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ADDIS ABABA UNIVERSITY COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

ISOLATION, IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY TEST OF *STAPHYLOCOCCUS AUREUS* FROM BOVINE MASTITIS IN LOME WOREDA OF OROMIA REGION, ETHIOPIA

BY

GETACHEW NGUSIE SHBESH

JUNE, 2020

BISHOFTU, ETHIOPIA

ISOLATION, IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY TEST OF *STAPHYLOCOCCUS AUREUS* FROM BOVINE MASTITIS IN LOME WOREDA OF OROMIA REGION, ETHIOPIA

A thesis submitted to College of Veterinary Medicine of Addis Ababa University in partial fulfillment of the requirements for the degree of **Master of Veterinary Science in Veterinary Microbiology**

BY

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DEPARTMENT OF VETERINARY MICROBIOLOGY, IMMUNOLOGY AND PUBLIC HEALTH

MSc IN VETERINARY MICROBIOLOGY

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As members of the examining Board of the final MSc open defense, we certify that we have read and evaluated the Thesis prepared by: Getachew Ngusie entitled, Isolation, "identification and antimicrobial susceptibility test of *staphylococcus aureus* from bovine mastitis in Lome woreda of Oromia region, Ethiopia"and recommend that it be accepted as fulfilling the thesis requirements for the degree of: Master of Science in Veterinary Microbiology.

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ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE

DEPARTMENT OF VETERINARY MICROBIOLOGY, IMMUNOLOGY AND PUBLIC HEALTH

Title: ISOLATION, IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY TEST OF *STAPHYLOCOCCUS AUREUS* FROM BOVINE MASTITIS IN LOME WOREDA OF OROMIA REGION, ETHIOPIA

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STATEMENT OF AUTHOR

First, I declare that this thesis is my bonafide work and all sources of material used for this thesis have been duly acknowledged. This thesis have been submitted in partial fulfillment of the requirement for advanced (MSc) degree at the Addis Abeba University, College of Veterinary Medicine and deposited at the University/College library to be available to borrowers under rules of library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of my academic degree, diploma or certificate.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BHI	Brain Heart Infusion Agar
CC	Clonal complexes
CLSI	Clinical and Laboratory Standards Institute
CM	Clinical Mastitis
CMT	California Mastitis Test
CNS	Coagulase Negative Staphylococcus
CSA	Central Statistical Agency
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EDTA	Ethylene diamine tetraacetic acid
FAO	Food and Agricultural Organization
HA	Hospital Associated
IMI	Intramammary infection
LA	Livestock Associated
MDR	Multi Drug Resistant
MHA	Mueller-Hinton Agar
MLST	Multilocus Sequence Type
MRSA	Methicillin Resistant *Staphylococcus aureus*
MSA	Mannitol Salt Agar
NAHDIC	National Animal Health Diagnostic and investigation Center
PAB	Purple Agar base
PBP	Pencilling Binding Protein
PCR	Polymerase Chain Reaction
QAC	Quaternary Ammonium Compounds
RNA	Ribonucleic acid
SCC	Somatic cell count
SCM	Subclinical Mastitis

SEs	Staphylococcal enterotoxins
TNase	Thermonuclease
TSST-1	Toxic shock syndrome toxin-1

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ABSTRACT

Staphylococcus aureus is a common causative agent of bovine mastitis in dairy herds worldwide and causes a huge economic loss in dairy industry. A cross-sectional study was conducted from November 2019 to May 2020 with the objectives of isolating and antimicrobial susceptibility test of *S.aureus* from bovine mastitis in Lume woreda of Oromia region, Ethiopia. Clinical examinations, California mastitis test (CMT) and bacteriological culture methods were used as diagnostic tools to carry out the study. Out of 200 randomly screened cows, mastitis was detected in 104 (52%) cows, 331 (41.4%) quarters, by California Mastitis Test (CMT) while 9 (9.5%) cows and 20 (2.5%) quarters were positive from clinical mastitis cows. From this, 31 (29.8%) cows and 40 (12.1%) quarters were found positive for *S.aureus* from subclinical cases whereas 5 (26.3%) cows and 8 (40%) quarters were culture positive from clinical mastitis. Eleven (13.8%) quarters which belong to 8 (19.5%) cows were found with blind teats. Furthermore, 34 out of 36 *Staphylococcus aureus* isolates were subjected to *in vitro* antimicrobial sensitivity test to nine commercially available antimicrobial agents. The antibiogram result of those isolates revealed a varying degree of susceptibility and resistance. *Staphylococcus aureus* isolates were resistant to at least one antimicrobial, 49.0% to two or more antimicrobials, and subclinical isolates showed more resistance to all tested antibiotics. *S. aureus* isolates were highly sensitive to Chloramphenicol (100%) followed by Ciprofloxacin and Sulphametoxazol-trimetoprime (97.1%), Cefotaxime (94.1%) and Cefoxitin (88%) whereas, the highest rate of resistance among the isolates was against penicillin G (91.7%) followed by tetracycline (76.5%). Gentamycin, Erythromycin and Cefotaxime were the antimicrobials that showed intermediate resistance to 20.6%, 14.7% and 2.9% respectively. In the present observation, 26.5% of *Staphylococcus aureus* isolates showed multiple drug resistant. Thus, the result of this study shows *Staphylococcus aureus* mastitis is an important concern for dairy farms of Lume Woreda of Oromia Region since this pathogen is becoming more resistant to commercially available antimicrobials and hence warrants for designing potential control measure to reduce the impact on dairy sector and public health risk.

Key words: *Antimicrobial susceptibility test, Bovine mastitis, California Mastitis Test, Lume Woreda, Staphylococcus aureus*

1. INTRODUCTION

Ethiopia has one of the largest livestock resources in Africa with a national herd estimated at 60.39 million cattle and 64 million sheep and goats (CSA, 2018). Female cattle constitute about 54.68% of the total cattle population. This huge potential together with increased number of crossed breed cows around major cities and towns have the capacity to satisfy country's milk need. However, productivity is suboptimal due policy and institutional challenges; low input husbandry practice; poor feeding, high stocking rate on grazing lands and wide spread livestock diseases (Mohammed *et al*., 2004;Tefera *et al*., 2010). Mastitis is one of the common widespread disease causing the biggest economic losses to the dairy industry (Mungube *et al*., 2004; Halasa *et al*., 2007; Schwarz *et al*., 2010).

Bovine mastitis, which is the inflammation of parenchyma of mammary glands regardless of the cause, is characterized by a range of physical and chemical changes in the milk and pathological changes in the glandular tissue (Radostits *et al*., 2007).The disease can be classified as clinical or subclinical mastitis depending on the degree of inflammation of the udder (Pumipuntu *et al.*, 2017). Clinical form of the disease may further be classified as mild, acute, per acute and chronic based on the severity of inflammatory response. It is characterized by sudden onset, swelling, and redness of the udder, pain and disturbance of function. All dairy herds even well managed ones as judged by somatic cell count and high level of milk production may suffer from clinical mastitis (Hogain *et al*., 1990).

Subclinical mastitis (SCM) refers to inflammation of mammary gland in the absence of visible gross lesions in the udder or its secretions with presence of pathogenic microorganisms and increased number of somatic cells in the milk (Smith, 1996; Radostits *et al*., 2007). It has special importance as it goes unnoticed and affects in a great extent the production animal (Bhati *et al*., 2016).There is lack of symptoms, with no apparent changes in the milk except for a drop in milk quality and quantity, which is not always detected by farmers. Due to its silent development, subclinical manifestations frequently evolve into chronic infections. As a result of bovine mastitis, there are low production levels, poor quality and/or discard of milk that generate economic losses in the dairy cattle industry (Viguier *et al*., 2009; Song *et al*., 2016).

Mastitis can be caused by physical or chemical agents but the majority of the causes are infectious and usually caused by bacteria. According to Ganda *et al*., (2016) most of mastitis infections are represented by bacteria including those belonging to the genera Staphylococcus, Streptococcus and coliforms. Staphylococcal mastitis, usually caused by *S. aureus*, is the most frequently isolated contagious pathogen in bovine mastitis worldwide. This pathogen is related to more than 80% of intramammary infections (Pellegrino *et al*., 2011; Song *et al*., 2016). *Staphylococcus aureus* causes predominantly subclinical intramammary infections (Radostits *et al*., 2007), resulting in increased somatic cell count and decreased milk production (Blowey and Edmondson, 2010).

Prevention and treatment of bovine mastitis in dairy cattle include the use of antimicrobials in an attempt to eliminate the disease (Pellegrino *et al*., 2011; Silva and Nogueira, 2010; Teixeira *et al*., 2014). However, widespread use of antibiotics on dairy farms and other food-producing animals could lead to emergence of antibiotic-resistant bacterial strains. Failure of antimicrobial therapy against Staphylococcus spp. has been recorded in the last decades (Vintov *et al*., 2003). And this therapeutic failure due to resistant *S. aureus* has been associated with intracellular location or micro abscess formation deep inside the udder tissues. *S. aureus* strains have showed resistance to different antimicrobial agents that are commonly used to treat mastitis (Güler *et al*., 2005).

Bovine mastitis becomes a great concern and is prioritized as one of the major diseases of dairy cows in Ethiopia (Arga *et al*., 2012). Field surveys of major livestock diseases have ranked mastitis as number one disease of dairy animals. Studies conducted in various corners of the country also showed that *S.aureus* is responsible for most bovine intramammary infections (Abera *et al*., 2010; Garedew *et al*., 2015; Tilahun *et al*., 2016; Abebe *et al*., 2016 ; Beyene *et al*., 2017; Biniam *et al*., 2017; Elemo *et al*., 2017; Asmamaw *et al*., 2017; Mekonnen *et al*., 2018; Dereje *et al*., 2018). Despite these findings, no research has been done in Lume district of Oromia region which relatively has high dairy production potential and better market access for milk and milk products. Knowing the exact pathogen and its antimicrobial susceptibility profile in dairy farms permits the treatment method to be specifically targeted to the causation pathogen and controlling the disease.

Hence, keeping the above facts in mind, the present study is designed with the following objectives:

- ✓ Isolation and identification of *Staphylococcus aureus* from mastitis lactating cows in Lume Woreda of Oromia Region.
- ✓ To determine the antimicrobial susceptibility profile of the *S.aureus* from mastitis cases in the study area

2. LITERATURE REVIEW

2.1. Historical background of Staphylococci

Staphylococci were first isolated from human pus in 1880 by the Scottish surgeon Alexander Ogston and the name derives from staphyle (“bunch of grapes”) and kokkos (“berry”) because the bacteria resembled bunches of grapes when viewed microscopically (Licitra, 2013). Ogston had noticed that non-virulent staphylococci were also present on the skin surface. Six years later, German surgeon isolated two strains of Staphylococcus (Rosenbach, 1884). One of these was *Staphylococcus aureus*, so-named because of the color of the pigmented colonies (aureus means gold-colored in Latin) and most of this strain was isolated from pyogenic lesions. The other strain he named was *Staphylococcus albus* which later renamed as *Staphylococcus epidermidis* isolated from normal skin producing white colonies on solid media. This strain is coagulase negative, mannitol non-fermenting and usually non-pathogenic strains (Plato *et al*., 2009).

2.2. Classification

The genus Staphylococcus comprises of several species and subspecies (Kwok and Chow, 2003). Currently, there are 47 species and 24 subspecies under this genus.The genus is also broadly grouped into two, namely, coagulase-positive and coagulase-negative Staphylococcus (CNS) (Sasaki *et al*., 2010; Becker *et al*., 2014). Among Staphylococcal species that has been described so far seven of them namely *S. aureus, S. intermedius, S. schleiferi* subsp. coagulans, *S. hyicus, S. lutrae, S. delphini and S. pseudintermedius* are known to exhibit coagulase-positive or variable reactions. CNS consists of a group of various Staphylococcus species that affect diverse host ranges. Some of them have evolved to cause mastitis in farm animals. *S. aureus, S. intermedius* and *S. hyicus* differ from other staphylococcus species by possessing coagulase enzyme and with greatest veterinary significance (Carter and Wise, 2004).

