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STUDY ON OCCURRENCE, ISOLATION AND ANTIMICROBIAL SUSCEPTILBILITY PROFILE OF *STAPHYLOCOCCUS AUREUS* FROM RAW COW MILK AND TRADITIONALLY PROCESSED DAIRY PRODUCTS IN ADAMA AND DEBREBERHAN TOWNS, ETHIOPIA

MSc THESIS

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DEPARTMENT OF VETERINARY MICROBIOLOGY, IMMUNOLOGY AND PUBLIC HEALTH

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A thesis submitted to the school of graduate studies of Addis Ababa University in partial fulfilment of the requirements for the degree of Master of Sciences in Veterinary Public Health

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STATEMENT OF THE AUTHOR

I declared that I am the author of this thesis and that all information sources used throughout the work were properly acknowledged. This thesis has been submitted in partial fulfilment of the requirements for an MSc degree at Addis Ababa University, College of Veterinary Medicine, and Agriculture, and is placed in the university or college library to be made available to borrowers in accordance with the library's rule. I honestly declare that this thesis is not being submitted to, or accepted by, any other institution for the purpose of receiving any academic degree, diploma, or certificate.

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LIST OF ABBREVIATIONS

BHIA	Brain heart infusion agar
BHIB	Brain heart infusion broth
BTS -QC	Bacterial test standard Quality control
CA-MRSA	Community acquired methicillin resistant *S. aureus.*
CDC	Centre for disease prevention and control
CNS	Coagulase negative *Staphylococcus*
CPS	Coagulase positive *Staphylococcus*
FBD	Food Borne Disease
HA-MRSA	Hospital acquired methicillin resistant *S. aureus.*
MALDI- TOF	Matrix assisted laser desorption time of flight.
MCC	Milk collection centres
MGE	Mobile genetic elements
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSA	Mannitol salt agar
PBP	Penicillin binding protein
PCR	Polymerase Chain reactions
SCC	Staphylococcal chromosomal cassette
SE	Staphylococcus Enterotoxine
SEA	Staphylococcus Enterotoxine A
SEB	Staphylococcus Enterotoxine B
SEC	Staphylococcus Enterotoxine C
SED	Staphylococcus Enterotoxine D
SFP	Staphylococcus Food Poisoning
VISA	Vancomycin intermediate *S. aureus*
VRSA	Vancomycine resistant *S. aureus*
VSSA	Vancomycine susceptible *S. aureus*
WHO	World Health Organization

ABSTRACT

Staphylococcal foodborne intoxication is among the most known food-borne illness with global distribution. Particularly, *S. aureus* antimicrobial resistance has rapidly grown into a major worldwide health issue. This study was intended to determine the prevalence of *S. aureus* and its antimicrobial susceptibility profiles in raw milk and traditionally processed dairy products in Adama and DebereBerhan towns. A total of 421 samples (188 raw milk, 80 traditionally processed dairy products, 73 milkers' hand swabs, 38 bucket swabs, and 42 bulk tank milk from collection centers) were sampled and analysed from December 2022 to May 2023. The samples were cultured to identify *S. aureus* following a standard microbiological procedure and using MALDI-TOF MS Bruker method. The antimicrobial susceptibility of the isolates was performed by a panel of 9 antimicrobials using the Kirby-Bauer disc diffusion technique. The association between the occurrence of *S. aureus* and potential risk factors was assessed using logistic regression. Resistance of the isolate to penicillin G and ampicillin were identified for the presence of *blaZ* gene using PCR. *S. aureus* was isolated from 32 (17.02%) of raw milk, 7(9.59%) of milkers' hand swab, 9 (21.43%) of bulk tank milk from collection centre, 3 (7.89%) of bucket swabs, 2 (5%) of yoghurt and 1(2.5%) of cottage cheese. A significant difference with a higher rate of contamination with *S. aureus* was observed among samples (p =0.021). Out of 54 *S. aureus* isolates subjected to 9 antimicrobials, 53(98.15%) isolates were developed resistance to penicillin G and Ampicillin. Twenty-three (42.6%) of the isolates were developed resistance to Tetracycline and 7 (12.96%) of the isolates were developed resistance to Erythromycin. In addition to this, 3 (5.56%) of the isolates were developed resistance to Clindamycin. However, among the 21 isolates subjected to DNA amplification for detection of resistance gene, only a single (4.76%) isolate was positive for *blaZ* gene. In conclusion, this study showed that raw milk and other dairy products were highly contaminated with *S. aureus.* Most of the isolates developed multi-drug resistance, which is a serious public health risk for the dairy consumers of the study area. Additionally, there is a lack of agreement between the phenotypic and genotypic (*blaZ* gene) detection, and this necessitates additional research to determine the mechanism by which *S. aureus* develops resistance to β-lactam drugs particularly penicillin, and ampicillin.

Keywords**:** *Antimicrobial resistance, Dairy products, Milkers hand, S. aureus, MALDI-TOF MS*

1. INTRODUCTION

Dairy products like milk have traditionally been considered essential foods for human physical and cognitive development due to the nutrients they contain, which are essential for growth and healthy development (Pereira, 2014). Raw milk and other milk derivatives are consumed in homes and even for business purposes as a supplement to regular meals (Maduka *et al.,* 2013). Despite milk and other dairy products having such significant benefits, they also serve as an excellent environment for microbial growth, which has a detrimental effect on consumers' health (Muehlhoff *et al*., 2013). Specifically, unhygienic, milking, milk handling and traditionally processing of milk products, especially those made from raw milk under unhygienic conditions, are prone to contamination and have the potential to cause foodborne infections and intoxication by organisms such as toxigenic *Staphylococcus aureus* (Kadariya *et al.,* 2014).

Staphylococci are normal inhabitants of mucous membranes and skin in both humans and animals (Boucher and Corey, 2008). It has been discovered that pathogenic strains of Staphylococci, which are typically coagulase-positive, can infect their hosts anywhere in the world (Larsen *et al.,* 2000). *Staphylococcus aureus*-related diseases in cattle can range in severity from mild toxic shock syndrome to simple abscesses and mastitis (Onasanya *et al*., 2003). *Staphylococcus aureus*, particularly animal associated strains, has regularly been found in raw milk and milk products all around the world (Hasman *et al.,* 2010). *S. aureus* contaminated milk can lead to severe infections and *staphylococcal*-related toxins (Grispoldi *et al.,*2021; Grispoldi *et al.,* 2019).

Staphylococcus aureus can be found in milk because of contamination during milking or from milk harvested from cows with subclinical mastitis cases (Grispoldi *et al.,* 2019). Furthermore, it may become contaminated during or after milking if inadequate hygiene practices are used, such as not washing hands properly before handling milk storage equipment or coughing or sneezing (Singh and PrAkASh, 2010). When contaminated food is consumed, *Staphylococcus aureus* is a major contributor to gastroenteritis in people. Stomach cramping, diarrhoea, nausea, vomiting, retching, and prostration are the most typical symptoms observed (Kadariya *et al.,* 2014).

Staphylococcal food poisoning (SFP) is one of the most common food-borne diseases worldwide, being second to salmonellosis in terms of prevalence (Acco *et al.,* 2003; Aycicek *et al.*, 2005). Staphylococci cause food contamination, decomposition, and a decrease in food quality and shelf life, as well as food poisoning through the formation of fatal enterotoxins (Smith *et al*., 2007). Incidence of Staphylococcus varies between farms and dairy products depending on how they are handled, stored, or milked. There are also genetic variations in disease resistance among the breeds in dairy farms (El-Malt *et al*., 2013; Lee *et al.,* 2012).

In the dairy industry, *S. aureus* contamination of dairy cows and raw milk is still a problem (Pal *et al.,* 2020). *S. aureus* is important for public health because of the numerous outbreaks of food-borne illnesses associated with contaminated dairy products (McMillan *et al.,* 2016). Dairy animals are probably the main source of contamination of raw milk with *Staphylococcus aureus* (Jørgensen *et al*., 2005).

To treat infections in humans and animals, antibiotic therapy is an essential tool, but unfortunately, it is facing the challenges of drug resistance and becoming less effective in treating illnesses in both humans and animals (Economou and Gousia, 2015). Due to their availability over the counter, lack of access to effective antimicrobial medication, unregulated supply chains, purchase without a prescription, and usage of incompetent individuals to treat animals, antibiotics are overused and abused in underdeveloped nations, including Ethiopia (Kimera *et al*., 2020). In addition, experts in human and animal health are contribute to the rise of antimicrobial-resistant staphylococci, which may be caused by the indiscriminate use of antibiotics by medical professionals, untrained practitioners, and pharmaceutical users (Pekana *et al*., 2017).

The increased usage of antibiotics facilitates the likelihood that bacterial infections may develop resistance to them and that these bacteria will spread to humans through the food chain (Guo *et al*., 2021). A significant contributor to the selection pressure and prevalence of antibiotic-resistant bacteria is the widespread use of antibiotics in both human medicine and agriculture, particularly in the prevention of disease and growth promotion in animal husbandry (Jamali *et al*., 2015). In Ethiopia, there has been a lot of drug use that deviates from recommended dosages, which encourages the emergence of drug resistant strain (Kifle and Tadesse, 2014). Even though a lot of finding have reported the contamination rate of

milk and milk products by *S. aureus* in various parts of the country; there is a lack of scientific information regarding the prevalence and associated risk factors for the occurrence of *S. aureus* in the study area, Furthermore, the antimicrobial resistance pattern of *S. aureus* and its prevalence in other dairy products were not well investigated, which is otherwise so crucial for establishing efficient food safety management and preventive measures along the dairy value chain. Therefore, this study was conducted to estimate the prevalence of *S. aureus* and determine the antimicrobial susceptibility profile of *Staphylococcus aureus* from dairy and environmental samples in Adama and DebreBerhan towns.

Specific objective of this study

- To assess the occurrence of *S. aureus* in raw milk and traditionally processed dairy products, milkers hand swab and swabs of milking equipment
- To evaluate antimicrobial susceptibility profile of *S. aureus* isolate
- To determine associated risk factors for the occurrence of *S. aureus* in raw milk samples

2. LITERATURE REVIEW ON *STAPHYLOCOCCUS AUREUS*

2.1. General Features of Staphylococci

The Scottish physician Sir Alexander Ogston discovered staphylococcus in pus from a surgical abscess in a knee joint for the first time in 1880. The Greek word *staphyle* (meaning bunch of grapes) and *kokkos* (meaning berry) were used to create the name staphylococcus (Khan, 2017; Gnanamani *et al.*, 2017). Staphylococcus is a genus of gram-positive, spherical bacterium that frequently causes mastitis, respiratory illnesses, food poisoning, and surgical and skin infections. In 1884, German physician Friedrich Julius Rosenbach distinguished the bacteria based on the colour of their colonies: *S. aureus* (from the Latin *aurum*, gold) and S. albus (from the Latin *albus*, white). Because it is so common on human skin, *S. albus* was later renamed *S. epidermidis* (Sejvar, 2013).

Staphylococcus is the coccoid, spherical, nonsporulating, and nonmotile bacteria. It can be seen under a microscope in pairs, short chains, or clusters that resemble grapes. They are Gram and catalase positive facultative aero-anaerobic bacteria (Harris *et al.*, 2002). Staphylococci are present everywhere in the environment, including the air, dust, sewage, water, natural surfaces, human, and animals (Hennekinne, 2018).

More than 50 species and 28 subspecies of staphylococci have been identified so far based on their ability to produce coagulase. This categorization distinguishes between coagulase-utilizing strains coagulase positive staphylococcus (CPS) and those that do not coagulase negative staphylococcus (CNS) (Podkowik *et al.*, 2013). *S. aureus, S. epidermidis,* and *S. saprophyticus* are three of the species that are frequently linked to infections in humas (Wandhare *et al.*, 2020).

Most coagulase-negative staphylococci are not harmful (Abebe *et al.*, 2020). Because it is well established that several CNS species are necessary for the fermentation of meat and dairy products, they are classified as food grade (Otto, 2010). It has long been controversial to discuss the CNS's possible enterotoxigenicity because of the CNS, enterotoxin-like genes or enterotoxin production have not been discovered despite extensive research (Becker *et al.,* 2001).

2.2. Staphylococcus aureus

Staphylococcus aureus is a spherical, 0.5-1.5 µm in diameter, catalase and gram-positive, bacterium. It is non-motile and non-spore forming bacterium (Harris *et al*., 2002). Gram-staining gives *S. aureus* a bluish/purple appearance, and it can be seen microscopically as *S. aureus* can thrive on media with up to 10% salt, and its colonies frequently have a golden or yellow colour (the Latin word *aureus* means "golden or yellow"). It is a facultative anaerobe that uses fermentation or aerobic respiration to obtain energy for growth. *S. aureus* divides through binary fission, with distinct planes serving as the sites for cell division. It grows best at temperatures between 18 to 40 °C (Taylor *et al*., 2022). According to Rasigade and Vandenesch (2014), *S. aureus* is positive for most biochemical tests including catalase, coagulase, mannitol fermentation, and novobiocin sensitivity.

Most healthy people have *S. aureus* on their skin and mucous membranes, most frequently in the nasal region. *S. aureus* can also be found in the environment and in typical human flora (Ganamani *et al.*,2017; Bergdoll and Lee Wong, 2006). On healthy skin, *S. aureus* usually does not cause infection; however, if the bloodstream or internal tissues are opened to the germs, several potentially harmful diseases may result (Lowy, 1998). *S. aureus* can also be discovered in milk when it causes mastitis, and under the right circumstances, it can quickly contaminate at high levels. Enterotoxigenic *S. aureus* strains can create thermostable enterotoxins when growing in food including milk, and these enterotoxins can then be consumed and result in gastrointestinal symptoms such as nausea, vomiting, and diarrhoea (Kashif *et al.,* 2019).

2.3. *Staphylococcus aureus* Biotypes and Reservoirs

S. aureus is a typical member of the flora that inhabits the skin and mucous membranes of mammals and birds (Boucher and Corey, 2008). According to report of Ganamani *et al.* (2017) and Bergdoll and Lee Wong (2006), at least 50% of healthy individuals also carry these bacteria in their skin, hair, and throat in addition to their nasal passages.

S. aureus can be isolated from animals, with the bovine being the most significant due to the role of staphylococci in mastitis (Haag *et al*., 2019). Staphylococci can be found in the air, dust, water, and animal and human waste, although they are primarily transferred between

people and animals (Bergdoll and Lee Wong, 2006). Several biotypes of the *S. aureus* species isolated from a range of hosts, including humans, chickens, cattle, sheep, and goats, have been reported. These biotypes demonstrate how well-adapted the bacteria is to its host (Hennekinne, 2018).

In case of infected udder, *S. aureus* can contaminate milk during milking with a density of 101 to 108 CFU/ml, most usually about 104 CFU/ml (Boynukara *et al*., 2008). According to Peles *et al*. (2007), *S. aureus* causes 30 to 40% of all mastitis cases worldwide. In primary production, the dairy environment, people, and the operational environment are the biggest contributors to product contamination, besides milk-producing animals. Because of this, bacteria are frequently found on human skin, in the gastrointestinal and urogenital systems, as well as in the nose, axilla, and umbilicus (Gnanamani *et al*., 2017).

The frequency of enterotoxigenic strains isolated from humans is high, varying between 40% and 60%. The organisms find their way into food through hands (infected wounds, skin lesions) or by coughing and sneezing (Lawrynowicz-Paciorek *et al.,* 2007). *S. aureus* can survive in milk because lactose phosphotransferase systems are present, as determined by genomic sequence analysis. Additionally, vitamin B (thiamine and nicotinic acid) and inorganic salts are necessary for the proliferation of staphylococci. Leucine, tyrosine, and glutamic acid are all necessary for the formation of enterotoxins but not for growth (Medveova and Valk, 2012).

2.4. The Prevalence and Importance of *S. aureus* in the Dairy Industry

The milking animal, animal handlers, milking environment, utensils, industry pipes, packing environment, and storage environment are all possible entry points for *S. aureus* into the milk supply chain from production to processing and storage (Lawrynowicz-Paciorek *et al*., 2007). The presence of *S. aureus* in the milk supply chain has a substantial impact on overall quality, customer safety, demand, and the global supply chain. Animals and food products made from animals have a big impact on public health, because food sourced from animals may include one or more staphylococcal enterotoxins that are already developed and can harm humans (Pal *et al*., 2020).

S. aureus contamination of processed milk products can result from several causes, including the direct use of raw milk, inadequate pasteurisation, post-pasteurization contamination, and the development of resistance (De Silva *et al*., 2021). Milk is a rich source of nutrients for the development of enterotoxins and the proliferation of *S. aureus. S. aureus,* including multi-drug resistant strains, is frequently discovered in raw milk, processed milk, and milk products (Zeinhom and Abed, 2020; De Silva *et al*., 2021).

The frequent reports of *S. aureus* contamination in milk products from all over the world are proof that the bacteria can transfer easily from raw milk to processed milk products. Cows with subclinical mastitis can serve as secondary *S. aureus* reservoirs, posing a risk of contamination to the dairy supply chain. Clinical and subclinical mastitis caused by *S. aureus* in cattle results in decreased milk production, spoiled milk, milk with lower nutritional value, milk with an unstable flavour, and milk with a shorter shelf life and yield of milk products (Pal *et al.,* 2020). In Ethiopia, *S. aureus* was present in raw cow milk with a prevalence of 30.7% (95% CI: 26.6-35) (Deddefo *et al*., 2022).

2.5. Characteristics of Staphylococcal Enterotoxins

The Staphylococcal enterotoxins are small proteins that are members of a wide family of pyrogenic toxin super antigens that are secreted in the medium and soluble in water and saline solutions (Aycicek *et al*., 2005). They are encoded by phage, chromosomal, or plasmid genes. Lysine, aspartic acid, glutamic acid, and tyrosine residues are abundant in them (Salandra *et al*., 2008). Most of them have a cystine loop that is necessary for correct conformation and is likely responsible for the emetic activity (Salandra *et al*., 2008).

