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ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

MOLECULAR CHARACTERIZATION OF ANTIMICROBIAL RESISTANCE AND VIRULENCE GENES IN *STAPHYLOCOCCUS AUREUS* ISOLATED FROM MILK WITH BOVINE SUBCLINICAL MASTITIS IN CENTRAL ETHIOPIA

BY

DESIYE TESFAYE TEGEGNE

JUNE, 2019

BISHOFTU, ETHIOPIA

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

BY

DESIYE TESFAYE TEGEGNE

DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND VETERINARY PUBLIC HEALTH

JUNE, 2019

BISHOFTU, ETHIOPIA

APPROVAL SHEET

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As Members of the Examining Board of the final MVSc open defense, we certify that we have read and evaluated the Thesis prepared by **Desiye Tesfaye Tegegne** entitled: "**Molecular characterization of antimicrobial resistance and virulence genes in *Staphylococcus aureus* isolated from milk with bovine subclinical mastitis in Central Ethiopia"** and recommend that it be accepted as fulfilling the thesis requirement for the degree of: **Master of Veterinary Science in Veterinary Microbiology.**

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TABLE OF CONTENTS

CONTENTS

TABLE OF CONTENTS(CONTINUED)

TABLE OF CONTENTS(CONTINUED)

STATEMENT OF AUTHOR

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

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LIST OF ABBREVIATIONS

Agr	Accessory gene regulator
bp	base pair
CI	Confidence Interval
*clf*A	clumping factor A
CMT	California Mastitis Test
CSA	Central Statistics Agency
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
EDTA	Ethylene diamine tetraacetic acid
eta	Exfolative toxin A
G+C	Guanine plus cytosine
H_2O_2	Hydrogen peroxide
hlb	hemolysin toxin B
MDR	Multiple Drug Resistant
MGEs	mobile genetic elements
mRNA	messenger Ribonucleic acid
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MSA	Mannitol Salt Agar
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
SCC	Somatic cell count
sea	*Staphylococcal* eneterotoxin A
TN	Transposons
TNase	Thermonuclease
TSB	Treptone soya broth
TSST	Toxic shock syndrome toxin
X^2	Chi-square

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

ABSTRACT

Staphylococcus aureus (*S.aureus*) is one of the predominant causative agents of mastitis in dairy herds. Mastitis has a negative impact through economic losses in the dairy sector across the globe. A cross sectional study design was conducted from November 2018 to May 2019 with the objectives to determine prevalence of bovine subclinical mastitis ,*S.aureus* and antimicrobial resistance profile and detect antimicrobial resistance and virulence genes in *S.aureus* isolated from milk with bovine subclinical mastitis in Central Ethiopia. A total of 265 lactating exotic zebu cross breed dairy cows were screened for bovine sub clinical mastitis using California Mastitis Test (CMT). Different biochemical tests and polymerase chain reaction (PCR) were used for the isolation and identification of *S.aureus* isolates. PCR was performed for detection of antimicrobial resistance genes; methicillin resistance (*mec*A) and erythromycin resistance (*erm*A, *erm*C and *mrs*A) genes and virulence genes (clumping factor A (*clf*A), enterotoxin A (*sea*), exfoliative toxin A (*eta*), beta-hemolysin (*hlb*), toxic shock syndrome toxin-1(*tst-1*) and intracellular adhesion D (*ica*D)). Out of 265 lactating dairy cows screened, 49.06% (n=130) of them were positive for subclinical mastitis. Out of 130 CMT positive samples,76.92% (n=100) of milk samples were positive for *S.aureus* and subjected to nine antimicrobials agents for antimicrobial resistance test. A large percent of the isolates were resistant to penicillin (93%) followed by ampicillin (87%) and tetracycline (75%). Sixty eight PCR confirmed *S.aureus* isolates were obtained from 100 phenotypic positive *S.aureus* isolates and from this, 17.64% (n=12) samples were contained the *mec*A gene. No amplification was observed for erythromycin resistance (*ermA*, *ermC*, and *msrA*) and virulence (*tsst-1*, *sea*, *ica*D and *hlb*) genes. The frequency of *clf*A and *eta* genes in *S.aureus* isolates were 25% (n=17) and 22.05% (n=15), respectively. The result obtained in this study suggests that poor management practices are responsible for the increase in *S.aureus* isolation. The high resistance of *S.aureus* to antimicrobials contribute in bovine mastitis in these farms may cause health problems in the community consuming raw milk purchased from these farms.

