**SCHOOL OF GRADUATE STUDIES**

# ISOLATION AND MOLECULAR DETECTION OF ANTIBIOTIC RESISTANCE GENE OF *STAPHYLOCOCCUS AUREUS* FROM COW MILK SAMPLE IN WOLKITE DISTRICT, ETHIOPIA

**MSc THESIS**

**AYANSA KEBENESSA MEDEKSA**

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# Isolation and molecular detection of antibiotic resistance gene *Staphylococcus aureus* from Cow milk sample in Wolkite district, Ethiopia

**A Thesis Submitted to School of Graduate Studies, in Partial Fulfillment of the Requirements for the Degree of general Biotechnology**

**Ayansa Kebenessa Medeksa**

**Major Advisor: BEAKAL MINDAYE (Ass. prof)**

**Co-Advisor: SEID MOHAMMED (Ph.D.)**

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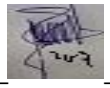
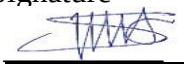
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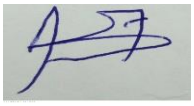
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| | | |
|---|---|---|
| **Dr. Beakel Mindaye (Ass.prof.)** |  | 18-11-2023 |
| Major Advisor | Signature | Date |
| **Seid Mohammed (Ph.D.)** |  | 18-11-2023 |
| Co-Advisor | Signature | Date |

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|---|---|---|
| Ayele Mandefro (PhD) |  | 1/16/2024 |
| Name of External Examiner | Signature | Date |
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Signature: _____

Department: **Biotechnology**

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## ABBREVIATIONS AND ACRONYMS

SFP…… *Staphylococcal* FoodPoisoning,

MRSA: …….Methicillin-Resistant *staphylococcus aureus*

CDC……...Center for disease control and prevention

TSB……Tryptone soya broth

PAB……Purple agar base

MSA…….Mannitol salt agar

BA……Blood agar

PSSI…….. Possible *Staphylococcus* species isolate

PSSI……..…. Possible *Staphylococcus* species isolate

WGS ……Whole genome sequencing

MGE…….. Mobile genetic elements

CDC…….center for disease control and prevention

SSCP ……The single-strand conformation polymorphism

APH……,..Aminoglycoside phosphotransferase

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## ABSTRACT

*Staphylococcus aureus* is one of the most frequent organisms known to cause food poisoning and other diseases including humans and animals. Raw milk is known to be a major means of transmission of this pathogenic bacterium to humans and animals. This study aims to isolate, characterize, and molecular detection of antibiotic resistance genes for *Staphylococcus aureus* from cow milk samples collected from wolkite, Ethiopia. A total of 385 samples of cow milk were collected randomly. Each of the samples collected was serially diluted, cultured on blood agar, and incubated at 37 $^{o}$C for 24hr and preliminary screening for possible *Staphylococcus* species isolate was conducted. The secondary screening for possible *Staphylococcus aureus* such as coagulase test, fermentation of mannitol salt agar, anti-biotic sensitivity tests, and molecular characterization for nuc gene and mecA genes were conducted. A total of 30 possible *Staphylococcus* species were isolated and 21 isolates were screened as *S. aureus* based on the advanced biological identification software. The rest of the isolates were found to be positive for catalase, triple sugar iron tests, cocci in shape, positive to gram staining, non-motile, and negative to vogues pressure tests. Some of them were coagulase-positive and 60% were positive to mannitol salt agar. All tested isolates were resistant to penicillin, 53.9% were multidrug-resistant and most of them were sensitive to clindamycin. Under the PCR detection of antibiotic resistance genes, 70% of the possible isolates were found to have the mecA gene while 80% were positive for the nuc gene. These isolates were predicted to be *S. aureus* subsp. *aureus*, *S. cohnii, S. intermedius, and S. vitulinus*in which nearly all of them have belonged to *S. aureus* subsp. *aureus* following the methods of advanced biological identification software identification tools. In conclusion, the recent isolates obtained from raw milk harbored specific genes responsible for disease-causing that were reported and available in the genomic DNA of *S. aureus* throughout the history of evolution.



**Key words: -** Antibiotics, PCR, Raw milk, *Staphylococcus*, Zoonotic disease

# 1. INTRODUCTION

## 1.1. Back background of the study

Milk is an important and common human food across the world containing good nutritive components (protein, vitamins, fats, and carbohydrates). The importance of milk in the diet of Ethiopians differs according to the farming systems and the socio-cultural setups. In the lowlands, especially where livestock keeping is the main occupation, milk is consumed by all groups of the society. In the highlands, the rural people are sedentary farmers raising both livestock and crops, with their diet consisting mainly of cereals (Abebe Bereda, 2014). Milk production is inextricably linked to the environment. Animals may become exposed to chemical substances during their production cycle which have been identified to date could come from drugs aimed at treating diseases or the application of chemicals for the control of weeds, insects, fungi and rodents has enabled agricultural productivity and intensity to increase. These chemicals are environmental contaminants linked to atmospheric pollution, feed, soil, and/or water. Uncontrolled processes interfering with the food-delivering ecological systems or with its natural compounds lead to residues in milk and result in risks to human and environmental health. Chemical residues that have been or are found in milk are chlorinated pesticides, organophosphates, herbicides, fungicides, anthelminthic drugs, and antibiotics (Samuel Aytenfsu, 2016). The milk is easily available for the growth of various pathogenic microorganisms when they get a chance to contact unhygienic handling and an environment that is not aseptic during processing. This increases the chance of transmission of disease-bringing agents to a human resulting in health problems which is highly difficult for consumers, decreased shelf life, and an economic loss to the country (Reta *et al.*, 2016).

The diseases of infectious /toxic features resulting from an intake of food or water are called food-borne diseases (FBD) according to WHO, and the pathogenicity of bacteria in contaminated foods depends on the toxin or intoxication after or before the intake of it. In terms of food poisoning *staphylococcus* ranked second next to Salmonella across the globe. It is caused mostly by the ingestion of traditionally

handled diets that contain certain heat-stable enterotoxins of Staphylococci. It is one of the great challenging FBDs worldwide (Addis *et al*., 2011). An important human pathogen called Staphylococcus aureus may cause a variety of infectious diseases in both community and nosocomial settings. A variety of virulence factors are employed by the Gram-positive pathogen to aid in the establishment of infections in their hosts. It is commonly recognized that the organism can develop resistance to several types of antibiotics(Arumugam Gnanamani, *et al*, 2017). Clinical and subclinical mastitis mainly caused by S. aureus is a major challenge in the majority of Ethiopian dairy farms (Enquebaher Tarekgne, 2015). The intake of *Staphylococci* enterotoxins which are formed before consumption in the food materials found to be due to *Staphylococcal* food poisoning (Thaker *et al*., 2013; Derebe Tessema and Solomon Tsegaye, 2017).

The genus *Staphylococcus* has the feature of Gram-positive coccus, positive in catalase test, negative oxidase, and facultative anaerobes exist in cutaneous and mucous membrane microbiota of mammals and birds (Ghebremedhin *et al.,* 2008). *Staphylococcus* is the most common genus, with 55 validly described species and 23 subspecies. Their cell wall is comprised of peptidoglycan, with the diamino acid l-lysine being the main component, and they develop in the presence of 10% NaCl (w/v) and at temperatures between 18 and 40 °C. Their cells form clusters that resemble grapes due to perpendicular division planes (Madhaiyan *et al*., 2020). They are traditionally split into two groups based on their capacity to cause blood plasma to clot (the coagulase response). The most harmful species of *staphylococcus* are those that produce coagulase enzymes. Over 30 more species of coagulase-negative S*taphylococci* (CNS) are currently recognized(Jana *et al*., 2022). These species are found to be in the family of *Micrococcaceae*. They include multiple drug-resistant *S. aureus* (MRSA) which occurred due to nosocomial infection (Ghebremedhin *et al*., 2008; Li *et al*., 2009; Thomson *et al*., 2022).

The use of antibiotic therapy to treat and prevent udder infections in cows is a key component of mastitis control in many countries. Unauthorized antibiotic use may result in residues of these substances in milk and tissues (Almashhadany, 2021).

Antimicrobial residues are small amounts of drugs or their active metabolites, which remain in milk after treating the cows (Syit, 2011). Because of the public health significance, milk and milk products contaminated with antibiotics beyond a given residue level are considered unfit for human consumption (Aytenfsu *et al*., 2016a). Problems associated with antibiotic residues in milk include the risk of allergic reactions, aplasia of the bone marrow, carcinogens, and increased resistance of pathogens toward antibiotics (Hussein Mohammed Roba, 2023). Isolates of *Staphylococcus* species showing multidrug-resistant e.g, Methicillin- Resistant (MRSA) were detected firstly in humans and then in animal samples is the thoughtful problem because of its transmission between them (Fufa Abunna *et al*, 2016; Lee *et al*., 2004). *Staphylococcus* species cause severe disease such as mastitis, arthritis, and urinary tract infection by introducing numerous virulence factors such as extracellular toxins and enzymes into animal species (Harris *et al*., 2002).

For humans, these organisms are the main causes of food poisoning, pneumonia, wound infections, and nosocomial bacteremia (Lee *et al*., 2004). These species have conventionally been categorized and distinguished based on the synthesis of the proteins (enzyme) such as: - coagulase, capable of converting fibrinogen into fibrin, a feature that is simply measurable in the laboratory and allows for a practical grouping. The *S. aureus*, *S. intermedius*, and *S. pseudointermedius* are coagulase-positive *Staphylococci* which are pathogenic most of the time among others (Thomson *et al*., 2022). *S. aureus* has been confirmed to be the causative agent of many cases of severe food poisoning; therefore, its presence in foods is of major concern (Götz *et al*., 2006).

The molecular determination methods are used in the successful effective isolation of *S. aureus* for the examination of its antibiotic susceptibility forms. There are many methods of genotyping the *S. aureus* bacteria including virulence gene profiling, agr typing, pulsed-field gel electrophoresis (PFGE), spa sequence typing, and multilocus sequence typing (MLST). Even today whole genome sequencing (WGS) remains unaffordable in resource-limited settings, however, its application has gained more acceptance in previous decades as technological advances have improved the accessibility and cost-effectiveness of high-throughput sequencing(Jana *et al*., 2022).

Real-Time PCR—qPCR uses a fluorescent probe to quantify a target species and to track the target DNA's real-time amplification. Automated Ribosomal Intergenic Spacer Analysis—ARISA employs a fluorescent primer to amplify the ribosomal intergenic spacers of microbes. It produces peaks that are related to discrete DNA fragments that may be found using a fluorescence detection system(Quigley *et al*., 2011).

The pathogenicity of the Saureus emanates from the synthesis of the numbers of the variable virulence factors. The virulence factors are useful for the colonization of the host tissue and discriminate the host‘s immune systems to get the nutrition and distribute the bacteria across the host‘s cells. There are many virulence factors synthesized by *S.aureus* including several enzymes and cytotoxins, like coagulase, collagenase, exfoliative toxins, hemolysins, hyaluronidase, leukocidins, lipases, nucleases, and staphylokinase(Schmidt *et al*., 2017). Maximum S. aureus strains are capable of yielding one or more pyrogenic toxin superantigens (PTSAgs) which include *staphylococcal* enterotoxins and the structurally related protein, toxic shock syndrome toxin-1 (TSST- 1). On the whole, these mentioned toxins show superantigenic action by associating with antigen-presenting cells and T-lymphocytes irrespective of the antigen specificity of the cells. This interaction leads to a huge proliferation of T-cells and the unrestricted release of pro-inflammatory cytokines that can lead to clinical signs in the host that include, fever, hypotension, and shock(Schmidt *et al*., 2017).

The mec A gene expression that encodes the penicillin-binding protein PBP2a which contains less affinity to β-lactam antibiotics is the main cause of MRSA. The mecA gene is not naturally found in S.aureus and it has a DNA segment of 2.1 kb which is inserted into the large block of exogenous DNA called the *Staphylococcal* cassette chromosome mec (SCCmec). A huge quantity of virulence factors has originated from *S. aureus* isolated from bovine mastitis, such as clumping factor (clfA), fibronectin-binding proteins (*fnbpA* and *fnbpB*), hemolysis (*hla* and *hlb*), thermonuclease (*nuc*), coagulase (*coa*), and toxic-shock syndrome toxin-1 (tsst-1), which is important for the bacteria to live and reproduce within the mammary gland (Dong Wang *et al*, 2016; Girmay *et al.*, 2020; Alsharif *et al*., 2021).

The information for the approximation of the extent of the distribution of *Staphylococcus* species and their AMR pattern for both humans and animals is hardly available in Ethiopia (Beyene *et al*., 2017). In developing countries, like Ethiopia, milk processing is held in a few dairy farms and conventional breeders in a way that it is blow the hygienic situation. There is no recognized information on FBD caused by *staphylococcus* species, and most of the time the occurrences are not reported to the public health authorities accurately even, in a country with enough investigation facilities (Addis et al., 2011). Therefore, this study intended to isolate, and molecular determination of the antibiotic resistance gene for *S. aureus* in milk samples collected from different areas of Wolkite.

### 1.1.1. Statement of the problem

Nearly in Wolkite, the documentation of information to milk producers regarding milk-borne zoonotic disease especially, for the distribution, effect, and which types of *Staphylococcus* species existing are not present and the absence of this awareness can face the milk providers, farm workers, and their entire households the series problem of zoonotic infections. Giving into account that many smallholders of milk farmers provide their milk to the public market and consumption of milk that is not pastoralized is general in these societies and this greatly enforces them for the milk derived diseases. Henceforth, the analyses of milk for Staphylococcus contamination [i]are vital for the protection of the animal, public health, and consumers as well as give alert to make the decisions to control and be aware of the prevalence in their settlements for not being affected.