This Exoproteins created in food and consumed by people result in acute gastroenteritis symptoms, which are the cause of SFP. The toxins are low-molecular-weight proteins with an amino acid composition only that are often produced by CPS species and range in size from 27 to 31 kDa (Chiang *et al*., 2008). *S. aureus* produce several heat stable enterotoxins (e.g., enterotoxin A) responsible for food poisoning; several cell membranes targeting toxins (alpha, beta, gamma, and delta and other extracellular proteins, such as, hemolysins, and leukocidins) (Kashif *et al.,* 2019).

Classical staphylococcal toxins (SE-A to SE-E) are responsible for more than 90% of food poisoning cases caused by *S. aureus* outbreaks because their great stability and resistance to most proteolytic enzymes, including pepsin and trypsin, staphylococcal enterotoxins maintain their activity in the digestive tract even after consumption; additionally, they can withstand temperatures of 100°C for at least 30 minutes and perhaps longer because of their strong heat resistance (Wang *et al*., 2018).

The heat-labile staphylococci cells are destroyed by pasteurization and cooking, but thermo-stable SEs usually maintain their biological activity (Aycicek *et al*., 2005). SE are more heat stable than staphylococci bacteria and it is possible to test a food product and get positive SEs results while getting negative results for staphylococci culture. Because of this, illnesses may manifest even when no viable bacteria can be isolated from the allegedly contaminated food (Soejima *et al*., 2007). According to Salyers and Whitt (2002), the composition of the food, competition from other microorganisms (the presence of other bacteria affects the production of enterotoxin apparently by limiting the staphylococci multiplication), temperature, and time all affect the number of enterotoxins produced.

The antigenic types (designated SE-A, B, C, D, and E) of the family of 14 different SE types, which have structural and sequence characteristics, have been identified and are most frequently found in SFP (Kerouanton *et al*., 2007). Because of the toxic shock syndrome toxin was mistakenly labelled as enterotoxin F (SEF) when it was initially discovered, there is no enterotoxin F (SEF) (Orwin *et al*., 2001; Walderhaug, 2007). In general, SE-A is recovered from outbreaks of food poisoning more frequently than any of the others, SE-D is second, and SE-E is associated with the lowest outbreaks. Low levels of staphylococcal enterotoxin B exposure might result in fever, coughing, headaches, vomiting, and some nausea (Shah, 2003).

SElJ, SElK, SElL, SElM, SElN, SElO, SElP, SElQ, SER, SES, SET, SElU, SElV, and SElW are recent additions to the SE family. Many of these recently found enterotoxins share structural similarities with classical enterotoxins, indicating that they could cause foodborne illness if taken in quantities that are high enough (Rall *et al*., 2008). Future studies and increased surveillance are needed to better understand the role of these SEs in producing foodborne poisoning (Rall *et al*., 2008; Ono *et al*., 2008).

A bioterrorist attack could use staphylococcal toxins as a biological agent by contaminating food or water or by dispersing them and inhaling toxins. High doses of the toxin have a much more serious effect (CDC, 2010). Due to the significance of these toxins for both public health and the food industry, an effective screening to find the presence of enterotoxin strains in foods is necessary. In fact, not all staphylococci produce SEs, and even when they do, the amount produced may not be enough to cause food intoxication (Morandi *et al*., 2007).

2.6. Resistance to the Microenvironment and Processing Conditions of *S. aureus*

The ability of *S. aureus* to quickly adapt to environmental changes influence its pathogenicity (Alreshidi *et al*., 2015). *S. aureus* has been proven to survive through a wide variety of environmental conditions such as cold, low water activity (Aw). Each environmental stress is known to affect the expression of several cellular functions, virulence factors, and antimicrobial resistance determinants which provide the organism capacity to survive under stressful circumstances (Anderson *et al*., 2006). In addition to this, *S. aureus* with other staphylococci forms a biofilm to increase their host defence mechanisms and environmental stress (Abdi *et al*., 2018).

These bacteria have a considerably wider water activity range than other food-associated infections. A minimum water activity of 0.83 (about 20% NaCl) is required for *S. aureus* to survive and grow. Depending on the type of toxin, the water activity conditions for SE generation are somewhat different from those for bacterial growth. When all other factors are favourable, *S. aureus* can proliferate since practically all water activity circumstances result in the formation of SEA and staphylococcal enterotoxin D (SED). Staphylococcal enterotoxin B (SEB) production is highly sensitive to water activity decreases; despite substantial growth, very little SEB is generated at water activity 0.93. The impact of water activity on SEC production follows the same pattern as SEB production (Qi and Miller, 2000).

The humectant used to reduce water activity, PH, atmospheric composition, and incubation temperature are other significant variables that have an impact on growth and generation of SE (Hajmeer *et al.,* 2006). The conditions for growth and production of SE in laboratory media and in food, may differ some extent. Studies on *S. aureus* osmoadaptive techniques have shown that when cells are cultured in a low water medium, they react by collecting

specific low-molecular-weight substances suitable solutes. According to research done on osmotically stressed *S. aureus* cells, the main compatible solutes that accumulate there are glycine betaine, carnitine, and proline. Their accumulation is due to sodium dependent transport mechanisms (Qi and Miller, 2000).

One of the most effective measures for inactivating *S. aureus in* food is heating (Bergdoll, 1989). After being exposed to the following temperatures and times: 57.2°C for 80 minutes, 60.0°C for 24 minutes, 62.8°C for 6.8 minutes, 65.6°C for 1.9 minutes, and 71.7°C for 0.14 minutes, *S. aureus* is entirely rendered inactive in milk. However, when considering heat inactivation in other dairy products, it's important to keep in mind that staphylococci likely get more heat resistant when the water activity is dropped until it reaches a water activity between 0.70 and 0.80, at which point resistance starts to decline (Troller, 1986; Bergdoll, 1989).

Skim milk was found to be the most thermally resistant substance for *S. aureus,* followed by Cheddar cheese whey, phosphate buffer, and whole milk (Walker and Harmon, 1966). Optimally, 80 ºC/20 minutes is required to kill *S. aureus* in dairy products (Yaniarti *et al*. 2017). But it was recently revealed by Yehia *et al*. (2019) that pasteurized camel milk sold in Riyadh City, Saudi Arabia, had heat-resistant *S. aureus* strains. Additionally, Staphylococcus aureus survivability at 80 ºC for 20 minutes and different dairy products still contained *S. aureus* after pasteurization was reported (Montanari *et al*., 2015).

When *S. aureus* was in the middle of its exponential growth phase, prolonged cold stress caused distinct metabolomic and proteomic profiles than when it was incubated at 37°C. In cells adapting to cold stress, there was an up-regulation of nine cytoplasmic ribosomal proteins as well as citric acid (Alreshidi *et al*., 2015).

The pH range where Staphylococcus species thrive is between 4.0 and 10.0, with an ideal PH of 6 -7. To survive under acid stress, genes for urease enzymes like urea have been revealed to be crucial. These genes may function by controlling PH homeostasis and urea consumption (Zhou *et al.,* 2019). *S. aureus* is halotolerant and can grow well in the presence of high salt concentrations, such as on skin surfaces which often have high NaCl concentration (10%). Low salinity causes an instantaneous release of tiny solutes that relieve physical tension, whereas high salinity causes a rise in compatible solutes such proline,

glutamate, glycine betaine, ectoine, and trehalose that balance out water efflux. (Omotoyinbo and Omotoyinbo, 2017). *S. aureus* may adapt to osmotic conditions by storing osmoprotectants such proline and glycine betaine (Hajmeer *et al.,* 2006).

2.7. Economic and Public Health Importance of *S. aureus*

Global health concerns related to food-borne infections are important and getting worse (Baron, 2007). According to the World Health Organization (WHO) and the US Centres for Disease Control and Prevention (CDC), many people acquire food-borne illnesses every year (Busani *et al*., 2006). An estimated 2 million people died from diarrheal illnesses worldwide in 2005; over 70% of diarrheal illnesses are foodborne. In some industrialized nations, it is estimated that up to 30% of the population contracts a foodborne illness every year (Jahan, 2012).

SFP is a serious concern in international public health initiatives for FBD (Hennekinne *et al.,* 2012). *Staphylococcus aureus* is one of the important foodborne pathogens that can cause a wide variety of diseases in humans, and its detection in milk and other dairy products poses serious public health risks (Bintsis, 2017). Hospitalization rates of up to 14% have been shown to be caused by Staphylococcal bacteria alone. Even though SE is not particularly fatal, it can cause mortality if significant amounts are consumed. According to Kerouanton *et al*. (2007), fatality rates range from 0.03% in the general population to 4.4% for more sensitive people such the elderly, children, and immunocompromised people. Although staphylococcal infections are common, the immune system often controls them at the point of entrance. Children, those with poor hygiene, those living in close quarters, and those who are overcrowded typically have the disease at its highest occurrence (Rho and Schaffner, 2007).

Since there is a shortage of FBD surveillance systems in developing nations, it is challenging to determine the true burden of the issue (Boschi-Pinto *et al*., 2008). However, the most recent report from the European Food Safety Authority revealed that *S. aureus* was the fourth most frequent cause of reported foodborne outbreaks in 2008, after Salmonella, foodborne viruses, and Campylobacter. This data was gathered from 27 European Union Member States and *S. aureus* was responsible for 291 foodborne outbreaks, which made up 5.5% of all outbreaks that were recorded in the European Union (Leuschner *et al*., 2010). Foods

associated with the outbreaks of staphylococcal food poisoning include meat and meat products, poultry and poultry products, egg, milk, and dairy products, salads, cream-filled bakery products, and sandwich (Rajkovic *et al*., 2020).

Antibiotic resistance has become a zoonotic issue that the public health is concerned, due to the bacterium continues to pose serious public health challenges (Pal, 2018). There are significant public health impacts of *S. aureus*, with respect to healthcare costs, and length of hospital stay increases when the isolates are resistant to antimicrobials, particularly MRSA compared to susceptible strains (Zhen *et al.,* 2020). The bacterium causes significant losses in milk yield, changes in its quality (impaired nutritive value of milk), fertility issues, and even systemic diseases that result in higher medical and production costs in livestock (Argudin *et al.,* 2010). Despite substantial studies, staphylococcal infections associated with livestock continue to affect many individuals (Lee *et al*., 2012).

Additionally, subclinical mastitis is more insidious form of the disease because it is invisible to the farmer, leading to delayed diagnosis and spreads widely among the dairy herds. This results in reduced milk quality and yield, which in turn leads to a reduction in the farmer's income as well as that of the dairy industry. Subclinical mastitis also causes serious damage to the udder and even systemic disorders leading to the culling of affected animals. Like dairy cows, mastitis in dairy sheep and goats can result in financial losses since it reduces milk production, decreases milk with high somatic cell counts, increases mortality, and increases treatment expenses. In chicken, poor weight gain, decreased egg production, lameness, mortality, and condemnation at slaughter can all lead to financial losses due to *S. aureus* (Halasa *et al.,* 2009). Because of the extensive consumption of raw milk, staphylococcal food poisoning is a severe public health concern in Ethiopia (Ayele *et al*., 2017).

2.8. Antibiotic Resistance Mechanisms of *Staphylococcus aureus*

S. aureus is one of the bacteria that is developing antibiotic resistance and is a severe public health concern. This bacterium develops antimicrobial resistance through a variety of mechanisms, including horizontal gene transfer of different mobile genetic elements (MGEs) like bacteriophages, plasmids, mutations, Staphylococcus cassette chromosomes (SCCs), transposons, and pathogenicity islands (Bitrus *et al*., 2018; Donham, 2010). MGEs may carry

genes that make bacteria resistant to antibiotics, depending on the size of their plasmids. Small plasmids may harbour tetracycline, erythromycin, and chloramphenicol resistance genes, whereas large plasmids harbour macrolides, beta-lactams, and aminoglycoside resistance genes. On the other hand, larger plasmids contain MGEs that work along with other genes to produce resistance to antibiotics like erythromycin, vancomycin, beta-lactams, trimethoprim, and spectinomycin (Bitrus *et al*., 2018; Bitrus *et al*., 2017). *S. aureus* can be characterized as a microorganism with enhanced potential to develop acquired antimicrobial resistance, in contrast to other microorganisms, as some Enterococcus species demonstrate inherent resistance to various antibiotics due to their natural metabolism (Klare *et al*., 2003).

2. 8.1 Resistance to beta-lactam antibiotics

All *S. aureus* isolates were almost universally sensitive to penicillin when it was initially used in clinical settings in the 1940s. However, by the end of the decade, 28% of the *S. aureus* strains discovered at Boston City Hospital were penicillin resistant (Maranan *et al*., 1997). Today majority of hospitals acquired *S. aureus* isolates are penicillin resistant, even though multiple new antibiotic classes have introduced. *S. aureus* has demonstrated a unique ability to quickly adapt to each new challenge by developing novel resistance mechanisms. Penicillin-binding proteins (PBPs), which facilitate transpeptidation and carboxypeptidation processes in staphylococci and are essential for the binding of the peptidoglycan backbone in the bacterial cell wall, are the biological targets of penicillin and other beta-lactam antibiotics. Acyl D-alanyl-D-alanine is the typical substrate for PBPs; Penicillin consequently interferes with the production of peptidoglycans, leading to the eventual death and lysis of the bacteria (Chambers *et al*., 1994).

There are two ways that *S. aureus* develops resistance to beta-lactam drugs: beta-lactam penicillinase and the *mec*A gene. The first pathway necessitates the development of plasmid-based, blaZ-encoded penicillinase or beta-lactamase enzymes (Gnanamani *et al*., 2017; Harris *et al*., 2002; IWG-SCC, 2009). This enzyme degrades the beta-lactam ring in the structure of the beta-lactam antibiotic, rendering it inactive (Gnanamani *et al.*, 2017). The second defence mechanism promotes bacterial cell wall formation even in the presence of beta-lactam antibiotics by requiring the mecA gene, which produces the PBP2a protein (Harris *et al*., 2002; Fuda *et al*., 2004; IWG-SCC, 2009). According to Hiramatsu *et al.*

(2002), all MRSA strains have a mobile genetic element that includes the mecA gene, which is the gene that causes methicillin resistance. MecA is a component of the staphylococcal cassette chromosome mec (SCCmec), a genomic island. Four distinct SCCmec components with sizes ranging from 21 to 67 kb have so far been characterised (Hiramatsu *et al.,* 2001).

Some MRSA strains have homologous of the *blaZ* regulatory genes that control the expression of resistance. Like how *blaZ* is controlled by the genes *blaR1* and *blaI* after exposure to penicillin, these genes, *mecI,* and *mecR1*, control the mecA response to β-lactam antibiotics. MecA expression is induced from loose alternative system because of *mecI, mecR1* sequence similarity to the *blaR1-blaI* regulatory genes (Archer and Bosilevac (2001). Mec is expressed permanently when *mecI* or the promoter region *of mecA* is deleted or altered (Niemeyer *et al*., 1996). Rosato *et al.* (2003) showed that one of the two *mecI* or *blaI* genes must be active in every MRSA, and they hypothesised that this might be a defence mechanism to prevent the over production of a harmful protein. Another group of genes known as the *fem* genes (factor essential for resistance to methicillin resistance) function in the cross-linking of peptidoglycan strands and contribute to the heterogeneity of methicillin resistance expression (Berger-Bachi, 1994). Additionally, SCCmec islands could contain insertion sequences, genes with unknown functions, and extra antimicrobial resistance genes. The two invertase/resolvase family members, *ccrA* and *ccrB*, are found in the four SCCmec and are responsible for site-specific integration and excision from the chromosome at *attBscc*, a region of an open reading frame (Hiramatsu et al., 2002; Ito *et al.,* 1999). Detail resistance mechanism of *S. aureus* to beta lactam drugs is described in figure 1.

Methicillin-resistant *Staphylococcus aureus* (MRSA) infections affect both humans and animals and range in severity from moderate to severe (Boswihi and Udo, 2018). Because subclinical mastitis in dairy cows can still transmit MRSA to milk without changing the organoleptic properties of the milk, it is conceivable to attribute the spread of the disease to people who handle cattle, prepare milk, and consume it (Basanisi *et al*., 2017). According to a study carried out in Addis Ababa, by Lemma *et al.* (2021) High levels of resistance to frequently used antimicrobials were found in *S. aureus* isolates from milk and other dairy products. (38.5%) of the isolates had cefoxitin resistance, which was referred to as MRSA (methicillin-resistant *Staphylococcus aureus*).

