al (2009) reported that the growth of *S. aureus* is inhibited at 8^{o}C. The same authors confirmed that the optimum growth can be occurred only at the optimum pH value. It was reported that Normanno et al (2005) no growth of *S. aureus* at low temperature combined with low pH which is a similar finding with the current our finding. Study conducted by Thapaliya et al (2017) showed that *Staphylococcus aureus* that isolated from commercially available meat was able to grow at 35^{o}C.

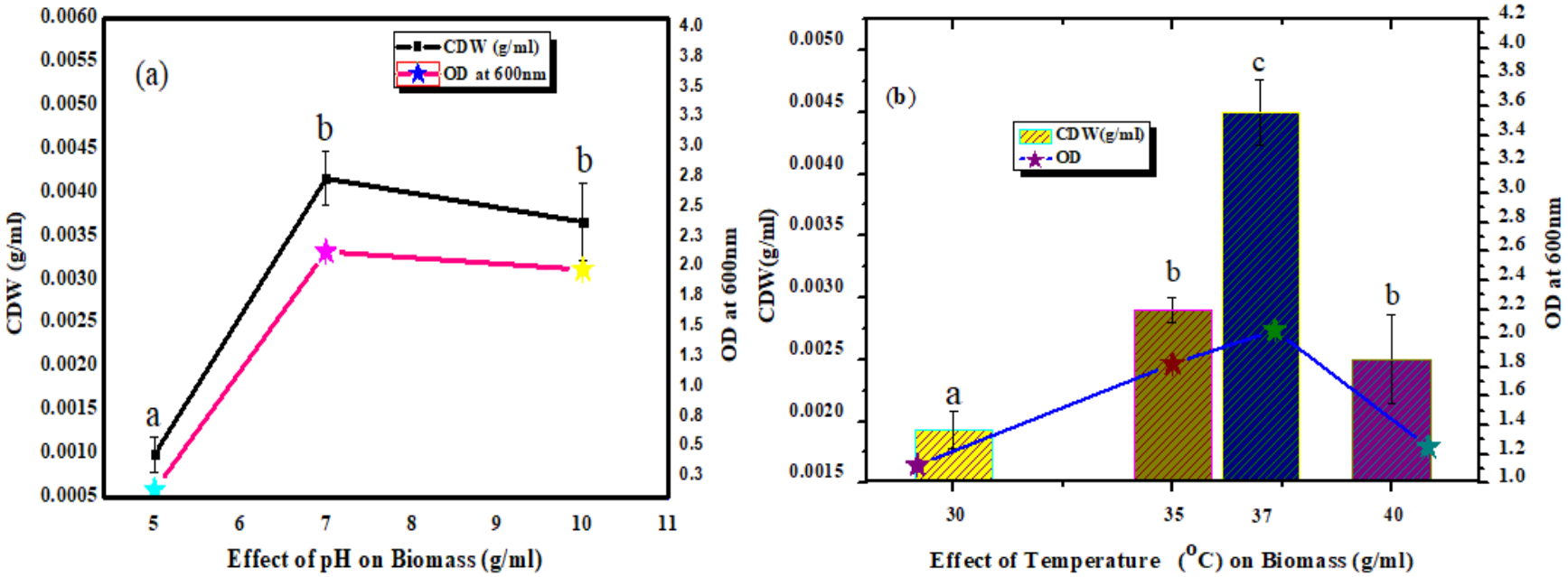

Figure 10: pH and temperature optimization for PSSI-D8

(a) Effects of pH value on the growth of *staphylococcus* species. **(b)** Effects of various temperature range on the growth of *Staphylococcus* species. Data represented mean ±SD of three replicate (n=3) of CDW (g/ml) and OD600*nm* for pH value and temperature. According to one way ANOVA, there is no significant variation at 95% confidence interval by using LSD test (*P*>0.05) with the same latter on line and bar for pH value and temperature.

4.5 Molecular detection of antibiotic resistance genes

4.5.1 Detection of *blaZ* gene in *Staphylococcus* species

In the current study, the presences of *blaZ* gene in *Staphylococcus* species strains were checked by using conventional PCR (Figure 11). It was confirmed that 2 (11.76%) of *Staphylococcus aureus* strains showed positive amplification for *blaZ* gene and 15 (88.24%) were negative for *blaZ* by PCR. The phenotypic study conducted by using disk diffusion method for antibiotic susceptibility testing showed that 15 isolates were resistant and 2 of them were susceptible to the penicillin antibiotics. As indicated in the Table 2, PSSI-D8 showed resistance to penicillin which demonstrated that the isolates harboured *blaZ* gene in their genomic DNA sequence. Similarly it was reported that *S. aureus* isolates that developed penicillin-resistant was positive for *blaZ* gene (Duran et al., 2012; Yılmaz & Aslantaş, 2017).

As indicated in the Figure 11, the *blaZ* gene was detected for isolate PSSI-D8 and PSSD-18 with approximately 750bp fragment size. The same gene fragment was also observed for *Staphylococcus aureus* ATCC 25923, which is a positive control. The *blaZ* gene was detected in 91.4% of *S. aureus* isolates which is a similar finding to our results (Yılmaz & Aslantaş, 2017). The authors further reported that the isolates were obtained from various clinical specimens such as blood cultures, wound, sputum, swabs, and urine which are in contrast to our source, NCG**s**.