Key words: *Antimicrobial resistance gene, Bovine Subclinical Mastitis, Central Ethiopia, Staphylococcus aureus, Virulence gene*

1.INTRODUCTION

Ethiopia has the largest cattle population in Africa with 7.2 million dairy cows (Shapiro *et al.,* 2015). Exotic zebu cross breed dairy cows are mainly kept in the Ethiopian highlands, which occupy the central part of the country (Fazzini *et al.,* 2015). Dairy production has great economic importance for Ethiopia. The sector contributes 45% of the agricultural production value, and 12-16% of the total national gross domestic product (Tegegne *et al.,* 2013) with a large number of people being employed in the sector. For individual farmers, dairy production leads to the availability of food, as well as to a regular cash flow. For rural people, livestock often is an important part of their assets. Even though the total milk production in Ethiopia is currently growing rapidly, there still a shortage of dairy products (Tangka *et al.,* 2002). In present day, there is a national drive to alleviate the existing food deficit by devising different agricultural strategies including improvements of the productivity of livestock sector by controlling some of the major infectious disease, has received little attention in the country, especially, mastitis, the common problem of dairy industry that is known by an inflammation of the mammary gland is the leading one, that can contribute to reduce, milk production (Mekonnen *et al.,* 2005).

Bovine mastitis is an infectious inflammation of the mammary glands that interferes with the normal flow and quality of milk (Radositis *et al.,* 2007). Pathogens invade the mammary glands, develop and multiply, producing some toxic substances that results in inflammation, reduced milk production and altered milk quality, leading to a clinical condition known as mastitis, which is either subclinical or clinical. It is one of the most economically important diseases of dairy cattle which is associated with financial implications and affects both cattle and human health (Petersson-Wolfe *et al.,* 2010). This disease can have an infectious or non-infectious etiology. Among infectious organisms, bacteria are the most commonly encountered causes of the disease (Quinn *et al.,* 2011). Of these bacteria, *Staphylococcus aureus* (*S.aureus*) has frequently been isolated from bovine mastitis (Quinn *et al.,* 2011)and it can directly be transferred by infected milk and/or dairy products or indirectly through environmental contamination of milk during handling and processing (Jagielski *et al.,* 2014).

Staphylococcus aureus is an important cause of clinical mastitis in dairy cows causing a huge economic loss worldwide (Zhu,2010).*S.aureus* can express a wide array of potential virulence factors, including surface proteins that promote adherence to damaged tissue and/or exotoxins and enzymes that can cause a variety of infections in skin and soft tissues, including intra-mammary infections, mastitis (Radositis *et al.,* 2007). Some evidence suggests that biofilm formation can be a virulence factor associated with *S.aureus* mastitis (Paape *et al.,* 2000). Furthermore, this organism can display resistance to several antimicrobials, making it difficult to treat mastitis (Inoshima *et al.,* 2011).The cure rate after antimicrobial treatment of clinical *S.aureus* mastitis is very variable due to both cow and bacterial factors such as parity of the cow, chronicity of the infection and bacterial genotype. To approach the appropriate treatment and control measures for bovine mastitis, it is important to study the antimicrobial resistance and epidemiology of *S.aureus* infections (Tong *et al.,* 2015).

Currently, in Ethiopia especially in the central highlands where most of the dairy farms are found, the information on the sensitivity of commonly used antimicrobials for treatment of *S.aureus* mastitis is scarce. Studies from Ethiopia reported *S.aureus* as the chief etiologic agent of mastitis in cattle (Mekibib *et al.,* 2010; Mekonnen and Tesfaye, 2010). Cumbersome prevention and control of mastitis caused by this bacterium can be achieved through proper isolation and molecular characterization of the strains, segregation of the infected animals, dry cow therapy, treatment of clinical cases during lactation and culling program. Thus, steadfast and speedy methods for detection of *S.aureus* in mastitic milk samples are crucial for the control of this disease, and economically sound udder health management (Viguier *et al.,* 2009).