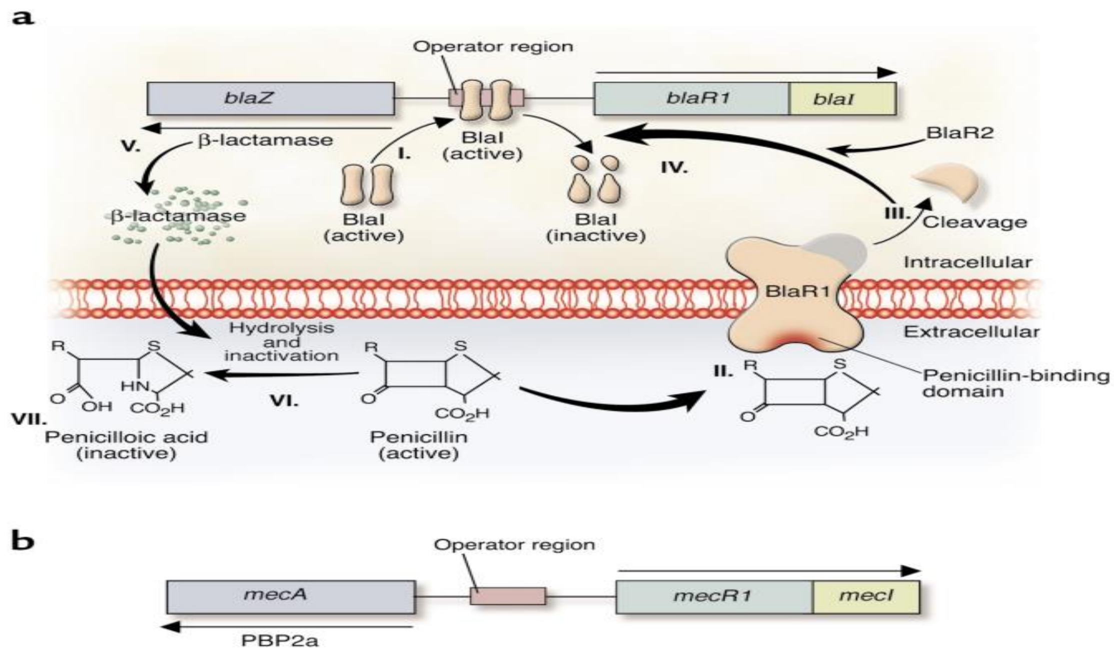

Figure 1: Beta lactam antibiotics resistance mechanism of *S. aureus*

Source: (Lowy, 2003)

2.8.2. Resistance to vancomycin and other glycopeptides

vancomycin was discovered in 1953 by Edmund Kornfeld (Khan, 2017). To treat infections caused by MRSA strains in hospital patients, vancomycin, which is a member of the glycopeptide antibiotic class, is the best option. According to Zeng et al. (2016), the vancomycin binds to the lipid II dipeptide D-Ala-D-Ala and inhibits peptidoglycan remodelling and prevents trans glycosylation and transpeptidation, which are processes carried out by PBP2 and PBP2a. Vancomycin resistance was first reported in 1996 in Japan. Vancomycin-intermediate S. aureus (VISA) was the name given to the strains since they exhibited intermediate vancomycin resistance (Khan, 2017; Harris *et al.,* 2002).

There are two routes that contribute to vancomycin resistance. Vancomycin treatment failure is frequently caused by strains that develop throughout the course of protracted treatment by accumulating numerous mutations in chromosomal genes that affect cell wall biosynthesis and homeostasis. These is called vancomycin-intermediate Staphylococcus aureus (VISA) and have a MIC of 4-8 μg ml^{-1}, whereas vancomycin-susceptible S. aureus (VSSA) has a MIC of ≤ 2μ g ml^{-1} (Howden *et al.* 2010; Park *et al.*, 2019). The acquisition of the *vanA*

gene is responsible for the second resistance mechanism seen in VRSA. According to Harris *et al*. (2002), vancomycin-resistant enterococci that were isolated from the environment transferred this gene to *S. aureus.* Additionally, this method is characterised by converting *D-ala-D-ala to D-ala-D-lac* by attaching to the pentapeptide C-terminal (Bitrus *et al*., 2018) (Figure 2).

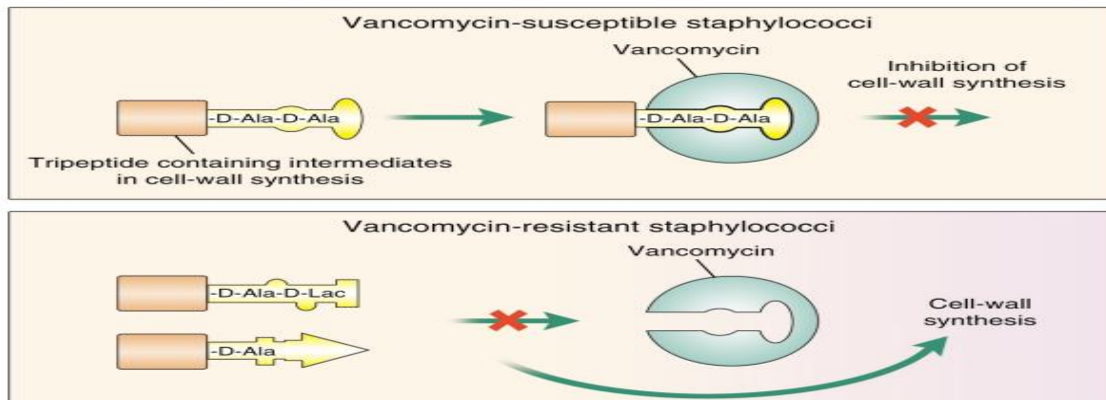

Figure 2: Vancomycin resistance mechanism of *S. aureus*
Source: (Murray, 2000)

2.8.3. Resistance to tetracyclines

Tetracyclines have been used extensively for many years to treat a wide range of illnesses because they are bacteriostatic and broad-spectrum antibiotics (Emaneini *et al*., 2013). By specifically targeting 30S ribosomal subunits and preventing tRNA, they prevent protein synthesis (Bitrus *et al., 2018*). Tetracycline resistance can be acquired in two ways: first by protecting the ribosome (encoded by the *tetM* and *tetO* genes) and second by developing the efflux pump system (encoded by the *tetK* and *tetL* genes transported by plasmid) (Emaneini *et al.,* 2013; Bitrus *et al*., 2018)

2.8.4. Resistance to fluoroquinolone

Immediately following the release of ciprofloxacin, *S. aureus* developed a quick resistance to fluoroquinolones, particularly the MRSA strain. Today, ciprofloxacin resistance is regarded as a distinguishing feature of HA-MRSA. Resistance results from spontaneous mutations that alter the amino acids sequence of either one or both DANA gyrase and Topoisomerase IV enzymes, which are necessary for DNA replication (Jones *et al*., 2004).

The DNA gyrase enzymes, encoded by the genes gyrA and gyrB, and topoisomerase IV, encoded by the genes *ParC* and *ParE*, are the targets of the fluoroquinolone antibiotic class, which prevents DNA synthesis. The target gyrase or topoisomerase IV can get mutated, or the antibiotics permeability to the bacterial cell can change, leading to fluoroquinolone resistance. The multidrug efflux pump system, which is controlled by the *norA* gene, also contributes to the emergence of fluoroquinolone resistance (Bitrus *et al*., 2018, Foster, 2017).

2.8.5. Resistance to clindamycin

Clindamycin is an antibiotic in the lincosamide class. By attaching to the 50S ribosomal subunit, it inhibits the synthesis of proteins in the bacterial cell (Adhikari *et al*., 2017). By methylating the ribosomes receptor binding site, lincosamide resistance develops, which then changes the target cells. According to Bitrus *et al*. (2018) the *erm* genes encode the methylase enzyme, which mediates methylation.

2.9. Application of Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry in Microorganisms' Identification

Currently, the most popular ionization methods for examining chemical structures in biological systems include laser desorption/ionization (LDI), matrix-assisted laser desorption ionization (MALDI), or surface-enhanced laser desorption/ionization (SELDI), as well as electrospray ionization (ESI) (Torres-Sangiao *et al*., 2021). With the advent of soft ionization in the late 1980s, protein analysis advanced quickly and revolutionized mass spectrometry. In the late 1990s, the groundbreaking use of mass spectrometry in microbiology (Griffiths, 2008) showed that entire bacterial cells could be differentiated using MALDI coupled to a time of flight (TOF) analyzer (Holland *et al*., 1996). These successes

accelerated the development of MALDI-TOF MS system techniques as potential instruments for the microbiological characterization of bacteria, fungi, viruses, and even nematodes (Perera *et al*., 2005).

Microbiological characterization and identification can be done quickly, precisely, and affordably using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). This technology creates distinctive mass spectral fingerprints, which serve as the individual signatures of each microorganism. These fingerprints are ideal for precise microbial identification at the genus and species levels, as well as the possibility of strain typing and identification. (Croxatto *et al*., 2012).

MALDI-TOF mass spectrometry is an analytical method based on the cellular proteome, which represents the organism's gene products and metabolic byproducts. This method is based on the examination of highly abundant proteins, primarily ribosomal ones, found in microorganisms with masses between 2,000 and 20,000 Daltons. To determine the mass-to-charge (m/z) ratio, these proteins are ionized into charged molecules either by the addition or removal of one or more protons (Vrion *et al*., 2018).

The sample is combined with an energy-absorbing substance termed "matrix" for analysis. The sample that is trapped inside the matrix crystallizes as well when the matrix is dried. In the following step, the sample is ionized by a laser beam, producing single protonated ions. The m/z ratio of these ions is then determined by timing how long it takes each ion to travel the length of the flight tube [time of flight (TOF)] when they are accelerated at a given potential, and as a result, they split from one another. A distinctive mass spectrum known as the "Peptide Mass Fingerprint" (PMF) is created using the TOF data. A database is then used to compare this PMF, which has peaks specific to genera and species distinct from different types of microorganisms. To identify the unknown organism at the family, genus, and species levels, the PMF of unknown microbial isolates is compared to those of known unknown microbial isolates stored in the database (Vrion *et al*., 2018; Nomura, 2015; Singhal *et al*., 2015).

Numerous microorganisms, such as bacteria, fungi, and viruses, have been characterized using MALDI-TOF MS (Giebel *et al*., 2010). MALDI-TOF has the potential to be used in a variety of fields, including medical diagnostics, biodefense, environmental monitoring, and

food quality control, thanks to its capacity to characterize microorganisms quickly. MALDI-TOF MS is an affordable, high-throughput alternative to traditional laboratory biochemical and molecular identification technologies for microbial identification. (Croxatto *et al.*, 2012).

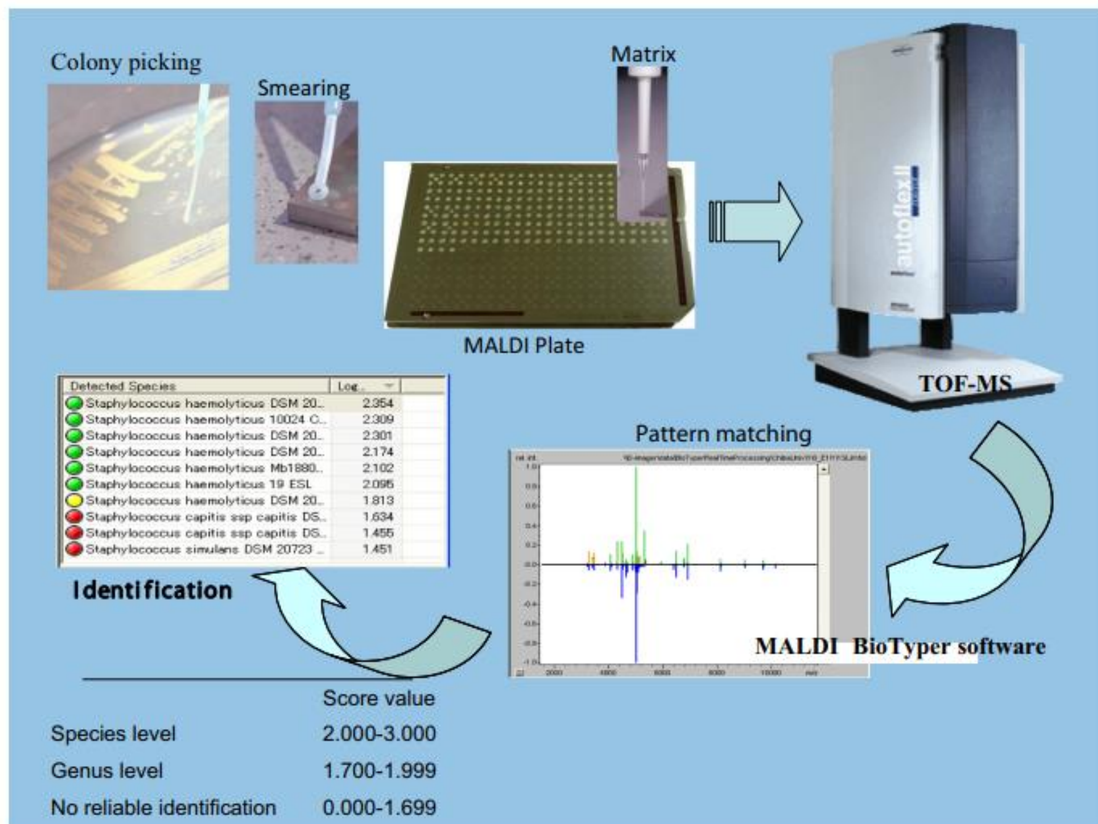

Figure 3: MALDI-TOF mass spectrometry workflow in the clinical microbiology laboratory with the MALDI Biotyper system.

Source: (Nomura, 2015)

2.10. Prevention and Control Measures of *S. aureus*

It is impossible to completely eradicate staphylococci from the environment due to their widespread distribution. The elimination or marked decrease of the bacterial burden in foodstuffs during harvest, processing, packaging, and storage prior to consumption has long been considered an effective mechanism of prevention (Dettenkofer and Spencer, 2007). When exposed to lethal or sublethal doses of heat, cold, drying, irradiation, or chemicals,

staphylococci may be destroyed or injured; however, while the destruction of these organisms may be ideal, sublethal injury may occur, thus giving the organism an opportunity to recover and proliferate, if conditions are conducive (Bennett and Monday, 2003; Bergdoll, 1989). Routine medical and laboratory tests for those who work with food have no value in preventing the spread of foodborne illnesses; nevertheless, it is important to train and educate everyone involved in food handling on the right hygienic practises (Argaw and Addis, 2015; CDC, 2011).

It is necessary to develop strategies for preventing different forms of transmission to avoid staphylococcal infections and poisoning. The development of personal hygiene practices among healthcare professionals and food handlers, the cleaning of tools, surfaces, and clothing, prudent antibiotic usage, correct food preparation and storage, and screening programs are only a few examples of these control programs (Alreshidi *et al*., 2015). Foods must be kept either cold (-10°C) or hot (45°C) to prevent the growth of the organism and the production of toxins, in addition, foods should be refrigerated in shallow layers or small portions to facilitate rapid cooling (Bennett and Monday, 2003).

2.11. Treatment of *S. aureus*

The two most important factors affecting how S. aureus infections treated are the nature of infection and whether drug-resistant strains are present. The type of illness and other factors play a significant role in determining the duration and form of antimicrobial therapy when it is necessary (Tong *et al*., 2015). For sensitive isolates (MSSA, or methicillin-sensitive S. aureus strains), penicillin remains the antibiotic of choice, whereas vancomycin is used for MRSA isolates (Boucher and Corey, 2008). Sometimes it's necessary to combine antibiotic therapy with alternative forms of treatment. Toxin-mediated sickness, for instance, frequently necessitates fluid replacement treatment, and infections linked with catheters or prosthetic valves typically necessitate the removal of foreign objects. MRSA infections are turning into significant diseases in both hospital and community settings since many MRSA strains are resistant to a wide range of drugs (Chambers, 2005).

3. MATERIALS AND METHODS

3.1. Description of the Study Area

The study was conducted in Adama town of East Shoa Zone and Debre-Berhan town of North Shewa Zone. Adama town is located at (8.54°N 39.27°E) at an elevation of 1712 meters, 99 km southeast of Addis Ababa. Its annual temperature ranges from 13.9 ºC – 29 ºC. The mean annual rainfall of the area is 1024 mm. The livestock population of the East Shoa was 1109685 cattle, 445120 sheep, 431649 goats and 310183 equine (CSA, 2021). DebreBerhan town is in the Semien Shewa Zone of the Amhara Region, about 120 kilometres northeast of Addis Ababa on the paved highway to Dessie, the town has a latitude and longitude of 9°41′N 39°32′E and an elevation of 2,840 meters. The livestock population of the North Shewa 1,704,407 cattle, 194,1024 sheep, 823,550 goat, and 704,128 equine (CSA, 2021).

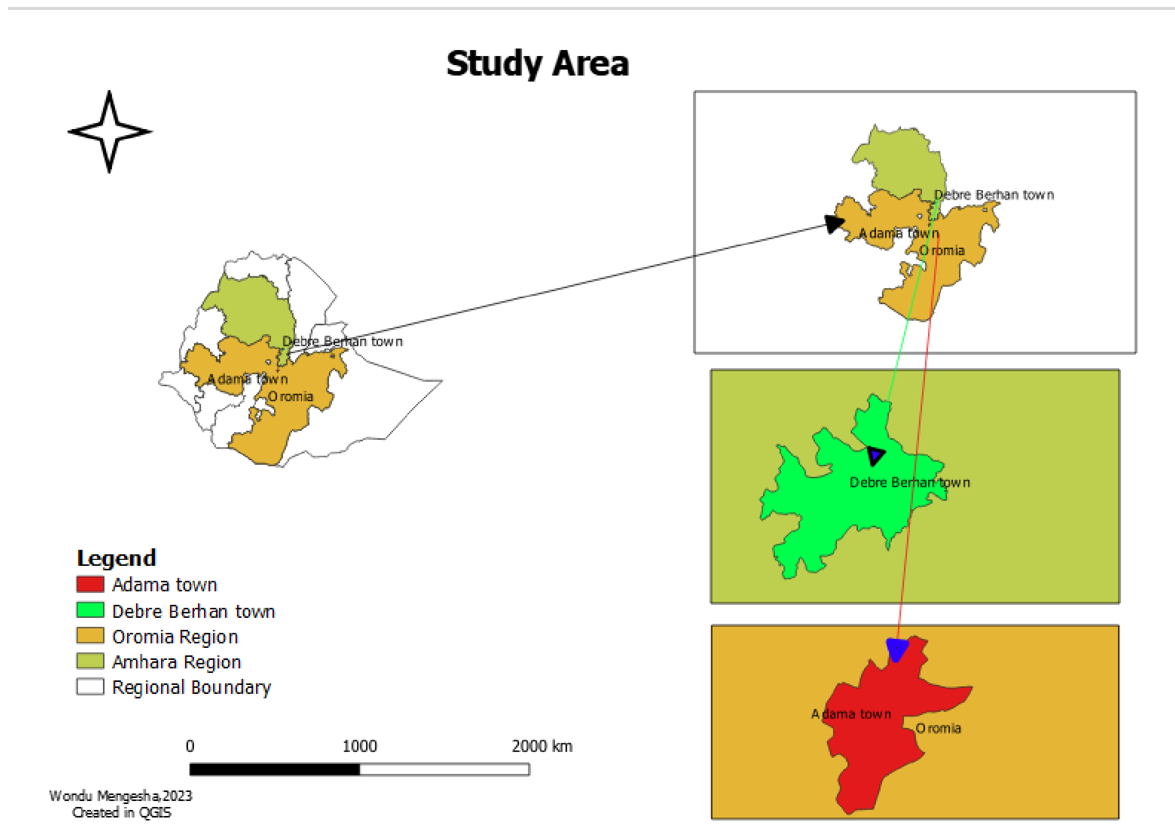

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Figure 4: District Map of Ethiopia showing the Study Areas (Developed with QGIS)

3.2. Study Setting

The study considers the milk value chain, starting with the intensive and semi-intensive dairy farms in and around the towns of Adama and DebreBerhan, milk collection centres, and retail shops that sell milk and traditionally processed dairy products (yogurt and Ethiopian cottage cheese) that are commonly consumed in the city. It also includes environmental samples such as swabs of milkers' hand and milk containers.