In line with the recent study, antibiotics resistance genes such as *blaZ, msrA, tetK, tetL*, and *vanB* were equally detected from *Staphylococcus aureus* that isolated from Milk in South Africa (Bissong & Ateba, 2020). This study indicated that the detected *blaZ* gene is conserved throughout evolutionary history for various strains of *Staphylococcus aureus* obtained from different sources. Certain heavy metal resistance gene such as *arsRBC, cadA, cadB, merA* and *merB* were reported for *Staphylococcus aureus.* Some other disease causing virulence genes such as *hysA, hlgA , hlgB, hlgC, hld, sak, sea, seb* & *sec, seb* & *sec, seq2* & *sek2, sep, spa, sspA, sspB,* and *tst* were also reported for *Staphylococcus aureus* (Turner et al., 2019).

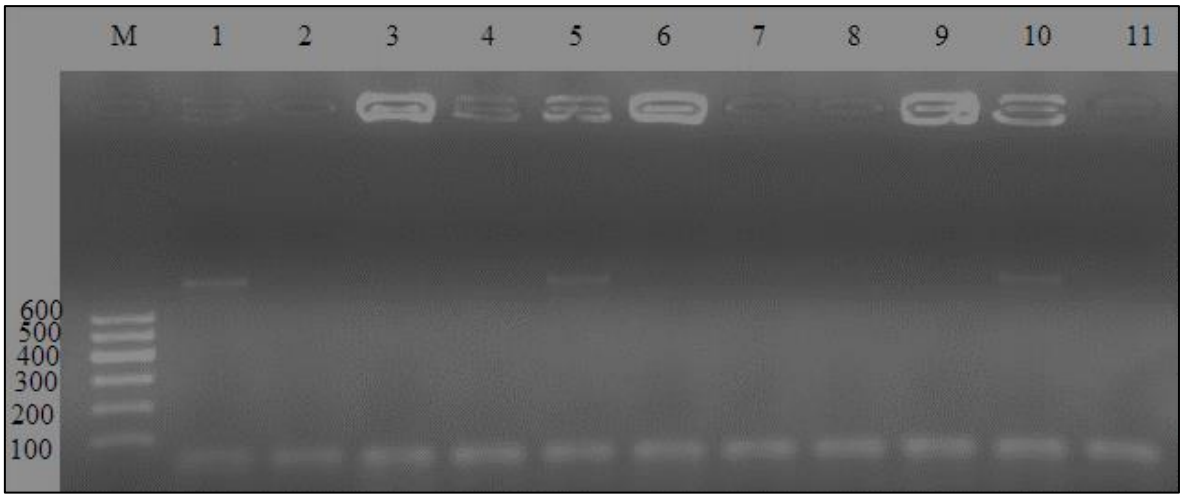

Figure 11: Gel electrophoresis of amplified for *blaZ* gene of *Staphylococcus* species isolated from the NCGs

4.5.2 Detection of *mecA* gene by PCR techniques

In this study, *mec*A gene (Figure 12) which is a methicillin resistance gene detected from bacterial isolates that obtained from the NCGs using conventional PCR techniques. As indicted in Figure, a total of 13 (76.47%) out of 17 isolates were identified as *Staphylococcus* species based on PCR amplification for *mecA* gene. In this study, the fragment size of methicillin resistance *mecA* gene was found to be 310bp for sample obtained from the NCGs. In agreement with this study, a total of 92 (56.8%) isolates were identified as a Methicillin resistance *Staphylococcus aureus* based on PCR amplification product of *mecA* gene and phenotypic methods (Sadeghi & Mansouri, 2014). Some authors confirmed that the expected amplicon size was detected at 532 bp which is different size to the recent finding for sample obtained from the NCGs. Hence, our current amplicon size of the *mecA* gene was similar with the finding of (El-Baz et al., 2021). This difference could be due to the primer design for amplification of this gene.

It was further confirmed that *mecA gene* which is methicillin resistance gene has been detected in 84.6% of *S. aureus* isolates from human clinical samples (Yılmaz & Aslantaş, 2017). In the current study, the *mecA* gene was also detected for various *Staphylococcus* species isolated from nasal cavity of goats. The PCR amplification product for *mecA* gene was not detected for four *S. simulans* strains out of 17 (23.53%) even though oxacillin and penicillin resistance was observed. From the positive PCR amplified for the *mecA* gene 12 (92.3%) isolated *Staphylococcus* strains that were resistant to oxacillin by disk diffusion

method and 1(6.7%) isolate was susceptible. The *mecA* gene amplification by PCR was used to confirm the MRSA in the isolated *Staphylococcus* species.