Rapid methods for accurate detection and susceptibility determination of *S.aureus* isolates are necessary to minimize patient suffering by identifying the antimicrobial agents to which the isolated strains may be sensitive to and hence provide treatment options (Duarte *et al.,* 2015). Owing to the poor discriminatory power of the phenotypic techniques, deoxyribonucleic acid (DNA) based identification and genotyping techniques are now considered the ideal methods for the detection of antimicrobial resistance and virulence genes of *S.aureus* (Perez-Roth *et al.,* 2001; Song *et al.,* 2015). In a previous study that was conducted in this study area, *S.aureus* isolates were identified using only

the phenotypic antimicrobial susceptibility test and identification of *S.aureus* (Duguma *et al.,* 2014; Marama *et al.,* 2016). Presently, there are limited published studies conducted in central Ethiopia concerning the harmful effects of mastitis in dairy cattle and human welfare in the consumption of raw milk. To the best of our knowledge limited number of similar work has been done in this area on the virulence genes and antimicrobials resistant genes in *S.aureus*. Therefore, the objectives of this study were:

General objective:

- To characterize antimicrobial resistance and virulence genes of *Staphylococcus aureus* in bovine mastitis

Specific objectives:

- To determine the prevalence of bovine subclinical mastitis using California mastitis test in the dairy cow of central Ethiopia
- To isolate and identify *S.aureus* isolates causing bovine subclinical mastitis using microbiological and PCR techniques
- To determine the antimicrobials resistance profiles of the identified of *S.aureus* isolates.
- To characterize antimicrobials resistance and virulence genes in the isolated *S.aureus*

Hypothesis

Staphylococcus aureus in milk with bovine subclinical mastitis possess antimicrobial resistance and virulence genes.

2.LITERATURE REVIEW

2.1.Background of *Staphylococcus aureus*

2.1.1. History and natural habitat

Staphylococci were first observed in human pyogenic lesions by Von Recklinghausen in 1871. Pasteur in 1880 obtained liquid cultures of cocci from pus and produced abscesses by inoculating them into rabbits. But it was Sir Alexander Ogston, a Scottish surgeon in 1880 who established conclusively the causative role of the coccus in abscesses and other suppurative lesions. He also gave the name *Staphylococcus* (Staphyle, in Greek meaning 'bunch of grapes': Kokkos, meaning a berry) due to the typical occurrence of the cocci in grape like clusters in pus and in cultures. Ogston had noticed that non-virulent staphylococci were also present on skin surfaces. Most staphylococcal strains from pyogenic lesions were found to produce golden yellow colonies, and the strains from normal skin, white colonies on solid media. In 1884, Rosenbach named them *Staphylococcus aureus* (*S.aureus*)and *S.albus* respectively. Later *S.albus* was renamed as *S. epidermidis* which were coagulase negative, mannitol non-fermenting and usually non pathogenic strains (Plata *et al.,* 2009).

Staphylococci are wide spread in nature although they are mainly found living on the skin, skin glands and mucous membrane of mammals and birds (Jarraud *et al.,* 2002). They may be found in the mouth, blood, mammary glands, intestinal, genitourinary and upper respiratory tracts of these hosts. *Staphylococcus aureus* generally have a benign or symbiotic relationship with their host; however they may develop the lifestyle of a pathogen if they gain entry into the host tissue through trauma of the cutaneous barrier, inoculation by needles or direct implantation of medical devices. Infected tissues of host support large populations of *Staphylococci* and in some situations they persist for long periods. The presence of enterotoxigenic strains of *S.aureus* in various food products is regarded as a public health hazard because of the ability of these strains to produce intoxication or food poisoning(Weese *et al.,* 2006).

2.1.2.Classification

Staphylococcus aureus is a bacterium, which belongs to the family *Staphylococcaceae* and thegenus *Staphylococcus* and as summarized in Table 1 below (Makgotlho, 2009). The genus *Staphylococcus* is Gram-positive bacteria that comprises of 41 known species and subspecies that are indigenous to humans (Makgotlho, 2009). Amongst the 41 species, only five are common in causing human disease such as *S.aureus*,*S. epidermidis*, *S. saprophyticus*, *S. haemolyticus and S .lugdunensis* (Trülzsch *et al.,* 2007). At least 30 species of staphylococci have been recognized by biochemical analysis. This is especially so with DNA-DNA hybridization. These Gram-positive bacteria can grow under both aerobic and facultative anaerobic conditions and form grape-like staphylococci clusters on solid media (Lowy, 1998).*Staphylococcus aureus* is the most virulent species of the staphylococci Other staphylococci can be human colonisers but rarely cause disease (Murray, 2005).