Table 1*:* Scientific classification of *Staphylococcus aureus*

Scientific classification	
Domain	Bacteria
Kingdom	Bacteria
Phylum	Firmicutes
Class	Cocci
Order	Bacillales
Family	Staphylococcaceae
Genus	Staphylococcus
Species (binomial name)	*Staphylococcus aureus*

Source: (Makgotlho, 2009)

2.3. Morphological and biochemical characteristics

Staphylococci are Gram-positive cocci, approximately 1 μm in diameter, which form irregular clusters resembling bunches of grapes. In exudates, they form clusters, pairs, or short chains. They do not form spores but are extremely resilient and can survive in harsh environments and on inanimate objects for long periods. The staphylococci do not produce flagella and are not motile. The bacterium forms fairly large yellow or white colonies on nutrient rich agar media. The colonies of bovine and human strains of *Staphylococcus aureus* are golden yellow. Colonies of some coagulase-negative staphylococci are also pigmented .The yellow colour of the colonies is imparted by carotenoids produced by the organism. The term 'aureus' is derived from latin, which refers to the colour of gold (Liu *et al*., 2005).

Staphylococcus aureus often haemolytic in blood agar due to production of different types of haemolysins. Some *Staphylococcus aureus* are α-haemolytic, β-haemolytic, α+β haemolytic, δ-haemolytic, non-haemolytic and even coagulase negative (Fox *et al*., 1996; Dinges *et al*., 2000).

Staphylococcus aureus is also catalase positive, oxidase negative and ferments maltose. The organism is salt tolerant, which is able to grow in mannitol-salt agar medium containing 7.5% sodium chloride (Quinn *et al*., 2004).

The ability to clot plasma is generally accepted criteria for the identification of S.aureus due to coagulase production. Free coagulase and slide test are the two coagulase tests used to detect free and bound coagulase or clumping factor respectively. While the tube test definitive, the slide test may be used as rapid screening test to identify *S.aureus*. Coagulase test is carried out using rabbit plasma containing EDTA (Cunha *et al*., 2004).. It is accepted that coagulase production correlates well with the pathogenicity of these bacteria and the coagulase-negative staphylococci (CNS) are deemed as "minor pathogens" (Otto, 2013). A heat stable staphylococcal nuclease (thermonuclease) that has endo and exonucleoltic properties and can cleave DNA or RNA produced by most strains of *S.aureus*. TNase can be demonstrated by the ability of boiled cultures to degrade DNA in an agar diffusion test or detect by using hetrochromatic+ agar diffusion procedure and DNase toludene blue agar (Quinn *et al*., 2011).

2.4. Cell components and virulence factors

The cell envelope of the staphylococci is highly complex. At its core, it consists of a thick, highly cross-linked peptidoglycan layer, with the cross-links themselves consisting of a unique pentaglycine bridge. These cross-links render staphylococcal species resistant to lysozyme but sensitive tolysostaphin, an enzyme currently being explored for therapeutic purposes. Other integral components include teichoic acids, which may be covalently linked to peptidoglycan (wall teichoic acids) or embedded in the cell membrane (lipoteichoic acids). Both of these forms contribute to the resistance of S. aureus and perhaps other staphylococcal species to cationic antimicrobial peptides, and in *Staphylococcus aureus* at least they serve as adhesins to promote colonization (McVey *et al*., 2011).

Staphylococcus aureus can produce more than 30 virulence factors that contribute to establishing and maintaining infection. The factors can be divided into two general groups, including surface associated factors and degradative enzymes, together with exotoxins. These virulence factors enable the organism to be successful as pathogen that causes wide range of human and animal infections. Virulence factors help in attachment to host cells, breaking down the host immune

shield, tissue invasion, causing sepsis and elicit toxin-mediated syndromes. This is the basis for persistent staphylococcal infections without strong host immune response. Based on their mechanism of action and role in pathogenesis, staphylococcal virulence factors are classified as described in table 2 (Quinn *et al*., 2016).

Table 2: Virulence factors of *Staphylococcus aureus* and their pathogenic effects

Virulence factor	Pathogenic effects
Coagulase	Conversion of fibrinogen to fibrin. Fibrin deposition may shield staphylococci from phagocytic cells.
Lipase, esterases, elastase, staphylokinase, deoxyribonuclease,hyaluronidase, phospholipase	These factors facilitate dissemination and enhance toxicity of the organism
Protein A	Surface component which binds the Fc portion of IgG and inhibits opsonization
Leukocidin	Cytolytic destruction of phagocytes of some animal species
Alpha-toxin (α-haemolysin)	The major toxin in gangrenous mastitis. It causes spasm of smooth muscle and is necrotizing and potentially lethal
Beta-toxin (β-haemolysin)	A sphingomyelinase which damages cell membranes
Toxic shock syndrome toxin (TSST)-1	Superantigen activity
Exfoliative toxins	Responsible for desquamation in staphylococcal ‘scalded skin syndrome’ in humans

Enterotoxins	Heat-stable toxins associated with staphylococcal food poisoning in humans

Source: (Quinn *et al*., 2016)

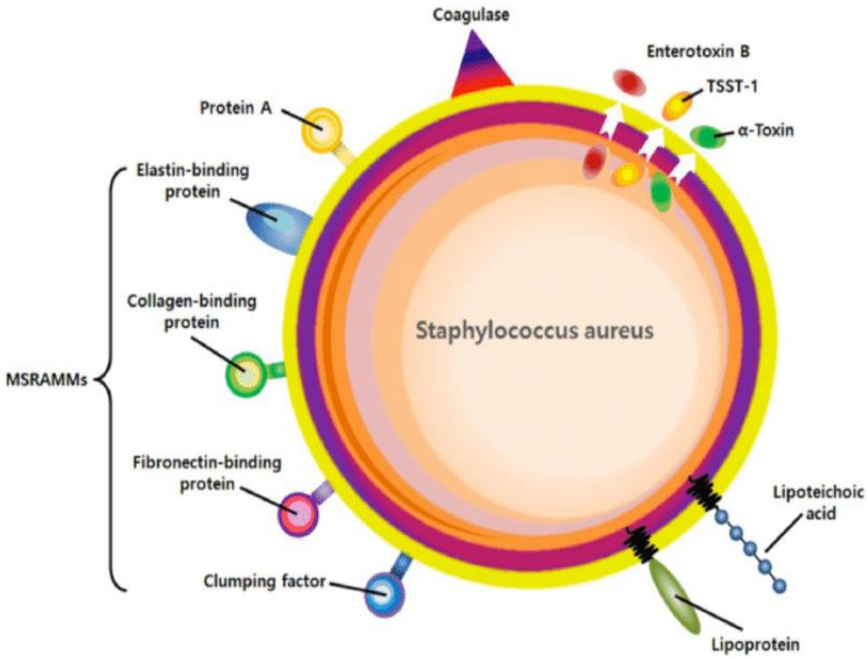

Figure 1: *S.aureus* virulence factors

Source: (Choi *et al*., 2014)

2.5. Methicillin resistant *S.aureus* (MRSA)

The MRSA are those *S. aureus* strains carrying a mecA gene, which codes for additional penicillin-binding protein, PBP2a. The beta-lactam antibiotics exert their antibacterial activity by inactivation of penicillin-binding proteins (PBPs), which are essential enzymes for bacterial cell wall synthesis. However, these antibiotics have only a low affinity towards PBP2a, thus this enzyme evades from inactivation and carry out the role of essential PBPs resulting in cell wall synthesis and survival of bacteria even in presence of beta-lactam antibiotics. Due to the presence of mecA, MRSA are resistant to nearly all beta-lactam antibiotics (Fuda *et al*., 2004).This MRSA strain group of

organisms is also frequently resistant to most of the commonly used antimicrobial agents, including the aminoglycosides, macrolides, chloramphenicol, and tetracycline (Lee, 2003).

Methicillin-resistant Staphylococcus aureus (MRSA) has been grouped into three namely livestock associated MRSA (LA-MRSA), hospital-associated MRSA (HA-MRSA) and community-associated MRSA (CA-MRSA) (Weber, 2005).The latter two groups are associated with human infections. Hospital-associated MRSA was first detected in a UK hospital in 1961, and was detected a few years later in U.S. hospitals and other healthcare facilities where the widespread use of antibiotics selected for bacteria carrying resistance gene (Gordon and Lowy, 2008). Until the 1990s, MRSA was almost exclusively an issue in hospitals and long-term care facilities, affecting surgical patients, other aged or ill residents, and some healthcare workers. Some MRSA infections occurred in non-hospitalized persons but these were traced to close contacts with persons who had been hospitalized. Central Disease Control classified MRSA infections as Hospital acquired). Because of high rate of antibiotic usage in healthcare facilities, HA-MRSA are often resistant to many classes of antibiotics like tetracycline, sulfa-drugs, and gentamycin and others in addition to the β-lactams (Lee, 2003).

There are five major lineages or clonal complexes (CC5, CC8, CC22, CC30, and CC45) that are originated from hospitals and spread globally. Most possess one of the larger SCCmec types I–III, which also carry genes for resistance to other antibiotics. Type II is most common in U.S. HA-MRSA, while type III is found more often in other countries (David and Daum, 2010; McCarthy *et al*., 2010).

LA-MRSA was first detected in 1972 in bovine mastitis milk (Vanderhaeghen *et al*., 2010; Pantosti, 2012; Bal *et al*., 2016. Since then, MRSA reports in different food and companion animals, such as pigs, cattle, chickens, dogs, cats, and horses, have increased *(*Vanderhaeghen *et al*., 2010; Fitzgerald, 2012). A novel strain of MRSA belonging to multilocus sequencing type (MLST) 398 (ST398) and related strains collectively grouped into clonal complex 398 (CC 398) have been frequently found in pigs, chickens, veal calves, dairy cattle, horses, dogs, and milk in various countries (Vanderhaeghen *et al*., 2010).

The emergence of methicillin-resistant *Staphylococcus aureus* on dairy farms can be responsible for the colonization or infection of farm personnel. It is also a source of contamination for consumers via the food chain (Papadopoulos *et al*., 2018). A large number of the reported staphylococcal food-poisoning outbreaks can be traced back to a human source harboring S. aureus producing certain staphylococcal enterotoxins (SEs). Livestock-associated MRSA (LA-MRSA) has created public health concerns because it is a source of antimicrobial-resistant bacteria, which can be transmitted to humans (Asiimwe *et al*., 2017). It is known that people working in close contact with animals, particularly on MRSA-positive farms, are at a higher risk of being colonized with LA-MRSA (Schmidt *et al*., 2017). In addition to the established risk of direct contact with animals, the presence of LA-MRSA in the human food chain is an additional potential route for the spread of the strain to the human population (Smith and Wardyn, 2015).

2.6. General overview of bovine mastitis

Mastitis, the inflammation of parenchyma of mammary glands, is a complex multi-etiological disease affecting dairy cattle worldwide (Radostits *et al*., 2007). It is characterized by physical, chemical and, usually, bacteriological changes in milk, and pathological changes in glandular tissues. The occurrence of disease is an outcome of interplay between infectious agents, host resistance, and environmental factors (Radostits *et al*., 2007; Gera and Guha, 2011). Based on the severity, the inflammation can be classified into sub- clinical, clinical and chronic forms, and its degree is dependent on the nature of causative pathogen and on the age, breed, immunological health and lactation state of the animal (Oliveira *et al*., 2015).