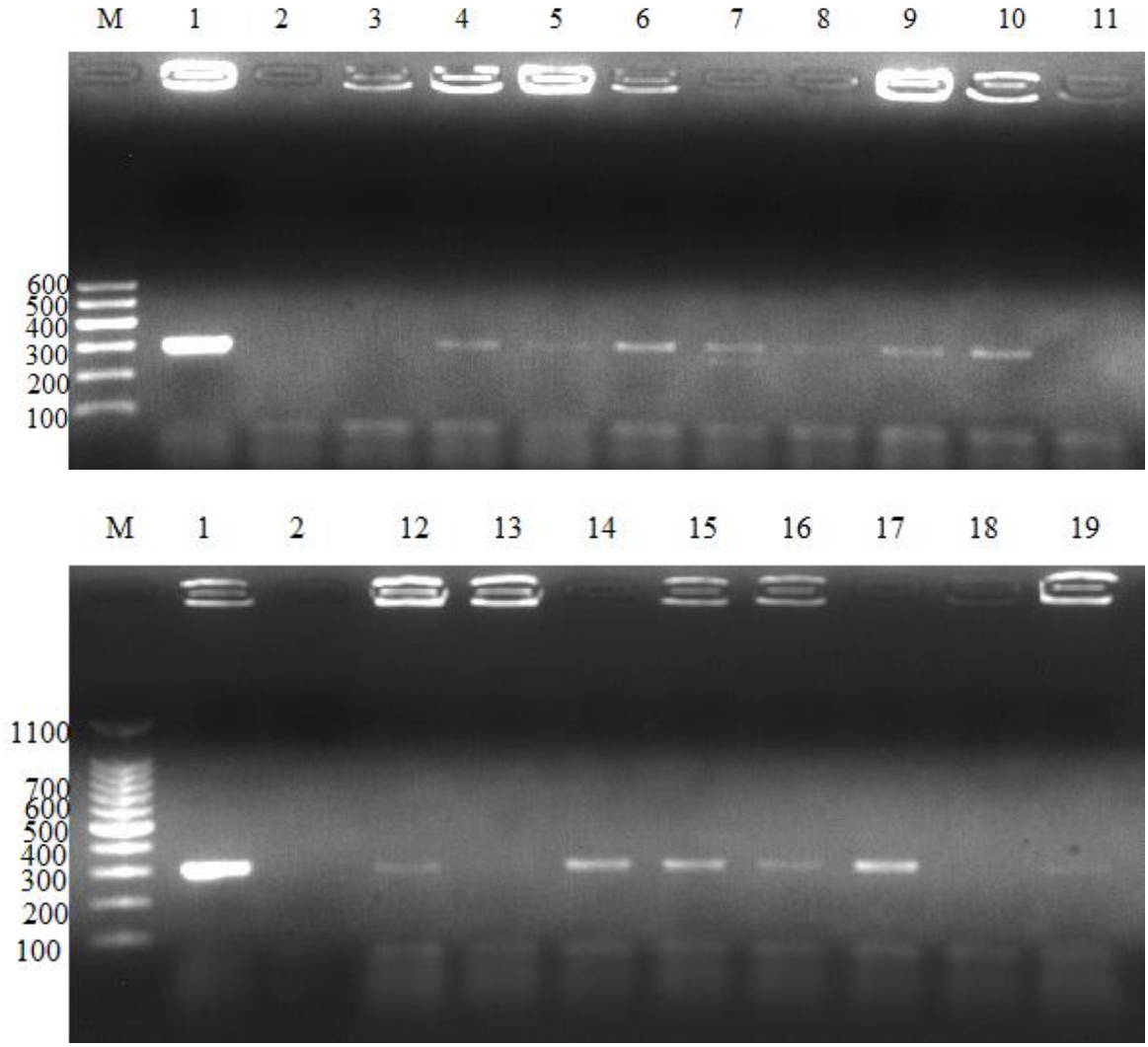

Figure 12: Agarose gel electrophoresis of PCR product of *MecA* gene

Lane M: DNA ladder 100 base pair (Qiagen, Germany); Lane 1: positive control of amplified 532-bp DNA (*S. aureus* ATCC 25923); Lane 2: negative control (DNA free water); Lane 3-19 *Staphylococcus* species samples isolated from the NCGs. Lane 3 (*S. simulans s*), 11 (*S. simulans*), 13 (*S. xylosus*), and 18 (*S. simulans*) are *Staphylococcus* species nasal swab isolates negative for the *MecA* gene. The remaining Lanes was positive for the *mecA* gene.

Our result showed that there is discordance between phenotypic antibiotic susceptibility testing (disc diffusion method) and genotypic analysis by conventional PCR method for the 17 methicillin-resistant *Staphylococcus aureus* (MRSA). The phenotypic antimicrobial susceptibility testing showed that *Staphylococci* isolate 2 (11.77%) and isolate 15 (88.23%) were susceptible and resistant to oxacillin, respectively when it was checked by using disk

diffusion method. All CoPS were resistant to oxacillin and they were also the carrier of *mecA* gene. It was recognized that CoNS 4 (36.37%) was negative for the *mecA* gene.

The *mecA* gene is found in bacterial resistance to antibiotics such as penicillin, methicillin, and penicillin-like antibiotics and encodes for penicillin-binding protein 2a (PBP2a) that has a low affinity to beta-lactam antibiotics (Xu et al., 2018). Currently, PCR is a gold standard method in molecular for the detection of a methicillin resistance gene from the isolates of *Staphylococcus* species strains (Chambers, 1997). Some investigators (Aires-de-Sousa et al., 2007; Virdis et al., 2010; Öztürk et al., 2019) did not were ineffective to identify the *mecA* gene in *S. aureus* isolates from goat milk. However, Salisbury et al. (1997) have identified the *mecA* gene from different strains of coagulase-negative *Staphylococci* isolated from goats with clinical mastitis were identified as MRSA by disc diffusion test in Turkey. Pekana & Green (2018) detected the presence of the *mecA* gene in *S. aureus* isolates by PCR.

4.5.3 Detection of *nuc* gene in newly isolated *Staphylococci* species

As indicated in (Figure 13), isolates PSSI-D4, PSSI-D8 and PSSI-D D18 were positive for *nuc* gene which indicates that these isolates could be most likely *Staphylococcus aureus*. In this study, it was observed that *nuc* gene was not amplified for *Staphylococcus* species (Figure 13) such as *S. sciuri*, *S. simulans*, and *S. xylosus.* This indicates that these isolates may not contain *nuc* gene in their genomic DNA. As it was previously reported, *nuc* gene was considered as a genetic marker for identification of *S. aureus* using conventional PCR Kateete et al (2001) which is a similar finding to our estimation. Similarly, it was reported that Zhou et al (2017) that *nuc* gene was detected in *S. aureus* strains that isolated from goats in Chongqing, China. In contrast to the current and other report Andrade et al (2021) the *nuc* gene has been identified in other species of *Staphylococci* isolates. It was suggested that non-*Staphylococcus aureus* may be acquired this gene as a result of horizontal transfer. Furthermore, it was confirmed that (van Leeuwen et al., 2008). The *nuc* gene naturally existed within most *S. aureus* isolates; however, some *Staphylococcus* isolates did not carrying this gene.