Table 1: Scientific classification of *Staphylococcus aureus*

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

Source:(Makgotlho, 2009)

2.1.3.Morphological and biochemical characteristics

The *staphylococci* are Gram positive, facultative anaerobic, non motile cocci in clusters and are non spore forming microorganisms (Quinn *et al.,* 2011). Depending on growth conditions, the colony pigmentation varies from grey, grey-white with yellowish to orange shades and a typical β-haemolysis on the blood agar (Medveďová and Valík, 2012). On Baird-Parker Egg Yolk Tellurite medium (OXOID, Hampshire, England), colonies appear grey-black, shiny and convex measuring 1-1.15 mm diameter (18 hrs) up to 3 mm (48 hrs) with narrow white entire margin surrounded by zone of clearing of about 2-5 mm. Mannitol salt agar (Hardy Diagnostics, CA, USA) is another growth media, where typical *S.aureus* appear as yellow colonies with yellow zones in the medium at 35-37°C after 24-48 hrs of growth (Quinn *et al.,* 2011).

Staphylococcus aureus ferment sugar producing acid but no gas. Mannitol is fermented anaerobically only by *S.aureus*. They are catalase and urease positive. They reduce nitrates to nitrites, liquefy gelatin and are Methyl red positive but indole negative. They are lipolytic when grown on medium containing egg yolk. They produce phosphatase which can be demonstrated by growing on nutrient agar containing phenolphthalein diphosphate. In a medium containing potassium tellurite, tellurite is reduced and black colonies are produced (Quinn *et al.,* 2011; Medveďová and Valík, 2012).

The ability to clot plasma is generally accepted criterion for the identification of *S.aureus* due to coagulase production by *S.aureus*. Two different coagulase tests are performed: a tube test for detecting free coagulase and slide test for bound coagulase or clumping factor. While the tube test is definitive, the slide test may be used as a rapid screening technique to identify *S.aureus*. Coagulase test is carried out using rabbit plasma containing EDTA (Cunha *et al.,* 2004). A heat stable staphylococcal nuclease (thermonuclease (TNase)) that has endo and exonucleolytic properties and can cleave RNA or DNA is 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 detected by using metachromatic agar diffusion procedure and DN*ase* toludene blue agar (Quinn *et al.,* 2011).

Staphylococcus aureus is capable of prolonged survival on environmental surfaces in varying conditions (Oliveira and de Lencastre, 2002). It can grow on the wide range of temperature (7 to 48°C) and pH(4 to 10) but temperature is 35 to 37 °C and optimum pH is 7 to 7.5(Stewart *et al.,* 2009). The bacterial cells are destroyed by heat but the toxin produced by them is extremely heat resistant, it can withstand heat at 60°C in 30 minutes, resulting in the food poisoning and the disease toxic shock syndrome (TSS) (Stewart *et al.,* 2009). Summary of biochemical test for *S.aureus* indicated in Table 2 below.

Table 2: Summary of biochemical test for *Staphylococcus aureus*

Biochemical test	**Test reaction**
Catalase test	Positive
Coagulase test	Positive
Oxidase test	Negative
Heamolysis	Positive
Mannitol fermentation	Positive
Maltose fermentation	Positive
DNase test	Positive
Urase test	Positive

Source : (Quinn *et al.,* 2011)

*2.1.4. G*enome organization

Staphylococcus aureus genome contains core genome, accessory component, and foreign genes. Core genome that constructs backbone of genome has main metabolic function. Core genome that constructs 25% of *S.aureus* genome contains mobile genetic elements (MGEs) such as transposons (Tn), chromosomal cassettes, pathogenicity islands (PIs), genomic islands, and prophages acquired horizontally between strains (Plata *et al.,* 2009)

(Figure 1). MGEs carry virulence genesthat are acquired horizontally by other strains (bacterial horizontal gene transfer (HGT)) (Novick and Subedi, 2007; Otto, 2010)