Clinical mastitis is characterized by sudden onset, alterations of milk composition and appearance, decreased milk production, and the presence of the cardinal signs of inflammation in infected mammary quarters. It is readily apparent and easily detected. In contrast, no visible signs are seen either on the udder or in the milk in case of sub-clinical mastitis, but the milk production decreases and the somatic cell count increases. It is more common and has serious impact in older lactating animals than in first lactation heifers (Khan and Khan, 2006). Because of the lack of any overt manifestation, the diagnosis of sub-clinical mastitis is a challenge in dairy animal management and in veterinary practice (FAO, 2014).

The mammary gland, because of its anatomical position, is exposed to several external factors that influence the physiology and pathology of the mammary parenchyma. Several microbial species, when enter into the udder, have the ability to infect the mammary parenchyma resulting in mastitis. Generally the udder defense mechanism clears off the infection, but when the animal is under immune-suppression (especially during immediate) post-partum period), the organism gets upper hand resulting in mastitis. Thus, occurrence of mastitis is an outcome of the interplay between the infectious agents and management practices stressing the defense of udder. Poor milking hygiene, milking machine faults, teat injuries and populations of pathogens on the cow's skin and epithelia and in its environment predisposes the cow to mastitis (Radostits *et al*., 2007).

Correct milking procedures such as milking mastitic cows last, and proper sanitation of utensils, milker's hands and udder before milking could help to improve the situation. The frequency of isolation of coliforms and other micro-organisms causing environmental mastitis is usually directly influenced by unhygienic housing conditions (Mekonnen and Tesafaye, 2010).

Of the several causes of mastitis, the microbes are the vital players in causing bovine mastitis. Although bacteria, fungi, yeasts and possibly virus can cause udder infections, the main agents are bacteria. The first known etiological agent of bovine mastitis identified belonged to the genus Streptococcus while most of the major pathogens causing mastitis were identified during 1940s (Sharma *et al*., 2012).Till date, more than 200 microbial species, subspecies and serovars have been isolated from the bovine mammary gland and this wide spectrum of pathogens are categorized epidemiologically in to contagious and environmental causing agents (Cervinkova *et al*., 2013).

Contagious pathogens are those for which udders of infected cows serve as the major reservoir. They spread from cow to cow, primarily during milking, and tend to result in chronic sub-clinical infections with flare-ups of clinical episodes. Contagious pathogens includes like Staphylococcus aureus, Streptococcus agalactiae, Mycoplasma spp. and *Corynebacterium bovis* (Radostits *et al*., 2007). On the other hand, environmental mastitis can be defined broadly as those intra-mammary infections caused by pathogens whose primary reservoir is the environment in which the cow lives (Smith et al., 1985). Environmental pathogens include E. coli, Klebsiella spp., *S.dysgalactiae* and *S. uberis* and the majority of infections caused by these pathogens are clinical and of short duration (Harmon, 1994).

2.7. Epidemiology of bovine mastitis

Mastitis has been reported in almost all domestic mammals and has a worldwide geographic distribution. Climatic conditions, seasonal variation, density and housing of livestock populations, and husbandry practices may affect the incidence and etiology. Mastitis continues to be the most economically important disease of dairy cattle, due to the expense of antibiotic treatment, along with the associated costs of decreased milk production and decreased fertility or, in cases where antibiotic treatment is ineffective, culling or death (Radostits *et al*., 2007).

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In most countries, the major mastitis pathogens are *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Streptococcus uberis*, *Staphylococcus aureus*, and *Escherichia coli*. But, the predominance of a bacterial species may vary according to the geographical region under scrutiny. S. aureus has been reported as the chief etiological agent of mastitis in most of the world countries by various researchers (Kang-Hee *et al*., 2001; Abdel-Rady and Sayeed, 2009; Sharma and Maiti, 2010; Rahman *et al*., 2010). *S. aureus* is ubiquitous organism and is capable of causing per acute, acute, subacute, chronic, gangrenous and sub-clinical types of mastitis (Sharma *et al*., 2012). The acute form of the disease usually occurs shortly after parturition and tends to produce gangrene of the affected quarters with high mortality. This infectious agent remains to be predominant and one of the significant pathogens causing mastitis in dairy ruminants in many countries, such as the Netherlands, Norway and Italy, Brazil, China, Egypt, India, Iran, Kenya, Turkey (De Oliveira *et al.*, 2000 ; Pengov, 2002) ; Shitandi *et al*., 2004;Turutoglu *et al*., 2005; Zadoks and Fitzpatrick, 2009; Li *et al*., 2009; Suelam *et* al., 2012 ; Sarkar *et al*., 2014. Schmidt *et al*., (2017).

2.7.1. Reservoir and mode of transmission

The infected mammary gland of lactating cows, teat canals and teat lesions are the major reservoirs and source of S. aureus. However, the organism have also been found on teat skin, muzzles, and

nostrils which may predispose to intramammary infection. Haveri *et al*., (2008) revealed that teat skin and especially the teat canals are important potential reservoirs of S. aureus causing intra mammary infections. The bacteria are spread to uninfected quarters by teat cup liners, milkers' hands, washcloths, and flies. Staphylococci do not persist on healthy teat skin but readily colonize damaged skin and teat lesions. The organisms multiply in infected lesions and result in increased chance of teat canal colonization and subsequent udder infection (Quinn *et al*., 2011).

Heifers infected during gestation that carry infections through calving represent an important reservoir from which S. aureus can spread to uninfected herd mates. Heifers with persistently colonized udder or teat skin, muzzles, and vaginas are the primary reservoir. Fresh heifers with colonized body sites can be a source of *S.aureus* when they are introduced into the herd (Radostits *et al*., 2007).

2.7.2. Risk factors

Many factors are involved for development of bovine mastitis most importantly management practice and infectious agents (Biressaw and Deme, 2015). In addition to management and the causative agents, other factors such as udder shape, genetics, (Sori *et al.*, 2005; Awale *et al*., 2012), parity, hygiene, age, lesions, milking practice, stage of lactation and tick infestation also play an important role in the development of mastitis (Dego and Tareke, 2003; Mungube *et al*., 2004; Joshi and Gokhale, 2006; Lakew *et al.*, 2009; Rahman *et al*., 2009; Islam *et al*., 2011; Awale *et al*., 2012; Girma *et al*., 2012; Moges *et al*., 2012)

2.7.3. Economic and public health significance bovine of mastitis

With the increasing intensification and commercialization of livestock and poultry industries, the economic implications of livestock diseases are becoming more important both at farm and national levels (McInerney, 1998), as animal diseases symbolize avoidable waste of scarce resources. Mastitis has been known to cause a great deal of loss or reduction of productivity by influencing the quality and quantity of milk yield, and culling of animals at an unacceptable age (Singh and Singh, 1994). It has been considered to be detrimental to a dairy farm's profitability, not only in terms of production loss and treatment costs, but also because of the loss of the cows themselves (Cobo-Abreu *et al*., 1979 ; Dohoo *et al*., 1983; Solbu, 1984; Bigras-Poulin *et al*., 1990; Rajala and

Grohn, 1998). Most estimates had shown a considerable reduction in productivity per affected quarter and a further reduction in production per cow/lactation, making the disease one of the most costly and serious problems affecting the dairy industry (Bartlet *et al.*, 1991).

Staphylococcus aureus has been associated food poisoning outbreaks and has also been reported in association with food products including milk from different parts of the world. Food poisoning due to staphylococcus is characterized by an acute onset of nausea, vomiting, abdominal cramps, and diarrhea (Le Loir *et al*., 2003). A large number of staphylococcal toxin genes are present in bovine S. aureus. More than half of the *S. aureus* harbored at least one of the enterotoxin coding genes, with sea being dominant, which pose a public health threat to consumers (Seyoum *et al*., 2016). Milk and dairy products are frequently contaminated with S. aureus and milk of infected animals is the main source of heat-resistant enterotoxins, which are responsible for staphylococcal food poisoning outbreaks. Thus, bovine mammary gland is a significant reservoir of enterotoxigenic strains of S. aureus. It can produce two different types of toxin with super-antigen activity: enterotoxins and toxic shock syndrome toxin (TSST-1). Staphylococcal food poisoning occurs when food is consumed that contains endotoxins produced by *S. aureus* (Argudin *et al*., 2010).

Food handlers carrying enterotoxin producing S. aureus in their noses or on their hands are regarded as the main source of food contamination via direct contact or through respiratory secretions. The udders and teats of cows are known sources of enterotoxigenic *S. aureus*, and the occurrence of *S. aureus* in unpasteurized milk and cheese is common. *S. aureus* is an important pathogen due to a combination of toxin-mediated virulence, invasiveness, and antibiotic resistance. This bacterium is a significant cause of nosocomial infections, as well as community-acquired diseases (Wisplinghoff *et al*., 2004).

2.7.4. The occurrence of bovine mastitis in Ethiopia

Different studies conducted in different parts of Ethiopia showed variable prevalence of mastitis depending on the type of farm and managements systems. In Ethiopia, over the years several studies have been carried out in which mastitis pathogens from different types of milk samples. *Staphylococcus aureus* is one of most common pathogen isolated from clinical and sub clinical

mastitis dairy cows (Terefe, 2018). Different studies conducted in different parts of Ethiopia showed variable prevalence of mastitis depending on the type of farm and managements systems. Different reports on the prevalence of mastitis are described in different districts of Ethiopia (Table 3).

Table 3: Prevalence of S. aureus in different districts of Ethiopia

District	Prevalence (%)		Major bacteria isolated	References
	clinical	subclinical		
Sebeta	16.11	36.67	*S.aureus, S.epidermidis* *P.aeruginosa*	Sori *et al*., (2005)
Addis Ababa	37.3	56.9	*S. aureus*	Garedew *et al*., (2015)
Addis Ababa			*S.aureus*	Beyene *et al*., (2017)
Sululta	-	15.3	*S.aureus*	Regasa *et al*., (2019)
	-	53.84	*S.aureus*	Wirtu *et al*., (2018)
Bishoftu	-	40.1	S.*aureus*, *S.intermedius,S. haicus*, other staphylococcus spp, *E.coli*	Birhanu *et al*., (2017)
Adama	10	36.7	*S.aureus*	Abera *et al*., (2010)
Holeta	5.37	65.05	*S.aureus*, *Str.agalactiaeStr.dysgalactiae, Str. Uberis, C.bovis, E. coli*	Dereje *et al*., (2018)

			P. aerogenosa and *K. pneumonie.*	
Kombolch	73.3	42	S.*aureus*	Tilahun *et al*., (2016)
Wolayita	2.6	26.9		Yohannis and Molla, (2013)
Sinna, Bale zone	4.95	31.77	*S.aureus, S. agalactiae, S. epidermidis,* CNS, *E.coli, S.dysgalactiae, C.bovis, K.pneumonia* and *B.cereus*	Elemo *et al*., (2018)
Hawassa	3.4	59.2	S.aureus	Abebe *et al*., (2016)

2.8. Pathogenesis

Many efforts have been made to understand the pathogenesis of bovine mastitis to reduce losses and promote animal welfare. S.aureus, to cause mastitis initially must gain access to the mammary gland through the teat canal and then has to avoid removal by the flushing of the fluids during the milking processes. Therefore, the ability to adhere to the epithelial cells and extracellular matrix proteins is instrumental to colonize the gland and develop the pathologic process. The adhesion mechanism of S.aureus is complex and includes multiple proteins able to specifically recognize components of the microbial surface that recognize adhesive matrix molecules, allowing bacterial anchorage in normal and inflamed tissues (Foster and Hook, 1998).