## 1.2. Objectives

### 1.2.1. General objective

- To isolate and characterize *S. aureus* from Cow milk samples as well as to detect, antibiotic-resistant genes for *S. aureus.*

### 1.2.2. Specific objectives

- To isolate and identify *Staphylococcus* species from raw milk samples.
- To conduct primary and secondary screening for isolated *Staphylococcus* species
- To determine the antibiotic resistivity characteristics for isolated *S. aureus*
- To detect antibiotic-resistant genes for isolated *S. aureus* isolates

## 1.3. Significance of the study

Nowadays, the greatest of the milk market is carried out informally and this increases the negative impact of microbial contamination on public health. In another way, especially in rural areas the maximum amount of milk that is consumed is under unhygienic handling, processing, and storage so awareness will give from the producer level to the consumer to avoid the risk following its usage. The molecular methods in this study were also used to determine the genes linked to antibiotic resistance. Determining which antibiotic or medications to provide and to overcome the dramatic increase of various resistant strains in milk samples are very important decisions. Therefore, this study was carried out to explore the status of *staphylococcal* contamination associated with milk and the health hazards interconnected with it and provides practical dealings concerned to reduce the problem.

# 2. LITERATURE REVIEW

## 2.1. History of the *Staphylococcus* bacteria

In 1880 the Scottish surgeon Sir Alexander Ogston discovered the *Staphylococci* from the pus of surgical abscesses and described them as *micrococci* for the first time. Because of the morphologic features include;- having a clustered arrangement, grape-like structure, and rounded shape he called it as *Staphylococcus*' which is the Greek word ―staphyle (bunch of grapes) and ―kokkos (berry) (Rasheed *et al*., 2021). The genus of S*taphylococcus* is different from streptococci because they are catalase-positive, and oxidase-negative, arranged in chains, and cause post-surgical infections. *Staphylococci* have the characteristics of Gram-positive, 0.5 – 1.5µm diameters and cocci in shape, greater than one cluster of grape-like structures, and non-motile of facultative in respiration (Harris *et al*., 2002). Ogston by 1880 experimented to find out the infection brought due to *S. aureus* by introducing *S. aureus* into subcutaneous tissues of guinea pigs and mice in the laboratory. In 1884, the *staphylococci* were identified, cultured, characterized classified based on the production of golden or yellowish colonies, naming the species ―-aureus‖ Latin word mean (golden), thus, *S. aureus* was distinguished from *S. epidermidis* (previously known as *S. albus*) by their golden and white colonies, respectively (Rasheed *et al*., 2021).

## 2.2. Classification and characteristics of *Staphylococcus*

The majority of species of the *Staphylococci* are mainly obtained from the cattle milk. The *staphylococci* are grouped into coagulase-negative *Staphylococci* (CNS)/non-*aureus staphylococci* (NAS) and coagulase-positive *Staphylococci*/, *Staphylococcus* (*S. aureus*). Coagulase-negative *Staphylococci* are a varied cluster having over 15 species identified about bovine mastitis (Wald *et al.*, 2019). Naturally, *staphylococci* are inhibiting common skins and mucous membranes of animals and humans. However, the strains are differently associated with different organisms based on the host they live with. For instance, *S. stepanovicii, is*

screened from wild small animals while S. *pseudintermedius*, from many domestic animals, including; - dogs, cats, horses, and parrots, and *S. agnetis, is* identified from bovine mastitis. Additionally, *S. felis* is normally isolated from feline, *S. pseudintermedius is* predominant in domestic dogs, and *S. caprae* is associated with intramammary infections in dairy goats(Rossi, 2020). It is difficult to identify *Staphylococci* through the help of only traditional bacteriology like biochemical and phenotypic characterization exactly at the species level. Coagulase-positive *Staphylococci* are well-thought opportunistic pathogens resulting in mastitis. From this *S. aureus* is one of the common zoonotic diseases mainly its way of transmission is cow-cow at the beginning (Wald *et al*., 2019).

## 2.3. Screening methods of *Staphylococcus* species from their samples

The classification of the species of *Staphylococci* can be done through biochemical tests(Rasheed *et al*., 2021). Using the blood agar media, the species of *staphylococcus* show yellow or white color, creamy, opaque, and convex colonies (Fernandes *et al*., 2021). To classify and differentiate *S. aureus* from other gram-positive microorganisms, several biochemical characterizations are used. The *S. aureus* is distinguished from others by having characteristics including, catalase-positive, and oxidase-negative, and produces a coagulase (coagulase-positive) enzyme that agglutinates blood. The other important *Staphylococcus* spp, like *S. epidermidis* and *S. saprophyticus*, normally show negative to coagulase test (Rasheed *et al*., 2021). Whereas *S. intermedius* and *S. hyicus*, *S. pseudintermedius*, *S. lutrae*, *S. schleiferi*, and *S. delphini* are usually inhabited animals and similarly coagulase positive. Because *S. aureus* is susceptible to novambicin it can be easily distinguished from *S. saprophyticus* which is novambicin-resistant. The DNase test important method to isolate and distinguish *S. aureus* from the collections of the *Staphylococci* species (Rasheed *et al*., 2021).

## 2.4. Factors affecting the growth of newly isolated *Staphylococcus aureus*

The tolerance to grow in the salt with up to 3.5M concentration is a rare characteristic of *S. aureus*. It has high $K^+$ in its cellular level and this cannot shift the more the stress of NaCl. A diversity of transport systems that are stimulated or induced by NaCl is accountable for the admission of osmoprotectants toward the cell. The *S. aureus* has several ways of the genes and protein expressions to tolerate the stress of the NaCl in addition to storing corresponding solutes to treat the turgor pressure. It is possible to understand the NaCl tolerance by S. aureus by making the NaCl-sensitive mutant *S. aureus* and identifying the gene associated with the phenomenon (Scybert *et al*., 2003). Several genes, like the branched-chain amino acid transporter gene *brnQ* and the arsenic operon regulatory gene *arsR*, collectively contribute to NaCl tolerance. Additionally, *Ebh* is the larger cell wall protein important in the tolerance of the passing hyperosmotic pressure (Tsai *et al*., 2011). The well-known spreading widely pathogenic *S.aureus* can live and reproduce in the environment of very aggressive. It inhibits the different areas of the body showing middle acidic conditions such as reproductive organs (vagina), skin, mouth, abscesses, and urine where it usually results in a series of infections because of its extraordinary resist various pH. The *S. aureus* additionally can live on and thrive in inner cellular acidic sections like phagolysosomes of phagocytic cells. These characteristics are very important to describe the frequent and worsening features of many infectious *Staphylococcus* bacteria (Baudoux *et al*., 2007).

## 2.5. Pathogenesis and genetic material related to antibiotic resistance in *S.aureus*

Multidrug-resistant *Staphylococci* are the greatest human health problem nowadays. This occurrence particularly methicillin-resistant *S. aureus* (MRSA) is the major challenge for controlling as well as for its treatment. In the case that MRS (Methicillin-resistant *staphylococci*) is resistant to the greatest of the antibiotics including, beta-lactams, aminoglycosides, and macrolides it is too difficult to treat

infections caused by it. The synthesizing of the beta-lactamase is the main way of resistance against penicillin antibiotics by degrading the ring of its beta-lactam. The penicillin-binding protein 2a (PBP2a), encoded by mecA and *blaZ* which encodes β-lactamase is the other way of resistance to penicillin (Duran *et al*., 2012). In the *Staphylococci* species, aminoglycoside modifying enzymes (AMEs) are major factors responsible for aminoglycoside resistance. Acetyltransferase (AAC), aminoglycoside phosphotransferase (APH), and aminoglycoside nucleotidyltransferase (ANT) are the recently identified three groups of AMEs. AMEs like aminoglycoside nucleotidyltransferases (APHs) are commonly important in the resistance of aminoglycoside in the *staphylococcus* bacteria. It is either plasmid or chromosome encoded (Duran *et al*., 2012).

The gene expression of the various virulence agents, for example, immune modulators, toxins, and exoenzymes are the basis of the development of the diseases or pathogenesis in *Staphylococcus aureus*. The unique genes have contributed to the controlling and coding of the expressions of these factors. Toxins are the greatest common factor used to defend the microbe by averting the potential destructing its host`s defense methods from the virulence agents in *S. aureus*. The various toxin expressions coded with numbers of genes result in many infectious diseases. For example, the gene tstH which is used to code the toxic shock syndrome toxin1 (TSST1), results toxic shock syndrome(Idrees *et al*., 2021). But the eta and etb genes code exfoliative toxin that results from the causes of scalded skin syndrome.

The *hla* and *lukDE* control haemolysin and leukotoxin which are used in the construction of pores and degradation of their host membrane. The extracellular proteins (extracellular enzymes) for instance lipases, proteases, nucleases, and the like are coded by their corresponding gene of Agr system and are the main cause of the tissue damage or degradation and metastasis disease. Leucocidin, phenol-soluble modulins are the immunomodulators accountable for resulting in the series of infections of the skin during its control by their associated genes such as *AgrA* and *Agr* (Idrees *et al*., 2021). The nuclease gene (nuc) was reported as a specific

target gene for detecting S. aureus. Also, methicillin- resistance mecA gene has been designated as a molecular marker gene extensively used for MRSA detection and typing(Alsharif *et al*., 2021). A huge quantity of virulence factors has originated from *S. aureus* isolated from bovine mastitides, such as clumping factor (*clfA*), fibronectin-binding proteins (fnbpA and fnbpB), hemolysins (*hla* and *hlb*), thermonuclease (nuc), coagulase (coa), and toxic-shock syndrome toxin-1 (tsst-1), which is important for the bacteria to live and reproduce within the mammary gland(Dong Wang *et al*, 2016).

## 2.6. Molecular and phenotypic detection of *Staphylococcus* species

By the use of the agar dilution method, the antibiotic resistance to penicillin, ceftaroline, oxacillin, gentamicin, teicoplanin, erythromycin, ofloxacin, and tetracycline was identified more of the times (Jana *et al*., 2022). Conventionally the bacteria are categorized depending on the examination of the physiological, morphological, and biochemical characteristics or using genetic analysis like DNA--DNA or RNA-DNA hybridization, determination of G+C content in DNA(Jana *et al*., 2022). The conventional biochemical assays and phenotypic methods for the identification of pathogenic microbes have the limitation of consuming the time, and the cost and sometimes may fail to distinguish between many *Staphylococcus* species. Regardless of their requirement of cost and time, the sequencing of 16S rRNA and 18S rRNA with the help of PCR and micro-nuclear magnetic resonance (μNMR) sequencing is hopeful and very sensitive for screening even at the species level (Manukumar & Umesha, 2017). The MALDI-TOF-MS (matrix-assisted laser desorption ionization time-of-flight mass spectrometry) is the rapid and robust tool currently developed to identify and diagnose microbes. Its method is quick, sensitive, and economically feasible. This method is highly acceptable since the MALDI-TOF-MS generated data is comparable with the well-characterized database. The assessment of the genetic material of organisms has key important contributions in many areas like biomedical fields (oncology and genetic diseases) in diagnostic applications. The polymerase chain reaction coupled single-strand conformation polymorphism (PCR-SSCP) assessment has been authenticated for

the screening of species and strains without performing the morphological characterizations. The single-strand conformation polymorphism (SSCP) depend on the principle of relies on the principle of the electrophoretic mobility of single-stranded DNA molecules in a non-denaturing gel based on the size and structure (Manukumar & Umesha, 2017).

## 2.7. Some Antibiotics for determining the resistivity of the *S. aureus*

Methicillin is a non-natural antibiotic categorized into the family β-lactams. Clinically it is important for the therapy of *S. aureus* because it inhibits the formation of the cell wall of bacteria resulting in the lysis of cells by attaching to the penicillin-binding protein. The methicillin sensitivity test is important determined to detection of MRSA (methicillin-resistant strains of Staphylococcus aureus). MIC determinations (Etest®, microdilution or microdilution), disk diffusion stand latex agglutination, chromogenic agar, automated methods, rapid screening methods and molecular are some of the tests used to analyze its performance. The encoding of the mecA or *mecC* genes is the result of resistivity against methicillin (Fernandes *et al*., 2021). These genes make an organism resistant to methicillin by uniting with the mobile genetic elements of the SCCmec (mec *staphylococcal* chromosome cassette) that can be found in screened MRSA isolates (Fernandes *et al*., 2021). Vancomycin is a drug grouped under the class of glycopeptides greatly helpful for the treatment of resistant Gram-positive cocci. Its performance is on the prevention of bacterial cell wall (peptidoglycan) synthesizing. The resistance to this drug is associated with the displacement of the transposon of vanA gene and Tn1546-containing plasmid (Fernandes *et al*., 2021).

## 2.8. Milk and its importance in Ethiopia

Milk is food that is composed of high nutritional components including carbohydrates, fats, minerals, proteins, and certain vitamins. It is veiled or secreted via epithelial cells in the mammary gland of mammals and has a yellowish-white colored liquid. Before they start the digestion of other additional food materials

milk is the most exact food for consumption at the beginning especially for non-matured (young) mammals(Getabalew *et al*., 2020). It is free from contaminants during the secretion to the alveoli of the udder but due to the case of unfair handling, storage, water activity, and nutritional components, various microorganisms use it as the best medium for their growth. Because of its nutritional value, in Ethiopia, milk and milk products are critically used for household consumption. Besides this, milk is the base for fundraising to buy farm requirements fertilizers, new crop varieties, and pesticides and also for purchasing their children‘s educational materials and their families‘ food requirements. However the quality of the milk is highly affected as a result of personal mismanagement which blows the hygienic situation and this makes the value of the milk unsafe for consumers and to store for further processing (Getabalew *et al*., 2020).

## 2.9. Production of dairy in Ethiopia

Milk production is the main for living many Ethiopian people settled in rural. There are large populations of cattle (59.5 million cattle, 30.70 million sheep, and 30.20 million goats) in Ethiopia, which can incapacitate the development of the huge dairy production and increase the salary of the minor holder as well as facilitate an employ and in the minimization of the poverty and nutritional inefficiency. The total annual milk production in the country governed by a small dairy production system is the traditional production method accounts for about 97-98% and is dominated by indigenous breeds. Even though there are many numbers of milk producers, the milk amounts are small and so low markets due to the varieties of limitations like biological and Technical, low rainfall, high temperature, low forage production, common plant association, the incidence of important livestock diseases and parasites, mainly defined in the lowland part of Ethiopia (Getabalew *et al*., 2019).