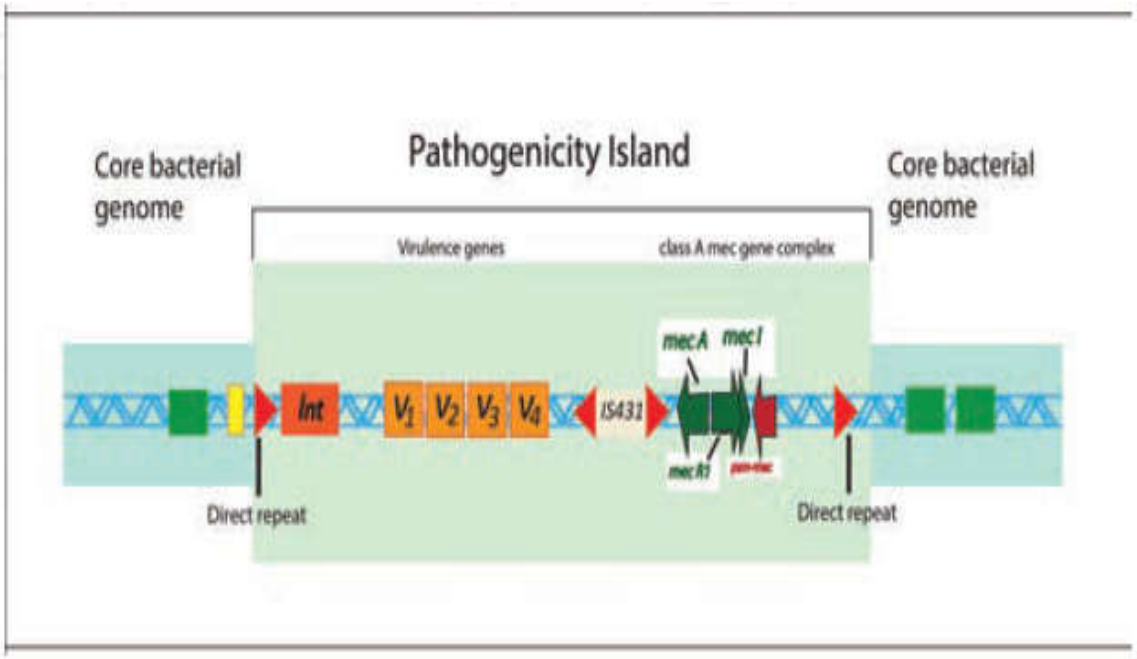

Figure 1:*Staphylococcal* genome organization

Source: (Novick and Subedi, 2007; Otto, 2010)

Each strain of *S.aureus* has virulence varied according to having mobile genetic elements (MGEs) of which genes encode for varied virulence factors and toxins (Chatterjee *et al.,* 2011). Genes of many secreted virulence factors such as exfoliative toxin A and B, superantigen toxins, toxic shock syndrome toxin (TSST), and enterotoxins are located in accessory genetic elements suchas transposons, plasmids, prophages, and pathogenicity islands (PIs) that are also referred as MGEs, whereas genes that encode toxins such as α-toxins present in whole *S.aureus* strains and are located in core genome (Otto, 2010; Chatterjee *et al.,* 2011).

2.1.5.Cell components and virulence factors

Cell components

The genome, the cell wall and the cell capsule of *S.aureus* forms important cellular components of the pathogen. A recent genome sequence study of *S.aureus* strain MI (HIP 5827) revealed that the chromosomal and plasmid genome size of *S.aureus* is 2,860,370 bp (G+C content 32.9%)and 55,980 bp (G+C content 29%), respectively (Novick, 2003). Another genome sequence study reported also that the genome of *S.aureus* was composed of a complex mixture of genes that mostly acquired through horizontal gene transfer mechanism. Moreover, the study identified three new classes of pathogenicity islands namely; the toxin-shock syndrome toxin island family, the exotoxin islands and the enterotoxin islands. The exotoxin islands and the enterotoxin islands were found linked with other genes forming a cluster that encodes putative pathogenic factors (Kuroda *et al.,* 2001).