Adhesive molecules are pivotal in the diffusion of *S.aureus* within and among herds, but they are only one of the several virulence factors involved in the pathogenesis of S.aureus infections. Staphylococcus aureus infections can occur in at all stage of lactation, but clinical mastitis is more common during drying off. Once the bacteria adhere to the milk fat inside the udder it can float upwards deeper into parenchyma tissue of the udder (Kloos and Bannerman, 1995; Janson, 2006).

Staphylococcus aureus has the ability to avoid phagocytosis by producing a polysaccharide containing mucus around itself causing the phagocyte not to recognize it. It is further shielded from the body's defenses by living intra-cellularty (Janson, 2006).

The extracellular defense mechanisms of the host cannot attack intra-cellular organisms and the lower intra-cellular pH reduces the efficacy of many antimicrobial drugs used for treatment of mastitis. Unlike most bacteria, S. aureus can resist the phagocytosis and can even multiply inside a phagocyte. It also uses the phagocyte as a vehicle to carry it deeper into udder tissue. When the phagocyte dies, the *Staphylococcus aureus* is released and it colonizes deep in the udder parenchyma (Fox and Gay, 1993; Pankey, 1989).

Certain strains of *Staphylococcus aureus* may produce enzymes like coagulase, deoxyribonuclease, hyaluronidase, fibrinolysin, lipase and protease. Enzymes produced by *Staphylococcus aureus* destroy oxygen radicals and protect the bacteria against oxidizing agents such as lactoperoxidase, one of the humoral defense mechanisms of the udder (Kloos and Bannerman, 1995); Janson, 2006). The presence of coagulase and deoxyribonuclease correlates positively with the virulence of the bacteria and is used for identification purpose. Various toxins are produced by S.aureus such as alpha, beta, gamma and delta haemolysin, leucocidin and enterotoxin gangrenous, of these the most destructive being alpha–haemolysin which can lead to gangrenous mastitis, which can be fatal to the cow (Anderson, 1976).

There is a high gene content similarity in the genomes of strains causing clinical or subclinical mastitis. However, a lipoprotein demonstrated a higher potential to distinguish clinical and sub clinical mastitis causing groups of bacteria, revealing that sequence variation among bovine S. aureus, and not only the presence/absence of virulence factors, is an important aspect to consider when comparing field isolates causing different mastitis outcomes. The several single nucleotide polymorphism detected on virulence factor might confer advantages to the subclinical strains to successfully evade the immune system without triggering immune responses, therefore facilitating the establishment of chronic and silent infections (Rocha *et al*., 2019).Nevertheless, there is still much to uncover in terms of *S. aureus* pathogenesis, which is hampering effective strategies to combat bovine mastitis (Rainard *et al*., 2018).

2.9. Diagnosis of bovine mastitis

Early diagnosis is extremely important due to the high costs of mastitis. If the lactating animals can be detected quite early before observation of any visual abnormality in milk, this can help the dairy farmers for taking adequate measures to prevent the animals from clinical mastitis. Diagnostic tests such as California mastitis test, culture test and polymerse chain reaction are used in the diagnosis of mastitis infection (Pyorella, 2009).

2.9.1. California Mastitis Test (CMT)

The California Mastitis Test (CMT) is a common indirect method for measurement of somatic cell count (SCC).It determines the quantity of DNA and thus the approximate number of leukocytes. The main advantages of CMT is that it is quick, cheap, simple, and can be used as a "cow-side" test. Owing to its simplicity and rapid reactivity, this test has been used extensively used as a rapid cow side test in the field. The CMT reagent reacts with the neutrophils and the mixture thickens or gels in proportion to the amount of cells that are present. High levels of neutrophils indicate infection (Table 4).The classification of cases based on SCC method and CMT score don't bear very precise relationship. It is mainly because each CMT score (negative, trace, 1, 2, 3) has a very wide and overlapping range of corresponding SCC score (0-2,00,000, 1,50,000-5,00,000, 4,00,000-15,00,000, 8,00,000- 50,00,000 and >50,00,000) respectively (Quinn *et al*., 2004).

Table 4: Interpretation of California mastitis test (CMT)

CMT score	Interpretation	Visible reaction
0	Negative	Milk fluid and normal
T	Trace	Slight precipitation
1	Weak positive	Distinct precipitationbut no gel formation
2	Distinct positive	Mixture thickens with a gel formation
3	Strong positive	Viscosity greatly increased. Strong gel that is cohesive with a convex surface.

Source: (Quinn *et al*., 2004).

2.9.2. Phenotypic methods

The standard method of diagnosing mastitis caused by Staph. aureus is microbiological culturing of milk from the infected quarter (Oliver *et al*., 2004). Bacterial culture has for some time served as the gold standard for the examination of phenotypic characteristics. Appropriate use of culture-enhancement methods can significantly increase sensitivity in the detection of mastitis-associated organisms in milk, and targeted use of selective media may offer significant improvements in sensitivity in composite cow samples and bulk tank milk culture (Britten, 2012).Phenotypic identification is based on an evaluation of morphology, growth characteristics, and ability to metabolize substrates, antimicrobial resistance, and other features that result from DNA expression (Zadoks and Watts, 2009).

Widely used medium for the isolation of S. aureus is Mannitol Salt Agar (MSA), which inhibits the growth of other bacteria due to the presence of 7.5% NaCl. *S. aureus* ferments mannitol and forms yellow colonies on the medium. In addition, blood agar is often used for the isolation of Staphylococcus spp. from clinical materials. *S. aureus* produces yellow, circular, and medium-sized colonies surrounded by a clear halo that indicates hemolytic activity on blood agar. Typical colonies obtained on the media described above are submitted to Gram staining to determine their purity and to observe their morphology and specific color. After confirmation of these characteristics, the isolates may be submitted to catalase and coagulase tests. The tube coagulase test for the detection of free coagulase continues to be the best for the identification of S. aureus and other coagulase-positive staphylococci. The test should always be analyzed after 4 h of incubation since some strains will produce fibrinolysin when incubated for 24 h, causing dissolution of the clot during the period of incubation, and may be erroneously identified as coagulase-negative staphylococci (CNS) if the test is read only after 24. Presumptive identification staphylococci is based on the bases of double zone haemolysis, cultural and morphological features table 5 (Carter and Wise, 2004).

Characteristic	S.aureus	S.hyicus	S.intermedius	S.epidermidis
Coagulase	+	V	+	+
Clumping factor	+	-	V	-
B-hemolysis	+	-	+	-
Pigment	+	-	-	-

Table 5: Some differential characteristics of important Staphylococci

Source: (Carter and Wise, 2004)

2.9.3. Molecular diagnosis of bovine mastitis

Several molecular typing methods have been used in order to investigate the bovine mastitis epidemiology at the level of subspecies. They include methods of comparative typing based on standard electrophoretic bands, typing methods of libraries and genome sequencing. Among the typing methods based on fingerprint, PCR-restriction fragment length polymorphism, random amplification of polymorphic, enterobacterial repetitive intergenic consensus polymerase chain reaction, and repetitive element sequence based PCR have been described as simple and quick techniques for taxonomic and epidemiological analyses of several species of the genus Staphylococcus (Bergonier *et al*., 2014; Idil and Bilkay, 2014; Rahman *et al*., 2016); Ławniczek-Wałczyk *et al*., 2017). Epidemiological molecular studies have contributed significantly to the understanding of the sources, routes of transmission and prognosis for many pathogens that cause bovine mastitis, as well as to understand the mechanisms of adaptation to the host and the causes of the disease (Supré *et al*., 2011; McMillan *et al*., 2016).

2.10. Treatment and prevention

Mastitis is among front line diseases that leads to the use of antimicrobials on dairy farms (Menéndez González *et al*., 2010). Control and eradication programs represent the most promising strategy for dairy owners to prevent contagious mastitis (Petersson-Wolfe *et al*., 2010). Implementation of such programs is reasonable because of the often unsatisfactory cure rate, which can be achieved with antibiotic therapy against Staph. Aureus in single cows (Sol *et al*., 1997; Sol *et al*., 2000; Gruet *et al*., 2001 ; Barkema *et al*., 2006). Additional reasons are the inappropriate use of antibiotics for mastitis therapy, as well as the limited efficacy of vaccination against *S.aureus* IMI (Peton and Loir., 2014; Landin *et al*., 2015).

Eliminate existing infections by using selective removal of chronic cases from the herd, dry cow treatment and therapy during lactation are the predominant methods used to eliminate existing S.aureus infections from dairy herds (Erskine, 2001).Culling of cows with chronic mastitis is one of the cornerstone recommendations of the original point of mastitis control programs (Philpot, 1979).

The use of long- acting intramammary treatment at the time drying-off is another means of mastitis control program. Dry cow treatment has a higher cure rate of existing infection than therapy during lactation. With the use of dry cow therapy before freshening, clinical mastitis at calving is reduced. Even with the use of long acting antibiotics, the risk of contamination of saleable milk is minimal. With all of these benefits, treatments of all quarters of all cows at drying off have become a standard recommendation (Schukken *et al*., 2003).

Controlling risk factors associated with the disease; reduction of repeated use of drugs; awareness creation among veterinarians, dairy farm owners and dairy workers on the effect of mastitis; identification of the causative agent and susceptibility test profile of pathogens before treatment; regular investigation of mastitis; culling of old aged and repeatedly infected cows and further investigation and molecular diagnosis on mastitis causative agents including S.aureus optimizes prevention and control options in different dairy farms (Terefe, 2018).

2.11. Antimicrobial resistance of *S.aureus*

The emergence of antibacterial resistance among pathogens that affect animal health is of growing concern in veterinary medicine as these resistant pathogens in animals have been incriminated as a potential health risk for humans (Moon *et al*., 2007). *S. aureus* exhibits resistance to a wide range of antimicrobial agents including disinfectants (Bjorland *et al*., 2001).

In the past, staphylococcal infections were treated using penicillin, but over the years this pathogen developed resistance to penicillin by building penicillinase. Methicillin was the next drug of choice as it is not cleaved by the penicillinase. While methicillin is very effective in treating most Staphylococcus infections, some strains have developed resistance to methicillin by production of penicillin binding protein and can no longer be killed by this antibiotic. These resistant bacteria are called methicillin resistant *Staphylococcus aureus* (MRSA) (Siegrist, 2011).

Resistance of this pathogen to antimicrobials is mainly due to several factors. One of these factors is formation abscesses within the udder that are surrounded by a thick fibrous capsule (Almeida *et al*., 1996). This prevents sufficient concentrations of antibiotic from entering the abscess itself and hence blocks the effective destruction of viable bacteria. Some strains of *S. aureus* can also live within cells such as macrophages. Most antibiotics are only able to circulate in the body fluids surrounding cells and cannot penetrate within cells themselves, hence these staphylococci are protected from the majority of antibiotics(Herbert *et al*., 2000).

Inherent ability of the bacteria to form biofilms is another possible way of deterring the action of antimicrobial agents (Melchior *et al*., 2006). Biofilm formation is accompanied by significant genetic and subsequent physiological changes in the microorganisms resulting, inter alia, in a loss of sensitivity to virtually all classes of antibiotics. The presence of extracellular matrix in biofilm acts as a barrier that slows down the infiltration, neutralizes, binds, and effectively diffuses to sub lethal concentrations of antimicrobial agents such as chlorine species, oxacillin, and vancomycin before they can reach cell targets.

Biofilm cells can also activate chromosomal β-lactamases and efflux pumps (e.g., QAC efflux system of *S. aureus*), induce mutations in antimicrobial target molecules or, indirectly, release extracellular DNA that promotes the synthesis of biofilm matrix to counteract the effect of antimicrobials (Anderson and O'Toole, 2008; Kaplan *et al*., 2012).