3.2.1. Study population

Dairy cows which were lactating were used for the investigation. There were both exotic cows and crossbred cows (crosses between exotic and zebu breeds) in the study population. Cows that weren't lactating during the time of the study's sampling were not included. Variables such as the ages of the animals, lactation stage, breed and socio-economic data were collected from the owners.

3.3. Study Design and Sample Size Determination

A cross-sectional study was conducted from December 2022 to May 2023 to isolate, identify and determine the prevalence, associated risk factors, and antibiotic susceptibility of *S. aureus* from milk in Adama and Debre Berhan towns. The sample size was estimated using the methods recommended by Thrusfield (2007). The required sample size for the present study was determined based on the recent prevalence studies of *S. aureus* in Ethiopia. According to Mekuria, *et al. (*2013) and Megersa (2018), the prevalence of *S. aureus* in the study area was 15.5% in raw milk and 5% in dairy products (yogurt and cottage cheese) as reported from a previous study.

Sample size for raw cow milk was determined using one-stage cluster sampling method (Thrusfield, 2007) taking dairy herd/farm as a cluster and every lactating cow in the selected cluster was sampled (composite milk).

$$g = \frac{1.962\{\mathrm{nVc} + \mathrm{Pexp}(1 - \mathrm{Pexp})\}}{nd^2}$$

Where: g = number of herds (clusters) to be sampled.

n = predicted average number of milking cows per herd = 3 (modification from Demissie *et al*., 2021).
Pexp = expected prevalence (Pexp = 0*:*155 from previous study- Mekuria *et al*. *(*2013))
d = desired absolute precision (d =0. 05).
VC = between- herd variance (VC = 0.02) (Demissie *et al*., 2021)

Fitting the assumed parameter values gives an estimated sample size of 73 farms. For large farms, a random selection of 10 lactating animals was made, for small farms, a random selection of 2, and for medium farms, a random selection of 5 lactating dairy cows was made. Therefore, 188 sample for raw cow milk, 80 sample for dairy products (40 ayib or cottage cheese and 40 ergo/ yoghurt) was collected. In addition to the raw milk and other dairy product samples, 42 bulk tank milk from collection centres, 73 milkers' hands swabs, 38 milking equipment (bucket) swabs was included in the study for greater study power. Overall, 421 samples were considered in the study.

3.4. Sampling Strategy

For this study farms with five and above cattle were included. The farms were classified as small [5-20], medium [21–40], and large herds [>40] following previous studies approach of Demissie *et al*. (2021). A list of 1,021 farms (421 Debre Berhan & 600 from Adama), data obtained from the respective cities' agriculture office was used as a sampling frame. To determine the number of farms to be selected from each category (stratum), proportional sampling was followed. The proportion of small, medium, and large farms in the study population estimated was 89%, 7% and 4%, respectively (Demissie *et al*., 2021). Taking this into account the number of farms selected from each category was presented below in Table 1.

Table 1: Sample size by farm category and study site

Farm category	*Debre Berhan*	*Adama*	*Total*
Large (>40)	1	2	3
Medium (21-40)	3	3	6
Small (5-20)	26	38	64
Total	30	43	73

3.5. Sampling Methodology

Representative dairy farms were selected from the city using systematic random sampling technique based on the list of dairy farm owners. The sampling frame used for selection of the dairy farms was a list of households registered by the Adama and DebreBerhan Farmers and Urban Agricultural bureau.

The number of cows was proportionally allocated to each farm based on the farm size. Cows' lactation stage grouped as early lactation stage between 0 and 3 months, mid lactation stage between 3 and 6 months, and late lactation stage from 7 months up to gestation. Cows were grouped into two age categories, young cows (those under five years old) and adults (those over five years old). Additionally, the cows were divided into three parity categories: few (1-2 calving), medium (3-5 calving), and many (more than 5 calving) based on Dabele *et al*. (2021). The California mastitis test (CMT) was performed in accordance with the guidelines given by Quinn *et al*. (2004). Using the CMT, randomly selected cows that didn't have evidence sign of inflammation on their teats or udder were checked for subclinical mastitis. Milk from each functional teat was squirted in each CMT paddle cup and then combined with the equal amounts of 3% CMT reagent. The presence of subclinical mastitis was determined in CMT positive animals if jelly (viscous) consistency was produced within 3-5 minutes.

Each sample of raw cow milk and bulk tank milk was collected directly from a dairy farm and milk collection centres respectively, whereas yoghurt, and cottage cheese (n = 80) were sampled from dairy product vendors in the study area. To include dairy product sellers, during trips to dairy farms, we looked for shops that sold traditionally processed yoghurt and cottage cheese in the area. Until the necessary sample size was reached, each additional shop along the road was considered.

3.6. Sample Collection and Transportation

Cow's udder and teats was first washed and dried using clean towels before sampling. The first few squirts of milk were discarded to clean the teat canal and composite milk sample (40 ml) was taken from each cow. Sterilized falcon tubes were used and labelled to gather milk samples from each cow. A sterile cotton swab moistened with Stuart transport media

was used for equipment surface and milkers' hand sampling. The surface of milking equipment and milkers' hands was swabbed with the wet swab and placed separately in the labelled test tube that had Stuart medium. After agitating the bulk tank milk sample was taken from the top of bulk milk from MCCs. Milker's hand swabs, bucket swabs, milk samples (40 ml) from individual cows, milk samples from collection centres (40 ml), and milk products (cottage cheese (Ayib) and yoghurt (ergo)) was transported to the Microbiology Laboratory of Animal Health Institute (AHI), Sebeta by keeping in icebox containing ice packs. Upon arrival, the collected samples were immediately stored at 4 °C until culturing for bacterial isolation.

3.7. Isolation of *Staphylococcus aureus* and Primary Biochemical Tests

Initial enrichment processes of the samples were conducted by adding 1ml of milk sample in to 9 ml of Brain heart infusion broth (BHIB) (HIMEDIA, INDIA) and incubate at 37ºC for 24hr. Then, a loopful of bacteria growth from brain heart infusion broth (BHIB) was taken and streak on mannitol salt agar and incubated at 37ºc for 24 hours. Next the plates were inspected for bacterial growth and mannitol fermentation. A bacterium which shows Golden-yellow colonies and grape like cluster were taken and streak on 10 % maltose salt (MPA) agar and incubated at 37ºC for 24 hours. The representative colonies which are typical grapes like structure and shows golden-yellow colour on MPA agar was further sub-cultured on brain heart infusion agar plates (HIMEDIA, INDIA) using wire loop and incubated at 37 °C for 24 hr. The pure colony on BHI agar was then stained by Gram stain and observed under microscope. The morphology and arrangements of the bacterial cells were determined. Gram positive (purple blue) cocci with grape like cellular arrangement was presumptively considered as staphylococci (Annex 4). In addition to this the primary biochemical tests such as catalase and coagulase test was done (Annex 5and 6). A Gram-positive bacterium with grape like cluster during Gram staining, yellow colour colonies on MSA and MPA, coagulase and catalase positive isolates were suspected of *S. aureus*. Finally, the pure suspected isolates were confirmed Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS, Bruker).

3.8. MALDI-TOF MS Based Identification of *S. aureus*

Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used for species identification; the procedure was performed as described by the company (Bizzini *et al.,* 2010). Briefly, after all suspected isolates was sub-cultured on brain heart infusion agar (HIMEDIA, INDIA) and incubated at 37 °C for 24hr overnight; Pure colonies were put in 300 µl of deionized water, vortexed, and then precipitated with 900 µl ethanol (96% vol/vol). After centrifugation for 2 min at 15,000 rpm, the pellet was resuspended with a volume of 25 µl of 70% (vol/vol) formic acid and 25 µl of acetonitrile. After centrifugation for 2 min at 15,000 rpm, 1µl of the supernatant was spotted onto an MSP 96 target Plate polished with Steel BC and allowed to air dry at room temperature. 1 µl of BTS was deposited onto each of the assigned BTS QC positions and allowed to air dry. A droplet of 1µl of matrix solution (HCCA) was overlayed on the entire spot, and again, the spot was completely dried at room temperature (Annex 8). Finally, analysis was performed after loading the MALDI target into the MALDI-TOF mass spectrometer using biotyper software.

3.9. Antimicrobial Susceptibility Test Using Disc Diffusion Method

After bacterial isolates were identified by MALDI-TOF (MALDI Biotyper, (Bruker Daltonik), the antimicrobial susceptibility test was performed using disc diffusion method (Kirby Bauer technique) (Khalili *et al*., 2012). Nine (9) antimicrobial discs were selected to determine the antibiogram characteristics of the *S. aureus* isolates, the prioritized discs were Ciprofloxacin (5µg), penicillin G (10µg), Erythromycin (15µg), Tetracycline(30µg), Trimethoprim-Sulphamethoxazole (25µg), Ampicillin(10µg), cefoxitin (30µg), Gentamycin (10µg), Clindamycin (2µg). Along with the national panel definitions of the pharmaceuticals, the antibiotics were selected based on their availability in the laboratory, distribution to adjacent veterinary clinics, and pharmacies.

Around 2-3 pure colonies of the isolates which was incubated at 37 ^{o}c for 24 hours were suspended in a sterile 0.85% NaCl solution and the turbidity was adjusted to 0.5 McFarland using McFarland Densitometer. In accordance with the standard procedure, a sterile cotton swab was dipped into the suspension and then wiped on Muller Hinton agar until the inoculum had sufficiently covered the plate surface. Then the antimicrobial disks were firmly

applied using antimicrobial disk dispensers and the plates were incubated at 37 °C for 18 hours. The zone of inhibition around each disk was measured using a calliper in the next day and the results were interpreted as sensitive, intermediate, and resistant using Clinical and Laboratory Standards Institute (CLSI) (2022) (Table 2).

Table 2: Antimicrobial Disc used for *S. aureus* susceptibility and diameter of zone of inhibition in millimetre with its interpretations.

Types antimicrobial discs	*Symbol (concentration)*	*Susceptible*	*Intermediate*	*Resistance*
Ciprofloxacin	CIP (5 µg)	≥21	16-20	≤15
Penicillin G	P (10 µg)	≥29	-	≤28
Trimethoprim-Sulfamethoxazole	SXT (25 µg)	≥16	11- 15	≤10
Cefoxitin	FOX (30 µg)	≥22	-	≤21
Gentamycin	CN (10 µg)	≥15	13-14	≤12
Erythromycin	E (15 µg)	≥23	14-22	≤13
Tetracycline	TE (30 µg)	≥19	15-18	≤14
Clindamycin	DN (2 µg)	≥21	15-20	≤14
Ampicillin	AMP (10 µg)	≥29	-	≤28

3.10 Resistance Gene Detection using conventional PCR.

3.10.1. Bacterial DNA Extraction

The DNA of overnight broth cultures of the *staphylococcus aureus* isolates, incubated at 37°C for 24 hours, was extracted using the lysis buffer and Qiagen DNease extraction kit (Thermo Scientific, Germany) in line with the manufacturer's instructions (Annex 9).

3.10.2. Gene Amplification Using PCR.

mecA, nuc, and blaZ genes of *S. aureus* isolates were attempted to be amplified by PCR. A 25 µl master kit mixture, consisting of 5µl of PCR buffer, 0.8 µl of dNTP, 2 µl each of

forward and reverse primers, 0.5 µl of Taq polymerase enzyme, 12.7 µl of RNase-free water, and 2 μl each of DNA components, was utilized for the operation. Sterile water was used as the negative control. The specific primers of *mecA*, *nuc* (Haque *et al*., 2018), and *blaZ* (Takayama *et al.*, 2018) were employed to target the cefoxitin resistant, thermostable, and penicillinase enzyme, respectively.

The procedure for amplifying the genes involved lightly labelling the PCR tubes after gently adding the mixture. A Flex thermal cycler (Biometra Gmbh, Germany) was used to process the PCR tubes with all their components. The thermal cycler was programmed as initial denaturation 94°C for 5 min and then the final denaturation at 94°C for 30 second, annealing at 55°C for 30 second, extension at 72°C for 1 minute, and a final extension at 72°C for 5-minute repeating for 40 cycles (Salisbury *et al.,* 1997) (Table 3).

Table 3: Specific primers used for the detection of resistance genes.

Forward and reverse primers	*Gene length*	*References*
F:5'GTAGAAATGACTGAACGTCCGATGA 3'	MecA	Haque *et al. (*2018)
R: 5'CCAATTCCACATTGTTTCGGTCTAA 3'	(163 bp)	
F: 5' GCGATTGATGGTGATACGGTT 3'	Nuc	Haque *et al. (*2018)
R: 5' AGCCAAGCCTTGACGAACTAAAGC 3'	(279bp)	
F: 5' TACAACTGTAATATCGGAGGG 3'	BlaZ	Takayama *et al.*
R: 5' ATTACACTCTTGGCGGTTTC 3'	(846bp)	(2018)

F (forward primer), R (reverse primer)

3.10.3. Gel electrophoresis and visualization of amplicons.

The ethidium bromide-stained PCR products were separated by electrophoresis on a 1.5% (w/v) agarose gel. The PCR product was put to the agarose gel slots after being combined with the loading dye (bromophenol blue). To determine the length of the amplicons, a maximum of 600 bp of DNA markers (Qiagen; Germany) with 100 bp interval DNA fragments were utilized. Electrophoresis was then run for one hour at 110V with 1X TBE buffer. Following electrophoresis, the separated PCR products were documented using a gel

documentation system (Gel DocTM XR+, BioRAD; Germany) and photographed under UV light (Annex 10).

3.11. Collection of Risk Factor Information

During sample collection from farms, information about animal such as herd size, age, parity, lactation stage, the farm hygiene, breed, teat status, use of disinfectants, use of towel, udder washing practices and management system was gathered through interviews and personal observations. Based on the data gathered from the literature and guidelines to assess the potential risk factors and the knowledge, attitude, and practises of study participants related to food safety training, handling, and consumption of raw milk in the study area, a semi-structured questionnaire was developed (Annex 2 and 3). A total of 114 actors in the milk value chain were interviewed (73 farmers, 21 milk collectors, and 20 retailers). This sample size was calculated based on the central limit theorem ($n = 0.25/SE^2$). Where SE is the standard error, which is 0.05 with a 95% confidence interval according to Arsham (2002).

3.12. Data Management and Analysis

Collected data was entered, cleaned, and coded using Microsoft-Excel sheet and analysis was done using STATA statistical software version 14.1. Descriptive statistics were used to summarize the prevalence of the *S. aureus* and antimicrobial susceptibility data. Using Pearson's chi-square or Fisher's exact test, the prevalence of *S. aureus* in raw milk and dairy product samples was identified, and the association between various risk variables and *S. aureus* sample positivity was determined. Univariable and multivariable logistic regression analyses were performed to assess the association between the prevalence of *S. aureus* and potential risk factors in raw cow milk. Noncollinear variables having a P value of less than 0.25 in the univariable analysis were chosen for the multivariable model. Finally, the results were considered significant at $P < 0.05$ at all levels of analysis.

3.13. Ethical Clearance

Before starting the research, a request was submitted to the Addis Ababa University College of Veterinary Medicine and Agriculture's animal research ethics and review committee,

explaining the purpose of the study. The research work was started after approval certificate was obtained with reference No.VM/ERC/09/02/15/2023 from committee (Annex 4).

4. RESULTS

4.1. Prevalence of *Staphylococcus Aureus*

Out of 421 samples tested, *Staphylococcus aureus* was detected in 54(12.83%) of the samples. Among the positive samples (12.83%), the highest prevalence was from bulk tank milk samples (21.43%) followed by raw milk samples (17.02%), hand swabs (9.59%) and bucket swabs (7.89%) whereas the lowest prevalence was in cheese (5%) and yoghurt (2.5%). As indicated in Table 4, a significant difference was observed in the isolation rate of *S. aureus* among the samples examined (P < 0.021).

Table 4: Prevalence of *S. aureus* from raw cow milk and milk products in the study area

Samples	*No. samples*	*No. positive*	*Prevalence (%)*	*95% CI*	*χ2*	*P value*
Raw milk	188	32	17.02	11.9-23.1		
Bulk_tank milk	42	9	21.43	10.2-36.8		
Bucket swab	38	3	7.89	1.66 - 21.38	13.3	0.021
Hands swab	73	7	9.59	3.94 - 18.76		
Yoghurt	40	2	5	0.61 - 16.91		
Cheese	40	1	2.5	0.06 - 13.15		
Overall	421	54	12.83	9.8 - 16.5		

CI (Confidence interval at (95%), P- value (probability value), χ2 (Chi square), No.(number).