About 50 % of the cell wall is composed of peptidoglycan layer by weight. Difference in the peptidoglycan structure of staphylococcal strains may contribute to variation in their capacity to disseminate intravascular coagulation and it is reported that the peptidoglycan layer has endotoxin activity (Kessler *et al.,* 1991). Ribitol teichoic acid and lipoteichoic acid are important components of the cell wall that form the peptidoglycan layer. Penicillin binding protein (PBP) structures are also located in the cytoplasmic membrane and are involved in the assembly of the cell wall (Lowy, 1998).

Capsular exopolysaccharides formed by some strains of *S.aureus* play important role in the pathogenesis and antibiotics resistance ability of the pathogen (Begun *et al.,* 2007). Eleven types of microcapsules could be produced by more than 90% of *Staphylococcus* species (Lee *et al.,* 1997). Staphylococci that could synthesis types 5 and 8 are responsible for 75 % of human infections. Most methicillin resistant staphylococci (MRSA) have type 5 microcapsule (Lee, 2006).

Virulence factors

Staphylococcus aureus has more than 50 virulence factors, with a wide range of biological activities. They are responsible for a variety of toxin-mediated and suppurative diseases (Ferry *et al.,* 2005a; Ferry *et al.,* 2005b).These virulence factors can be divided into two main categories as cell surface-associate (surface proteins) and secreted proteins (exotoxins) (Lowy, 1998). The different surface proteins and secreted proteins as well as their expression time during the growth phase of the bacteria are summarized in figure 2.

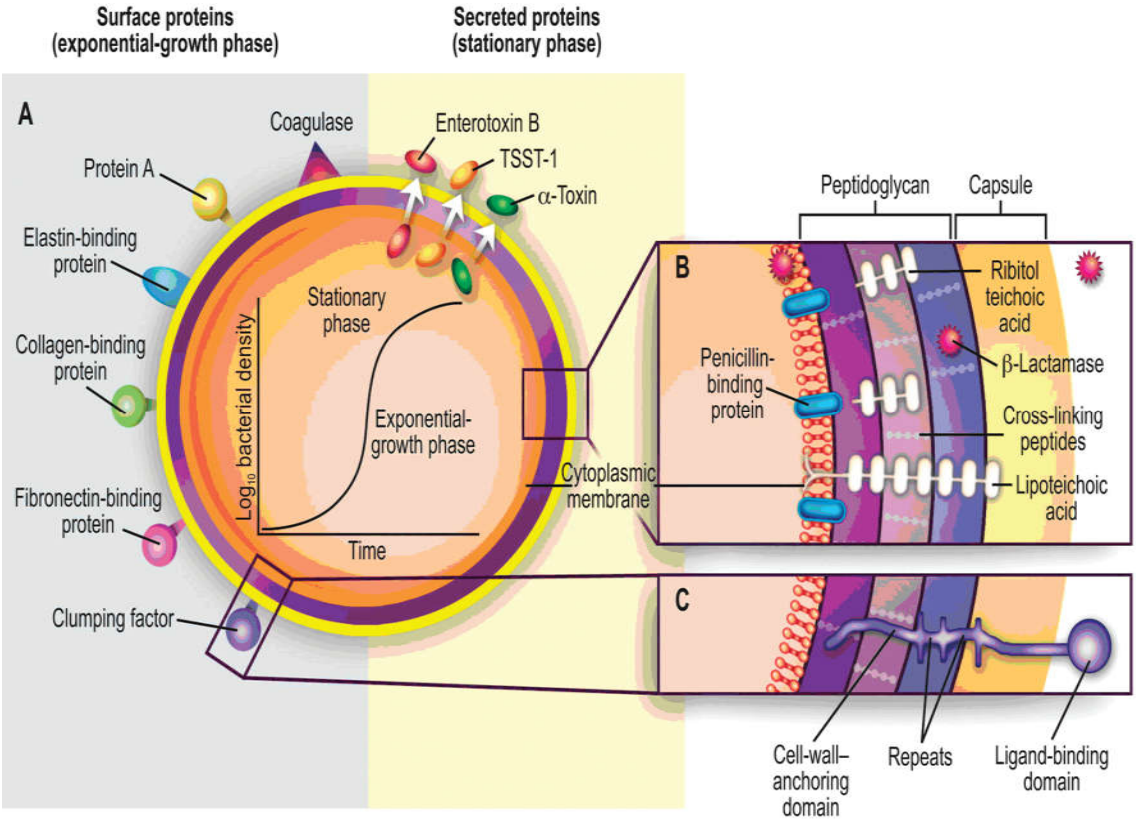

Figure 2: Pathogenic factors of *S.aureus* with structural and secreted products both playing roles as virulence factor