In this study we carried out detection of *SNase* activity (*nuc*) gene from the NCGs by using PCR method and the *nuc* gene was encountered in 3 (17.64%) of the isolates. The enzymatic test for *SNase* activity was positive with all of *S. aureus* strain and non *SNase* activity were tested in 14 (82.36%) *Staphylococci* strains isolates. Figure 13 shows the result obtained from the amplified *nuc* gene by PCR method and *S. aureus* strains were recognized by primer

and amplified, while other strains like *S. simulans*, *S. sciuri*, and *S. xylosus* were not amplified and recognized by primer.

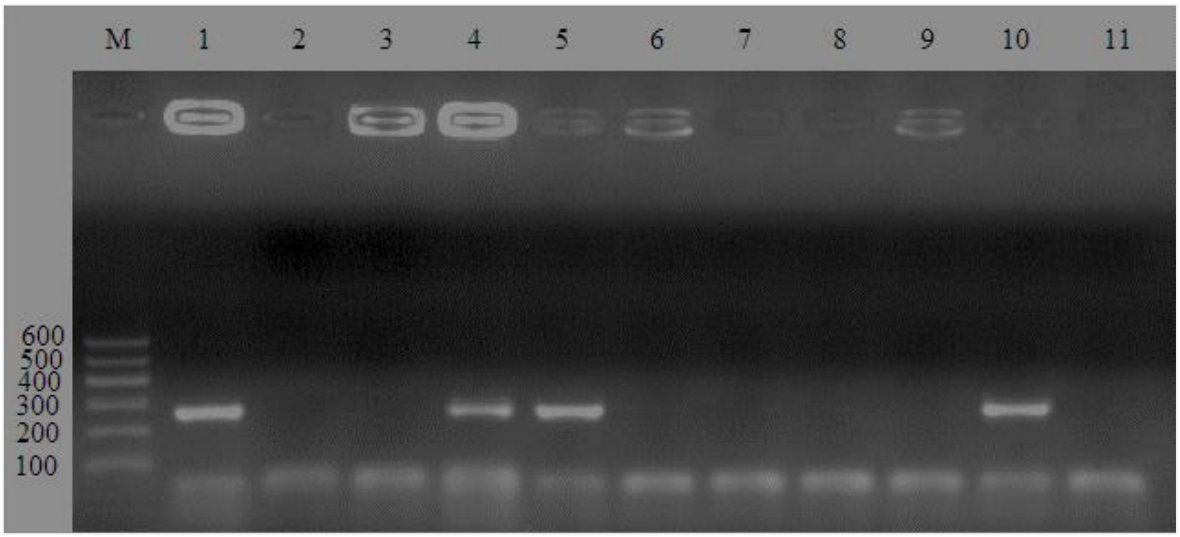

Figure 13: Gel electrophoresis of amplified for *nuc* gene of *Staphylococcus* species these isolated from the nasal cavity of goats.

Lane M: DNA ladder (Qiagen, Germany); **Lane 1**: positive control (ATCC 25923) of amplified 297bp DNA; **Lane 2**: negative control (DNA free water); **Lane 4** (PSSI-D4), 5 (PSSI-D8) and 10 (PSSI-D18) are species of *Staphylococcus aureus* these are positive *nuc* gene.

As indicated in Figure 16, the PCR amplification products for *nuc* gene appeared as a single DNA band with a size closest to 279bp. In our study, 88.23% of *Staphylococci* species isolates were phenotypically resistant to penicillin antibiotic and 11.77% of isolates were fully susceptible to penicillin antibiotics. *Staphylococcus* species contain other virulence gene beside *nuc* gene. For instance, their virulence genes such as *can, clf, icaA, hla, hlb, sdrC,sdrD, sdrE, seg, sea,* and *sei* were reported earlier (Yu et al., 2012).

4.6 Phylogenetic tree for newly isolated PSSI-D8

The 16S rRNA analysis showed that the isolates closest similarity with *Staphylococcus aureus* strains (Figure 14). A sequence comparison by BLAST analysis for the isolated strain had shown the highest sequence similarity with *Staphylococcus aureus* strains with 99.47%. Based on 16S rRNA sequence similarity, the isolate designated as *Staphylococcus aureus* NCG8. . In line with this study, our strain of *Staphylococcus aureus* that obtained from NCGs showed sequence similarity with strain of *Staphylococcus aureus* BARS001 Salauddin et al (2020) that isolated from Bovine mastitis milk in Bangladesh.