4.2. Risk Factors Associated with *S. aureus* Occurrence in Raw Milk at Farm Level

Out of the total 188 milk samples collected, 32 (17.02%) were *S. aureus* positive. According to a univariable logistic regression analysis age of animals, use of disinfectants, mastitis positivity, farm size and farm hygiene were associated with the prevalence of *S. aureus* at the farm levels, which was statistically significant (P< 0.05). Higher prevalence was detected in mastitis positive dairy cows (46.2%), in large farm size (30%) and (23.8%) in hygienically poor farms. This logistic regression analysis shown that, there is not statistically significance

difference in prevalence between the two towns ($P > 0.05$). Except for farm hygiene and disinfectant use, all independent variables at the farm level were non-collinear to one another, according to the multicollinearity matrix. Farm hygiene was used as the collinear variable for the multivariable model. Based on the multivariable logistic regression analysis result, farm hygiene, farm size, housing floor, and mastitis positivity were strongly associated with the prevalence of *S. aureus* at the farm level, which was statistically significant ($P < 0.05$) (Table 5).

Table 5: Factors associated with the prevalence of *Staphylococcus aureus* in raw milk.

Variables	*Categories*	*No. of tested*	*No. of positive %*	*UOR [95% CI]*	*P value*	*AOR [95% CI]*	*P - value*
Farm size	Small scale	128	15 (11.72)	1.0			
	Medium	30	8 (26.67)	2.74 (1.04 - 7.24)	0.042	3.75 (1.24 - 36)	0.133
	Large	30	9 (30)	3.23 (1.25 - 8.34)	0.015	23.38 (3.03 -134.4)	0.003
Town	DebereBerhan	77	12 (15.58)	1.0			
	Adama	111	20 (18.02)	1.19 (0.54 - 2.6)	0.663		
Management	Intensive	165	27 (16.36)	1.0			
	SemiIntensive	23	5 (21.73)	1.42 (0.48 - 4.15)	0.522		
Breed	HFX	177	28 (15.82)	1.0			
	Jersey	11	4 (36.36)	3.04 (0.83 - 11.1)	0.092	1.06 (0.04 - 3.14)	0.962
Age(years)	>5	68	5 (7.35)	1.0			
	≤5	120	27 (22.5)	3.65 (1.34 - 10.0)	0.012	4.12 (0.66 - 16.2)	0.107
Parity status	1 -2 calving	66	10 (15.15)	1.0			
	3 – 5 calving	79	12 (15.18)	1.00 (0.40 - 2.49)	0.99		
	>5 calving	43	10 (23.25)	1.69 (0.64 - 4.50)	0.28		
Lactation stage (Month)	≥7	17	1(5.88)	1.0			
	0 -3	53	5 (9.43)	4.5 (0.57 - 35.71)	0.152	9.64 (0.16 - 20.6)	0.158
	3 -6	118	26 (22.03)	1.66 (0.18 - 15.5)	0.652	13.4 (0.3 - 20.9)	0.084

Teat status	All normal	160	24 (15)	1.0			
	One blind	17	4 (23.5)	1.74 (0.52 - 5.79)	0.365		
	Two blinds	8	3 (37.5)	3.4 (0.76 - 15.17)	0.109		
	Three blinds	3	1 (33.33)	2.83 (0.25 - 32.5)	0.403		
Housing floor	Concrete	170	26 (15.29)	1.0			
	Soil	18	6 (33.33)	2.77 (0.95 - 8.04)	0.061	14.4 (0.66 - 40.6)	0.026
Farm hygiene	Moderate	100	11 (11)	1.0			
	Poor	88	21(23.8)	2.57 (1.16 - 5.71)	0.022	10.0 (1.2 - 28.5)	0.011
Use of Dis infectant	yes	30	9 (30)	1.0			
	No	158	23 (14.55)	2.52 (1.03 - 6.17)	0.044		
Udder & Teat washing before milking	Yes	118	18(16.1)	1.0			
	No	70	13(18.57)	1.18 (0.55 - 2.58)	0.66		
Water source	Tap	170	26 (15.29)	1.0			
	Ground	18	6 (33.33)	2.76 (0.95 - 8.04)	0.06	4.14 (0.6 - 23.9)	0.191
Milking techniques	Hand milking	176	29 (16.47)	1.0			
	Machine	12	3 (25)	1.69 (0.43 - 6.63)	0.452		
Individual towel Use	No	178	29 (16.29)	1.0			
	Yes	10	3 (30)	2.20 (0.54 - 9.01)	0.27		
Mastitis	Negative	123	2 (1.62)	1.0			
	Positive	65	30 (46.15)	51.8 (11.8 - 227.7)	0.00	87 (14.9 - 517.0)	0.000

4.3. Prevalence of *S. aureus* from Milkers' Hand Swab and Possible Risk Factors

The current study shows that 7/73 (9.59%) of the isolates were positive for *S. aureus* which was statistically not significant ($p>0.05$). 36(49.3%) of milkers had no experience of washing their hands before milking and a high percentage (11.11%) of *S. aureus* was isolated from milkers' hands who had a habit of fingering their nose as compared to those who did not experience fingering their nose. The highest prevalence (33.33%) of *S. aureus* was identified from milkers who can't read and write (12/73(16.4%) (Table 6).

Table 6: prevalence of *S. aureus* from milkers' hand swab and possible risk factors

Variable	*Category*	*Number of samples*	*No. positive*	*Prevalence (%)*	*χ2*	*P value*
Age	15-25	13	1	7.7	0.4525	0.798
	26-45	46	4	8.7		
	>45	14	2	14.3		
Sex	Female	23	1	4.4	1.06	0.302
	Male	50	6	12.0		
Towns	Adama	43	3	7.0	0.8236	0.364
	DebereBerhan	30	4	13.33		
Education level	Illiterate	12	4	33.33	10.1691	0.017
	Primary	41	3	7.32		
	Secondary	10	0	0		
	Above secondary	10	0	0		
Food_safety training	No	54	4	7.41	1.1390	0.286
	Yes	19	3	15.80		
Hand_washing before miking	Yes	47	5	10.64	0.1676	0.682
	No	26	2	7.70		
Nose_fingering habit	No	37	3	8.10	0.1898	0.663
	Yes	36	4	11.11		

In current study, 114 milk value chain actors including farm owners and milk collection centres were interviewed. A high percentage of the respondents 97 (85.1%) had a knowledge of milk borne disease and only 26(22.8%) of the respondents had awareness on the staphylococci food poisoning. Additionally, 35(30.7%) of the respondents had a habit of consuming raw milk. Among all respondents only 33(29%) of them have taken a food safety training course (Table 7).

Table 7: Descriptive statistics of questioner survey on food safety awareness and training in the study area

Variables	*Categories*	*Number of respondents*	*Percentage*
Raw milk consumption habit	No	79	69.3
	Yes	35	30.7
Knowledge on milk borne disease	No	17	14.9
	Yes	97	85.1
Awareness of staphylococci food poisoning	No	88	77.2
	Yes	26	22.8
Food safety training	No	81	71
	Yes	33	29

4.4. Antimicrobial Susceptibility Profile of the *Staphylococcus aureus* Isolated

From 54 isolates of *S. aureus* subjected to antimicrobial susceptibility tests, 54(100%) isolates were susceptible to Trimethoprim-sulfamethoxazole, Ciprofloxacin, cefoxitin and Gentamycin drugs. However, 53(98.15%) of the isolates were developed resistant to beta lactam antibiotics particularly penicillin G and Ampicillin. Additionally, 7(12.96%), 23(42.6%) and 3(5.56%) isolates were developed resistant to Erythromycin, Tetracycline and Clindamycin respectively. Whereas a single isolate was resistant to none of the drugs (Figure 4).

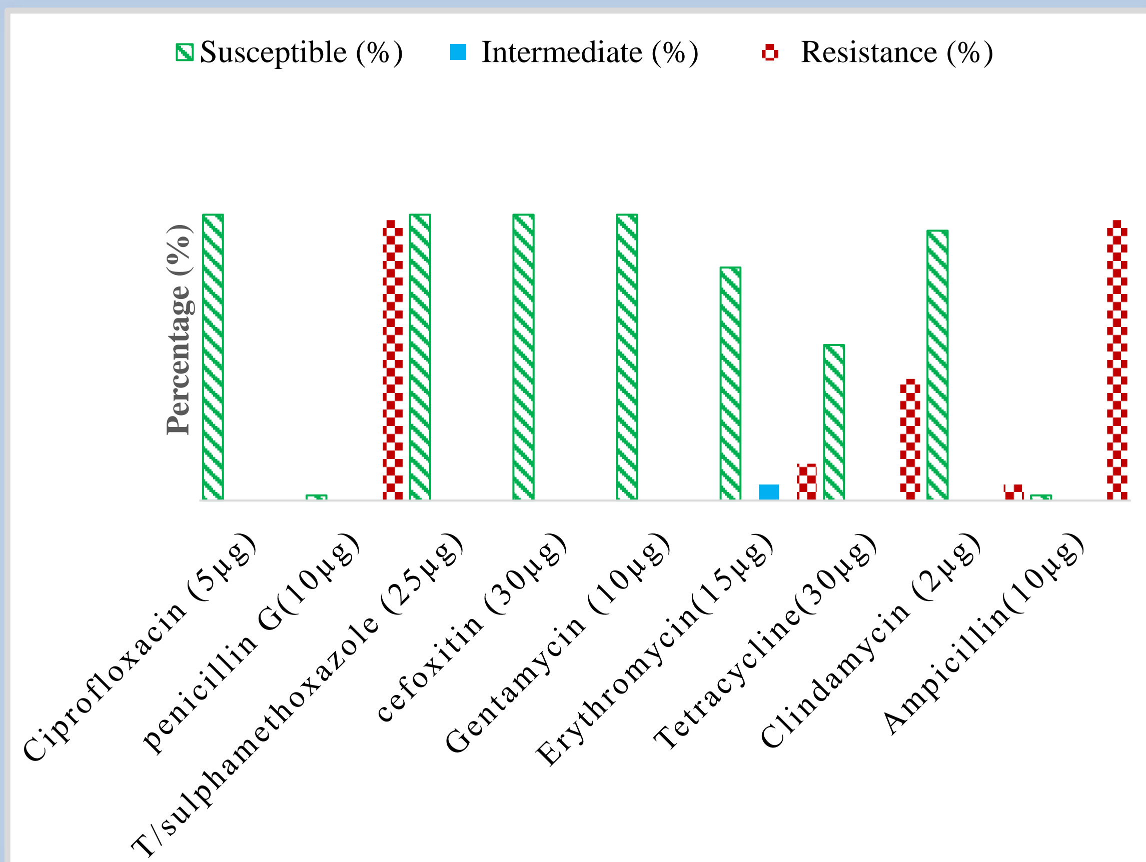

Figure 5: The result of the antimicrobial pattern of S. aureus isolates against nine antimicrobial discs.

4.4.1. Multidrug resistance

In the current study, the isolates have developed multidrug resistant to two to four drugs. A total of 24 isolates were resistant to two drugs (penicillin G and Ampicillin), three isolates were developed resistance to four drugs (penicillin G, Ampicillin, Erythromycin, and clindamycin). Similarly, 26 isolates have developed resistance for three drugs namely, penicillin G, Ampicillin and Tetracycline (Table 8).

Table 8: Multi drug resistance pattern of *S. aureus* isolates in the study area.

No. of antibiotics	*Resistance pattern*	*Number of isolates*	*Percent (%)*
Two	P, AMP	24	44.44
Three	P, AMP, E	26	48.15
	P, AMP, TE		
Four	P, AMP, E, DA	3	5.56
Total		53	98.15

P(penicillin), AMP (ampicillin), E(Erythromycin), TE (tetracycline), DA(Clindamycine)

4.5. Antibiotic Resistance and blaZ Gene Detections

Among the isolates of *S. aureus* that had developed antibiotic resistance, 21 isolates were examined for the presence of the drug resistance genes *blaZ, mecA, and thermonuclease (nuc) genes*. According to the conventional PCR results, the 846 bp long *blaZ* gene was detected in a single isolate of *S. aureus* examined. However, none of them were discovered to have the 279 bp and 163 bp *long nuc and mecA genes*, respectively (Figure 5 and 6).

BlaZ gene = *846bp*

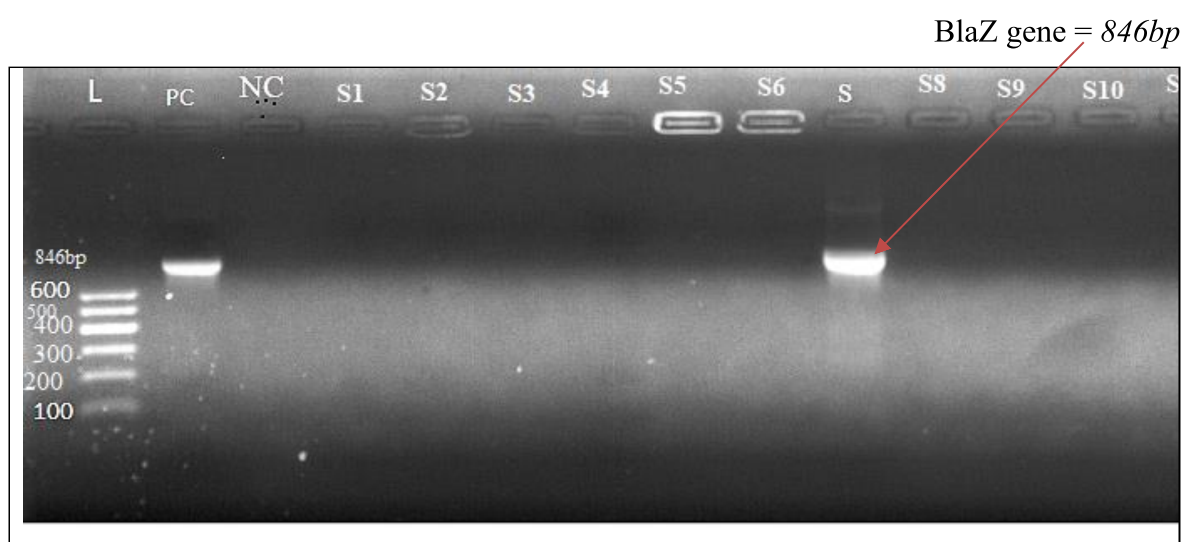

Figure 6: Conventional PCR amplification of blaZ gene imaged with a UV-illuminator after electrophoresis in a 1.5% agarose gel. L=100bp interval DNA ladder, PC= positive control, NC= negative control, bp=base pair, length of blaZ gene =846 bp. Only sample No.7 was positive.

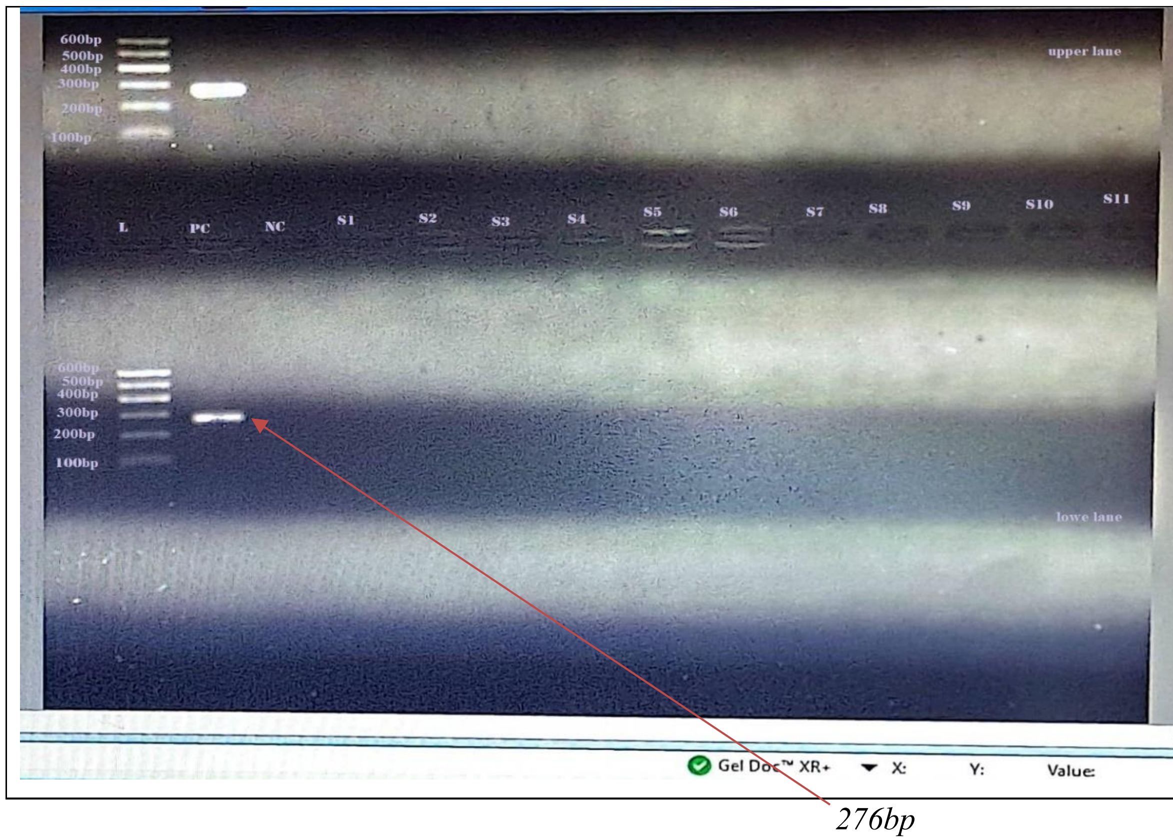

Figure 7: Conventional PCR amplification of nuc gene imaged with a UV-illuminator after electrophoresis in a 1.5% agarose gel. L=100bp interval DNA ladder, PC= positive control, NC= negative control, bp=base pair, length of *nuc* =279 bp. All samples were negative.