3. MATERIALS AND METHODS

3.1. Description of the study area

The study was conducted in Lume woreda, East Shoa Zone, Oromia region, central Ethiopia. The woreda which, is located in the Great Rift Valley, is bordered on the South by the Koka reservoir, on the West by Ada‟a Chukala, on the North West by Gimbichu and on the North by East Adama town. The capital town of the woreda is Modjo which is located 70 kms South-East of Addis Ababa. The altitude of the district ranges from 1500 to 2300 meter above sea level and the annual rainfall range from 500-1200 mm. The minimum and maximum annual mean temperatures are 14 and 27^0C respectively.

Livestock is considered as an important component of the prevailing crop-livestock mixed farming systems of the study district. The study area owned various livestock species such as; cattle, sheep, goat, chicken and equines. Dairy cattle are mostly reared in small, medium and large scale dairy operations, in which animals are managed both intensively and extensively. Dairying is the first income source for about 62% of the dairy producing households in Lume district (Melesse, 2005**)**. According to the district Agricultural office of Lume district, the study district is reported to have a total population of 33,797 for cattle, 10,953 for sheep and goat, 12,699 for equine, 31, 984 for chicken, which (26,852 local and 5132 cross and exotic breed chicken). Available reports showed that Gimbichu is arable or cultivable, 3% pasture, 2% forest and the remaining 20 % is considered degraded or otherwise unusable. There are 260 dairy farms in Lume Woreda according to agricultural office of Lume district and the Woreda is known for its crop and livestock production.

3.2. Study population

The study population was lactating dairy animals that are apparently healthy and clinical ones in Lume woreda. The study population comprises both exotic and cross breeds in which herd size ranges from 10-230 managed under intensive and extensive management conditions. Dairy Cows which have treated for mastitis either intramammary or systemic route during the study period were excluded.

3.3. Study design

A cross-sectional study was conducted from November 2019 to May 2020 to isolate the occurrence of *S.aureus* and assess the antimicrobial susceptibility profile of the isolates from clinical and subclinical mastitic cows.

3.4. Sample size

3.4.1 Sample size determination and sampling strategy

The sample size was calculated according to the formula given by (Thrusfield, 2007) with expected prevalence of 16.2% at 95% confidence interval and significance level of 5%. Simple random sampling method were considered to select the individual apparently healthy cow. The sample size of dairy cows was determined by taking prevalence of *S. aureus*, 16.2% isolated from mastitic dairy cows conducted by Mekuria *et al*., (2013), in central Ethiopia. Accordingly, the calculated value for sample size is equal to 208. However, the sample size of this study (200) is lower than our reference sample size (208) as our objective was isolating *S.aureus* from bovine mastitis.

$$N = \frac{1.96^2 \text{ pexp } (1—\text{pexp})}{d^2}$$

Where

1.96 =the value of Z at 95% confidence interval

N= number of sample size

Pexp= expected prevalence = 16.2%;

d2= absolute precision = 5%

CI= confidence interval (95%)

3.5. Methodology

3.5.1. Clinical inspection of the udder

The udder of lactating cows were examined clinically, using visual, then through palpation to detect the presence of any abnormality. Besides, milk was withdrawn and checked from each quarters for any change in color and consistency of cow's milk (Quinn *et al*., 2004). Cows with clinical cases were diagnosed on the bases of manifestation of visible signs like inflammation characterized by swollen and warm with painful upon palpation and other chronic lesion such as atrophied, hard and fibrotic quarters of the udder.

3.5.2. California Mastitis Test (CMT)

California mastitis test was used as a screening test for subclinical mastitis according to the procedure described by Quinn *et al*., (2004). A squirt of milk from each quarter of the udder was placed in each of four shallow cups in the CMT paddle and an equal amount of the CMT reagent was added. A gentle circular motion was applied to mixtures in a horizontal plane for about 10 seconds. Quarters samples that showed gel formation within a few seconds were considered as positive. The CMT results were scored as 0 (negative), T (trace), 1(weak positive), 2(distinct positive) and 3(strong positive) based on gel formation. Cows that showed CMT score >1 with at least one quarter was considered as positive or all quarters with CMT score T (trace) (Quinn *et al*., 2004).

3.5.3. Sample collection and transportation

Aseptic procedure was followed while collecting milk samples in order to reduce or prevent contamination with microorganisms (Quinn *et al*., 2004). Before collecting the sample from each quarter, teats were disinfected. Accordingly, teat ends were wiped thoroughly with cotton soaked

in 70% ethyl alcohol after washing and drying a quarter with tap water. After the discharge of the first three milking streams, approximately 10 ml of individual quarter milk samples were collected from clinical and CMT positive cows into horizontally held universal bottles. At sampling, the near teats were sampled first and then followed by the far ones. All collected sample from each quarter were labeled and placed in an icebox and transported to National Animal Health Diagnosis and Investigation Center (NAHDIC) for microbiological analysis. Samples that were not inoculated immediately were kept at 4°C until cultured on appropriate media for isolation of the organism.

3.5.4. Bacterial isolation and identification

Bacteriological culturing and identification was done at National Animal Health Diagnosis and Investigation Center (NAHDIC) of microbiology laboratory. Both clinical and CMT positive milk samples from each quarters were enriched with brain heart infusion broth (Preethirani *et al*., 2015). Before enriched with brain heart infusion broth, refrigerated milk samples were warmed at room temperature for 25min and then homogenized using a Vortex mixer. Aloopfull of brain heart infusion (BHI) broth culture were streaked on mannitol salt agar (Difco, USA) and incubated at 37˚C for 24-48h. Presumptive colonies producing yellow pigment on the media were selected and sub cultured on nutrient agar overnight to get pure culture (Quinn *et al*., 2004). Gram's stain was made from nutrient agar and observed under a light microscope for Gram's reaction, size, shape and cell arrangements. The presence of Staphylococcus was confirmed based on Gram's stain and colony pigmentation on mannitol salt agar (Quinn *et al*., 2004).

Then suspected culture from nutrient agar was subjected to catalase test, coagulase test, hemolysis and cultured on purple agar base (PAB).The culture to be tested for catalase test was picked with sterile inoculating loop from the nutrient agar plate and mixed with a drop of 3% H_2O_2 on a clean glass slide and the formation of oxygen bubbles was observed within a few seconds. Tube coagulase test was performed by adding 0.5 ml of horse plasma to 0.5ml BHI culture broth then mixed and incubated for 24 h at 37 °C. The mixture with in tube was followed at 4 h interval if any degree of clotting was visible within the tube when tilted. The suspected culture was also inoculated on PAB media with 1% of maltose and incubated at 37 °C for 24 h. Samples were considered positive for *S. aureus* when the suspected isolates were catalase positive, coagulase positive and

showed rapid fermentation of maltose on PAB (Quinn *et al*., 2004).Biochemically confirmed isolates were preserved until confirmed by using molecular technique.

3.5.5. Antibiotic susceptibility test

Antimicrobial susceptibility of *S.aureus* isolates was evaluated by Kirby-Bauer disk diffusion method on Meuller-Hinton agar (MHA) (HiMedia Laboratories, India) plates according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2012). Small inoculum of each bacterial isolate was selected and emulsified in 3 ml sterile normal saline solution in a sterile test tube. The density of bacterial suspension was measured to 0.5 with McFarland densitometer (Cambs, England) in order to standardize the size of inoculum. A sterile cotton swab was then dipped into the standardized suspension of the bacterial culture, squeezed against the sides of the test tube to remove the excess fluid and evenly inoculated into Mueller-Hinton agar and allowed to dry the flood. Nine commercially available antimicrobial agents were selected from ten different antimicrobial classes or groups. Thereafter, antimicrobial discs with following drug contents: penicillin G [10 IU], cefoxitin [30 µg], cefotaxime [30µg], gentamycin [10 µg], erythromycin [15 µg], tetracycline [30 µg], ciprofloxacin [10 µg], sulphamethoxazole-trimethoprim [25 µg] and chloramphenicol [30µg]were placed on the agar with sterile forceps and gently pressed down to ensure contact. The inoculated agar plates with antimicrobial discs were left at room temperature for 30 min followed by incubation at 37^0c in inverted fashion for 18h. The results were classified as sensitive, intermediate and resistant according to the standardized table supplied by the manufacturer (CLSI, 2012) as described on table 16. *Staphylococcus aureus* ATCC 25923 was used as quality control.

Table 6: Antimicrobial susceptibility test interpretive criteria for *S.aureus*

Antimicrobial agent	Symbol	Disc content	Susceptible	Intermediate	Resistant
Pencillin G	P	10IU	≥ 29	-	≤ 21
Cefoxitin	FOX	30 µg	≥ 22	-	≤ 21
Cefotaxime	CTX	30 µg	≥ 23	15-22	≤ 14
Gentamycin	CN	10 µg	≥ 15	13-14	≤ 12
Erythromycin	E	15 µg	≥ 23	14-22	≤ 13
Tetracycline	T	30 µg	≥ 19	15-18	≤ 14
Ciprofloxacin	CIP	10 µg	≥ 21	16-20	≤ 15
Sulphametoxazol-trimetoprime	SXT	23.75/1.5 µg	≥ 16	11-15	≤ 10
Chloramphenicol	C	30 µg	≥ 18	13-17	≤ 12

3.5.6. Data Management and Statistical Analysis

Data were entered to a Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA, USA). Data were summarized using descriptive statistics. The prevalence of *S.aureus* in clinical and subclinical samples were estimated using standard formula. The number of positive samples were divided by the total number of samples examined multiplied by 100.R statistical software Version 3.5.2 (R Core Team, 2018) was used to analyze the data (Annex). Pearson chi-square. P-value less than 5% ($P<0.05$) was considered statistically significant. For antimicrobial susceptibility test the results were interpreted according to Clinical and Laboratory Standards Institute (CLSI, 2012) 'interpretive criteria for *S.aureus* (Table16).

3.6. Constraints of the study

The research was initially proposed to isolate, molecularly characterizing Staphylococcus in terms of virulence factors and also aimed to look association between phenotypic and genotypic antimicrobial resistance determinants of S.aureus isolates recovered from dairy farms of the study area, however, the objective doesn't met due to pandemic of COVID-19 which was initially started in Wuhan, China. Due to this reason postgraduate research conducted in some laboratories in Addis Ababa has been suspended since the 4th cases of COVID-19 in Ethiopia. Similarly, the National

Animal Health Diagnostic and Investigation Center (NAHDIC) which I was conducted my research stopped its routine diagnostic activities the as institute was assigned as one of testing laboratory for screening of the COVID-19 in Ethiopia. Hence, due to this reason our initial objective doesn't met and we are forced to do our work to the isolation level only.

4. RESULTS

4.1. Proportion of mastitis

In total, 800 quarters from 200 cows were included in the study. Of these, 11 quarters (13.8%) belonging to 8 cows were blind. Hence, a total of 789 functional quarters was examined by California Mastitis (CMT). Of lactating dairy cows examined for mastitis using CMT screening test and clinical examination, 123 (61.5%, 95% CI: 0.59, 0.64), 104 (52%, 95% CI: 0.45, 0.59) subclinical and 19 (9.5%, 95% CI: 0.05, 0.14) clinical cases were found positive. From cows having blind quarters, 5 (62.5%, 95% CI: 0.60, 0.65) cows had only one blind, 3 (37.5%) cows had two blind quarters. Two (18.2%), 2 (18.2%), 5 (45.5%), and 2 (18.2%) of the blind quarters were at the right front, right hind, left front and left hind positions respectively.