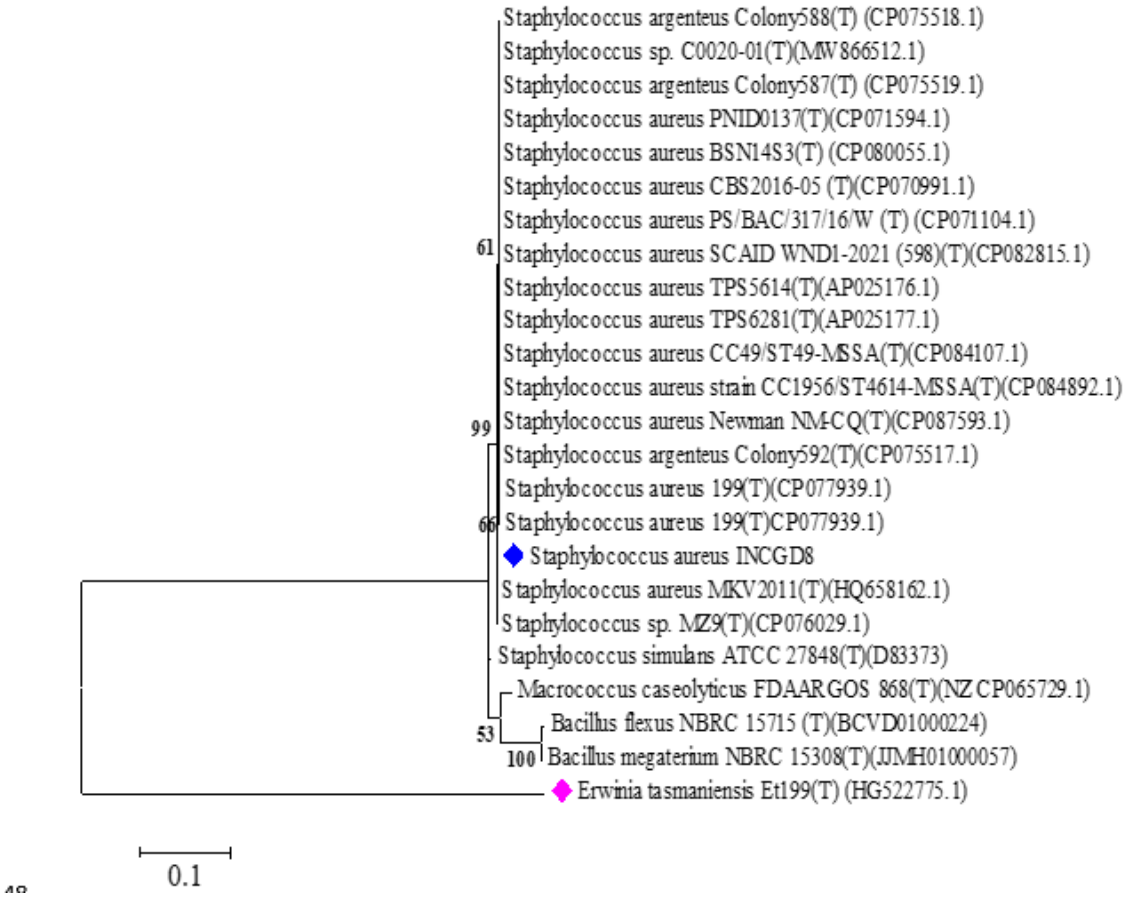

Figure 14: Phylogenetic tree analysis of INCGD8 strain**.**

The identification of *Staphylococcus* species from different samples were commonly reported using biochemical and phenotypic methods. In the present study, however, its identification was performed by using 16srRNA analysis which is the most trustful identification methods for newly isolated microbial cells. As indicated in the (Figure 15), the closest bacterial species are belonging to various strains of *Staphylococcus aureus*. In agreement with this study, the 16S rRNA sequence for strain C20R and E63 were also displayed 100% sequence similarity with gram positive strain of *Staphylococcus cohnii* (GTC) 728^{T}. Furthermore, it was confirmed that the sequence similarity between E4 and *Staphylococcus arlettae* (ATCC) (43957^{T}) sequences are 100% identical. It was found that the sequence similarity between *Micrococcus luteus* AUH1 that tend to degrade hydrocarbon shown 100% sequence similarity with E36 & E51strains (López-Cortés et al., 2008).

5 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

In this study, the following conclusions were drawn from the recent study: In the present study, *Staphylococcus* species are the most common inhabitants of the nasal cavity of goats. Seventeen isolated from NCGs and these isolates were found to be closest to *Staphylococcus* species based on morphology, phenotype, genotype and phylogeny. MALDI-TOF MS analysis was carried out to identify the recent PSSI and these isolates were identified as *Staphylococcus aureus, Staphylococcus sciuri, Staphylococcus simulans* and *Staphylococcus xylosus*. *Staphylococcus simulans* accounts for higher proportion and *Staphylococcus sciuri* are the least commonly inhabit the nasal cavity of goats. The newly isolated PSSI-D8 grown on optimum temperature at 37^{o}C, salt 1.28M, uses glucose as sole carbon sources and optimum pH7 to produce CDWg/ml. The *Staphylococcus* species isolated from the NCGs were tested for various antimicrobials using disk diffusion and chloramphenicol and vancomycin are the most effective drug to treat the isolates.

The result obtained from conventional PCR showed that the *mecA* gene was the most dominant antibiotic resistant gene present in the four species of *Staphylococcus* isolated strain in this study. The *nuc* gene encodes for the thermo tolerant gene that only present in PSSI-D8 isolated strain and *blaZ* genes were also identified as a potential strain type of PSSI-D8. In the main time, 16S rRNA gene sequence was conducted to identify the recent isolate obtained from NCGs. Based on sequence similarity, source of samples, the PSSI-D8 designated as *Staphylococcus aureus* isolated from NCGs (*Staphylococcus aureus* INCGD8). To conclude, the 16S rRNA analysis of *Staphylococcus aureus* INCG8 showed that the isolate was affiliated with various strains of *S. aureus*.