## 2.10. Milk Borne Bacterial Pathogens:

Fresh milk is provided to the market both formally and informally from rural and

urban milk producers(Yigrem *et al*., 2008)**.** In several societies of different farms and with varieties of animals, the milk is not taken into concern to examine either the presence or the absence of zoonotic diseases, adulterants, and contaminants before being taken to the collection center and these results in the spoilage of the whole constituents of the collected milk. In milk processing clarity and healthfulness are the major vital considerations but there is the low-cost substitution of skimmed milk powder which is under the standard so it is worries for the health(Getabalew *et al*., 2019).

Most Ethiopian societies keep their animals near and within their house with very low management and under hygienic conditions; therefore the high probability of the transmission of zoonotic or infectious diseases between animals and humans is a great challenge. Like Ethiopia food consumers in developing countries are affected with food-borne bacterial sicknesses habitually *Bacillus cereus, Staphylococcus spp*., *Salmonella spp*., and *Shigella spp*. . Even though there are varieties of pathogenic microorganisms causing food-borne illnesses, more than 90 % of illnesses and deaths reported to the Centers for Disease Control and Prevention (CDC) are from bacteria(Zelalem Ayana, 2015).

There are so many milk-borne disease sources starting from the individual milker and the other bases like the milk tank, the animal itself, milking equipment, milk pipelines, and manufacturing environments. The main and newly emerged zoonotic pathogens isolated from the row milk samples reported recently are *Brucella* ssp., mainly *B. melitensis*, *Listeria monocytogenes*, *Salmonella* ssp., *Mycobacterium bovis*, *Yersinia enterocolitica*, *Streptococcus pyogenes*, and *S. agalactiae*, *E.coli* O157:H7, *E. sakazakii*, hepatitis A virus, *M. avium* subsp. *paratuberculosis*, *S. zooepidemicus* (B-hemolytic *Streptococcus* Lancefield C group), *Campylobacter jejuni*, *Citrobacter freundii*, *Corynebacterium ulcerans*, and *Cryptosporidium parvum* (Velázquez-ordoñez *et al*., 2019).

## 2.11. Staphylococci and Staphylococcus food poisoning

The species of *staphylococci* are non-spore forming facultative bacteria having the feature positive when gram stained; morphologically cocci, grape-like clustered in

shape, and non-motile. Out of 32 species of the genus of *staphylococcus, staphylococcus aureus,* and *epidermis* are the most studied and favorably affecting humans. More numbers of them need relative nutritional elements and greatly the organic source of N2 (Ghebremedhin *et al*., 2008; Harris *et al*., 2002). These groups are distinguished from genus *Streptococci*, by having catalase-positive and oxidase-negative characteristics. They are high salt-tolerant heat (Harris *et al*., 2002). Pathogenic *Staphylococci* clot the blood because they form coagulase. SFP is the most well-recognized foodborne disease due to the presence of toxins in the diet; the effect of an organism (illness) is not direct, but the type of food poisoning caused by toxin production in the diet. There are nine family major serotypes of heat-resistant *Staphylococcus*, which are enterotoxins (A-E and G-j). Out of these, Enterotoxins A and C are the main reasons for food poisoning (Ghalehnoo, 2019). The most common FBD challenge worldwide is the *Staphylococca*l food-borne disease. Due to the development of the multi-drug resistant *Staphylococcus* species, the number of infected humans has increased and the treatment also become too problematic in the 20 years before. *S.aureus* has different growth environments including dry and stressful conditions, kindness to living, and multiply in multiple numbers of foodstuffs like salinized food materials, milk and milk products (commonly), meat and meat products, egg products and poultry, bakery products, salads, cream-filled pastries, sandwich filling and cakes (Tarekgne, 2016).

## 2.12. Causes and problems of Antibiotic resistance

Antibiotic resistivity is highly increasing and it has become a public health problem across the world. Thus known bacteria are grouped as multiple drug resistance rather than single. This phenomenon shows many treatments present for the treatments of different diseases are not properly working according to the WHO 2014 annual report; resulting in essential changes in the microbial ecosystems of animals and the human environment (Tarekgne, 2016). There are four basic reasons for antibiotic resistance to take place which are; Natural (Intrinsic, Structural) resistance, Acquired resistance, Cross-resistance, Multi-drug resistance, and pan-resistance. Natural resistance is related to neither heredity nor the use of antibiotics

rather than the bacterial structural characteristics. The occurrence is due to resistance naturally or the bacteria either has no the structure of the target antibiotic, or antibiotics not reaching its target.

The resistance of S. aureus to almost all the previous antimicrobial drug groups is facilitated by the elements obtained through the horizontal genetic transfer. The methicillin and vancomycin resistance are the most serious caused by horizontal trait exchanging. The resistances which are result from the random mutation and section under the antibiotic stress is currently playing a role in clinical and presenting a higher base of resistance to antimicrobials especially vancomycin (for the intermediate level of resistance), fluoroquinolones, daptomycin, linezolid and others(Sanchini, 2007).

The acquired resistance is because of the modification in genetic information not being exposed to antibiotics before as a result of structural of either chromosomal or extrachromosomal like plasmid, transposons, and the like. Chromosomal resistance develops from the spontaneous change in chromosomes through chemicals or physical mutagens which result in structural changes in the cells of organisms (Cesur & Demiröz, 2013). Therefore this can change the permeability of the antibiotics or its target. The extrachromosomal resistance arises because of the extrachromosomal genetic materials enabling it to be passed in various means(plasmid transposons, etc.) eg plasmids carry the genes which encode for the enzymes of deactivating the antibiotics and these can be transmitted through transduction, transformation, conjugation, and transposition ways. Cross-resistance occurs by the time certain microorganisms act by the same or related mechanism to resist some drugs and is most of the time seen in structurally similar antibiotics (Cesur & Demiröz, 2013).

In multi-drug resistance and pan-resistance, bacteria are resistant to the antibiotics applied to control them so that particular antibiotics have no extra ability to kill. This may be due to either the existence of multiple genes each code for resistance to a single antibiotic which is present on resistance plasmid or multiple drug resistance by gene expression coding for three or more groups of antimicrobials(Cesur & Demiröz, 2013). The features of resistance to antibiotics

result in accelerated rates of infections and increase the expansion of further virulent bacterial pathogens causing severe infections. Worldwide overdose use and misuse is the key cause of the development of antibiotic resistance. Using antibiotics in animals is the other main difficulty of the current antibiotic resistance issue across the continent. This circumstance developing countries are highly endangering because of the increase in the usage of antibiotics and their easy availability without prescription, under hygienic states(Tarekgne, 2016).

## 2.13. Mechanisms of resistance to antibiotics

On mobile genetic elements, many genes coding antibiotic resistance are present which can be exchanged between strains from animals and humans inhabiting the equivalent ecological niche(Nhatsave *et al*., 2021). The resistivity against antibiotics emanates from the synthesizing of the enzymes degrading the antibiotics applied to treat the bacteria. Some of these enzymes are classes of β-lactamases or aminoglycoside modifying enzymes that modify the antibiotic targets, and decrease intracellular antibiotic concentration, either by limiting the penetration of the antibiotic or facilitating its expulsion. The *S. aureus* developed resistance to about all antibiotics to defend against so it is one of the most special pathogens in case of antibiotic resistance. *S. aureus* has steadily acquired new mechanisms of resistance, allowing it to become resistant to all β-lactams, tetracyclines, aminoglycosides, fluoroquinolones, clindamycin, trimethoprim-sulfamethoxazole, vancomycin, daptomycin and linezolid starting from the beginning of 19400‘s, at the time of the explanation that *S. aureus* resistant to penicillin (KakoKakoullis *et al*., 2021).

The modification in the ribosomal target of the microorganisms is one of the antibiotic resistances that occur repeatedly. The change in the penicillin-binding proteins (beta-lactamase enzymes) and *S. pneumoniae*, *Neisseria meningitidis*, *St. aureus*, and *E. faecium* causes resistance against the antibiotic penicillin. There are different mechanisms of antibiotic resistance. The first is a change in the structure of the target, beta-lactam, quinolones, glycopeptides, macrolides, tetracycline, and rifampicin resistance is an important mechanism in the development of antibiotic

resistance(Cesur & Demiröz, 2013). The second one is the enzymatic inactivation of antibiotics by the greatest numbers of Gram-positive and Gram-negative bacteria producing the antibiotic hydrolyzing proteins. Most of the time this is the common mechanism of resistance to antibiotics by which microbes have inactivated the activity of the drugs. For instance, beta-lactamases, aminoglycosides, modifying enzymes (acetylase, fosforiazadenilaz, and enzymes) hydrolysis the beta-lactam antibiotics and frequently upgrade their amount of which deactivates enzymes consisting of chloramphenicol and erythromycin(Cesur & Demiröz, 2013).

The third mechanism of resistance to antibiotics is the decrease of the internal and exterior membrane permeability. These formations of variation decline the uptake of the drug towards the cell or rapidly disconnect from the active resistance of the drive systems. As a result of a change in membrane permeability decreased porin mutations in resistant strains can occur in proteins. In the resistance of the quinolones and aminoglycosides, the decrease in permeability of the external membrane might have a significant role (Cesur & Demiröz, 2013). The use of alternative metabolic pathways is the fifth mechanism of antibiotic resistance. This is by developing a new pathway to escape the action of the antibiotics against bacteria. For example, the sulfonamide and trimethoprim resistance are developed in this mechanism. Bacteria can obtain folate from the natural environment rather than producing it by itself (Cesur & Demiröz, 2013).

## 2.14. Diagnosis and Control of SFP

Clinically within 30 minutes after the food is eaten, the projectile vomiting and diarrhea signs are recommended as *Staphylococcus* intoxication. However, to prove this, microbiological, molecular examinations, and immunological tests of food or vomit, alone or in combination, should be approved (Tarekgne, 2016). Everywhere there are *S. aureus* but approximately, 20- 75% hosts of them are humans. Under unhygienic processed and non-refrigerated foods have the chance of being easily contaminated with *Staphylococcus* species. Hence, reheating of diet next to management by humans is an important method of decreasing the SFP. However, the diet must be kept refrigerated for some time because *S. aureus* can

develop at room temperature and not be demolished later by heating temperature (Tarekgne, 2016).

# 3. METHODOLOGY

## 3.1. Description of Study area

The study was conducted at Wolkite University, Biotechnology from November to January 2023. The university is found in Wolkite, the administrative center of the Gurage Zone of the central Ethiopia regional state. Welkite is a town and separate woreda in southwestern Ethiopia, 158 km from Addis Ababa, the capital city of Ethiopia. Wolkite is the administrative center of the Gurage Zone of the central Ethiopia regional state; its latitude and longitude are 8°17′N 37°47′E and an elevation between 1910 and 1935m above sea level (Desalegn *et al*., 2018).

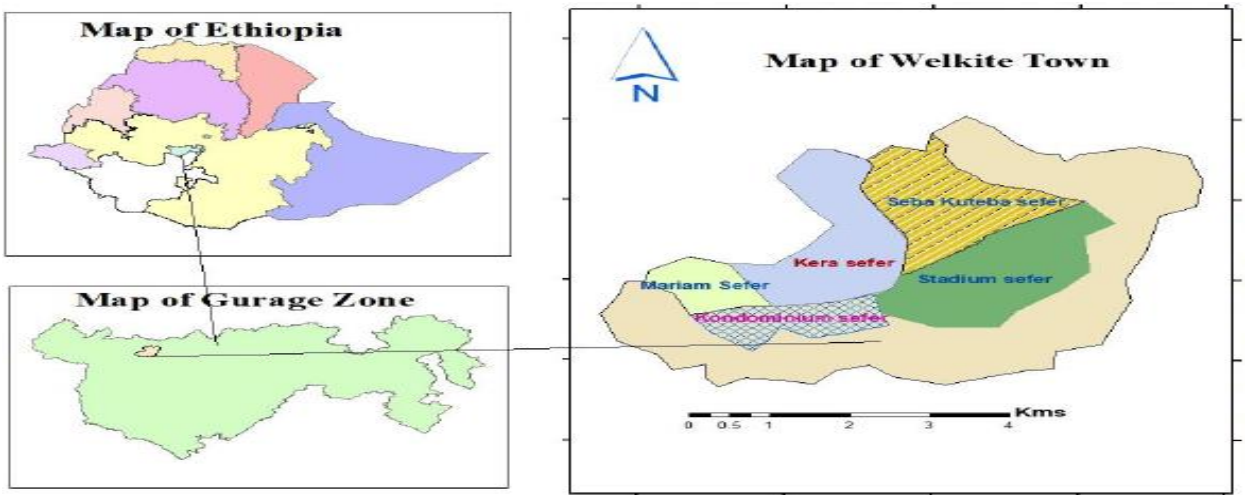



Figure .a. The study area location

## 3.2. Sample collection and transportation

Raw milk samples were collected using purposive sampling techniques and aseptically collected from, restaurants, milk sellers, and milk collection centers using sterile falcon tubes from different areas of Wolkite. Then the samples were transported to the Wolkite University Biotechnology Department Laboratory using an ice box and kept at 4 $^{o}$C in a refrigerator. The sample size was determined as follows; n = $1.96^2$*pexp (1-pexp)/ d2 = $(1.96)^2$ (0.5) (0.5)) / $(0.05)^2$ = 385, where d is the intended absolute precision of 5%, pexp is the predicted prevalence of the organism, and n is the necessary total sample size.

## 3.3. Primary screening for *Staphylococcus* species

### 3.3.1. Isolation and identification of Staphylococcus bacteria:

A total of 385 samples each 5ml were collected from different areas of Wolkite. The 1ml of the milk sample was vigorously mixed with 9ml of sterilized distilled water and the serial dilution was done. The sample was then transferred into blood Agar plate medium (g/L) (0.5% Peptone, 0.3% beef extract/yeast extract, 1.5% agar, 0.5% NaCl distilled water, 5% Sheep Blood, and pH at 7.2 -7.6). It was incubated at 37 $^{o}$C for 24 h. The plates were examined for the identification of *Staphylococcus* species using morphological features such as opaque, creamy, grapelike irregular, greyish, white, or yellow colonies, and hemolytic patterns on the surface of blood agar plates. Presumed *Staphylococcal* colonies were then sub-cultured on nutrient agar plates and incubated at 37 °C for 24 to 48 h to get a pure culture (Addis *et al*., 2011).