Source: (Costa *et al.,* 2013)

Except for the toxin-mediated diseases such as toxic shock syndrome (TSS), the causation of staphylococcal infections is not due to a single virulence factor. The different virulence factors participate, in a stepwise manner, in the pathogenesis of the various diseases. In each step, one or more virulent factor may be involved (Ferry

*etal.,*2005). First, a virulence determinant that aids the bacterium to adhere to the host surface or tissue, and virulence factors that avoid or invade the immune system of the host will be functional. For this purpose, the surface proteins such as protein A and collagen binding proteins will be expressed during the exponential growth phase. In the meantime, the second step, that involves the secretion of exotoxins or proteins that cause a harmful toxic effect to the host will follow (Costa *et al.,* 2013). The major virulence factors and their putative function as described in table 3 below.

Table 3: The major virulence factors and their putative function

Virulence factor	Putative function
Staphylococcal protein A	The protein binds to IgG, thus interfering the phagocytosis activity of the host immune system
Collagen-binding protein	Adherence to collagenous and cartilage parts of the host tissue
Clumping factor proteins	Mediate clumping and adherence to fibrinogen in the presence of fibronectin.
Superantigens	Massive activation of T cells and antibody-presenting cells
β-hemolysin and αhemolysins	Induce lysis of erythrocytes, monocytes and platelets
Leukocidin	Kills leukocytes
Hyaluronidase	Breaks down hyaluronic acid
Toxic shock syndrome toxin-1	Shock, rash and desquamation
Epidermolytic toxins A and B	Epidermal splitting and exfoliation

Source: (Costa *et al.,* 2013)

2.1.6. Regulation of genes involved in virulence

The genes coding for virulence factors are regulated in a tightly coordinated manner that is synchronized with the biological cycle of *S.aureus*. The production of factors involved in virulence is controlled by quorum sensing mechanism. In *S.aureus*, genes coding for

surface proteins are down regulated during early stages of the growth whereas genes that encode secreted proteins are up regulated in late exponential phase. This pattern of gene expression in which surface proteins involved in adhesion and defense against host's immune system (protein A, coagulase, fibronectin binding proteins, among many others) are synthesized before production of secreted hemolysins, cytotoxins, proteases and other degradative enzymes seems to reflect a strategy of *S.aureus* in which the pathogen first establishes itself in the host and only then attacks it. This regulation is, in large part, due to the accessory gene regulator (*agr*) two component system (Novick and Geisinger, 2008).

2.2.General overview of bovine mastitis

Derived from the Greek word *mastos* (breast), mastitis refers to an inflammatory disease in the mammary gland, affecting dairy herds worldwide. Based on the severity of disease, mastitis is divided into clinical (symptomatic) and subclinical (asymptomatic) mastitis (Jones and Bailey, 2009).

Causes of inflammation range from physical trauma to chemical irritants, but the most common cause of disease is pathogenic microorganisms (Rall *et al.,* 2014). The teat skin cells act as a first line of defense against these infectious agents by producing keratin, a fibrous protein combined with lipid components that have bacteriostatic effects. However, during inadequate milking procedures, lesions can occur and the teat canal becomes highly vulnerable. Furthermore, after milking, the teat canal remains dilated for 1-2 hours, increasing the likelihood of bacterial infection (Jones and Bailey, 2009). When pathogens enter the teat canal, they multiply and release toxins, enzymes and surface proteins which are responsible for adherence to the host's extracellular matrix. Altogether these induce an inflammatory response from the host, increasing the number of polymorphonuclear neutrophils, phagocytes and other leukocytes (Jones and Bailey, 2009; Ote *et al.,* 2011). The immune response can vary greatly, depending on the causative agent, lactation stage, age and health status of the cow (Harmon, 1994). Due to this somatic cell increase, milk quality and composition is significantly altered, reducing its economic value. Thus, mastitis is considered one of the costliest diseases of the dairy

industry (Kitchen, 1981), causing, for instance, an annual financial loss of 1.7-2 billion dollars in the U.S (Jones and Bailey, 2009).