Out of 789 quarter milk samples examined, 331quarters were CMT positive and 20 quarters had CM. The remaining 458 milk samples were found CMT negative. From CMT positive cows (52.5%, n=104), 46 (4.4%) showed trace reaction with more than one quarter, 30 (28%), 16 (15.4%), and 12 (11.5%) were showed weak, distinct and strong reactions respectively.

4.2. Occurrence of *S.aureus*

Among a total of 123 lactating exotic and crossbred cows with either clinical or subclinical mastitis examined for the presence of *Staphylococcus aureus*, *Staphylococcus aureus* was isolated at rate of 29.8% and 26.3% of the sub- clinical and clinical cases, respectively. The overall isolation rate of *Staphylococcus aureus* recorded in this study was (29.3%, 95% CI: 0.21, 0.37). Similarly, out of 331quarter CMT positive and 19 clinical milk samples, 48 (14.5%) samples were positive for *S.aureus*. From this, 8 (40%) isolates were from CM and the remaining 40 (12.1%) were isolated from SCM (table 8). Our present findings also revealed that the number of *Staphylococcus aureus* isolates were statistically significant with the categories of mastitis (p-value <0.05) as shown in table 7.

Table 7: *Staphylococcus aureus isolated in cows by the mastitis type*

Form of mastitis	No. of positive samples	S. aureus (%)	X-squared	p-value
Clinical	19	5 (26.3)		
Subclinical	104	31(29.8)	27.616	<0.05
Over all	123	36 (29.3)		

The number of isolates from SCM also varied according udder quarter location, and remained highest in left front (LF;15 (37.5%) quarters, followed by right front (RF; 13 (27.1%) quarters, right hind (RH; 10 (20.8.0%) quarters, left hind (LH; 10 (20.8) SCM) quarters, respectively. 4 (8.3%), 2 (4.1%), 1 (2.3%) and 1 (2.3%) of clinical isolates were isolated from right front, right hind, left front and left hind quarter respectively..

Table 8: Cow and quarter-level occurrence of *Staphylococcus aureus* mastitis (clinical and subclinical) in large and small holder exotic and crossbred lactating cows

Observation level		Clinical mastitis		Subclinical mastitis	
	No.examined	+ve cases (%)	S.aureus (%)	CMT +ve (%)	S.aureus (%)
Cow level	200	19 (9.5)	5 (26.5)	104 (52.5)	31(29.8)
Quarter level	800	20 (2.5)	8 (40)	331(41.4)	40 (12.8)

4.3. Antimicrobial sensitivity test

Out of 36 isolates of *S.aureus* 34 were subjected to *in vitro* antimicrobial sensitivity test to nine commercially available antimicrobial agents. The antibiogram result of the isolates from bovine mastitis revealed a varying degree of susceptibility and resistance. The degree of susceptibility ranges from 2.9 % up to 100% whereas resistance ranges from 2.9% up to 91.2%. *S.aureus* isolates were highly susceptible to Chloramphenicol (100%), followed by Ciprofloxacin (97.1%) and Sulphametoxazol-trimetoprime (97.1%), Cefotaxime (94.1%) and Cefoxitin (88.2%). About 91.2% and 76.5% of *S.aureus* isolates were highly resistant for Pencillin G and Tetracycline respectively. Gentamycin and Erythromycin also showed low level of resistance to 7 (20.6%) and 5 (14.7%) isolates respectively as shown table 8.

Table 9: Antimicrobial susceptibility test result of *S.aureus* isolates

		Susceptibility and resistance profile of 34 isolates to respective antimicrobials		
Antimicrobial agent	Disc content	Susceptible (%)	Intermediate (%)	Resistant (%)
Pencillin G	10 IU	1 (2.9)	2 (5.9)	31 (91.2)
Cefoxitin	30 µg	30 (88.2)	0 (0)	4 (11.8)
Cefotaxime	30 µg	32 (94.1)	1 (2.9)	1 (2.9)
Gentamycin	10 µg	21 (61.8)	7 (20.6)	6 (17.6)
Erythromycin	15 µg	25 (73.5)	5 (14.7)	4 (11.8)
Tetracycline	30 µg	8 (23.5)	0 (0)	26 (76.5)
Ciprofloxacin	10 µg	33 (97.1)	0 (0)	1 (2.9)
Sulphametoxazol-trimetoprime	23.75/1.5 µg	33 (97.1)	1 (2.9)	0 (0)
Chloramphenicol	30 µg	34 (100)	0 (0)	0 (0)

Key: %= Percent

All 34 *S.aureus* isolates were resistant to the seven antimicrobials tested except to that Sulphametoxazol-trimetoprime and Chloramphenicol as indicated in table 9. Most of the isolates showed resistance against two or three antimicrobials while only one isolate showed resistance against four antimicrobials tested as indicated in table 10. Regarding the multidrug resistance profile, 26.5% of isolates were found to be multi-drug resistant (MDR).

Table 10: Antimicrobial resistant pattern of isolates

	Antimicrobial resistance pattern	Total number of resistant isolates
For one	P (31), FOX (4), CTX (1), CN (5), E (4), TE (26), CIP (1),	72
For two	P TE (15), P FOX (4) , E TE (3), TE CIP (1), P CTX (1), CTX E (1), FOX TE (4), CN TE (3)	32
For three	P CTX TE (1), P FOX TE(3), P E TE (2), P CN TE (2)	8
For four	P FOX E TE (1)	1

Key: Pencillin G (P), Cefoxitin (FOX), Cefotaxime (CTX), Gentamycin (CN), Erythromycin (E), Tetracycline (TE), Ciprofloxacin (CIP), Sulphametoxazol-trimetoprime (SXT) and Chloramphenicol (C)

5. DISCUSSIONS

5.1. Proportion of mastitis and isolation rate of *S.aureus*

The present study has given a due attention to isolate *Staphylococcus aureus* and determine the occurrence of antimicrobial resistance of this pathogen. Accordingly, the present study revealed an overall occurrence of mastitis of 61.5% and 41.4% at cow and quarter respectively which were determined by the CMT and clinical examinations. The finding of present study at cow level (61.5%) was relatively comparable with the previous reports of Abebe *et al*., (2016), Zeryehun and Abera, (2017) and Elemo *et al*., (2017) who reported 62.6% in Hawassa in South Ethiopia, 64.3% in Eastern Harrarghe Zone and 65.36% in Asella in Southern Eastern Ethiopia, respectively. However, the present finding is relatively higher than the reports from other studies (Marama *et al*., (2016), Asmamaw *et al*., (2017). This variability in prevalence of mastitis irrespective of the cause between different reports could be attributed to differences in farms management practice or to differences in study methods agro-climatic condition. Detail comparison and discussion with other previous was not established as small samples were collected in this study.

According to the microbiological finding of this study, 29.3% of *S.aureus* isolates were recovered from both clinical and subclinical mastitic cows. This is relatively in line with previous findings of Zeryehun *et al*., (2013) and Yohannis and Molla, (2013) who reported 28.8% *S. aureus* isolates in and around Addis Ababa, 30% in Wolaita Sodo, Southern Ethiopia respectively. Shitandi and Sternesjo, (2004) in Kenya reported an equivalent prevalence rate (30.6%) of *S. aureus*. However, the present finding is higher than other studies (Bitaw *et al*., 2010; Abebe *et al*., 2013). Similarly, it is by far higher than the findings of Hussein *et al*., (1997) in Addis Ababa, Ethiopia (10%), Mekuria *et al*., (2013) around Addis Ababa, Ethiopia (16.2%), and Sania *et al*., (2018) in Addis Ababa, Ethiopia (5.2%). On contrary, higher isolation rate than the present result was reported by others (Mekonnen *et al*., 2005; Sori *et al*., 2011; Birhanu *et al.*, 2013; Garedew *et al*., 2015; Abebe *et al*., 2016; Mitiku *et al*., 2017).

The frequency of *S.aureus* in subclinical mastitis (29.8%, n= (31/104) was higher compared to clinical mastitis, (26.3% (5/19) with statistical significant difference ($P < 0.05$). This isolation rate of *S.aureus* in subclinical mastitis higher than the clinical one was slightly similar with previous

finding that showed *S. aureus* is the principal causative agent of subclinical mastitis (Asmamaw *et al*., 2017). This result further supported by many other studies in different region of the country which have proved that subclinical mastitis is more frequent than clinical one (Sori *et al*., 2005; Getahun *et al*., 2008; Belayneh *et al*., 2013; Abebe *et al*., 2016; Biniam *et al*., 2017). On the contrary, Tilahun *et al*., (2016) reported higher isolation rate of S.aureus in clinical (73.3%) than subclinical mastitis (42%).

Higher prevalence rate of subclinical mastitis, which strengthen this study, was also reported by Ali *et al*., (2018); Wald *et al*., (2019) from Egypt and Austria, respectively. This might be due to *S. aureus* capacity to survive in the udder and usually establishes chronic and subclinical infection of long duration from which it is shaded through milk serving as sources of infection for other healthy cows and transmitted during the milking process (Radostits *et al*., 2007).

5.2. Antimicrobial resistance test

In Ethiopia, there have been reports on the drug resistance of *S.aureus* isolates from bovine mastitis (Abera *et al*., 2010; Tilahun *et al*., 2016; Beyene *et al*., 2017; Mekonnen *et al*., 2018). The antimicrobial susceptibility tests carried out in this study indicates that *S.aureus* isolates from clinical and subclinical mastitic cows displayed differences in the antimicrobial susceptibility patterns. This current study revealed the highest sensitivity of most of S. aureus isolates towards Chloramphenicol (100%), followed by Ciprofloxacin (97.1%) and Sulphametoxazol-trimetoprime (97.1%), Cefotaxime (94.1%) and Cefoxitin (88.2%). The absence of resistance against Chloramphenicol is in agreement with the previous findings of Gizat, (2004) and Abera *et al*., (2013) who indicated 100% sensitivity of *S.aureus* isolates to chloramphenicol. In other investigation conducted by Wang *et al*., (2014), a good efficacy to chloramphenicol (100%) was exhibited by *S. aureus* isolates. This might implies that such antibiotics can be used to treat infections caused by *S. aureus*.

This study also demonstrated 97.1% susceptibility of *S. aureus* to Ciprofloxacin and Sulphametoxazol-trimetoprime. This is relatively comparable with the findings of Daka *et al*., (2012) that reported 0% and 7.7% resistance around Hawassa to Ciprofloxacin and

Sulphametoxazol-trimetoprime respectively. The reason why these antimicrobials were very effective against S.aureus in this experiment might be that they are not used in the study area in veterinary clinics and even may not be the first choice for treatment of mastitis in most part of the country hence the chance to develop resistance will be minimum.

Low level of resistance to Gentamicin (17.6%) and Erythromycin (11.8%) was recorded in the study area. Resistance to Gentamicin was in line with study of Elemo *et al*., (2017) who reported 17.8% in and around Asella town, Arsi Zone, South Eastern Ethiopia . Emeru *et al*., (2019) reported low level of resistance of Gentamycin (3.3%) and erythromycin (3.3%) which were far below the current study.

The isolates of *S.aureus* were found to be highly resistant among others to penicillin G (91.7%) followed by tetracycline (76.5%). This was in parallel with the report of Abebe *et al*., (2013) who recorded resistance of *S. aureus* to penicillin G and tetracycline found to be 94% and 73.8%, respectively, around Addis Ababa. Kalayu *et al*., (2020) also reported that 91.7% milk isolates of *S.aureus* isolated in dairy farms of Mekelle town were resistant to pencillin G which is in harmony with this report. The resistance of *S. aureus* to penicillin may be attributed to the production of beta lactamase, an enzyme that inactivates penicillin and closely related antibiotics. It is believed that around 50% of mastitis causing *S. aureus* strains produce betalactamase (Green and Bradely, 2004).