### 3.3.2. Catalase test

Using the clean glass slide the pure culture was taken with the help of an inoculation loop and mixed with a drop of 3% $H_2O_2$ (v/v). Then the bacteria forming the bubbles was selected as positive and considered as *Staphylococcus* species (Addis *et al.,* 2011).

### 3.3.3. Urease test

The bacterial isolate was inoculated in a tube having the urea broth or slant and incubated at 37 $^{o}$C for 24 h. Then the reddish pink or red color was recognized as a positive result of *staphylococcus* (Pervin *et al*., 2019).

### 3.3.4. Indole test

This experiment tested the ability of microorganisms to convert the amino acid tryptophan into indole. The Koves reagent was given to the culture media after the bacteria had been incubated for at least 24 hours, and it worked effectively after

that. Within seconds of introducing the reagent, the reagent layer on top of the medium developed a pink to cherry-red color, indicating a successful indole test. When a culture was indole-negative, the reagent layer remained yellow (Pervin *et al*., 2019).

### 3.3.5. Methyl Red test

A small amount of culture from the pure test organism was added to 5 ml of the sterile MR-VP broth, and the mixture was cultured for 24 hours at 370 °C. Five drops of methyl red solution were added after incubation, stirred, and color development was then checked. The developments of the red and yellow colors indicate the results were positive and negative outcomes, respectively (Sandeep Kumar *et al*, 2012).

### 3.3.6. Voges proskauer (VP) test

The overnight pure culture was inoculated into MR-VP broth and incubated at $37^{o}$c for 24 hours. Then the 6 drops of 5% alpha-naphthol & and 2 drops of 40% potassium hydroxide were added and mixed well. The mixture was left for 30 min and the development of pink red color was considered a positive result (Sandeep Kumar *et al*, 2012).

### 3.3.7. Motility test

SIM medium was used for this motility test and the 24 h fresh bacteria were taken by sterile inoculating needle. Then it was stabbed down into the test tube having the sterilized SIM media. Lastly, it was incubated at 37 °C and examined daily for up to three days (Sneha Bhat, *et al*, 2017).

### 3.3.8. Citrate agar test

This test is based on the ability of microorganisms to use citrate as their source of carbon and ammonia. The overnight culture was streaked over a slant of Simmon‘s citrate agar and incubated for 24 h at 37 $^{o}$C. The change in color to blue said as positive to citrate (Numrah & Hafsa, 2014).

### 3.3.9. Starch hydrolysis

Until heavy growth took place, bacterial isolates were cultured at 30°C after being streak-plated on nutritional agar plates containing 0.2% soluble starch (w/v). After incubation and growth evaluation, plates were treated with Gram's iodine for 5-8 minutes, and after draining off the iodine, they were examined for clear zones (disappearance of starch) indicative of positive starch hydrolysis(Evans *et al.*, 2004).

## 3.4. Secondary screening for *Staphylococcus aureus*

### 3.4.1. Gram staining

Gram staining was performed following the methods of (Boone *et al*., 2001) with little modification. One drop of sterile distilled water was placed in the center of the microscopic slide using a micropipette. Using an aseptic technique a very small part of a single colony was taken and placed on the slide. A thin smear was carried out and allowed to air dry at room temperature. The heat-fixed smears were covered with crystal violet for 30 seconds. The stain was washed off gently with tap water and covered with Gram‘s iodine for 1 min. Then the iodine stain was removed with tap water. The smear was held at 45 $^{o}$C and decolorized with 95% ethyl alcohol (v/v) until the decolorizer was colorless and it was immediately washed with water. The smear was covered with safranin for one minute and washed with tap water. Finally, the smear was blotted and air dried to examine under the oil immersion objective.

### 3.4.2. Mannitol salt agar (MSA) medium for *S. aureus*

It was performed according to previous methods (Peterson & Kaur, 2018) with slight modifications. A loop full of the enriched sample was aseptically spread on freshly prepared Mannitol Salt Agar (MSA) medium (Protease peptone, 10 g/l; Sodium chloride, 75 g/l; D-mannitol, 10 g/l; Beef extract, 1 g/l; Phenol red, 0.025 g/l; and agar, 15 g/l) (Oxoid, UK). These all plates were incubated aerobically at 37 $^{o}$C for 24-48h. 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.

### 3.4.3. Coagulase test

The 0.5ml grown bacteria at 37$^{o}$C for 24h on tryptone soya broth (TSB) (Pancreatic digest of casein, 17g/l; enzymatic digest of soybean, 3g/l; NaCl, 5g/l; $K_2HPO_4$, 2.5 g/l and glucose, 2.5 g/l at 37$^{o}$C and pH6.8±0.2) was added to 0.5ml of citrated plasma followed by gentle mixing and then incubated at 37$^{o}$C. As a negative control, the tube contains 0.5ml of sterile TSB and 0.5ml of plasma clotting incubated at the same time and temperature. Clotting was evaluated at 30-minute intervals for the first 4 h of the test and then after 24 h incubation (Addis *et al*., 2011). If cloting changed from a loose to solid, it can be considered as positive and if not it it can be considered as negative.

## 3.5. Antimicrobial susceptibility testing for *S. aureus*

An antimicrobial susceptibility test was performed on selected 13 *Staphylococcus aureus* isolates according to the criteria of the Clinical and Laboratory Standards Institute (Normanno *et al*., 2007). Briefly, two colonies were suspended in sterile saline. Then using the spreading plate method the suspension was applied on Mueller Hinton agar(Beef infusion from buffalo, 300g/l; acid hydrolysate casein, 17.5 g/l; starch, 1.5 g/l and agar,17 g/l) and allowed the agar to dry. Using the autoclaved forceps, antimicrobial layered discs were placed on the agar layer which was covered with and then the incubation was placed aerobically at 37 $^{o}$C for 18 h according to the manufacturers and CLSI instructions (Gizaw *et al*., 2020). For the susceptibility test, one antimicrobial from each subclass of antimicrobials that were commonly used for the treatment of bovines or considered as important antimicrobial agents for humans was selected for antibiogram based on the criteria of Clinical and Laboratory Standards Institute. Thus, antimicrobials included in this study was clindamycin (2μg), erythromycin (15μg,), cefazolin(30 μg), penicillin G

(10units, _29mm) and tetracycline (30µg,). With the help of a ruler, the diameters of the zone of inhibitions around the disks were measured to the nearest mm then the isolates were classified as susceptible, intermediate, and resistant. The isolates having the characteristics of resistance to three or more antimicrobial subclasses were taken as multidrug-resistant (Fufa Abunna *et al*, 2016).

## 3.6. Molecular characterization for newly isolated *Staphylococcus aureus*

### 3.6.1. Genomic DNA Extraction

Gnomic DNA extraction extraction from bacterial isolates was conducted at the Institute of Animal Health Sebeta, Ethiopia. At the Institute of Animal Health Sebata, Ethiopia, the genomic DNA of bacterial isolates was extracted. Thermo Scientific, Germany's Qiagen DNeasy extraction kit was used to extract the DNA from the possible *S. aureus* strain following the manual's recommendations. To demonstrate, two loop-full colonies were grown overnight at a temperature of $37^{o}$c in microcentrifuge tubes containing 800ul of LB broth (Tryptone 10g/l, yeast extract 5g/l, and sodium chloride 10g/l) with a final pH of 7. Ten bacterial isolates were initiated on nutrient agar throughout an overnight period at 37 °C. After centrifuging each tube for 5 minutes at 20,000 x g, the bacterial cells/pellets were then collected by discarding the supernatants. The cells were gently mixed with 240 l of lysis buffer before being added, and they were then incubated at 37 °C for a half-hour in a water bath. 32u l of proteinase K and 260 l were added, carefully mixed, and then incubated at $56^{o}$C for 45 min. Then, 260 ul of pure ethanol was added, mixed with a vortex, introduced to the spin column, and centrifuged there for one minute at 10,000 x g. After adding 660 mL of AW1 buffer, the column tubes were put in a clean collection tube and the collection tube was discarded. The sample was then centrifuged at 10,000 x g for 60 seconds. Once more, the columns were removed, and placed in clean collecting tubes, 660 ul of AW2 buffer was added, and it was centrifuged 20,000 times in 3 minutes. 660 ul of AE buffer was then added to the column after it had been transferred to a 1.5 ml tube. It took the

column one minute to stand at room temperature. Pure DNA was then extracted and kept at - 20$^{o}$C until it was needed for further analysis after the column had been centrifuged 10,000 times for 1 minute at 25$^{o}$C.

### 3.6.2. PCR amplification for antibiotic resistance genes

The PCR assays were performed to identify antibiotic resistance elements in the *staphylococcus aureus*. PCR amplification for Antibiotic resistance genes was performed. Using a PCR master kit with 25 µl mixtures comprising 2 µL of each primer, 1.5 µl of 25 mM of MgCl2, 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, 10 genomic DNA isolates from *S.aureus* isolates were subjected to PCR to amplify the resistance genes. For the negative control, sterile double distilled water was used in place of nucleic acids. In this study, the antibiotics resistance genes such as *mecA* and *nuc* gene were used for amplification. The specific primers for each were: F-5′GTA GAA ATG ACT GAA CGT CCG ATA A3′ and R-5′CCA ATT CCA CAT TGT TTC GGT CTA A3′ for mecA gene (band size of 310 bp) (Girmay *et al*., 2020). The *nuc gene* nuc F: GCGATTGATGGTGATACGGGT and R: AGCCAAGCCTTGACGAACTAAAGC were used (Dong Wang *et al*, 2016). The PCR protocol comprised an initial denaturation phase at 94°C for 4 minutes, 35 cycles of primer extension at 72°C for 1 minute, annealing at 37°C for 45 seconds, and final denaturation at 72°C for 8 minutes.

### 3.6.3. Preparation of agarose gel electrophoresis and gel documentation analysis

Agarose gel was prepared by mixing 1 g of agarose powder in 100 ml of Tris-Acetate-EDTA buffer to obtain a 1% concentration of the gel with some modifications (Tilahun *et al*., 2020). The mixture was completely dissolved by boiling in a microwave oven until a clear solution appeared. At about 50$^{o}$c, 2 µl of ethidium bromide was added and mixed well by simple shaking. The agarose was then poured into the horizontal electrophoresis casting equipment. The 5µl of the PCR products was mixed thoroughly with 1 µl of 6X loading dye on aluminum foil and loaded in the wells of the agarose gel followed by loading 10 µl of a 1 kb DNA ladder and the three selected antibiotic- resistant genes in a next in parallel wells.

The horizontal gel electrophoresis was carried out at a constant voltage of 100V for 30 minutes. The amplicons of genes of the *staphylococci* were separated and the image was captured using the gel documentation analysis (Gel DocTM XR+, BioRAD; Germany) at the Institute of Animal Health, Sebeta, Ethiopia.

# 4. DATA ANALYSIS

The laboratory outcomes of the gathered samples were entered into Microsoft Excel 2010 and analyzed using the SPSS 20.0, and the species were identified by the advanced biological identification software tool (ABIS)

# 5. RESULT

*S. aureus* was obtained from the raw milk as illustrated in Figure 1. This pathogenic bacterial isolate was Gram-positive with cluster type. The colonies of these specific bacterial isolates were golden when inoculated on the Mannitol salt agar. However, when the same isolate was inoculated onto nutrient agar (NA), the colony was white (Figure 1c).

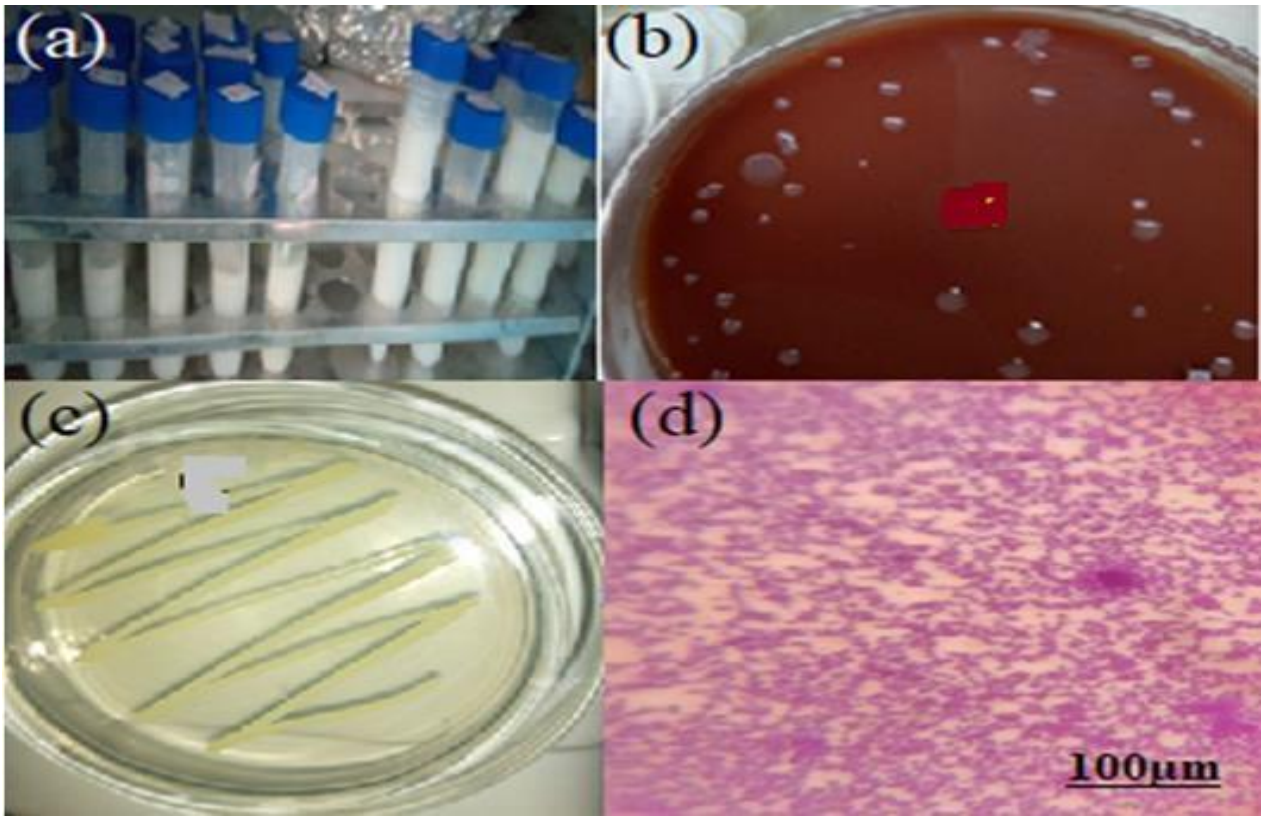



**Figure 1 Sample collection and isolation**

**(a) Milk samples were collected in a sterilized culture tube. (b) represents bacteria grown on blood agar media. (c) represents pure colony inoculated on Nutrient agar. (d) Gram stain for possible *Staphylococcus* species isolate under electronic microscope**

## 5.1. Primary screening methods for *Staphylococcus* species

On blood agar medium with 5% human blood (v/v) (Figure 1b), 30 isolates(Table-1) of *Staphylococcus spp.* were detected with characteristic features of grape-like structure, creamy, grayish, white, and yellow. The same isolates were shown

hemolytic features as indicated in the Figure 1b.