5. DISCUSSIONS

The current study found an overall prevalence of 12.83% S. *aureus* in milk and dairy products which was comparable with the report of Umaru *et al*. (2014) 12.6% from Nigeria, Jamali *et al*. (2015) 12.4% from Malaysia and Basanisi *et al*. (2017) 12.9% from Italy. Similarly, comparable prevalence ranging from 10.7% to 14.9% have been reported by Mekibib *et al*. (2010) from Holeta, Ethiopia, Abunna *et al*. (2016) from Asella Ethiopia, and Gebremedhin *et al*. (2022) reported 13.8 %, 14.9 %, and 10.7%, respectively.

However, the current finding was higher than that of Wang *et al*. (2014) 8.2% from China, Rahimi (2013) 5.8% in Iran, and Riva *et al*. (2015) 9.1%. In contrary other studies have reported higher prevalence of *S. aureus* compared to the current finding such as 19.6% by Ayele *et al*. (2017), 40.6% by Daka *et al.* (2012), 24.9% by Deddefo *et al*. (2023), 35.71% by Abera *et al.* (2013) and 36% by Haftu *et al*. (2012), Borena *et al.* (2023) 15.64% and 37.14% by Biniam *et al*. (2017) from different parts of Ethiopia. Such variation in *S. aureus* prevalence between studies might be linked to the study's geographic location, the study protocols (procedures) followed by farm management practices, sample size, study methods, and hygienic practices followed in farms and milk collection facilities. When screening approaches like the California Mastitis Test are used to select positive samples for subsequent culture, *S. aureus* is more likely to be identified in milk (Gebremedhin *et al*., 2022).

The prevalence of *S. aureus* in individual cow level milk in the current study (17.02% or 54 out of the total 188). This finding was in accordance with the findings observed in Addis Ababa 16.2% by Mekuria *et al*. (2013), Gizaw and Duguma (2014) 17.3% in Bishoftu Ethiopia and Feyissa *et al*. (2023) 16.7% in West Shewa Zone, Ethiopia and 15.29% Borena *et al*. (2023) from Ambo and Bako towns, Oromia, Ethiopia. However, the current finding is slightly higher than the previous prevalence of 8.64% by Gebremedhin *et al*. (2022) in Holeta, Central Ethiopia, 13.9% by Marami *et al*. (2022) in Central Oromia, Ethiopia and Thaker *et al*. (2013) who reported 6% prevalence from Anand, Gujarat, India. It is also not uncommon to find a high prevalence of the organism such 50% by Beyene *et al*. (2017) in Addis Ababa Ethiopia.

Variations in the size of the prevalence may be caused by a variety of circumstances, including the sampling procedure, sanitary milking, handling techniques, and the use of common towels for udder drying. Additionally, observations made during sample collection at the farm level indicate unsanitary milking and handling procedures. *S. aureus* is mostly found on the udder or teat surface of infected cows and is the primary route of transmission between uninfected and infected udder quarters. This infection frequently occurs during milking (Abebe *et al*., 2016). Poor hygiene practices during milking may increase the risk of *S. aureus* intramammary infection (Azevedo *et al*., 2016). According to Abebe *et al*. (2016) the significant risk variables that increased *S. aureus* contamination was people working on dairy farms because of milkers thoroughly wash their hands before milking cows.

In the present study 46.15% of *S. aureus* was identified from mastitis positive dairy cow. In this case *S. aureus* was 51 times more likely to be found in milk from cows with mastitis than in milk from cows without mastitis. One of the main causes of mastitis in cattle has been found to be *S. aureus* (Svennesen *et al*., 2019. Additionally, this bacterium can be found in teat canal and on the outer surfaces of the udder and teats. Because it belongs to the microflora, it can occasionally infect animal as an opportunistic pathogen because of mechanical damage to the teat and other stressors (Harris *et al*., 2002).

The current study also identified cow age as a risk factor for the prevalence of *S. aureus* which is in accordance with the findings of Zenebe *et al.* (2014), who reported that older cows were more affected than young ones. Also, this research revealed a high occurrence of *S. aureus* in jersey breed (36.36%) and Holstein Frisian hybrid (15.82%) animals. In this case *S. aureus* was 3 times more likely to be found in jersey breeds than Holstein Frisian hybrid dairy cattle. This could be due to the position of the teat and udder anatomy of teat canal in these cattle. Additionally, this could be explained by the fact that, cows with the most pendulous quarters appear to be the most susceptible to mammary infections. This is because bacteria can easily adhere to the teat and enter the udder, exposing the teat and udder to potential damage (Awale *et al*., 2012).

In the current study *S. aureus* was shown to be significantly (P 0.05) associated with farm size and the likelihood of S. aureus occurrence was 23.4 times higher in large scale dairy farms as compared to small scale dairy farms. The prevalence of *S. aureus in* large scale

dairy farms 30.0% which was lower than report from Holeta Ethiopia 68.0% by Gebremedhin *et al*. (2022), West Shewa Zone, Ethiopia 33.33% by Feyissa *et al.* (2023), in and around Asella town, Ethiopia 76.19% Kemal *et al.* (2017). However, the current result was higher than the reports in China 12.2% by Wang *et al*. (2018).

This high prevalence of *S. aureus* in dairy cattle could be due to the high stocking density, unclean ground, contaminated utensils, inadequate ventilation, and high humidity levels in the environment, there may be a higher likelihood of mastitis occurrence. In addition to this Haque *et al*. (2018) put clear evidence that, high prevalence of *Staphylococcus aureus* in dairy cattle farms may be caused by hygienic and management issues, including breed, farm size, lack of teat dipping prior to and after milking, inability to diagnose subclinical and chronic mastitis, lack of dry cow therapy and diagnostic resources, and practice of hand milking. To stop the disease from spreading, farms should closely adhere to biosecurity and management procedures (Feltrin *et al*., 2016). It was challenging to milk infected cows at the end of the milking session and to use a different milking unit on these cows, particularly in herds where several personnel are involved in the milking process. This may increase the prevalence of *S. aureus* in large farms (Gebremedhin *et al*., 2022).

In the present study, the prevalence of *S. aureus* was 16.36% in intensive management systems and 21.73% in semi-intensive management systems, which was in accordance with Borena *et al.* (2023) who reported a higher prevalence in semi- intensive management (28.57*%)* than intensive management (23.53%) in Ambo and Bako towns, Oromia, Ethiopia. Abebe *et al*. (2016), also reported a higher prevalence in semi-intensive management (63.2%) compared to intensive management type (36.8%) in Hawassa Southern Ethiopia, suggesting the role of management in the occurrence of *S. aureus*. This difference in prevalence between different management system could be due to the comprehensive management system expose the animal to stress and tick infestation while grazing in the field, which in turn subjects the teats to harmful germs like *S. aureus.*

The prevalence of *S. aureus* in bulk tank milk at collection centre was 21.4% in the current study, which agrees with the 21.1% prevalence report from Greece (Papadopoulos *et al*., 2019), and other reports from Ethiopia such as 23.08% from Holeta Ethiopia (Gebremedhin *et al*., 2022), 24.6% from Addis Ababa, Ethiopia by (Lemma *et al*., 2022) and 21.1% from Alage Atvet College (Tessema and Tsegaye 2017). The study findings, however, differ from

those of South-West Uganda 12.1% by Asiimwe *et al.* (2017), and 3.33% by Thaker *et al.* (2013) from Gujarat, India, 80.0% from Sebeta by Ayele *et al*. (2017), 19.3% by Abunn *et al*. (2016) from Asella and 13.9% by Marami *et al*. (2022) from Ambo and Guder towns, Ethiopia. The observed difference could be due to improper handling during transportation from farms to collection centres and milk collection facilities, as well as cross-contamination of the milk during bulking (Addis *et al*., 2011). In addition to this, the possible causes for this high degree of milk contamination in the research area include the use of unpasteurized milk for commercial purposes, poor hygiene habits, insufficient refrigeration, and a lack of facilities that meet standards for milking, storing, and transporting milk. There is a lot of proof that microbial contamination in the milk market value chain can result from a sick cow, improper milking techniques, poor personal hygiene, dirty utensils and/or milking equipment, unsanitary storage conditions, and a lack of pure water supply (Lubote *et al*., 2014).

The current finding also reveals that, mid-lactation cows had considerably higher frequencies of *S. aureus* than early lactation cows did. The risk of S. aureus incidence was 13.4 times higher in the mid-lactation stage compared to the late lactation period. The results of this study were comparable to those of Mureithi and Njugun (2016), who found that *S. aureus* prevalence was considerably greater in mid-lactation stage. However, Gebremedhin *et al*. (2022) and Abebe *et al*. (2016) reported a higher prevalence of *S. aureus* in the early stages of lactation, which is the opposite of the current finding. The differences in the age and breed of the animals used for sampling between studies may be the cause of this variability, additionally the rise in *S. aureus* at the mid-lactation stage is related to production since the animal is at its peak of production at this time. Dairy cows with high milk production are more likely to contract disease causing microorganisms because their glandular tissues are more prone to infection (Radostits *et al.,* 2000).

Out of 38 bucket swabs collected from milk collection centres at study area 3 (7.89%) were found to be *S. aureus* positive, which was in line with the reports 9.0% by Marami *et al*. (2022), and a 11.1% Ayele *et al*. (2017). However, Parmar *et al*. (2014) reported a higher occurrence (18.8%) of *S. aureus* in swabs of milking equipment of dairy farm in Anand Gujarat, India. This variability difference could be due to the difference in the hygienic status of the equipment sampled. Additionally, the finding of *S. aureus* on milking equipment could be due to ability of heat stable and biofilm formations and resistance to insufficient cleaning

S. aureus persists and proliferates in milk buckets, in collecting and storage tanks (Matallah *et al*., 2019).

The isolation rate of *S. aureus* in present study from milkers' hand was 7.59%, Unlike the present results, other studies observed higher isolation rate of *S. aureus* from milkers' hand such as 32% *by* Ayele *et al*. (2017), 32.5% by Tondo *et al*. (2000), but Parmar *et al*. (2014) found low prevalence (2.55%) of *S. aureus* from milkers' hand. This study also reveals that 11.1% (n = 4) of the *S. aureus* prevalence was identified in those milkers who had the habit of fingering their nose. These made it very evident that milk handlers and milk buckets might be possible *S. aureus* contamination sources for milk. Additionally, this could be explained by the fact that staphylococci are widespread organisms and that at least 50% of people have the organism in their throat, nasal passages, and sneezes or coughs (Bergdoll and Lee, 2006) and they can also infect their hands (Gwida and EL-Gohary, 2013). In addition, this difference in prevalence between studies may be caused by farms, milk collection facilities, and milk product handlers' failure to adhere to regular foodborne pathogen prevention and control procedures.

The prevalence of *S. aureus* in traditionally processed dairy products (Ayib and Ergo) was 3.75 % in the current finding, and this result was similar with the 5% prevalence report by Megersa (2018) from Ambo and Guder towns, Ethiopia. But other studies found a relatively higher prevalence of 11.3% (Lemma *et al*. (2021), 31.15% (Borena *et al*. 2023) and 14.3% prevalence (Argaw and Addis, 2018) have been reported from traditionally processed dairy products. Traditionally cheese is produced by heating milk from which butter has been removed after churning, whereas traditional yoghurt is produced by allowing the milk to ferment at room temperature without pasteurisation and by adding microbes to start the fermentation process (Berhe *et al.*, 2017). So, presence of *S. aureus* in these dairy products, especially yogurt, could be due to tolerance of S. aureus to lactic acid produced by competent bacteria. But the presence of the bacterium in cottage cheese could be due to post-processing contamination as the milk is often subjected heat treatment. Improving food handlers' and equipment hygiene, as well as the application of cold chain facilities, is required in the milk chain to protect the consumer from milk-borne hazards (Tarekgne *et al.*, 2015). Dhanashekar *et al*. (2012) also reported that traditional or contemporary ways of heating or fermenting dairy products may be able to minimise staphylococcal contamination.

The current study found a strong correlation between *S. aureus* prevalence and floor and farm hygiene like reports by Biffa *et al*. (2005) in Southern Ethiopia and by Abera *et al*. (2013) in Asella town, where significant incidence was seen in farms with muddy floors as opposed to those with concrete floor types. This could be because of shared barns that are filthy and muddy encourage the spread of infection in dairy farms. *S. aureus* can withstand vast temperature and moisture ranges due to its environmental toughness. The organism is also easily attracted to teat orifices, where it damages the roughened epithelium (NMC, 1990).

According to the current finding, 10.6% of farmers failed to wash their hands before milking, and 37.2% of farmers didn't wash the cows' udders and teats before milking, like observation of Hofi (2011), in which several farmers did not properly wash their hands and udders before milking. These practices, along with the presence of *S. aureus* on milkers' hands and milk buckets, showed that farmers are ignorant of the value of good milking procedures in reducing microbial contamination of milk, generating public health concerns. However, Desissa *et al.* (2012) found that appropriate milking methods and pre-milking udder preparation are important in reducing *S. aureus* transmission at the farm. For instance, among interviewed individuals (n = 114), about 85.1% (n = 97) of dairy farm owners were aware of the occurrence of foodborne diseases due to the consumption of raw milk, but very few of them 22.8% (n = 26) had awareness of staphylococcal food poisoning associated with consumption of raw milk and milk products. Among the 77.2% (n = 88) who had no awareness of food poisoning, 30.7% (n = 35) of them had a habit of consuming raw milk, and only 29% (n = 33) of them had food safety training.

Antimicrobial resistance in veterinary medicine is becoming more common and of a serious concern at a global scale. As a result, the spread of antimicrobial resistant staphylococci is increasingly a challenge for both human and animal health professionals (Al-Thani *et al.,* 2012). The current study on antimicrobial susceptibility testing revealed that *S. aureus* was 98.15% resistant to penicillin G and ampicillin. This prevalence supported by findings of Sori *et al*. (2011) who reported that *S. aureus* isolates from bovine mastitis in a dairy farm in Jimma town Southwest Ethiopia, were 98% of the isolates were developed resistant to penicillin G and 67% resistant to ampicillin. Similarly, this finding was in line with Marami *et al*. (2022) who reported that 100% isolates were developed resistant to penicillin G and ampicillin in a dairy farm in central Oromia Ethiopia, Abera *et al.* (2010) reported 94.4%

resistant to penicillin G in Adama town and lemma *et al.* (2021) reported 94.2% ampicillin resistance of *S. aureus* in Addis Ababa, Ethiopia.

Tetracycline and penicillin G are the two antimicrobials that are most frequently used in Ethiopian veterinary practice to treat infections or mastitis, which accounts for the high level of resistance (Reta *et al*., 2016). Furthermore, penicillin resistance is plasmid-based and spread to numerous other strains very quickly (McCarthy and Lindsay, 2012). In addition to this scientific evidence during farm visits we observed that farmers and even animal health professionals in the study area were using these veterinary medications excessively and improperly to treat chronic clinical mastitis in dairy cattle.

This study showed that considerable proportion of the *S. aureus* isolates showed resistance to Tetracycline (42.6%), Erythromycin (12.96%) and Clindamycin (5.56%). Observed resistance to Tetracycline showed similarity with other studies i.e., Marami *et. al*. (2022) reported 40% of *S. aureus* show showed resistance to tetracycline, and Lemma *et al.* (202) reported 46.2% *S. aureus* resistance to tetracycline and 25% of the showed resistance to Erythromycin. In another study, a relatively higher proportion of the isolated were found to be resistance to Tetracycline (56.1%), resistance to Clindamycin (11.9%) and Erythromycin (7.9%) (Jamali et al. 2015). Similarly, 78% (Al-Thani *et al*., 2012) and 83.3% (Borena *et al*., 2023) of *S. aureus* isolates have been also reported to resistant to tetracycline.

In the current finding, among 54 isolates checked for the antimicrobial susceptibility, three of them were developed resistant to the four of the most used veterinary drugs in the study area specifically, Penicillin G, Ampicillin, Erythromycin and Clindamycin. However, all the 54 *S. aureus* isolates were susceptible to Trimethoprim-Sulfamethoxazole, Gentamycin, Ciprofloxacin and Cefoxitin. But only a single isolate was susceptible to all class of antibiotics used for this study.

Reasonably, there are several problems that are either directly or indirectly associated to the increased prevalence of MDR dominant bacterial infections. Although the emergence of resistance among different bacterial strains is a normal evolutionary process for microorganisms, it has been greatly exacerbated by the on-going use of antimicrobial drugs to treat infections (Laxminarayan *et al*., 2013; O'Brien, 2002). More than 50% of medications are allegedly prescribed, sold, or distributed without adhering to established

protocols, and the problem is even worse in developing nations (WHO, 2002). Particularly in low-income countries, the use of antibiotics is frequently based on clinical judgment rather than the use of specific diagnostic tools. This practice invariably results in the rapid evolution of drug-resistant strains, which is primarily caused by the irrational use of antibiotics (Shears, 2001; Vialle-Valentin *et al.*, 2012). Similarly, according to Odeyemi and Sani (2016), the widespread unrestricted use of antibiotics in underdeveloped nations is a major contributor to the development of resistant bacterial strains.