Moreover, the present finding was comparable with the result of Tilahun *et al*., (2016) who found resistance of 77.4.% isolates to tetracycline. Sania *et al*., (2018) reported relatively similar results around Kombolcha who recorded 78.6% to tetracycline. On the other side , the resistance of *S.aureus* to tetracycline in this report (76.5%) was relatively higher than the earlier studies conducted by others (Asmamaw *et al*., 2017; Emeru *et al*., 2019; Regasa *et al.*, 2019; Kalayu *et al*., 2020). High tetracycline resistance could be associated to the fact these drugs specifically tetracycline is commonly used in the treatment of infections in the current study area than the previous area. Lack of stringent regulation and monitoring in the dispensing and use of antimicrobials in the country also might contribute to the occurrence of high antimicrobial resistance to these drugs.

The present study has demonstrated the existence of alarming level of resistance of *S. aureus* to commonly used antimicrobials (pencillin G and tetracycline) in the study area. These results were

further supported by findings from previous studies in other countries (Malinowski *et al*., 2002; Jamali *et al*., 2014; Islam *et al*., 2016). This suggests the fact that prolonged and indiscriminate usage, and frequent prescriptions of particular drugs often leads to possible resistance development in the animals, which were also revealed by other authors (Gentilini *et al*., 2000; Edward *et al*., 2002; Jaims *et al*., 2008).

Cefoxitin disk diffusion method using cefoxitin disc was used to detect methicillin resistant *Staphylococcus aureus* isolates in the study area. It far superior to most of the currently recommended phenotypic methods like oxacillin disc diffusion and is now an accepted method for the detection of MRSA (CLSI, 2012). Cefoxitin disk test showed that low rate (11.8%) of MRSA in this study which contradicts reports from the other previous studies (Tigabu *et al*., 2015; Asmamaw *et al*., 2017; Biniam *et al*., 2017; Elemo *et al*., 2017). All cefoxitin resistant *S. aureus* isolates in this study were also found resistant to penicillin.

According to the definition of Magiorakos *et al*., (2012) for multidrug resistance, 26.5% isolates were multidrug resistant to at least three or more different antimicrobials. Eight (8) *S.aureus* isolates (23.5%) were resistant to three (3) antimicrobials followed by one (1) isolate (2.9%) which was resistant to four (4) antimicrobials as shown in table 9. In the present study, multi-drug resistance pattern of *S.aureus* isolate were found. Based on analysis of multidrug resistance patterns, 23.5% isolates exhibited resistance with the combination of P CTX FOX, CTX FOX TE, and CN CTX TE, P FOX TE, P E TE and P CN TE. Meanwhile, 2.9% of isolates showed resistance to combination of Pencillin, Erythromycin and Tetracycline. In addition, all isolates have showed resistance to all antibiotics tested except Chloramphenicol. The probable explanation could be S.aureus strains have the capacity to change their resistance behavior to the exposed antimicrobial.

Results from this study indicates that Chloramphenicol is the drug of choice for *S.aureus* treatment, since all isolates were susceptible to this drug. This study helps for suggesting the use of antibiotics.

6. CONCLUSION AND RECOMMENDATIONS

The overall isolation rate of *Staphylococcus aureus* in the study area was 29.3%, which was 29.8% and 26.3% in clinical and subclinical mastitis respectively. This proportion *S.aureus* in the study area will undoubtedly has an adverse effect on productivity of dairy farms. Antimicrobial susceptibility test showed *S. aureus* isolates were highly sensitive to Chloramphenicol (100%) followed by Ciprofloxacin and Sulphametoxazol-trimetoprime (97.1%), Cefotaxime (94.1%) and Cefoxitin (88%) whereas, the highest rate of resistance among the isolates was against penicillin G (91.7%) followed by tetracycline (76.5%). Besides, 26.5% *S. aureus* isolates from mastitic cows showed multidrug resistance to commonly used antibiotics. Resistance to the some of these tested antibiotics indicates that they are no longer effective against *S. aureus*. Moreover, the presence such multi drug resistant (MDR) *S.aureus* in milk may impose public health hazard in the study area. This ensures that the right use of antibiotics of choice is mandatory in line of treatment and control of the infections caused by *S. aureus*. Based on the above conclusion, the following recommendations were forwarded,

- ✓ There should be a regular antimicrobial sensitivity testing, not only in study area but, also at a national level to select effective antibiotics against this pathogen.
- ✓ Use of Chloramphenicol should be encouraged to treat bovine mastitis caused by *S.aureus* infected cows in the study area.
- ✓ Management factors that could potentially associated with *S.aureus* infection should be studied in study area for more comprehensive control of mastitis due to this pathogen
- ✓ Further advanced molecular studies should be conducted to characterize *S.aureus* to detect the presence of antibiotic resistance determinants which may provide full figure of genotypic antimicrobial resistance pattern in addition to phenotypic one.

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8. ANNEXES

Annex I: Sample record sheet for mastitis type

Cow code	Type of mastitis	Quarter code
	Clinical	RF
1.		RH
2.		LF
3.		LH
	Sub clinical	RF
1.		RH
2.		LF
3.		LH

RF= right front, RH= right hind, LF= left front, LH= Left hind

Annex II: Interpretation California mastitis test result

CMT score	**Interpretation**	**Visible reaction**
0	Negative	Milk fluid normal
T	Trace	Slight precipitation
1	Weak positive	Distinct precipitation but no gel formation
2	Distinct positive	Mixture of thickness with gel formation
3	Strong positive	Viscosity greatly increased, strong gel with cohesive with convex surface

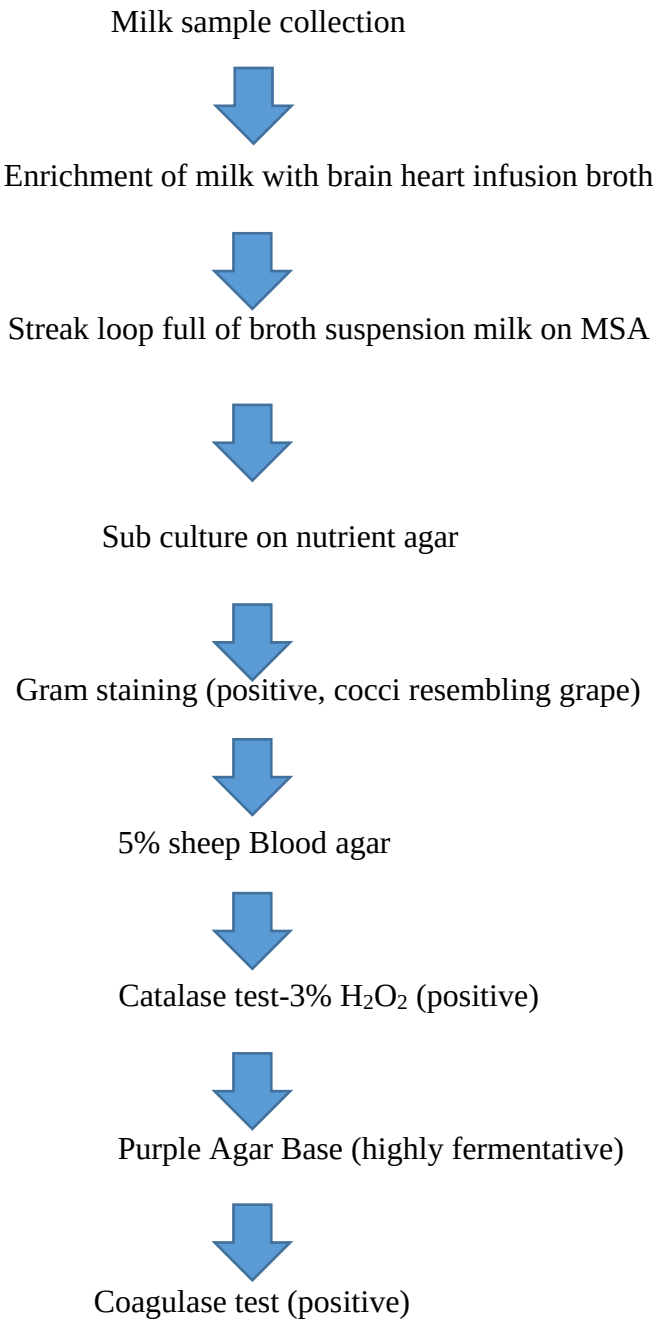
Source: (Quinn *et al*., 2004)

Annex III: Record sheet for laboratory isolation and identification of S.aureus

Cow ID		Quarter ID	Enrichment with BHI	Colony characteristics on MSA	Gram stain	Catalase test	Coagulase test	Haemolysis	PAB (1% maltose)	S.aureus
1										
2										
3										
4										
5										
6										
7										
8										
9										
10										

MSA =Mannitol salt agar, PAB= Purple agar base, BHI= Brain heart infusion broth

Annex IV: Flow chart showing procedure for the isolation and identification of S.aureus from mastitis milk samples



Annex V: Type and preparation of media used for isolation and identification of S.aureus

Table 11: Brain Heart Infusion Broth (BHI broth) (Cat.1400.00, CONDA, Madrid, Spain)

Ingredients	**gms/litre**
Gelatin peptone	10.0
Beef heart infusion	10.0
Calf brain heart infusion	7.5
Sodium chloride	5.0
Disodium phosphate	2.5
Dextrose	2.0
pH at 25 ^{0}c	7.4+/-0.2

Preparation: Suspend 37gms of the medium in one litre of distilled water. Mix well and dissolve by heating with frequent agitation. Boil for one minute until complete dissolution. Dispense in to appropriate containers and sterilize in autoclave at 121^{0}c for one minute. For best results the medium should be used in same day or if not heated in a boiling water bed to expel the dissolved oxygen and left cool before using.

Table 12: Nutrient Agar (CM 0003, OXOID, Basingstoke, England)

Ingredients	**gms/litre**
'lab-lecmo' powder	1.0
Yeast extract	2.0
Peptone	5.8
Sodium chloride	5.0
Agar	15.0
pH at 25^{0}c	7.4+/-0.2

Preparation: Suspend 28g in one litre of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121^{0}c for one minute. Cool the media up to 45^{0}c.

Annex VI: Mannitol Salt Agar (Cat. no.:C6231, Santa Maria, USA)

Ingredients	gms/litre
Mannitol	10.0
Proteose peptone	10.0
Sodium chloride	75.0
Beef extract	1.00
Phenol Red	25.0
Agar	15.0
pH at 25 ^{0}c	7.4+/-0.2

Preparation: Combine 111gm of the medium with one litre of deionized water and stir to mix thoroughly.

Table 13: Purple Agar Base (HIMEDIA, India)

Ingredients	gms/litre
Peptone, special	10.0
Beef extract	1.0
Sodium chloride	5.0
Bromo cresol purple	0.02
Agar	15.0
pH at 25^{0}c	6.8+/-0.2

Preparation: Suspend 31.02gms in one litre of distilled water. Add 5-10 gms of the carbohydrate to be tested. Dispense in to tubes as desired and sterilize by autoclaving at 15Ibs pressure (121^{0}c) for 15 minutes.

Annex VII: Media and antibiotic discs used during the antimicrobial sensitivity testing with their respective concentration

Table 14: Mueller Hinton Agar (cat. no.: c 66421, Santa Maria, USA)

Ingredients	gms/litre
starch	1.5
Casein Acid hydrolysate	17.5
Beef extract	2.0
Agar	17.0
pH at 25 ^{0}c	7.3+/- 0.1

Directions: Combine 38.04gm of medium with one liter of deionized water. Stir to mix thoroughly. Boil to dissolve completely and don't over heat. Autoclave at 121^{0}c for 15 minutes.