### 5.1.1. Catalase test:

Out of 30 isolates, 30(100%) (Table 1) were catalase-positive and there were no catalase- negative isolates (Table 1). The catalase- positive isolates produced foam when hydrogen peroxide was applied on the clean slide containing the targeted S. *aureus* isolate as illustrated in Figure 3a

**Table 1 Biochemical tests result against the presence of S. *aureus* in number/percent (%)**

| S.N | List of biochemical | Number/(%)of positive | Number /(%) of negative |
|---|---|---|---|
| 1 | Catalase | 30/100 | - |
| 2 | Urease | 24/80 | 6/20 |
| 3 | Gram | 30/100 | - |
| 4 | Mannitol salt agar | 18/60 | 12/40 |
| 5 | Coagulase | 13/43.33 | 17/56.67 |
| 6 | VP Test | 4(13.3) | 26(86.7 |
| 7 | Citrate agar | 24(80) | 6(20) |
| 8 | motility | - | 30(100) |
| 9 | Starch hydrolysis | 5(16.7) | 25(83.3) |
| 10 | Triple sugar iron | 30(100) | - |
| 11 | Indole test | - | 30(100) |
| 12 | Methyl red | 28(93.3) | 2(6.7) |

### 5.1.2. Urease test:

From the total isolates of the recent isolates of *S. aureus*, 24/80% were urease positive and the left 20% were found to be urease negative (Table 1).

### 5.1.3. Citrate agar test:

Certain species of the recent isolates could show citrate positive (Figure 3 M&N) when citrate was employed as the main carbon source. This test shows whether or

not a living thing can use citrate (citric acid) as a food source. Bromthymol blue, a pH indicator, is also included in the medium. The indicator bromthymol blue turns green below pH 7.6 and becomes blue above pH 7.6. The medium will change from green to blue and the pH will increase if citrates are used.

### 5.1.4. Indole test:

For the indole activity, the possible species of the isolates were examined and in this test, all isolates were detected to be negative against indole biosynthesis which means they do not show the red color at the upper portion of the test tubes (Figure 3D)

### 5.1.5. Methyl Red test:

Most of the isolates were changed the media to the red color but for example, isolates GU -6 & GU - WO2D were negative (Figure 3 I & J).

### 5.1.6. Motility test:

The motility test was performed in this investigation to determine whether the bacteria isolated motile or non-motile. In this study, all isolates were not motile which means unable to spread across the media (Fig. 3K)

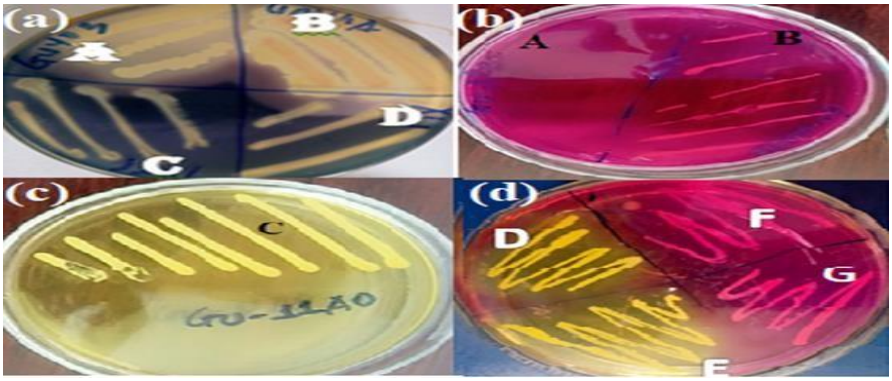



**Figure 2 Starch and mannitol salt agar tests**

(a) Starch hydrolysis test (A &B represent positive while (C&D is negative against starch hydrolysis). (b-d) Mannitol salt agar test (A ) represents mannitol salt agar negative results while B, F & and G are positive but couldn't ferment the mannitol salt agar and C, D & and E are positive and able to ferment the mannitol salt agar).

### 5.1.7. Starch hydrolysis:

After the bacteria were grown on nutrient agar containing 0.2 starches, the iodine solution was added, and most of the isolates did not form the clear zone (blue-black) as indicated in (Figure 2A). There is also a yellowish clear zone for specific isolates which is positive for starch hydrolysis (Figure 2A). In this study, 83.3% of the total isolates were negative to starch hydrolysis (Table 1).

### 5.1.8. Vogues proskauer (VP) test:

Except for a few isolates, more isolates were found to be negative against the vogues Proskauer test (Table 3). For instance, GU -D4, GU -D0, GU-8, and GU -W4 positive (Figure 3 F & G, and Table 3). Even isolates B, C, D, E, F & and G were grown on mannitol salt agar but isolates C, D & and E were more yellowish golden in color which means they can ferment the mannitol (Figure 2. C, D & E). From the salt mannitol test of the expected *Staphylococcus* species, 60% were positive and 40% were negative (Table 1).

## 5.2. Secondary screening for *S. aureus*

### 5.2.1. Gram staining:

Out of 30 isolates allowed for the Gram staining test, 30(100%) isolates were positive and no isolates were negative (Table 1). All isolates were found to be cocci in shape looking like grape structures (Figure 1d).

### 5.2.2. Coagulase test

In a recent study, isolates of *Staphylococci* species were found to be coagulase-positive. These isolates were able to coagulate blood when coagulase tests were done suggesting that these species of Coagulase isolate contained coagulase enzyme.

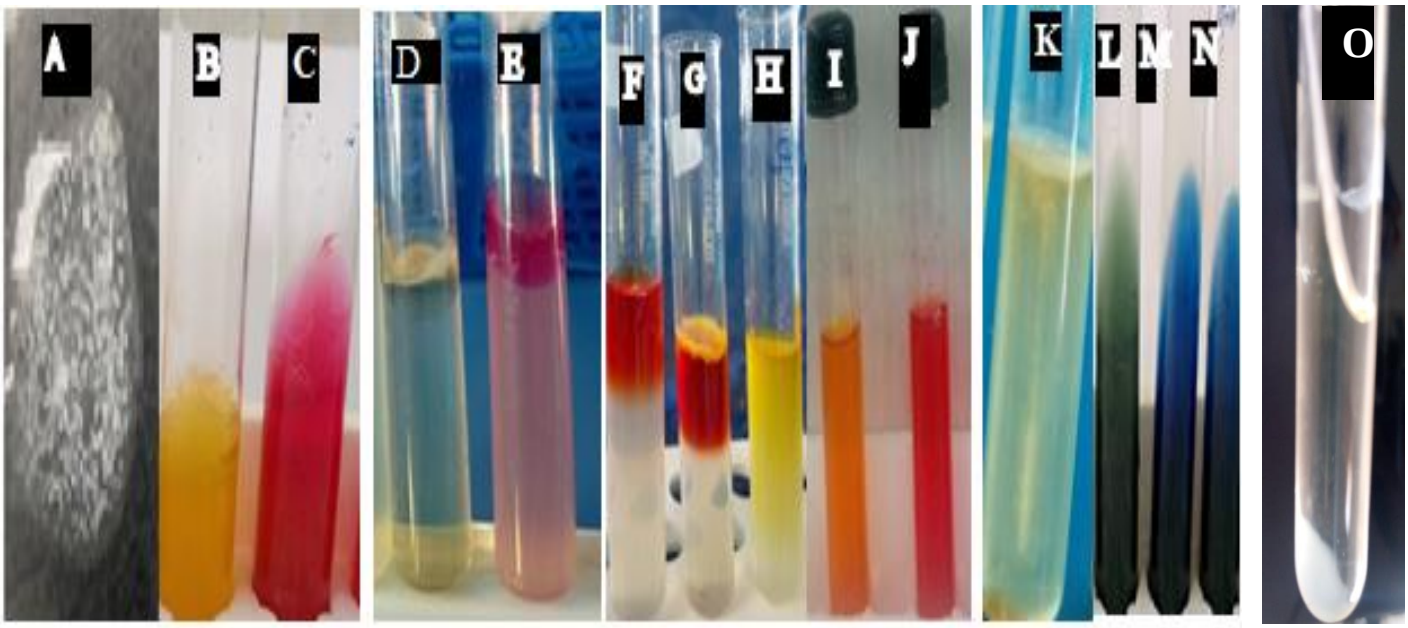



**Figure 3: Biochemical tests**

**"**a" represents catalase positive, "b" urease negative, "c" urease positive, "d" indole negative, "e" indole positive, "f" &"g" represent Voges prausker's positive "h" Voges prausker's negative "i"& "j" represent methyl red positive "k" motility negative "l" simmon citrate negative, "m" &"n" represent positive in Simmon citrate test "o" coagulase positive test. The isolate GUAB2 IS positive for to coagulase test while the isolate GUW4 is negative

## 5.3. Antimicrobial susceptibility testing for *S. aureus*

In this study, the antibiotic susceptibility (figure 4) result was classified as S (susceptible), I (intermediates), and R (resistant). From this study, 8(61.54%) isolates of *S. aureus* were found to be susceptible to clindamycin, 2(15.4%) were against vancomycin, 8(61.54%) were against cefazolin), and 7(53.85%) was against erythromycin (Table 2B). It was observed that *S. aureus* was not susceptible to penicillin. The susceptibility was higher in clindamycin and cefazolin but lower in penicillin. The susceptibility test against clindamycin 5(38.5%), and vancomycin, 2(15.4%) intermediate were found to be intermediate for *S. aureus. E*rythromycin (0(0%) and penicillin (0, 0%) were not intermediate against *S. aureus* (Table 2B). No isolate resisted clindamycin (0%), However, about 13(100%) isolates were resistant to penicillin and 11(84.62%), 3(23.1%), and 6(46.15%) were resistant against vancomycin, cefazolin, and erythromycin, respectively as indicated in the (Table 2). The six isolates such as GU-WO2D, GU-UshA, GU-11AO, TOR5, GUN1, and GuTor5D were found to be multidrug-resistant (Table 2A).

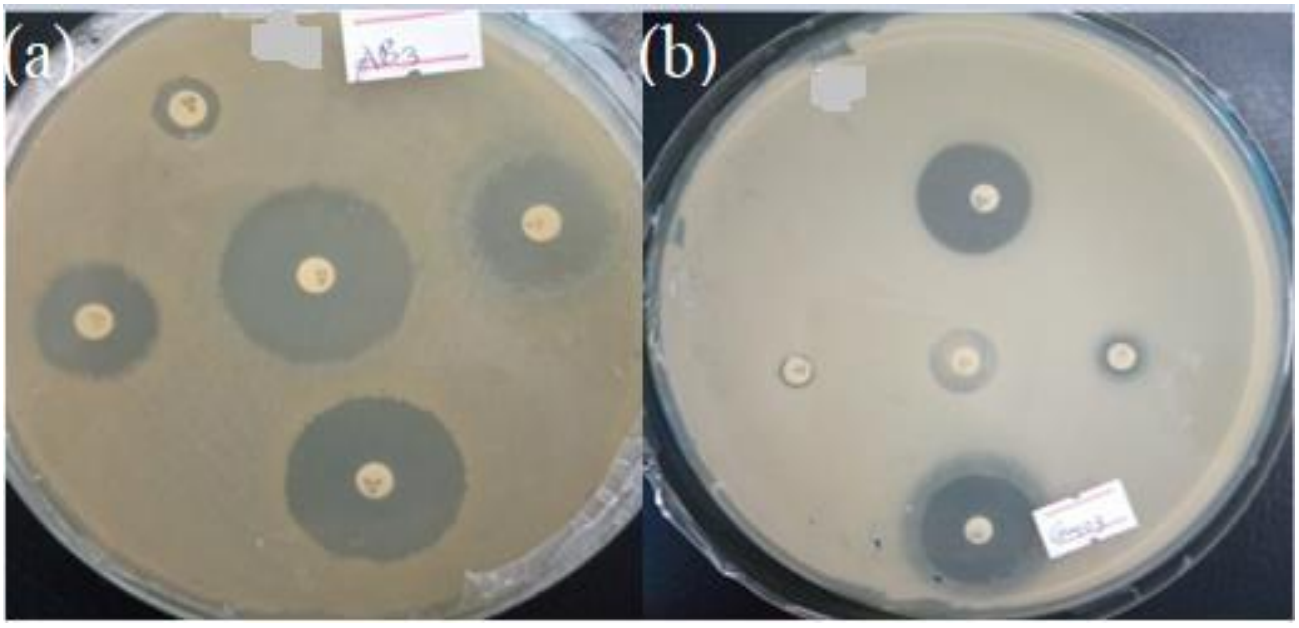



**Figure 4 Antibiotic-resistant tests for *S. aureus* by using different antibiotic disks**

**Table 2A. The diameter of the zone of inhibition measured in (mm) and classified as S, I, and R**

| Isolates ID | Types of Antibiotic Feedback | | | | | |
|---|---|---|---|---|---|---|
| | Clin | Van | Cef | E | P | MDR |
| **GU-AB33** | S | S | S | S | R | non |
| **GU –UNSH1D** | S | R | S | S | R | non |
| **GU –W1B1** | S | R | S | S | R | non |
| **GU –GU7B1** | I | R | S | S | R | non |
| **GU –WO2D** | I | R | R | S | R | yes |
| **GU –UshA** | I | R | I | R | R | yes |
| **GU -11AO** | I | R | I | R | R | yes |
| **GU –E8** | S | R | S | S | R | non |
| **GU –TOR5** | S | R | S | R | R | yes |
| **GU –AB2** | S | S | S | R | R | non |
| **GU –N1** | S | R | R | R | R | yes |
| **GU –TOR5D** | S | R | R | R | R | yes |
| **GU –UNSH1E** | I | R | S | S | R | non |

**Cli** (clindamycin), **Van** (vancomycin), **Cef** (cefazolin), **E** (erythromycin), **P**(penicillin), **S** (susceptible), **R** (resistant) and **I** (intermediate), ―**non**-represent not multi-drug resistant ―MDR(multi-drug resistant) –yes represent (multi-drug resistant).