5.2 Recommendations

Based on this finding, the following recommendations were forwarded

- Because of different reasons this study was not extensively covered the dairy goat herds in the town, the study was carried out on a limited sample size to identify the antibiotic resistance gene, thus similar studies should be carried out on a large number of dairy goat herd goat in the town using numerous primers to identify resistance gene for different antibiotics.
- Further study should be carried out on mechanisms of antibiotic resistance gene and transferring from animals to humans

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7 APPENDICES

Appendix 1: Illustrative pictures during sample collection and processing

Sample collection from the NCGs

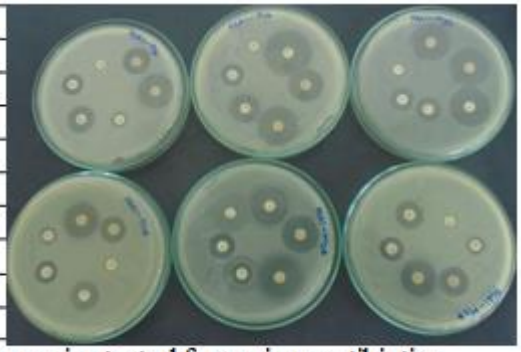
Primary identification by biochemical tests

Identification of isolates by using MALDI TOF MS

Appendix 2: AST result for the *Staphylococcus* species isolate from NCGs

Sample code	Diameter of zone of inhibition									MALDI-TOF Identification Spps.
	K	C	PEN	VAN	NAL	AMX	ST	OX	AMP	
	30	30	10	30	30	2	25	1	10	
PSSI-D4	16.9	19.5	9	13.3	14.7	7.3	19.3	7.4	7.2	*S. aureus*
PSSI-D2	18.4	21.7	7	15.7	7.4	7.1	10	7.1	6.6	*S. simulans*
PSSI-M23	25.2	19.3	6	15.8	6	6.4	23.3	7.1	7.6	*S. xylosus*
PSSI-D9	20.4	23.5	11.2	14.8	6.9	8.8	16.6	8.4	6.9	*S. simulans*
PSSI -D20	19.8	18.4	25.8	15.3	8.1	18.6	16.2	10.6	6.6	*S. simulans*
PSSI-D8	17.1	22.4	6.4	13.7	19	6	18.3	7.1	8.6	*S. aureus*
PSSI-M29	19.6	24	30	13.5	10.4	13.6	17.6	8.5	8.3	*S. simulans*
PSSI-M37	22.6	25.5	20.2	15.2	11.2	12	20.8	8.2	6.9	*S. sciuri*
PSSI-M31	23.5	20.4	28.1	14.7	9.6	19.8	17.7	7.2	7	*S. simulans*
PSSI-M22	12.1	20.1	10.4	12.8	14.8	9	17.6	13.8	7	*S. xylosus*
PSSI-D14	14.2	18.6	8.2	13.4	14.9	6.2	19.7	7.2	7.5	*S. xylosus*
PSSI-D16	21.8	23.4	27.5	14.4	7.6	17.7	19.9	7.0	8.8	*S. simulans*
PSSI-D13	18.8	20.4	6	14.5	6	6	18.7	8.3	7.6	*S. simulans*
PSSI-D18	16.9	17.8	7.6	15.4	16.1	6.4	18.6	15.3	8.7	*S. aureus*
PSSI-D19	23.7	22	9.8	15.2	9.2	20.6	20.7	7.7	8.8	*S. simulans*
PSSI-M32	24	24.6	23.8	15.3	9.8	16.9	18.8	6	7.3	*S. simulans*
PSSI-M24	20.5	24.1	22.7	14.9	7.8	11.2	19.9	7.1	8	*S. sciuri*

ANTIBIOTICS	S	I	R
KAN	≥18	14-17	≤13
CHL	≥18	13-17	≤16
PEN,G	≥29	-	≤28
VAN	≥12	10-11	≤9
NAL	≥19	14-18	≤13
AMX	≥20	-	≤19
STR	≥15	12-14	≤11
OX	≥13	11-12	≤10
AMP	≥29	-	≤28



Zone of size interpretation chart and *Staphylococcus* species tested for various antibiotics

Appendix 3: Ethical clearance approval letter

ADAMA SCIENCE AND TECHNOLOGY UNIVERSITY

RESEARCH AFFAIRS DEAN OFFICE

UNIVERSITY ETHICAL REVIEW BOARD (UERB)

Phone: +251-0221-11-07-80

Fax: +2510221-10-00-46

Email: raff@astu.edu.et

CERTIFICATE OF APPROVAL

To: Leta Guta Inki

Department: Applied Biology

Program: M.Sc

Study Title: Isolation, Molecular Characterization and Screening for Antibiotics Resistance Potential of Staphylococcus for the Sample Collected from the Nasal Cavity of Goats in Adama, Ethiopia

Approval Date: February 10, 2021

Certificate Ref. No: RECSoANS/BIO/05/2021

Based on the request, the University Ethical Review Board (UERB) members reviewed the proposal taking into consideration the following key points:

1) Whether the intended research project have any risk to the research team, to the participants, data collectors, the data to be collected, the institutes involved in the research, project partners, funders involved, viz.
2) How, where, and by whom participants will be identified, approached, and recruited.
3) Type of people recruited.
4) The consequences of the research, risk of the data, how will it be protected and addressed
5) Consideration of anonymity and confidentiality

In light of the above key points, the School Ethical Review Board and University Ethical Review Board (UERB) members identified no any risk to any of the participants, institutions, data collectors and viz. Therefore, UERB recommend the implementation of the project as per the approved proposal.

Finally, I congratulate you for securing this Ethical Approval Certificate to precede your project.

Wishing you all the best!