Table 15: Antibiotic discs, disc content and interpretive criteria for S.aureus

Antimicrobial agent	Symbol	Disc content	Susceptible	Intermediate	Resistant
Pencillin G	P	10IU	≥ 29	-	≤ 21
Cefoxitin	FOX	30 µg	≥ 22	-	≤ 21
Cefotaxime	CTX	30 µg	≥ 23	15-22	≤ 14
Gentamycin	CN	10 µg	≥ 15	13-14	≤ 12
Erythromycin	E	15 µg	≥ 23	14-22	≤ 13
Tetracycline	T	30 µg	≥ 19	15-18	≤ 14
Ciprofloxacin	CIP	10 µg	≥ 21	16-20	≤ 15
Sulphametoxazol-trimetoprime	SXT	23.75/1.5 µg	≥ 16	11-15	≤ 10
Chloramphenicol	C	30 µg	≥ 18	13-17	≤ 12

Source: (CLSI, 2012)

Annex VIII: Miscellaneous pictures during field and laboratory work of this study

A. B.

Figure 2: Clinical mastitis with clots and discoloration of milk from visited dairy farms

A. Addition of equal amount CMT reagent to CMT paddles

B. Left hind quarter showing gel formation (CMT score 2)

Figure 3: Screening of bovine mastitis with California mastitis test

Figure 4: Media preparation

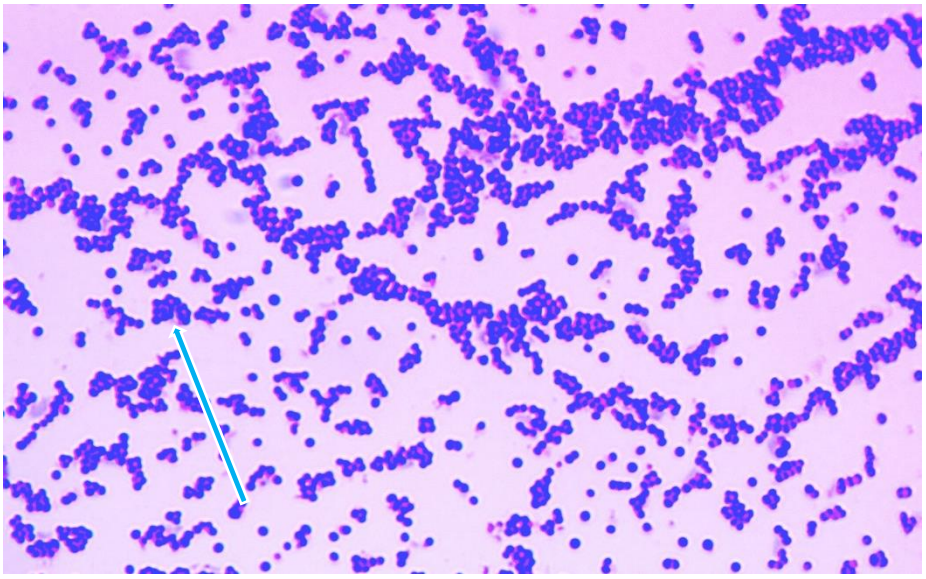


Figure 5: *S.aureus* isolate in a Gram stained smear from overnight culture

Figure 6: Appearance of *S.aureus* in mannitol salt agar

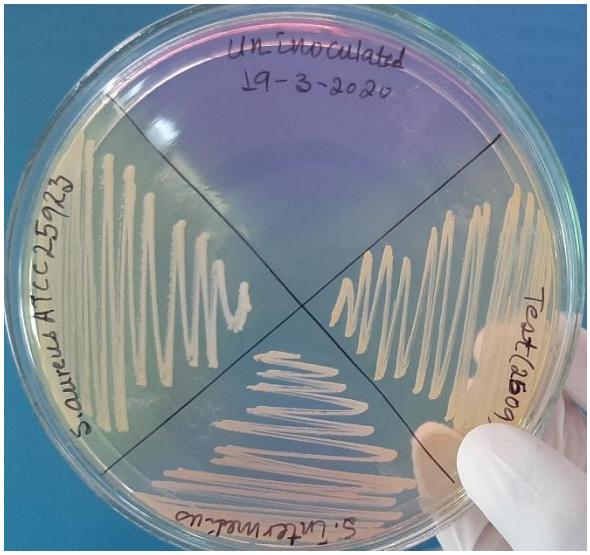


Figure 7: Purple agar base containing one percent maltose. Both positive control (*S.aureus* ATCC 25923) and test (2509) rapidly ferments sugar within 24hr which produce yellow color colonies but *S.intermidius* (bottom) gives delayed reaction. **A**. Uninoculated purple color (top), **B**. Test (right), **C**. *S.entermidius* (bottom) and **D**. S.aureus ATCC 25923 (left)

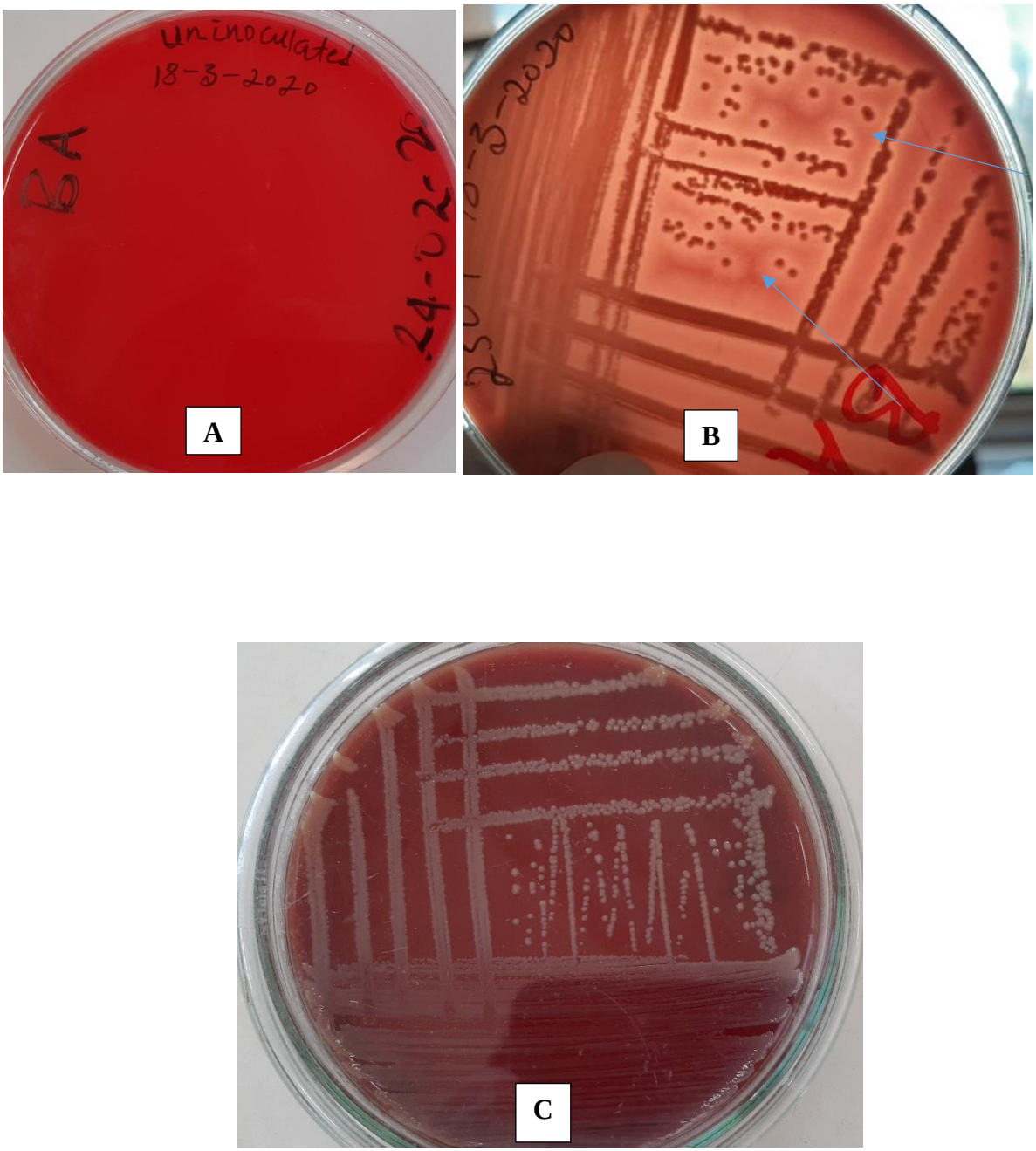


Figure 8: Hemolysis test on a 5% sheep blood agar. A. Uninocultad blood agar B. *Staphylococcus aureus* isolate cultured on a 5% sheep blood agar showing β-haemolysis and (agar side view) C. *Staphylococcus aureus* isolate cultured on a 5% sheep blood agar showing (beta hemolysis)

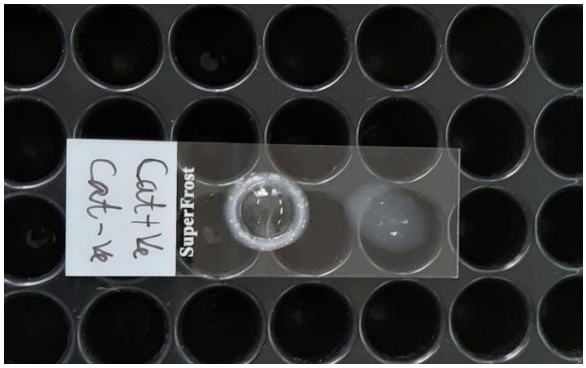


Figure 9: Catalase test A. Formation of oxygen bubble within seconds (Catalase positive) B. No formation oxygen bubbles (negative)

Figure 10: Tube coagulase test: S.epidermidis ATCC 12228 (top); sample (middle) and *S.aureus* ATCC 25923 (bottom)

A. Sterile saline without bacterial suspension B. Sterile saline inoculated with S.aureus

Figure 11: Measurement and adjustment of bacterial suspension to 0.5 using McFarland Densitometer

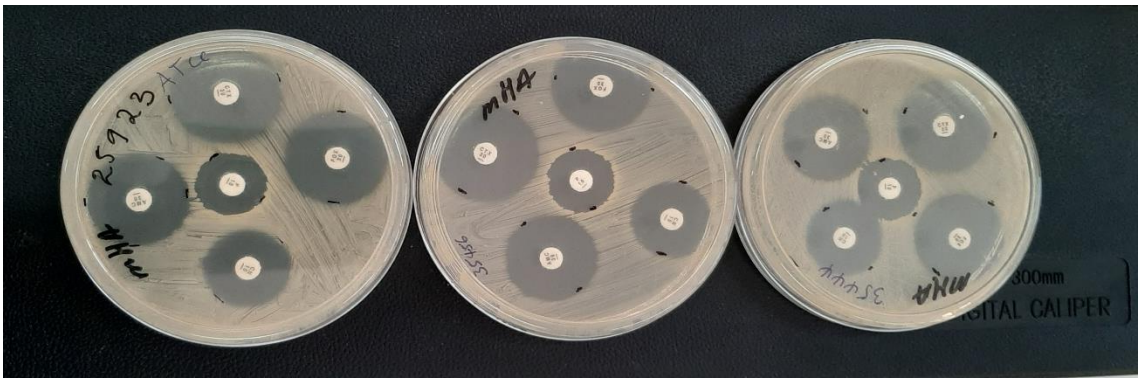


Figure 12: Antimicrobial sensitivity test showing different degrees of zone of inhibition