**Table 2. B Antibiotic resistance patterns in percent**

| S.N. | List of antibiotics | Antibiotics and zone of inhibition in Percent (%) | | |
|---|---|---|---|---|
| | | Susceptible | Intermediate | Resistant |
| 1 | Cli | 66.67% | 33.33% | - |
| 2 | Van | 5.56% | 16.67% | 33.33% |
| 3 | Chef | 55.56% | 1.11% | 33.33% |
| 4 | E | 50% | 11.11% | 38.8% |
| 5 | P | - | - | 100% |

**Cli** (clindamycin), **Van** (vancomycin), **Cef** (cefazolin), **E**(erythromycin), **P**(penicillin), **S** (susceptible), **R** (resistant) and **I** (intermediate).

## 5.4. Biochemical identification

The recent isolates of *Staphylococcus* species (Table 3) were identified following the methods of ABIS online identification tools which are available at https://www.tgw1916.net/bacteria_abis.html. In Table 3, these isolates were mainly identified as *S. aureus* subsp. aureus. A few of them were identified as *S. cohnii, S. intermedius, and S. vitulinus* from the raw milk. As indicated in Table 3, these isolates are mainly able to grow on mannitol as the main carbon source in the presence of salt concentration. All are found to be Gram-positive and urease positive. Few of these isolates (Table 3) are unable to grow on mannitol salt agar. These isolates were predicted as *S. cohnii, S. intermedius, and S. vitulinus*

**Table 3. Indicated biochemical test against the recent isolates of *Staphylococcu*s obtained from raw milk**

| | Biochemical tests | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **List of Isolates** | C | IT | U | MR | TSIT | SHT | Gram | shape | VPT | Ci | M | MSA | Co | Presumptive identified |
| GU-D | + | - | + | + | + | - | + | C | - | + | NM | + | + | *S. aureus* subsp. aureus |
| GU -1 | + | - | + | + | + | - | + | C | - | - | NM | - | - | *S. aureus* subsp. aureus |
| GU -7B1 | + | - | + | + | + | - | + | C | - | + | NM | + | + | *S. aureus* subsp. aureus |
| GU -2 | + | - | + | + | + | - | + | C | - | + | NM | - | - | *S. aureus* subsp. aureus |
| GU –W1B1 | + | - | + | + | + | - | + | C | - | + | NM | + | + | *S. aureus* subsp. aureus |
| GU -3 | + | - | + | + | + | + | + | C | - | - | NM | - | - | *S. intermedius* |
| GU -4 | + | - | + | + | + | - | + | C | - | + | NM | - | - | *S. intermedius* |
| GU -5 | + | - | + | + | + | + | + | C | - | + | NM | - | - | *S. intermedius* |
| GU -7 | + | - | + | + | + | - | + | C | - | + | NM | - | - | *S. intermedius* |
| GU –AB | + | - | + | + | + | - | + | C | - | + | NM | + | - | *S. aureus* subsp. aureus |
| GU –11AO | + | - | + | + | + | - | + | C | - | + | NM | + | + | *S. aureus* subsp. aureus |
| GU – AB33 | + | - | + | + | + | - | + | C | - | + | NM | + | + | *S. aureus* subsp. aureus |
| GU –UshA | + | - | + | + | + | - | + | C | - | + | NM | + | + | *S. aureus* subsp. aureus |
| GU -AO2 | + | - | + | + | + | - | + | C | - | + | NM | + | + | *S. aureus* subsp. aureus |
| GU -W4 | + | - | + | + | + | - | + | C | + | - | NM | - | - | *S. aureus* subsp. aureus |
| GU - Unis1E | + | - | + | + | + | - | + | C | - | + | NM | + | - | *S. aureus* subsp. aureus |
| GU –AO | + | - | + | + | + | - | + | C | - | + | NM | + | + | *S. aureus* subsp. aureus |
| GU -8 | + | - | + | + | + | + | + | C | + | + | NM | + | - | *S. aureus* subsp. aureus |
| GU –E8 | + | - | + | + | + | - | + | C | - | + | NM | + | + | *S. aureus* subsp. aureus |
| GU –TOR5D | + | - | + | + | + | - | + | C | - | + | NM | + | + | *S. aureus* subsp. aureus |
| GU –AB2 | + | - | + | + | + | - | + | C | - | + | NM | + | + | *S. aureus* subsp. aureus |
| GU –N1 | + | - | + | + | + | - | + | C | - | - | NM | + | + | *S. aureus* subsp. aureus |
| GU - WO2D2 | + | - | + | - | + | + | + | C | - | - | NM | + | - | *S. cohnii* |

**(Cont..)**

| List of Isolates | Biochemical tests | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C | IT | U | MR | TSIT | SHT | Gram | shape | VPT | Ci | M | MSA | Co | Presumptive identified |
| GU - WO2D | + | - | + | - | + | + | + | C | - | - | NM | + | - | S. aureus subsp. aureus |
| GU -D0 | + | - | - | + | + | - | + | C | + | + | NM | - | - | *S. vitulinus* |
| GU-TOR5 | + | - | + | + | + | - | + | C | - | - | NM | + | + | S. aureus subsp. aureus |
| GU - GYO3 | + | - | + | + | + | - | + | C | - | + | NM | + | - | S. aureus subsp. aureus |
| GU -D1 | + | - | + | + | + | - | + | C | - | + | NM | - | - | *S. intermedius* |
| GU -D3 | + | - | + | + | + | + | + | C | - | + | NM | - | - | *S. intermedius* |
| GU -D4 | + | - | - | + | + | - | + | C | + | + | NM | - | - | *S. intermedius* |

―+‖ represents positive,-― represents negative ―C cocci, and ―NM represents nonmotile, SHT-indicated Starch hydrolysis test, MSA- Mannitol salt agar, M- Motility, Co- Coagulase, MR-Methyl red, U-Urease, C-Catalase, Ci-Citrate, IT-Indole test, TSIT-TSI test, VPT- VP test

## 3.4. Molecular characterization for newly isolated S. aureus

PCR *mecA* and nuc gene detection: From the assumed possible isolates, 70% of isolates were found to have the *mecA* gene while the 30% possible isolates were negative (Figure 5a). For instance, lane 1 (GUN1), lane 9 (GUTor5), and lane 10(GU11O) are negative to this desired gene but, lane 2(GUW1B1), lane 3 (GU7B1), lane 4 (GUAB33), lane 5 (GUD), and lane 6 (GU UnishA) were showing that there is *mecA* gene (310bp) (Figure 5A). In the detection of the *nuc* gene 279 bp, (80%) of possible isolates were found with the *nuc* gene positive while only 20% were negative for the *nuc* gene (Figure 5B).

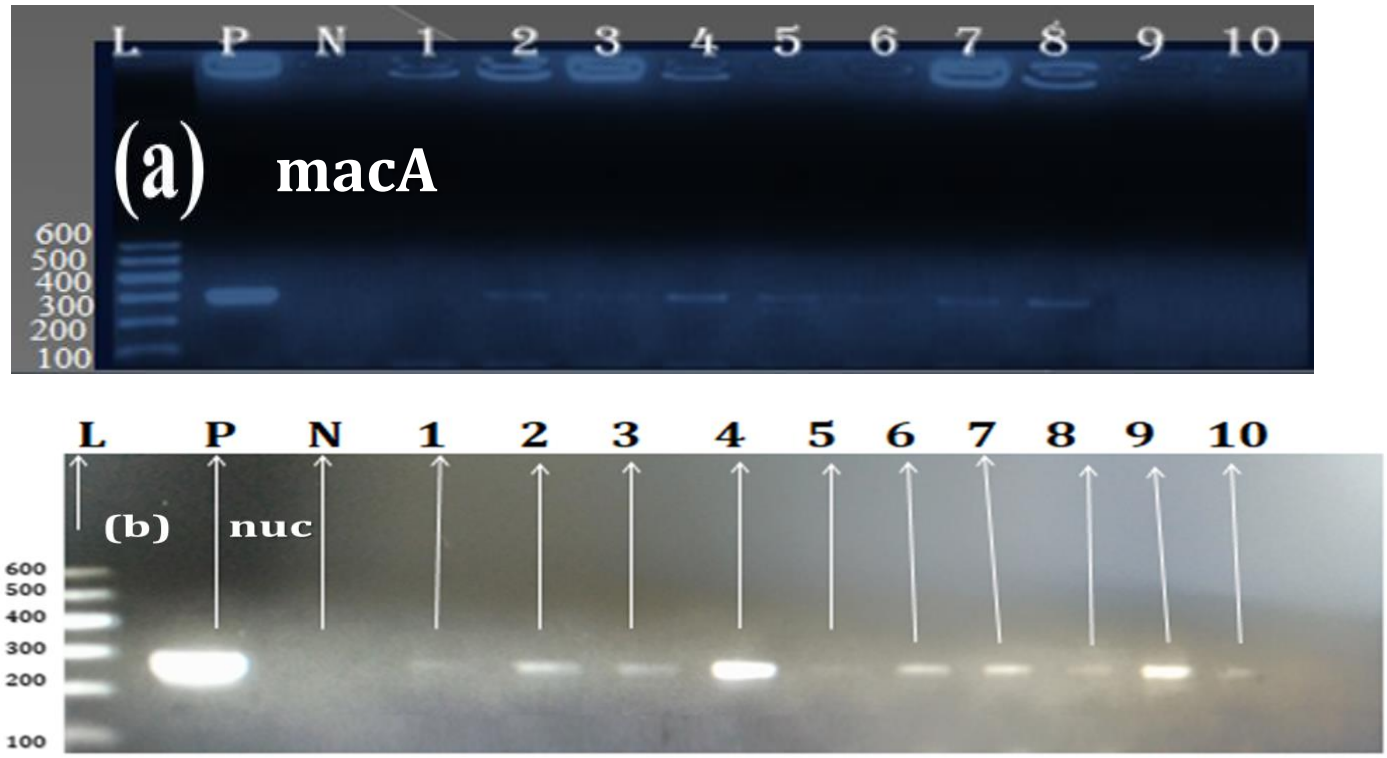



**Figure 5. The PCR amplicon separated by Gel electrophoresis**

(A) represents "*mecA*" gene products while (B) represents "*nuc*" gene products. L(marker(100bp)), N (negative control) lane 1 (GUN1), 2(GUW1B1) 3 (GU7B1), 4 (GUAB33), 5 (GUD), 6 (GU -D), 7(GU UnishA), lane 8 (GUE8) lane 9 (GUTor5),and lane 10(GU11O).

# 6. DISCUSSION

In this study, our isolates were found to be positive for catalase tests. When $H_2O_2$ was applied to the culture, the bubble gas was formed. This indicated that the recent isolates of *Staphylococcus* isolates might have contained catalase enzyme that was employed for bubble gas formation. In line with this study, it was reported that *Staphylococcus* species were able to produce catalase enzyme and employed as a distinguishing tool between *Staphylococcus* and *Streptococcus* genera (Fernandes *et al*., 2021). (Ghayyib et al , 2022) stated that certain bacterial isolates tend to produce catalase enzymes that enhance the degradation of $H_2O_2$ in water ($H_2O$) and oxygen. There was an investigation that the *Staphylococcus* isolates produce the catalase enzyme that can break down $H_2O_2$ to produce oxygen and water which leads to the development of foams (Jahan *et al*., 2015) similar results to the current research.

In this study, the majority of the most recent isolates of *Staphylococcus* isolates were shown positive for citrate test. It was observed that these positive isolates of *Staphylococcus* were shown a blue color. Similarly, it was confirmed that (Sd *et al.* 2020) the media's pH level has increased, due to the color changing from green to brilliant blue. Morphologically, these isolates of *Staphylococcus* species showed gram-positive, grapelike structure, and cocci in shape. A corresponding study shows that (Getenet *et al*., 2019) *S. aureus* is a gram-positive coccus causing severe food-borne diseases. According to Regasa *et al*., (2019), the gram-stained smears from typical colonies of expected *Staphylococci* species that show gram-positive yellow pigment on the media are considered to be *S. aureus*.

It was found that the majority of the most recent isolates were found to be negative against *Staphylococcus* isolates after the voges-Proskauer test had been carried out. These isolates might be not converting the organic acids produced by the metabolism of glucose into neutral end products like acetyl methyl carbinol. However, in the presence of strong alkali and ambient oxygen, some isolates changed acetylmethyl carbinol to diacetyl when Barritt reagent (α- naphthol) was added to the broth. According to Shoaib *et al*. (2020), substances present in the broth's peptones including diacetyl subsequently condensed to generate a reddish-pink Polymer. In the blood agar medium that supplied 5% human blood, the

current isolates of *Staphylococcus* were found to show grape-like structure in terms of morphology. The same isolates were shown creamy, grayish, white, and yellow and hemolytic features. These mentioned features are the typical characteristics of *Staphylococcus* species to distinguish them from other species (Addis *et al*., 2011).

Those isolates, which tend to be grown on the Mannitol salt agar, ferment and produce a yellow color, are expected to be *S. aureus* as it was also stated by (Scybert *et al*., 2003). With the same author, it was stated that *S. aureus can* tolerate high salt concentrations (3.5M). It has high $K^+$ in its cellular level and this cannot shift the more the stress of NaCl. A diversity of transport systems that are stimulated or induced by NaCl is accountable for the admission of osmoprotectants toward the cell (Scybert *et al.*, 2003). The *S. aureus* displays genes and protein expressions to tolerate the stress of the NaCl in addition to storing corresponding solutes to treat the turgor pressure (Scybert *et al*., 2003). Most of these isolates were golden and yellowish which is in line with the finding of (Rasheed *et al*., 2021), features used to distinguish them from *S. epidermidis*.