Alemu Disassa Kuletu
Research Affairs Dean

Alemu Disassa (Ph.D), Chairman

Appendix 4: Mean, SD, and SEM for Carbon source, pH, Salt concentration, and temperature of PSSI-D8

Carbon sources (OD and Biomass)								
Replications	**Glucose (OD)**	**Biomass**	**Fructose (OD)**	**Biomass**	**Sucrose (OD)**	**Biomass**	**Mannitol (OD)**	**Biomass**
R1	1.56	0.003	1.22	0.0029	0.34	0.0019	1.12	0.0024
R2	1.54	0.0034	1.23	0.0024	0.32	0.0021	1.13	0.0026
R3	1.55	0.0029	1.23	0.0025	0.34	0.0024	1.12	0.0023
Mean	**1.55**	**0.0031**	**1.22667**	**0.0026**	**0.33333**	**0.00213**	**1.12333**	**0.002433333**
SD	**0.01**	**0.00026**	**0.00577**	**0.00026**	**0.01155**	**0.00025**	**0.00577**	**0.000152753**
SEM	**0.005773503**	**0.00015**	**0.00333**	**0.00015**	**0.00667**	**0.00015**	**0.00333**	**8.81917E-05**

pH						
Replications	**pH5 (OD)**	**Biomass**	**pH7 (OD)**	**Biomass**	**pH10 (OD)**	**Biomass**
R1	0.07	0.0008	2.05	0.0041	1.92	0.0037
R2	0.06	0.001	2.05	0.0045	1.89	0.0041
R3	0.07	0.0012	2.06	0.0039	1.91	0.0032
Mean	**0.06667**	**0.001**	**2.05333**	**0.00417**	**1.90667**	**0.00367**
SD	**0.00577**	**0.0002**	**0.00577**	**0.00031**	**0.01528**	**0.00045**
SEM	**0.00333**	**0.00012**	**0.00333**	**0.00018**	**0.00882**	**0.00026**

Salt concentration						
Replications	**7.50% (OD)**	**Biomass**	**10% (OD)**	**Biomass**	**12.50% (OD)**	**Biomass**
R1	2.07	0.0043	1.96	0.0025	1.7	0.0019
R2	2.08	0.0039	1.95	0.0018	1.71	0.0014
R3	2.09	0.0045	1.86	0.0023	1.7	0.0022
Mean	**2.08**	**0.00423**	**1.92333**	**0.0022**	**1.70333**	**0.00183**
SD	**0.01**	**0.00031**	**0.05508**	**0.00036**	**0.00577**	**0.0004**
SEM	**0.005773503**	**0.00018**	**0.0318**	**0.00021**	**0.00333**	**0.00023**

Temperature								
Replications	**30ºC (OD)**	**Biomass**	**35ºC (OD)**	**Biomass**	**37ºC (OD)**	**Biomass**	**38ºC (OD)**	**Biomass**
R1	1.12	0.0018	1.83	0.0029	2.05	0.0042	1.26	0.0026
R2	1.13	0.0019	1.81	0.003	2.05	0.0046	1.24	0.0021
R3	1.12	0.0021	1.83	0.0028	2.07	0.0047	1.25	0.0028
Mean	**1.123333333**	**0.00193**	**1.82333**	**0.0029**	**2.05667**	**0.0045**	**1.25**	**0.0025**
SD	**0.005773503**	**0.00015**	**0.01155**	**0.0001**	**0.01155**	**0.00026**	**0.01**	**0.000360555**
SEM	**0.003333333**	**8.8E-05**	**0.00667**	**5.8E-05**	**0.00667**	**0.00015**	**0.00577**	**0.000208167**

Appendix 5: Sequences producing significant alignments

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Staphylococcus aureus strain MJ015 chromosome, complete genome	Staphylococcus aureus	2745	16428	100%	0.0	99.47%	2784836	CP038183.1
Staphylococcus aureus strain MJ163 chromosome, complete genome	Staphylococcus aureus	2745	16456	100%	0.0	99.47%	2746673	CP038229.1
Staphylococcus aureus strain PMB179-1 chromosome, complete genome	Staphylococcus aureus	2745	16438	100%	0.0	99.47%	2741492	CP050690.1
Staphylococcus aureus strain M2024 chromosome, complete genome	Staphylococcus aureus	2745	16465	100%	0.0	99.47%	2791940	CP047021.1
Staphylococcus aureus strain SA1428 chromosome, complete genome	Staphylococcus aureus	2745	16460	100%	0.0	99.47%	2752113	CP048431.1
Staphylococcus aureus strain UP_274 chromosome, complete genome	Staphylococcus aureus	2745	16465	100%	0.0	99.47%	2768425	CP047852.1
Staphylococcus aureus strain UP_620 chromosome, complete genome	Staphylococcus aureus	2745	16471	100%	0.0	99.47%	2779486	CP047843.1
Staphylococcus aureus strain UP_644 chromosome, complete genome	Staphylococcus aureus	2745	16471	100%	0.0	99.47%	2738812	CP047841.1
Staphylococcus aureus strain UP_522 chromosome, complete genome	Staphylococcus aureus	2745	16471	100%	0.0	99.47%	2781568	CP047847.1
Staphylococcus aureus strain UP_830 chromosome, complete genome	Staphylococcus aureus	2745	16465	100%	0.0	99.47%	2747791	CP047790.1
Staphylococcus aureus strain UP_1150 chromosome, complete genome	Staphylococcus aureus	2745	16471	100%	0.0	99.47%	2790390	CP047786.1
Staphylococcus aureus strain UP_883 chromosome, complete genome	Staphylococcus aureus	2745	16465	100%	0.0	99.47%	2774422	CP047835.1
Staphylococcus aureus strain UP_1484 chromosome, complete genome	Staphylococcus aureus	2745	16465	100%	0.0	99.47%	2709643	CP047815.1
Staphylococcus aureus strain UP_1539 chromosome, complete genome	Staphylococcus aureus	2745	13726	100%	0.0	99.47%	27468	