According to the current study finding, the isolates were 98.15% Penicillin G and ampicillin resistant. But, the conventional PCR, results showed that only a single isolate have harboured the *BlaZ* gene among the 21 isolates subjected to DNA amplifications. *BlaZ* gene is responsible for hydrolysing the beta-lactam antibiotics such as penicillin and ampicillin because it encodes the *β*- lactamase enzyme (González-Domínguez *et al*., 2020). However, in the current study the *mecA or the* thermostable *(nuc)* gene was not observed. The lack of *mec-A* in the isolates was because of susceptibility of the *S. aureus isolates* to cefoxitin, and the absence of *nuc* indicated that the isolates were not *thermostable* (González-Domínguez *et al*., 2020).

Limitation of this is study is that the detection of enterotoxin genes and molecular characterisation were not done. Additionally, due to a lack of funding and a lack of primers for resistance gene detection, only 21 out of 54 isolates were treated to DNA amplification for resistance gene detection. Therefore, it is preferable to include molecular characterisation, resistance gene and enterotoxin gene detections in subsequent investigations of this kind.

6. CONCLUSIONS AND RECOMMENDATIONS

The current study has demonstrated that raw milk, milkers' hands, milking equipment, and milk products in Adama and Debere-Berhan towns are frequently contaminated with *Staphylococcus aureus*. The results showed a high rate of isolation of *S. aureus,* suggesting existence of high risk to the public's health given the extensive intake of raw milk and milk products. The results of the questionnaire survey revealed widespread consumption of raw milk, a lack of training in food safety, and unsanitary milking procedures in the research area. The antibacterial susceptibility characteristics of *S. aureus* revealed that they were extremely resistant to the locally common antibiotics used in veterinary services, and most of the isolates developed multi-drug resistance. Based on the PCR amplification results the isolates were harboured BlaZ gene which is responsible for hydrolysing the beta-lactam antibiotics such as penicillin and ampicillin because it encodes the *β*- lactamase enzyme.

Based on the conclusion drawn from this study, the following recommendations are forwarded:

- Strategies should be prepared for the *S. aureus* prevention and control, which will subsequently lower the disease's prevalence.
- Providing antimicrobials with stronger therapeutic responses to the areas should be required.
- Antimicrobial drug usage should be prudent and antimicrobial resistance should be regularly monitored to prevent antimicrobial resistance.
- Awareness should be given to the farmworkers on the importance of good hygienic practices.
- Food safety training should be given to the consumers and milk product handlers on the importance of milk hygiene and its related zoonotic disease.
- Further research is required to determine the mechanism by which *S. aureus* develops resistance to β-lactam drugs, particularly penicillin and ampicillin, as there is a lack of consistency between phenotypic and genotypic detection of the *blaZ* gene.

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8. ANNEXES

Annex 1: Informed Consent Form for Research

Title of the study: Prevalence, isolation, and antimicrobial susceptibility profile of *staphylococcus aureus* from raw cow milk and traditionally processed dairy products in and around Adama and Debre-Berhan towns, Ethiopia.

Principal Investigator: Dr. Wondu Mengesha (MSc student at AAU College of veterinary Medicine and agriculture, Bishoftu, Ethiopia

Some general rules you should know about this research.

Participation in a research project is requested of you. It is entirely optional for you to take part in this study. You are free to decide whether to take part in this study, and you are not subject to any consequences if you decide not to. Gaining more knowledge about a certain topic or issue is the goal of research investigations. Being a part of a study does not assure you of any personal benefits. Detailed information on the study in which you are being requested to participate is included in this consent form. This consent form includes a description of the study in which you are being asked to take part. You have the right to ask the researcher for clarification or further information if you do not understand something in this form. We'll provide you with a copy of this consent form. Please feel free to get in touch with the above-mentioned researcher if you ever have any questions concerning your involvement.

purpose of this study: The health of humans and animals can be impacted by a variety of food-borne illnesses. Ethiopia suffers from many these diseases. The ability to focus on these issues in upcoming preventative and treatment programs is important. This research project focus on isolation, identification, characterization, and antibiotic resistance profile of *staphylococcus aureus* from raw cow milk and traditionally processed dairy products and what the community is practicing assess the KAPs of community related to handling and consumption of milk in the study area. If you accept to take part in this study, you will be required to respond orally to questions from a survey I will be giving out. You'll need to finish the questionnaire in about 15 minutes.

Risks: This study has very little risk. You will not be able to identify yourself from this study because no personal information about you will be collected.

Benefits: Identifying the causes of Staphylococcus aureus contamination in raw milk and milk products as well as suggesting a method of transmission between animals and the general population are two advantages of this research. Future control initiatives to combat bacterial zoonotic diseases in underdeveloped nations like Ethiopia will be aided by the knowledge provided by this study.

Confidentiality**:** To the fullest extent permitted by law, study-related data will be kept private. Neither verbal nor written reporting will include any allusions to the study that might identify you. On any study materials, you won't be required to write your name, ensuring that no one can connect your answers to you personally.

Compensation**:** There is no compensation for taking part in this study. You can reach me by phone at **+251912118026** or email at **wendumengesha@gmail.com** at any time if you have any questions concerning the study.

What if you have questions about your rights as a research participant

Through my principal advisor, Prof. Bekele Megersa, or the Regulatory Compliance Administrator of Ethiopia, you may reach out to the AAU College of Veterinary Medicine and Agriculture with your concerns if you feel that you have not been treated as described in this form or that your rights as a research participant have been violated during this project.

Consent to Participate: I was given the opportunity to hear or read the material above, and I understood it. This form was delivered to me. I hereby consent to participate in this study with the understanding that I may withdraw at any time without penalty or loss of any advantages to which I am otherwise entitled.

Name of participant___________ Date___________ Signature_______Phone №_______

Annex 2: Questionnaire for the dairy farm workers on Hygienic Handling Practices

Basic information

Date____

Questionnaire Code___________________________

NO.	Questions	Responses
	1. General characteristics of individuals	
1.1	Age	-----------
1.2	Sex	Male [] Female [ ]
1.3	Level of Education	Illiterate [] Informal Education [ ] Primary Education [ ] Secondary Education [ ] degree [Other (Specify)………………
1.4	Your role at the farm?	Veterinarian [] attendant [ ] milker [ ] Other (specify)______________
1.6	What type of Management system used	Intensive [] extensive [] Semi- intensive []
1.7	What type of Bedding condition used	Concrete [] Clean floor [] Muddy soil floor [] Other specify___
1.8	Breed of animal	Local [] HF Cross [] Jersey [] other ____
1.9	Age of animal	<5 years [ ] >5 years [ ]
1.10	Herd size	5-20 [] 21-40 [ ] > 40[ ]
1.11	Parity status of the cow	1-2calving [] 2-3 calving [] >5 calving []
1.12	Lactation stage of the cow	0-3month [] 3-6month [ ] ≥7month [ ]
1.13	How many teats are functional	All [ ] one [ ] two [ ] three [ ]
1.14	have you used disinfectant in the farm	Yes [ ] No[ ]
1.15	Do you wash your hand before milking	Yes [ ] No[ ]
1.16	Have you hand dying cloth after washing	Yes [] No []

1.7	Practice of hand washing	Before & after milking [ ] only before milking [ ]
1.18	Do you wash udder and teat before milking the cow	Yes [ ] No[ ]
1.19	If yes for Q1.18 what type of water source used	Tap water [] ground water[ ] river water [ ]
1.20	Teat and udder washing practice used in the farm	Cold water [] Cold water and detergent [ ] Warm water [ ] Warm water and detergent [ ]
1.21	What type of milking procedure used	Manual [ ] machine [ ]
1.22	how often clean the Barn?	daily [ ] Once per week [ ] More than two per a week [ ]
1.23	What type of milking utensils used	Wide necked-aluminium [] Wide necked-plastic Narrow necked plastic containers [ ] Other(s) specify [ ]
1.24	The Source of water used for cleaning milk equipment's.	Pipelines (tap) [ ] Wells (pond) [ ] tap water River [ ] (other)___
1.25	how often clean the milk utensils?	before milking [ ] after miking [ ] before and after milking [ ] other______
1.26	What type of practice used for cleaning milk equipment's?	Cold water and detergent [ ] Warm water and detergent [ ] Cold water only [ ] Warm water only [ ]
1.27	Do you check milk quality test at farm level?	YES [ ] NO [ ]
1.28	If yes for Q.27 what type of test used	organoleptic test [ ] clot on boiling test [Alcohol test [ ] Others specify __________ lactometer test [ ]

1.29	Have you done Routine Mastitis check in the farm?	Yes [ ] No [ ]
1.30	Do you wash hand between milking	YES [ ] NO [ ]
1.31	Do you use towel for drying teat	YES[ ] NO [ ]
1.32	How do you use dying cloth	Common cloth [ ] Individual cloth [ ]
1.33	Have you received any food safety training	YES [ ] NO [ ]
1.34	Do you have a habit of fingering your nose while milking	YES [ ] NO [ ]
1.35	Do you have any information on milk borne disease	YES [ ] NO [ ]
1.36	Do you have a habit of consuming raw milk	YES [ ] NO [ ]
1.37	Do you have a refrigerator for storage of the milk that remains from daily sale?	YES [ ] NO [ ]
1.38	Have you received any food safety training	YES [ ] NO [ ]
1.39	Do you have any information about staphylococci food poisoning	YES [ ] NO [ ]

Annex 3: Questionnaire for milk collectors and retailers on hygienic practices

Basic information

Date____________________

Questionnaire Code_________

Questions	**Response**
Age	____________
Sex	Male [] Female [ ]
Level of Education:	Cannot read and write [] Primary Education [] Secondary Education [] Other (Specify)……………………….
What type of product(s) do you sell?	Raw milk [] Fermented milk [ ] Other (s) specify []
Where do you get your milk from?	Dairy farms [] Milk selling cooperatives [] Households []
Materials used for collection of milk.	Wide necked aluminium [] Wide necked plastic [] Narrow necked plastic containers [] Other(s) specify []
How is your cleaning routine for the milk containers?	Cleaning just before putting in milk [] Cleaning after delivery of milk [] Twice a day (before putting in milk and after delivery of milk [] Other(s) specify []
Source of water used for cleaning of milk utensils	Tap water [] ground water[ ] river [ ]
Water used for Milk Equipment/bucket/ washing material.	Cold water and detergent [] Warm water and detergents [] Cold Water only [ ] Warm water only [ ]

Do you ever test the milk when you receive the milk from the producer?	YES [] NO [ ]
Type of milk quality test performed at collection centre	organoleptic test [] clot on boiling [ ] Alcohol test [] others, specify [ ] lactometer test []
Type of container used to fetch milk from the larger container.	A cup with a handle [ ] A cup without a handle [ ]
How long does it for the milk to finish	3 hrs after collection [] 9 hrs after collection [ ] 12 hrs after collection [] 6hrs after collection [ ]
Do you have a refrigerator for storage of the milk that remains from daily sale?	YES [] NO [ ]
Other product of milk sold at shop	Pasteurized milk [] Yogurt [ ] Both
Have you received any food safety training	YES [] NO [ ]
Do you have any information on milk borne disease	YES [] NO [ ]
Do you have a habit of consuming raw milk	YES [] NO [ ]
Do you have any information about staphylococci food poisoning	YES [] NO [ ]

Annex 4: Approved Ethical Clearance

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine and Agriculture
Bishoftu

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/09/02/15/2023

Name and affiliation of applicant: **Wondu Mengesha (DVM, MSc student)**
Department of Microbiology, Immunology and Veterinary Public Health, College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: *isolation, identification, Characterization and antibiotic resistance profile of Staphylococcus aureus from raw cow's milk and traditionally processed dairy products in Adama and Debrebirhan towns*

Date of application:	**December, 2022**
Nature of the project:	**Field investigation**
Target animal species:	**Dairy cattle**
Number of animals involved:	**210**
Study area:	**Adama and Debrebirhan, Ethiopia**

Minutes No. and date of review: **VM/ERC/02/15/022, 23/12/2022**

The Animal Research Ethical Review Committee of the College of Veterinary Medicine and Agriculture of Addis Ababa University has reviewed the above research project and unanimously approved the application of **Wondu Mengesha.**

Professor Getachew Terefe (DVM, PhD)
Chairman

Signature

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Please quote Our Ref. No. When replying

ፋክስ Fax 251-11-4339933 ስልክ Tel. +251 114338450 ፖ.ሣ.ቁ P.o.x. Box 34 ቢሾፍቱ ኢትዮጵያ Bishoftu, Ethiopia

Annex 5: Principle and Procedure of Gram Staining

Most bacteria can be differentiated by the gram reactions due to difference in their cell wall structure. The gram-positive cell wall has a multitude of layers of peptidoglycan (up to 40) which resists decolorization better than the thin (often only 2 layers) gram-negative cell wall. The gram-negative cell wall also contains lipoprotein and lipopolysaccharide that can be verified through chemical analysis. Gram-negative bacteria are decolorized by the alcohol, losing the colour of the primary stain, purple blue (crystal violet-iodine complex). Gram positive bacteria are not decolorized by alcohol and will remain as purple blue. After decolorization step, a counterstain is used to impart a pink colour to the decolorized gram-negative organisms.

Procedure

1. Slide was marked with permanent marker to the opposite side on which the smear was made.
2. A smear was made, by taking young colonies growing on purple base agar.
3. A smear was dried with air.
4. The smear was fixed with heat by passing the slide on a low flame, by facing up the Smeared side.
5. Smear was placed on a staining rack over a sink and apply crystal violet to smear by flooding slide and stayed for 2minutes.
6. tap water tap was Rinsed on smear.
7. Gram's iodine was Applied on smear by flooding slide and wait for 2 minutes.
8. The slide was rinsed with tap water and decolorized with 95 % Ethanol.
9. The smear was Rinsed with tap water.
10. The smear was Flooded with safranin (safranin) and leave for 1 minute.
11. The smear was Rinsed with water and blot dried with tissue paper.
12. Finally, the smear was Observed under microscope using oil immersion (100X Objective)

Annex 6: Principle and Procedures of Catalase Test

The catalase enzyme works on breaking of the hydrogen peroxide into water and oxygen. Therefore, the hydrogen peroxide is added on bacterial colony on the glass slide if it has catalase enzyme, it breaks the H_2O_2 into H_2O and O_2 and then bubble is produced.

Procedure:

1. A pure colony of bacteria cultured on BHIA was taken by sterilized loop and placed on glass slide

2. The two drops of 3% H_2O_2 was added onto colony of bacteria on the glass slide

3. The slide was then observed for bubble formation

Annex 7: Principle and Procedures of Coagulase Test

Certain organisms have coagulase enzyme that can be able to coagulate rabbit plasma, so coagulase test is used to differentiate the coagulase-positive from the coagulase-negatives. Bound coagulase was detected by the slide coagulase test, whereas free coagulase is detected by the tube coagulase test. Bound coagulase adsorbs fibrinogen from the plasma and alters it, so it precipitates on the staphylococci, causing them to clump resulting in cell agglutination. The tube coagulase test detects both bound and free coagulase. Free coagulase reacts with a substance in plasma to form a fibrin clot.

Procedure

1. 0.5 ml of rabbit plasma is placed in small tube(7ml)
2. Two drops of overnight broth culture of staphylococcus were added.
3. The tube was gently rotated to mix the contents.
4. The tube was incubated aerobically at 37°C overnight.
5. Finally, the test was checked for formation of coagulase or broth sedimentation at the bottom of the tube.

Annex 8: Standard procedure for identification of *S. aureus* using MALDI_TOF

1. 300 µl of HPLC-grade water was transferred into an Eppendorf tube.
2. Isolated pure colonies from the cultured plate were mixed with HPLC-grade water, and a complete suspension was made.
3. The addition of 900 µL of pure ethanol was followed by centrifugation at 15000 rpm for two minutes to mix the suspensions.
4. The supernatant was discarded using a pipette.
5. The tubes were centrifuged at 15000 rpm for two minutes to avoid residual ethanol.
6. The tubes were air-dried at room temperature for 5 minutes.

7. After 25 µL of 70% formic acid was added the pellets were resuspended,
8. The addition of 25 µL of 100% acetonitrile was followed by centrifuging at 15000 rpm for two minutes.
9. *1*µl supernatant was deposited onto a vacant sample position on a cleaned MALDI-target plate and left the plate to air dried for 15minutes.
10. 1 µL of BTS was deposited onto each of the assigned BTS QC positions and air dried.
11. 1µLof HCCA matrix solution was overlayed on each sample position and BTS QC position.
12. The spots were air-dried at room temperature.
13. Finally, the target plate was loaded into the mass spectrometer after a specific bar code was scanned.

Annex 9: DNA extraction procedures

1. A sample of each isolate confirmed with MALDI-TOF was harvested using a sterile loop, and 1.75 ml of bacteria cultured were preserved in the Eppendorf tube containing BHIB and incubated at 37 °C for 24 hours.

2. The tubes were centrifuged at 20,000 rpm for 5 minutes.
3. The supernatant was discarded, and 180µl of lysis buffer (ATL) was added to the tubes.
4. After being vortexed for half a minute, the tubes were incubated at 37°c for 30 minutes.
5. The addition of 25 µl of proteinase K and 200 µl of lysis buffer (AL) was followed by vertexing for 2 minutes.
6.The tubes were incubated at 56 °C for 30 minutes.
7. 200 µl of 100% ethanol was added, and tubes were vortexed for 1 minute.
8 The entire contents were transferred to the labelled DNase main spin column (Qiagen DNease extraction kit) (Thermo Scientific, Germany) using a micropipette, and tubes were centrifuged at 10,000 rpm for 1 minute.
9. The column was removed from the collection tubes and placed in new collection tubes.
10. 500µl of wash buffer one (AW1) was added and centrifuged at 10,000 rpm for 1 minute.
11. After the column was removed from the collection tube, 500 µl wash buffer two (AW2) was added and centrifuged at 20, 000 rpm for 3 minutes.