Isolates agglutinated in the liquid blood serum were assumed to be *S. aureus* (fig.3 O). Similar to our current finding Public Health England (2020) stated that the coagulase test separates *S. aureus* from other *Staphylococcus* species. Out of the total isolates tested for the coagulase activity, about 43.33% were positive in the coagulase test as shown. Similarly, documented Wald *et al*.(, 2019) stated that *S. aureus* is coagulase-positive, which is well-thought as an opportunistic pathogen that results in mastitis. In line with the study, it was confirmed that (Rasheed *et al*., 2021) this bacterial species can produce coagulase enzymes that are used to change the state of the plasma from loose to solid structure. The coagulase enzyme, which creates clots in plasma or blood by converting fibrinogen to fibrin, is what causes coagulation. It is believed to be involved in the formation of abscesses in host tissues and the pathogen's capacity to cause fatal sepsis(Ghayyib *et al*, 2022). In contrast with this our current study (Milion Weldeselassie *et al*, 2020), was isolated 26.2% of coagulase-positive *S. aureus* out of 146 raw milk samples. The present study is also higger than the report by (Tesfaye *et al*. 2021) that 30.6% of coagulase-positive bacteria were isolated from milk samples. These differences could be because of discrepancies in sample sites, sample gathering systems, and knowledge regarding safety in handling milk.

In the antibiotic disc diffusion determination methods, for the erythromycin antibiotics, 53.85 % of the isolate were susceptible this is relatively similar to Ebru & Aslantas, (2017). All isolates were resistant to penicillin (100%) which is similar to the work of Adugna *et al*.,( 2018); and Mama *et al*., (2019). One of the main reasons for this antimicrobial resistance may be the long-term use of antimicrobial drugs. Penicillin, which is a widely used drug, has been preferred for treating staphylococcal diseases for many years, and this has resulted in a relatively stable level of resistance in S aureus bacteria. As a result, most of these bacteria (MRSA and MSSA) contain a type of penicillin encoded by the bacterium blaZ which can hydrolyze penicillin. In contrast with this study, (Bagcigil *et al*. 2012), (61.4%) Penicillin resistance has been stated in Estonia. This difference may be due to time duration, location, and variation in environmental conditions. Other S. aureus virulence factors may be connected to penicillin resistance, which could help resistant clones spread (Bagcigil *et al*., 2012). There is no resistant isolate against clindamycin. Most of the isolates are susceptible to clindamycin (61.54%) while all are recorded as resistant to penicillin. An intermediate value was documented against clindamycin and no intermediates against erythromycin, penicillin, and vancomycin. Some of the current isolates including GU –WO2D, GU –UshA, GU -11AO, GU –TOR5, GU –N1, and GU –TOR5D are multidrug resistant. These *Staphylococcus aureus* isolates were examined, and showed signs of multidrug resistance, meaning that the isolates could not withstand the effects of three or more antibiotics. This resulted from the widespread development of multidrug resistance in S. aureus strains.

The presence of the *mecA* gene determines the existence of methicillin resistance (Ahmed *et al*., 2020). *Staphylococcal* cassette chromosome *mec* (SCCmec), a mobile genetic element, is acquired by horizontal transfer and mediates methicillin resistance(Turner *et al*., 2019). The mecA gene, which is part of a family of various but connected *Staphylococcal* chromosomal cassette (SCC) elements, encodes PBP2a(Foster, 2017). In this study there were 70% of possible isolates were found with the mecA gene when the PCR amplicon was separated by gel electrophoresis and magnified under gel documentation analysis. For instance, GUW1B1, GUGy7B1, GUAB33, GUAB2, GUD, GU UnishA, and GUE8 (well 2 to 8 respectively) contained the *mecA* gene. On the other hand, 16.6% of the mecA gene was found in the milk sample by (Hamid *et al*. 2017). These variations could result from the

various study places, animal species, and animals' physical conditions (i.e., well or ill). Similar results of the mecA gene were reported in the milk samples investigated (Tarekgne, 2016). In contrast, 30% (GUN1, GUTor5, and GU11AO) well 1, 9, and 10 respectively were negative to this gene (fig.5A). It was reported (Hamid *et a*l., 2017) that the *mecA* gene was isolated from *S. aureus* which isolated from milk sample.

When isolates were characterized using the PCR amplification, as shown in Figure 5B, isolates 1,2, 3, 4, 6, 7, 8, and 9 were positive for the *nuc* gene(fig. 5B). 80% of the possible isolates showed the presence of the nuc gene amplicons under gel separations. This result is below the result stated by (Mbindyo *et al.*, 2021) which was 100% after the separation of the amplified nuc gene through the gel-electrosis. It was stated by (Seyoum *et al*., 2015) that the presence of the nuc gene is a main important element as the marker for the conformation of *S. aureus*. Therefore based on this, positive isolates can be assumed as they are *S. aereus*. In parallel to our current analysis (Kim *et al.*, 2001) reported that the nuc gene was isolated from *S. aureus* which is isolated from a bovine milk sample. The other suggestion by (Nhatsave *et al*., 2021) stated that the *nuc* gene is present in *S. aureus*.

# 7. CONCLUSION

From this study, different biochemical tests which are important for the isolation of *staphylococcus* species were performed and 30 possible *staphylococcus* species were isolated. To screen possible *staphylococcus aureus* phenotypically as strain level from possible *staphylococcus* species, 21 possible *staphylococcus aureus* were identified based on an online advanced biological identification software. Molecularly, the antibiotic resistance genes such as; meA and nuc genes were determined for selected isolates of *S. aureas.* Both *the* mecA gene (70%) and nuc gene(80%) were found in possible isolates selected for PCR detection. Thus since the high degree of raw milk contamination with the *s. aureus*, hygienic practices during collecting, transporting, and selling are commended.

# 8. RECOMMENDATIONS

This investigation was conducted with a limited sample range to identify the antibiotic resistance gene, thus comparable research would be performed on a more area of the zone with additional parameters for many other genes encoding for antibiotic resistance. Currently, all isolates of *S. aureus* are resistant to penicillin so the use of these antibiotics for the treatment of *S. aureus* infection is not productive around the study area. However, clindamycin is more active than the all antibiotics used in this current study. To identify the emergence and distribution (spread) of innovative resistance traits it is important to detect the ways and genetic determinants of resistant organisms. An advanced study should be carried out to reduce the transmission of these resistance genes between humans and animals.

# 9. REFERENCE


Addis, M., Pal, M., & Kyule, M. N. (2011). Isolation and Identification of *Staphylococcus Species* from Raw Bovine Milk in Debre Zeit , Ethiopia. *Veterinary Research 4*(2), 45–49.

Adugna, F., Pal, M., & Girmay, G. (2018). Prevalence and Antibiogram Assessment of *Staphylococcus aureus* in Beef at Municipal Abattoir and Butcher Shops in Addis Ababa, Ethiopia. *Hindawi BioMed Res Int 2018: 1–7. doi: org/10.1155/2018/5017685*

Alsharif, S. M., El-sayed, W. S., & Hanafy, A. M. (2021). Geographic distribution and prevalence of potential asymptomatic *Staphylococcus spp*. In the nasopharyngeal cavity of elementary school boys. *Journal of Taibah University for Science*, 15(1),122-132. https://doi.org/10.1080/16583655.2021.1892991

Ahmed, M., Ahmed, H., & Abd Al-azeem, M. (2020). Molecular detection of antibiotic resistance and virulence genes in s*taphylococcus* species isolated from human and poultry. *SVU-International Journal of Veterinary Sciences*, *3*(1), 100–122. https://doi.org/10.21608/svu.2020.25542.1045

Annalisa Pantosti, Andrea Sanchini & Monica Monaco (2007). Mechanisms of antibiotic resistance in *Staphylococcus aureus*. *Future Microbiology*, *2*(3), 323–334.

Arumugam Gnanamani, Periasamy Hariharan and Hariharan and Maneesh Paul-Satyaseela Maneesh Paul-Satyaseela ( 2017). Staphylococcus aureus: Overview of Bacteriology, Staphylococcus aureus, Clinical Diseases, Epidemiology, Antibiotic Resistance and Therapeutic Approach. *Frontiers in Staphylococcus Aureus.4:* 1-26. doi.org/10.5772/67338.

Bagcigil, A. F., Taponen, S., Koort, J., Bengtsson, B., Myllyniemi, A., & Pyörälä, S. (2012). Genetic basis of penicillin resistance of *S. aureus* isolated in bovine mastitis. *Acta Veterinaria Scandinavica*, 54(1): 1–7.

Baudoux, P., Bles, N., Lemaire, S., & Tulkens, P. M. (2007). Combined effect of pH and

concentration on the activities of gentamicin and oxacillin against S*taphylococcus aureus* in pharmacodynamic models of extracellular and intracellular infections. *Journal of Antimicrobial Chemotherapy,59(2)*: 246–253. https://doi.org/10.1093/jac/dkl489.

Beyene, T., Hayishe, H., Gizaw, F., Beyi, A. F., Abunna, F., Mammo, B., Ayana, D., Waktole, H., & Abdi, R. D. (2017). Prevalence and antimicrobial resistance profile of *Staphylococcus i*n dairy farms , abattoir and humans in Addis Ababa , Ethiopia. *Hindawi BioMed Research International,* 2018 1–7. https://doi.org/10.1186/s13104-017-2487-y.

Cesur, S., & Demiröz, A. P. (2013). Antibiotics and the Mechanisms of Resistance to Antibiotics. *Medical Journal of Islamic World Academy of Sciences, 21*(4): 138–142. https://doi.org/10.12816/0002645.

Derebe Tessema and Solomon Tsegaye,(2017). Study on the Prevalence and Distribution of *Staphylococcus aureus* in Raw Cow Milk Originated from Alage Atvet College Dairy Farm , Ethiopia. *journal of food nutrition and food sciences* 7(2): 1-4. https://doi.org/10.4172/2155-9600.1000586

Dong Wang, Limei Zhang, Xuezhaang Zhou, Yulong He, Changfu Yong, M. S. O. S. and B. H. (2016). Antimicrobial susceptibility , virulence genes , and randomly amplified polymorphic DNA analysis of *Staphylococcus aureus* recovered from bovine mastitis in Ningxia , China. *American Dairy Science Association* , 99(*1)*:9560–9569. https://doi.org/10.3168/jds.2016-11625

Duran N, Ozer B, Duran GG, Onlen Y, Demir C (2012) Antibiotic resistance genes & susceptibility patterns in *staphylococci. Indian J Med Res*, 135: 389–396

Ebru, S. Y., & Aslantas, O. (2017). Antimicrobial resistance and underlying mechanisms in S*taphylococcus aureus* isolates. *Asian Pacific Journal of Tropical Medicine Journal, 10*(11):1059–1064. https://doi.org/10.1016/j.apjtm.2017.10.003

F., Bannerman, T., & Schleifer, K.-H. (2006).The Genera *Staphylococcus* and *Macrococcus.The Prokaryotes,4: 4-75*.https://doi.org/10.1007/0-387-30744-3_1.

Fernandes G, Moraes Q, Cordeiro LV, De FP (2021) Main laboratory methods used for the isolation and identification of *Staphylococcus* spp . Resumen Principales métodos de laboratorio utilizaa dos para el aislamiento Resumo Principais métodos laboratorais utilizados para o isolamento e identificação de Stap. *Rev Colomb Cienc Quím Farm* , 50: 5–28. doi: org/10.15446/rcciquifa.v50n1.95444

Foster, T. J. (2017). Antibiotic resistance in *Staphylococcus aureus* Current status and future prospects. *jouurnals investigating in science*1–20. https://doi.org/10.1093/femsre/fux007

Fufa Abunna, Tekeste Abriham, Fikru Gizaw, Takele Beyene, Ashenafi Feyisa, Dinka Ayana, B. M. and R. D. (2016). *Staphylococcus*: Isolation, Identification and Antimicrobial Resistance in Dairy Cattle Farms, Municipal Abattoir and Personnel in and Around Asella, Ethiopia . *Journal of Veterinary Science & Technology*, *7(383):1-7*. https://doi.org/10.4172/2157-7579.1000383

Gan, T., Shu, G., Fu, H., Yan, Q., Zhang, W., Tang, H., Yin, L., Zhao, L., & Lin, J. (2021). Antimicrobial resistance and genotyping of *Staphylococcus aureus* obtained from food animals in Sichuan Province, China. *BMC Veterinary Research*, *17*(1): 4–13. https://doi.org/10.1186/s12917-021-02884-z

Getabalew, M., Alemneh, T., & Akeberegn, D. (2019). Dairy Production in Ethiopia—Existing Scenario and Constraints. *biomedical journal of scientific and technical research*, 12304–12309. https://doi.org/10.26717/BJSTR.2019.16.002903

Getabalew M, Dairy H, Alemneh T (2020) Review paper Review on Milk and Milk product Handling Practices , Utilization and Microbial quality in Ethiopia. *International Journal of Dairy Sci ence andTechnol* ogy, 4: 218–224

Getenet Beyene, Girma Mamo, Tesfaye Kassa, Getnet Tasew STM (2019) Nasal and Hand Carriage Rate of *Staphylococcus aureus* among Food Handlers Working in Jimma Town , Southwest Ethiopia. *Ethiop Journal of Health Science,* 29: 605–610. doi: org/ 10.4314/ejhs.v29i5.11

Ghayyib Abbas Arif, Ahmed A ,Ahmed k, (2020).Isolation, molecular identification, and

antimicrobial susceptibility testing of staphylococcus aureus isolates.*HIVNursing*.22(2); 278-283.doi.org/10.31838/hiv22.02.56.