Bruker MALDI Biotyper

Identification Results

Appendix 6: MALDI-TOF MS identification result

Run info:

Run identifier:	211101-1425-1011027872
Comment:	Staphylococcus spp. Suspected sample from Adama
Operator:	tof-user@MBT-WIN10
Run creation date/Time:	2021-11-01T14:42.39.812
Number of tests:	29
Type:	Standard
BTS-QC	passed
BTS-QC Position	C5:0
Instrument ID:	8604832.05381
Server version	4.1.100 (PYTH) 174 2019-06-158_01-16-09

Result overview

Sample Name	Sample ID	Organisms (best match)	Score Value	Organisms (second best match)	Score value
A1 (+) (C)	30740 (Standard)	Staphylococcus simulans	1.88	Staphylococcus simulans	1.80
A2 (+) (B)	3072 (Standard)	Staphylococcus simulans	1.85	Staphylococcus simulans	1.77
A3 (+) (C)	30737 (Standard)	Enterococcus gallinarum	1.87	Enterococcus gallinarum	1.83
A4 (+) (B)	30728 (Standard)	Enterococcus gallinarum	1.81	Enterococcus gallinarum	1.76
A5 (+) (B)	30738 (Standard)	Staphylococcus simulans	1.77	Staphylococcus simulans	1.74
A6 (+) (C)	30729 (Standard)	Staphylococcus aureus	1.92	Staphylococcus aureus	1.93
Result overview table-continued on next page					

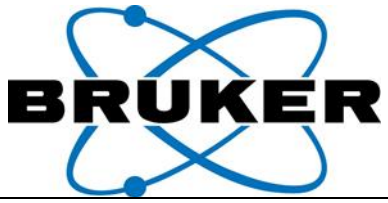
Bruker MALDI Biotyper

Identification Results

Result overview table-continued from previous page					
Sample Name	**Sample ID**	**Organisms (best match)**	**Score Value**	**Organisms (second best match)**	**Score value**
A7 (+++) (A)	30732 (Standard)	Staphylococcus xylosus	2.15	Staphylococcus xylosus	2.08
A8 (+) (C)	30741 (Standard)	Enterococcus gallinarum	1.89	Enterococcus gallinarum	1.86
A9 (+++) (C)	30744 (Standard)	Staphylococcus sciuri	2.05	Staphylococcus sciuri	1.80
A10 (+) (C)	30745 (Standard)	Bacillus cereus	1.87	Bacillus cereus	1.83
A11 (+) (B)	30734 (Standard)	Staphylococcus sciuri	1.81	Staphylococcus sciuri	
A12 (+) (B)	30743 (Standard)	Enterococcus faecium	1.92	Enterococcus faecium	1.90
B1 (+++) (B)	30736 (Standard)	Enterococcus faecium	2.00	Enterococcus faecium	1.84
B2 (+++) (B)	30724 (Standard)	Enterococcus faecium	2.00	Enterococcus faecium	1.94
B3 (+) (B)	30739 (Standard)	Staphylococcus simulans	1.87	Staphylococcus simulans	1.80
B4 (+) (B)	30721 (Standard)	Staphylococcus aureus	1.87	Staphylococcus aureus	1.86
B5 (+) (B)	30731 (Standard)	Staphylococcus simulans	1.72	Staphylococcus simulans	1.72
B6 (+) (B)	30733 (Standard)	Staphylococcus xylosus	1.99	Staphylococcus xylosus	1.94
B7 (+) (B)	30746 (Standard)	Enterococcus faecium	1.96	Enterococcus faecium	1.82
B8 (+++) (C)	30722 (Standard)	Staphylococcus aureus	2.07	Staphylococcus aureus	2.00
Result overview table-continued on next page					

Bruker MALDI Biotyper

Identification Results



Result overview table-continued from previous page					
Sample Name	**Sample ID**	**Organisms (best match)**	**Score Value**	**Organisms (second best match)**	**Score value**
B9 (+++) (C)	30735 (Standard)	Enterococcus faecium	2.10	Enterococcus faecium	2.02
B10 (+) (B)	30725 (Standard)	Staphylococcus simulans	1.88	Staphylococcus simulans	1.79
B11 (+) (A)	30730 (Standard)	Staphylococcus simulans	2.00	Staphylococcus simulans	1.81
B12 (+) (B)	30747 (Standard)	Bacillus cereus	1.93	Bacillus cereus	1.80
C1 (+) (B)	30742 (Standard)	Enterococcus faecium	1.99	Enterococcus faecium	1.88
C2 (+++) (C)	30723 (Standard)	Staphylococcus simulans	2.01	Staphylococcus simulans	1.88
C3 (+) (B)	30733 (Standard)	Staphylococcus xylosus	1.80	Staphylococcus xylosus	1.78
C4 (+++) (C)	30727 (Standard)	Staphylococcus simulans	2.21	Staphylococcus simulans	2.10
C5 (+++) (A)	BTS (BTS)	Escherichia coli	2.23	Escherichia coli	2.18

Key:

Range	Interpretation	Symbol	Colour
2.00-3.00	High confidence identification	+++	Green
1.70-1.99	Low confidence identification	+	Yellow
0.00-1.69	No organism identification possible	-	Red