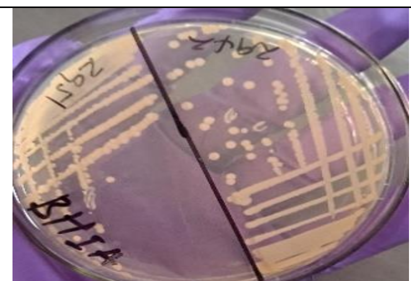
12. Then the extracted DNA was transferred to a 1.5ml tube column, and 200 µl elution buffer (AE) was added to the column and left at room temperature for 1 minute.

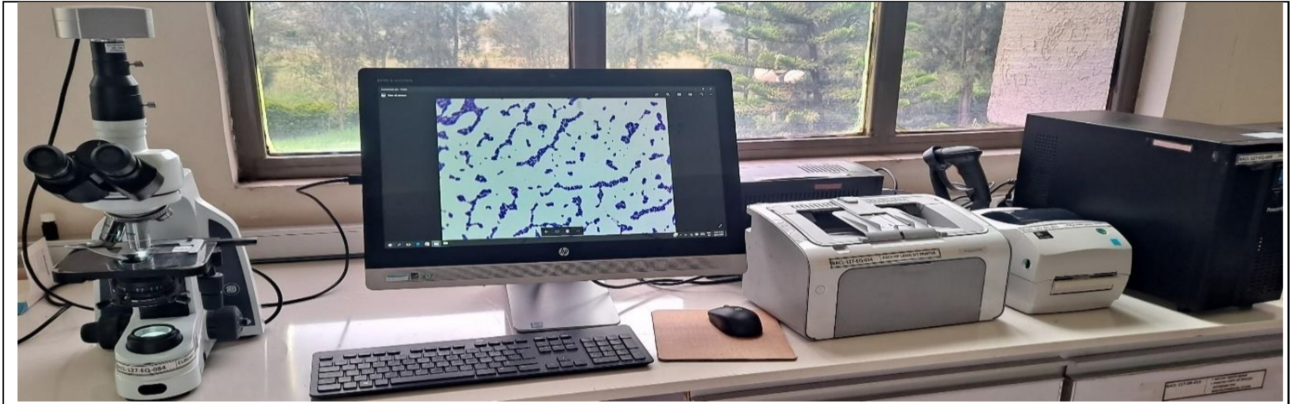
13. Finally, the DNA extracts were centrifuged at 10,000 rpm for 1 minute and stored at -20 ^{o}C until further extraction was conducted.

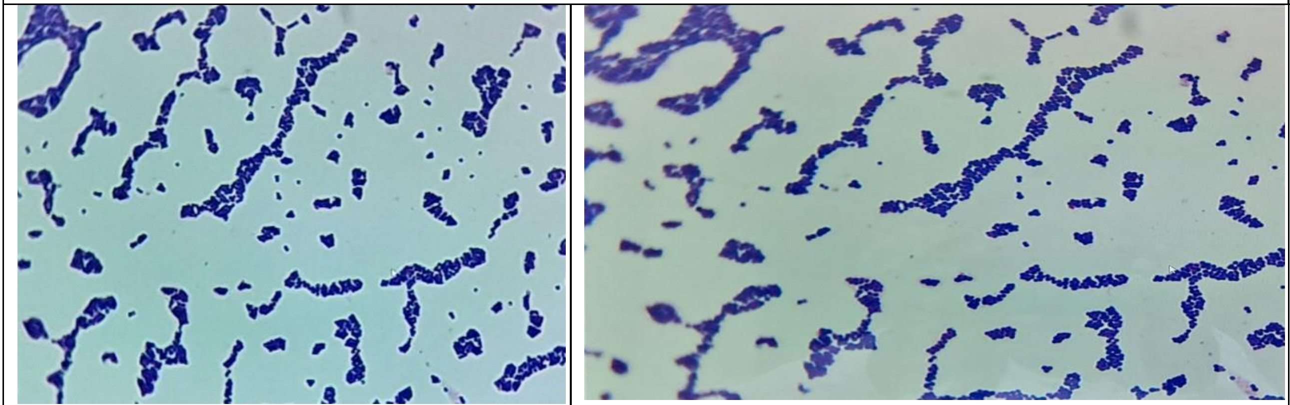
Annex 10: Pictures during sample collection, laboratory work and test results

Debereberhan holland MCC	*Sample reception at AHI*
Media preparation	Sample enrichment with BHIB

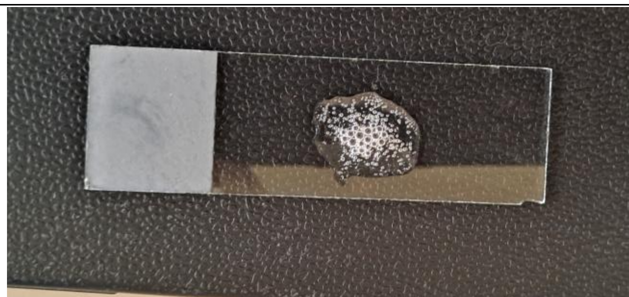
Colony Characteristic of S. aureus on mannitol, purple and brain heart infusion agar.

Bacterial colony on MSA	*Bacterial Colony on MPA*	 *Bacterial Colonies on BHIA*
Golden yellow colonies on the MSA& MPA is the indicative		Yellow colonies and clustered

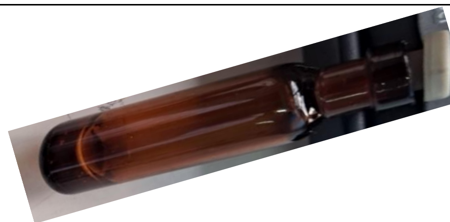




*The fotograph taken after EUROMEXMICRO projection**(B)***



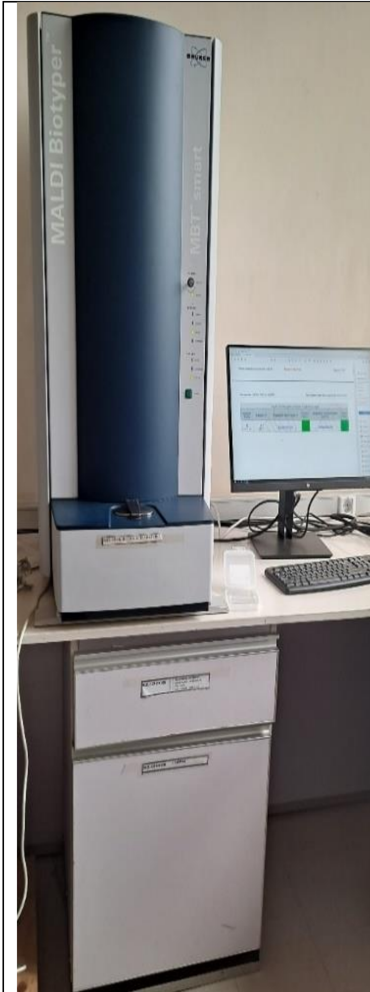
*Slide Catalase positive **(A)***



*Tube coagulase positive**(B)***

Sample inoculation

Sample preservations



MALDI-TOF Bruker

Bruker MALDI Biotyper
Identification Results

Run Info:

Run Identifier:	230311-1857-1811006658
Comment:	Isolation and Identification of S.aureus for AMR Surveillance
Operator:	Admin@MBT-WIN10
Run Creation Date/Time:	2023-03-11T19:11:19.827
Number of Tests:	23
Type:	Standard
BTS-QC:	passed
BTS-QC Position:	C7:0
Instrument ID:	8604832.05381
Server Version:	4.1.100 (PYTH) 174 2019-06-158_01-16-09

Result Overview

Sample Name	Sample ID	Organism (best match)	Score Value	Organism (second-best match)	Score Value
A1 (+++) (A)	8566 (Standard)	Staphylococcus aureus	2.15	Staphylococcus aureus	2.05
A2 (+++) (A)	8575 (Standard)	Staphylococcus aureus	2.22	Staphylococcus aureus	2.22
A3 (+++) (A)	8607 (Standard)	Staphylococcus aureus	2.33	Staphylococcus aureus	2.27
A4 (+++) (A)	8569 (Standard)	Staphylococcus aureus	2.26	Staphylococcus aureus	2.25
A5 (+++) (A)	8608 (Standard)	Staphylococcus aureus	2.34	Staphylococcus aureus	2.28
A6 (+++) (A)	8565 (Standard)	Staphylococcus aureus	2.32	Staphylococcus aureus	2.16
A7 (+++) (A)	8564 (Standard)	Staphylococcus aureus	2.28	Staphylococcus aureus	2.25

Result overview table--continued on next page

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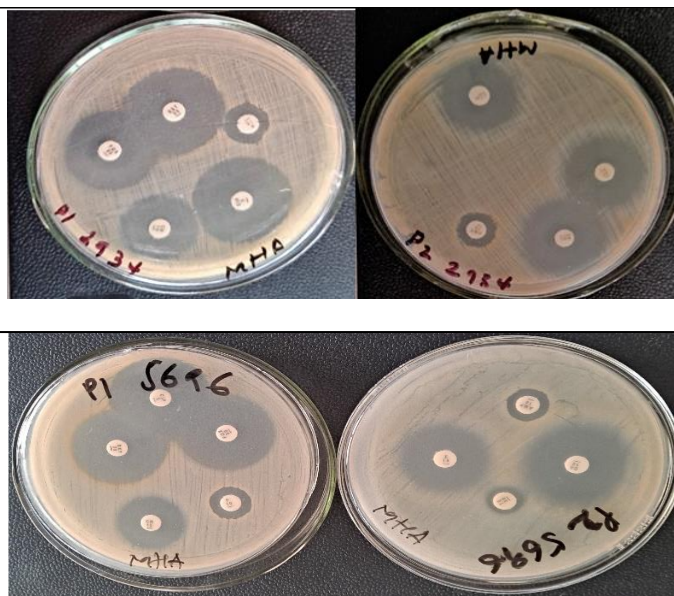
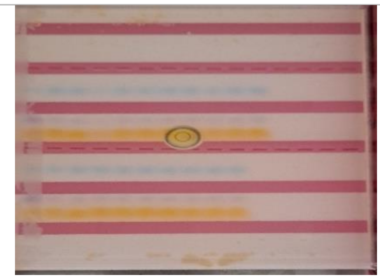
MALDI- TOF out put results

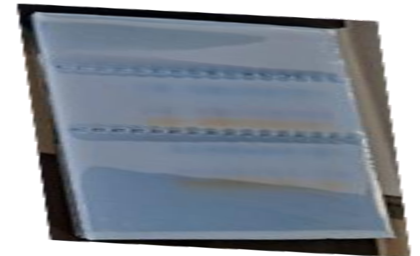
Antimicrobial susceptibility testing



Antimicrobial test results

Flex thermal cycler(upper)

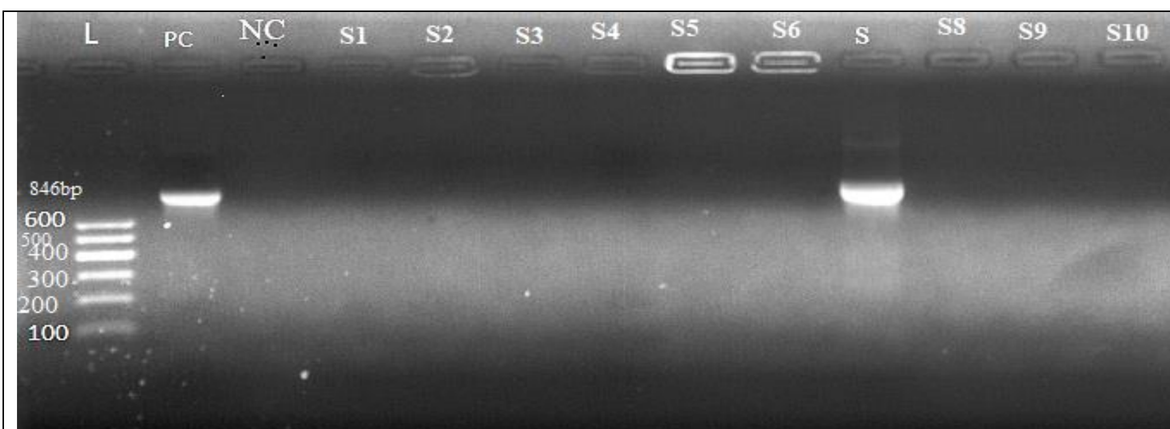




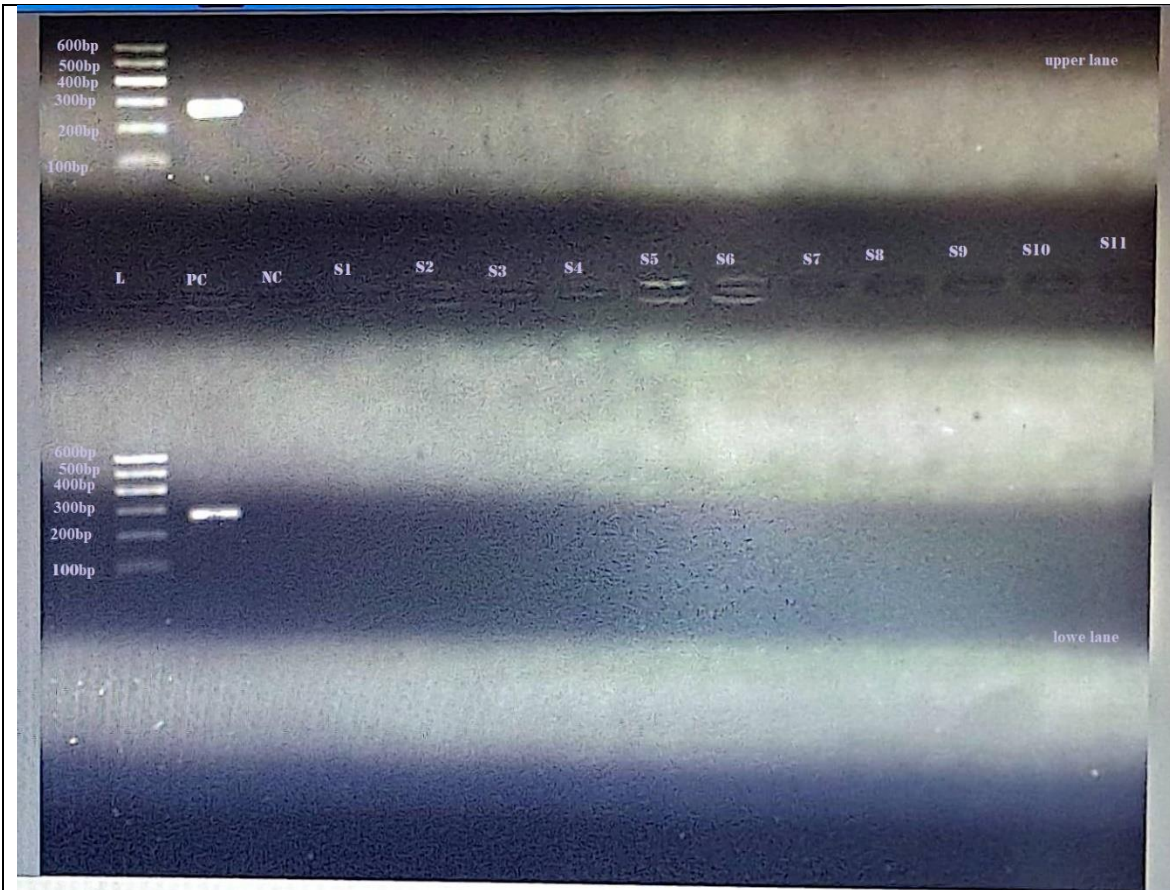
Agarose gel after electrophoresis

Gel documentation

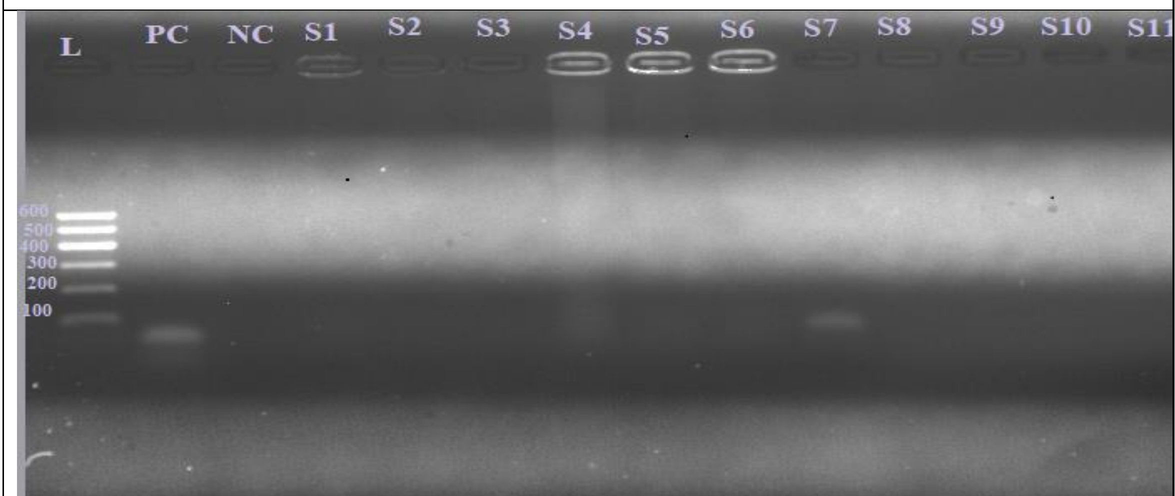
McFarland Densitometer



Imaged with UV-illuminator after electrophoresis in a 1.5% agarose gel, conventional PCR amplification of the blaZ gene



Imaged with a UV-illuminator after electrophoresis in a 1.5% agarose gel, conventional PCR amplification of the nuc gene



Imaged with a UV-illuminator after electrophoresis in a 1.5% agarose gel, conventional PCR amplification of the mecA gene