Ghebremedhin, B., Layer, F., König, W., & König, B. (2008). Genetic classification and distinguishing of *Staphylococcus* species based on different partial gap, 16S rRNA, hsp60, rpoB, sodA, and tuf gene sequences. *Journal of Clinical Microbiology*, *46*(3):1019–1025. https://doi.org/10.1128/JCM.02058-07

Girmay W, Gugsa G, Taddele H, Tsegaye Y, Awol N, Ahmed M, Feleke A (2020) Isolation and Identification of Methicillin-Resistant *Staphylococcus aureus* (MRSA) from Milk in Shire Dairy Farms, Tigray, Ethiopia. *Veterinary Medicine International,* 2020: 1–5. doi: 10.1155/2020/8833973

Gizaw F, Kekeba T, Teshome F, Kebede M, Abreham T, Hayishe H, Waktole H, Beyene T, Mammo B, Ayana D (2020) Heliyon Distribution and antimicrobial resistance pro fi le of coagulase-negative staphylococci from cattle , equipment , and personnel on dairy farm and abattoir settings. *Heliyon,* 6: 1–11. doi: 10.1016/j.heliyon.2020.e03606

Hamid S, Bhat MA, Mir IA, Taku A, Badroo GA, Nazki S, Malik A (2017) Phenotypic and genotypic characterization of methicillin-resistant *Staphylococcus aureus* from bovine mastitis. *Veternary World,* 10: 363–367. doi: 10.14202/vetworld.2017.363-367

Harris LG, Foster SJ, Richards RG (2002) An introduction to staphylococcus aureus , and techniques for identifying and quantifying *S . aureus* adhesins in relation to adhesion to biomaterials. *European Cells and Materials,* 4: 39–60. doi: 10.22203/eCM.v004a04

Idrees M, Sawant S, Karodia N, Rahman A (2021) *Staphylococcus aureus* biofilm : morphology , genetics , pathogenesis and treatment strategies. *Int Journal of Environment Research and Public Health* 18: 1–20. doi: org/10.3390/ijerph18147602

Jana V, Regecov I, Zigo F, Semjon B (2022) Antimicrobial Resistance of *Staphylococcus*

sp . Isolated from Cheeses. *Animals,* 12: 1–11. doi: org/10.3390/ ani12010036

Lee JH, Jeong J myung, Park Y ho, Choi S sun, Kim Y hwan, Chae J seok, Moon J san, Park H, Kim S, Eo S kug (2004) Evaluation of the Methicillin-Resistant *Staphylococcus aureus* ( MRSA ) -Screen Latex Agglutination Test for detection of MRSA of Animal Origin. *Journal of Clinical Microbiolology,* 42: 2780–2782. doi: 10.1128/JCM.42.6.2780

Li, M., Wang, X., Gao, Q., & Lu, Y. (2009). Molecular characterization of *Staphylococcus* epidermidis strains isolated from a teaching hospital in Shanghai, China. *Journal of Medical Microbiology*, *58*(4), 456–461. https://doi.org/10.1099/jmm.0.007567-0

Madhaiyan M, Wirth JS, Saravanan VS (2020) Phylogenomic analyses of the *Staphylococcaceae* family suggest the reclassification of five species within the genus *Staphylococcus* as heterotypic synonyms , the promotion of five subspecies to novel species , the taxonomic reassignment of five Staphylococ. *international journal of systematic and evoulutionary microbiology*,70: 5926–5936. doi: 10.1099/ijsem.0.004498

Manukumar HM and Umesha S (2017) MALDI-TOF-MS based identification and molecular characterization of food associated aureus. *Scientific Reports*, :7(11414), 1–16. doi: 10.1038/s41598-017-11597-z

Nhatsave N, Garrine M, Messa A, Jr. , Arsénia J. Massinga AC, Vaz R, Ombi A, Zimba TF, Alfredo H, Tchamo IM and C (2021) Molecular Characterization of Staphylococcus aureus Isolated from Raw Milk Samples of Dairy Cows in Manhiça District ,Southern Mozambique. *Microorganisms,* 9: 1–11. doi: org/10.3390/ microorganisms9081684.

Normanno G, Salandra G La, Dambrosio A, Quaglia NC, Corrente M, Parisi A (2007) Occurrence , characterization and antimicrobial resistance of enterotoxigenic Staphylococcus aureus isolated from meat and dairy products. *Int J Food Microbiol* 115: 290–296. doi: 10.1016/j.ijfoodmicro.2006.10.049

Pervin R, Rahman A, Zereen F, Ahmed R (2019) Isolation , Identification and Characterization of Staphylococcus Aureus from Raw Milk in Different Places of Savar , Bangladesh. *International Journal of Sciences: Basic and Applied Research*, 48: 1–25.

Peterson, E., & Kaur, P. (2018). Antibiotic resistance mechanisms in bacteria: Relationships between resistance determinants of antibiotic producers, environmental bacteria, and clinical pathogens. *Frontiers in Microbiology*, *9*, 2928.

Public Health England N (2020) UK Standards for Microbiology Investigations. *UK Stand Microbiol Investig* , 3(4): 1–26

Rasheed NA, Hussein NR (2021) Staphylococcus aureus : An Overview of Discovery , Characteristics , Epidemiology , Virulence Factors and Antimicrobial Sensitivity. *Eur J Mol Clin Med* 08: 1160–1183

Regasa, S., Mengistu, S., & Abraha, A. (2019). Milk Safety Assessment, Isolation, and Antimicrobial Susceptibility Profile of Staphylococcus aureus in Selected Dairy Farms of Mukaturi and Sululta Town, Oromia Region, Ethiopia. *Veterinary Medicine International*, *2019*. https://doi.org/10.1155/2019/3063185

Reta, M. A., Bereda, T. W., & Alemu, A. N. (2016). Bacterial contaminations of raw cow ' s milk consumed at Jigjiga City of Somali Regional State , Eastern Ethiopia. *International Journal of Food Contamination*. https://doi.org/10.1186/s40550-016-0027-5

Rossi, C. C., Pereira Monalessa Fábia, and M. G. (2020). Underrated Staphylococcus species and their role in antimicrobial resistance spreading. *Genetics and Molecular Biology*, *43*(1), 1–10. https://doi.org/org/10.1590/1678-4685-GMB-2019-0065

Sage Desalegn LI and KW (2018) Analysis of physico-chemical properties and heavy metals content of drinking water from selected areas in. *African journal of pure Applied Chemistry* 12: 9–13. doi: 10.5897/AJPAC2017.0729

Sandeep Kumar Dash, Subhankari Prasad Chakraborty, Debasis Mandal And Somenath Roy, (2012). Isolation and characterization of multi drug resistant uropathogenic *escherichia coli* from urine sample of urinary tract infected patients. *Life science*.2(1); 25-39.

Schmidt, T., Kock, M. M., & Ehlers, M. M. (2017). *Molecular Characterization of Staphylococcus aureus Isolated from Bovine Mastitis and Close Human Contacts in South African Dairy Herds: Genetic Diversity and Inter-Species Host Transmission*, *8*(511) : 1-15. https://doi.org/10.3389/fmicb.2017.00511

Scybert, S., Pechous, R., Sitthisak, S., Nadakavukaren, M. J., Wilkinson, B. J., & Jayaswal, R. K. Ã. (2003). *NaCl-sensitive mutant of Staphylococcus aureus has a Tn917-lacZ insertion in its ars operon*. *Federation of European Microbiological Societies* , *222*, 171–176. https://doi.org/10.1016/S0378-1097(03)00312-4.

Sd S, Ghosh S, Meghanachowdary M, S MM (2020) isolation and molecular characterization of plastic degrading bacteria from dumped garbage soil. *Innovative journal of life science*,8(5): 1–5.

Seyoum, E. T., Asrat, D., Mihrete, T. S., Adkins, P., & Ababa, A. (2015). *Phenotypic and genotypic characterization of Staphylococcus aureus isolates recovered from bovine milk in central highlands of Ethiopia*. *African Journal of Microbiology Research*https://doi.org/10.5897/AJMR2015.7562

Shoaib M, Muzammil I, Hammad M, Bhutta ZA, Yaseen I (2020) A Mini-Review on Commonly used Biochemical Tests for Identification of Bacteria. i*nternational Journal of Research Publications*54. doi: 10.47119/ijrp100541620201224.

Sneha Bhat, Nichith K R, Kiran Y, Nagendra M, Pallavi S L, Shreya S, Pruthvi B and Madhumita Ghosh Dastidar, 2017. Production of bioplastics from microorganisms.*International journal of advanced research*. 5(2), 2710-2716. doi: 10.21474/ijar01/3465

Tarrekgne EKT (2016) Staphylococcus aureus from Milk and Milk Products in Ethiopia : Prevalence , Enterotoxigenic Potential , Antibiotic Resistance and spa types,

Volume 48. 1–20 pp.

Tesfaye Kaleab, Gizaw Zemichael, Feleke Akalu (2021) Prevalence of Mastitis and Phenotypic Characterization of Methicillin-Resistant Staphylococcus aureus in Lactating Dairy Cows of Selected Dairy Farms in and Around Adama Town , Central Ethiopia. *Environ Health Insights* 15: 1–8. doi: 10.1177/11786302211021297.

Thaker, H. C., Brahmbhatt, M. N., & Nayak, J. B. (2013). *Isolation and identification of Staphylococcus aureus from milk and milk products and their drug resistance patterns in Anand , Gujarat*. *Veternary World*, 6(1): 10–13. https://doi.org/10.5455/vetworld.2013.10-13

Thomson, P., Garc, P., Miles, J., Isla, D., Camilo, Y., Santib, R., Andrea, N., Flores-y, C., & Cuadra, F. (2022). *Veterinary sciences Isolation and Identification of Staphylococcus Species Obtained from Healthy Companion Animals and Humans. veternary science9(79):1-11*. doi.org/10.3390/ vetsci9020079.

Tilahun, B., Yinur, D., Zenabu, D., & Menesha, F. M. (2020). *Isolation and identification of Enset wilt disease causing bacteria using 16S rRNA Gene Sequence samples collected from Gurage zone , Ethiopia*. J*ournal of Life Science and Biomedicine*, *10*(4): 51–58. DOI: https://dx.doi.org/10.36380/scil.2020.jlsb7Isolation

Tsai, M., Ohniwa, R. L., Kato, Y., Takeshita, S. L., Ohta, T., Saito, S., & Hayashi, H. (2011). *Staphylococcus aureus requires cardiolipin for survival under conditions of high salinity*. *BMC Microbiology,11: 1-12. http://www.biomedcentral.com/1471-2180/11/13.*

Turner, N. A., Sharma-kuinkel, B. K., Maskarinec, S. A., Eichenberger, E. M., Shah, P. P., Carugati, M., Holland, T. L., & Fowler, V. G. (2019). Methicillin-resistant Staphylococcus aureus: an overview of basic and clinical research. *Nature Reviews Microbiology*, *Nature Reviews Microbiology,17*: 203–218. doi.org/10.1038/s41579-018-0147-4.

Velázquez-ordoñez, V., Valladares-carranza, B., Tenorio-borroto, E., Talavera-rojas, M.,

Varela-guerrero, J. A., Acosta-dibarrat, J., Puigvert, F., Grille, L., & Revello, Á. G. (2019). Microbial Contamination in Milk Quality and Health Risk of the Consumers of Raw Milk and Dairy Products. *intech open,1-25. DOI: 10.5772/intechopen.86182*

Wald, R., Hess, C., Urbantke, V., Wittek, T., & Baumgartner, M. (2019). *Characterization of Staphylococcus Species Isolated from Bovine Quarter Milk Samples*. *animals*,*90*, 1–16. doi:10.3390/ani9050200.

Yigrem, S., Beyene, F., Tegegne, A., & Gebremedhin, B. (2008). Dairy production, processing and marketing systems of Shashemene–Dilla area, South Ethiopia. *IPMS (Improving Productivity and Market Success) of Ethiopian Farmers Project Working Paper*, *9*.

zelalem Ayana, M. Y. and Z. A. (2015). *Food-Borne Bacterial Diseases in Ethiopia*. *Academic Journal of Nutrition*, *4*(1):62–76. https://doi.org/10.5829/idosi.ajn.2015.4.1.95168.

Kim, C., Khan, M., Morin, D. E., Hurley, W. L., Tripathy, D. N., Jr, M. K., Oluoch, A. O., & Kakoma, I. (2001). Optimization of the PCR for Detection of Staphylococcus aureus nuc Gene in Bovine Milk. *Journal of Dairy Science*, *84*(1), 74–83. https://doi.org/10.3168/jds.S0022-0302(01)74454-2

Madhaiyan, M., Wirth, J. S., & Saravanan, V. S. (2020). Phylogenomic analyses of the Staphylococcaceae family suggest the reclassification of five species within the genus Staphylococcus as heterotypic synonyms , the promotion of five subspecies to novel species , the taxonomic reassignment of five Staphylococ. *International Journal of Systematic and Evoulutionary Microbiology*, *70*, 5926–5936. https://doi.org/10.1099/ijsem.0.004498

Mama, O. M., Dieng, M., Hanne, B., Ruiz-ripa, L., Gueye, C., Diop, M., & Torres, C. (2019). *Genetic characterisation of staphylococci of food-producing animals in Senegal . PVL detection among MSSA*. 1–6.

Milion Weldeselassie, Getachew Gugsa, Ashwani Kumar, Yisehak Tsegaye , Nesibu Awol

, Meselu Ahmed NA and HT (2020) Isolation and Characterization of Staphylococcus aureus From Food of Bovine Abstract : *Open Microbiol J* 14: 234–241. doi: 10.2174/1874285802014010234.

Nhatsave, N., Garrine, M., Messa, A., & Massinga, J. (2021). Molecular Characterization of *Staphylococcus aureus* Isolated from Raw Milk Samples of Dairy Cows in Manhiça District. *Microorganisms*,9: 1–11. doi.org/10.3390/ microorganisms9081684.

Quigley, L., Sullivan, O. O., Beresford, T. P., Ross, R. P., Fitzgerald, G. F., & Cotter, P. D. (2011). International Journal of Food Microbiology Molecular approaches to analysing the microbial composition of raw milk and raw milk cheese. *International Journal of Food Microbiology*, *150*(2):81–94. https://doi.org/10.1016/j.ijfoodmicro.2011.08.001.

Tarekgne, E. K. T. (2016). Staphylococcus aureus from Milk and Milk Products in Ethiopia : Prevalence , Enterotoxigenic Potential , Antibiotic Resistance and spa types. In *Norwegian University of Life Sciences*, 48.