A significant number of microbial organisms have been isolated from the bovine mammary gland, indicating that mastitis infections can be caused by over 150 different species, belonging mostly to three major groups of organisms: *Staphylococcus, Streptococcus* and coliforms. Other mastitis-causing agents have been identified, albeit less frequently, including *Enterococcus*, *Mycoplasma*, pseudomonas, algae and yeasts (Quinn *et al.,* 2011).

Pathogenic agents can be found either in the udder (contagious pathogens) or in the cow's surroundings (environmental pathogens) and this distinction is correlated to their behaviour in dairy herds. Longer and more prevalent infections are caused by contagious organisms that spread during the milking process, whereas environmental agents typically cause a more clinically severe case of mastitis (Jones and Bailey, 2009).

The most common contagious pathogens are *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus dysgalactiae,* whereas the most prominent environmental agents are *Streptococcus uberis, Escherichia coli* and *Klebsiella pneumonia* (Radositis *et al.,* 2007). Recently, with the development of more precise diagnostic techniques, the classical distinction between contagious and environmental agents is in question. Studies indicate that some bacterial strains within a species can display a contagious transmission pattern while others present an environmental origin (Muñoz-Planillo *et al.,* 2009).

2.3.Epidemiology and economic importance of bovine mastitis

2.3.1.Reservoirs and transmission

Dairy cows, among other domestic artiodactyls, are considered to be temporary hosts of *S.aureus*, in which the microbe is frequently present as a contaminant that can multiply and persist for short periods. *S.aureus* has been isolated from practically all external surfaces of healthy cows udder skin being the preferred site. Long-term colonization by *S.aureus* on teat skin and several other body sites, primarily mucosal external orifices, have been observed in heifers suggesting persistent colonization (Roberson *et al.,* 1994).

It's likely that a calf has its first contact with *S.aureus* already at birth via the genital area of the mother (Kloos *et al.,* 1997).

Infected milk is generally considered to be the primary source of the microbe, and milking liners are the main vector of transmission, since they have been frequently shown to be contaminated with similar *S.aureus* strains to those in infected milk (Zadoks *et al.,* 2002). Traumatized sites such as abrasions on teats, legs, bends and navel, typically infected by *S.aureus*, are regarded as secondary sources of *S.aureus* causing intra-mammary infections. *S.aureus* is regarded as a clonal organism as the populations consist of groups of genetically related strains with a common ancestor. *S.aureus* diversifies more often by point mutation than by horizontal gene transfer (Feil *et al.,* 2003). Most studies have demonstrated that a given herd usually harbours a limited number of *S.aureus* strains, often with one or a few strains predominating (Rabello *et al.,* 2007; Mørk *et al.,* 2010).

2.3.2.Ability to survive and grow in the environment

The *Staphylococcus aureus* can survive indefinitely in the nasal passages and throats of humans and animals. From these sources they can be transferred to meat and other foods. In foods that provide a satisfactory medium they can grow to sufficient numbers to produce enterotoxin if the foods are not refrigerated. These organisms can be transferred to equipment; if the equipment is then not adequately cleansed before use, the organisms can be transferred to foods. A common source of contamination of dairy products is cows' udders, particularly in animals with *Staphylococcal* mastitis. The organisms are destroyed when the milk is pasteurized, but any enterotoxin in the milk will not be inactivated (Evenson *et al.,* 1988).

2.3.3.Economic importance of bovine mastitis

Mastitis still remains the most costly disease in the dairy industry worldwide (Radositis *et al.,* 2007). The economic losses are due to decreased milk production, increased replacement cost, discarded milk, treatment cost, extra labor cost, and negative effects on milk quality (Seegers *et al.,* 2003). In a study on clinical mastitis cases, the average estimated cost was 179$ because of milk yield losses, increased mortality, and treatment

cost. In dairy heifers, elevated somatic cell counts may negatively affect milk production during the first lactation (Braem *et al.,* 2014). Prepartum antibiotic treatment of heifers will reduce the prevalence of Intra-mammary infection after treatment (Demon *et al.,* 2012) and is effective to reduce the rate of clinical mastitis in heifers during lactation.

Subclinical and clinical mastitis, especially during the first 90 days of lactation, have been implicated in decreasing reproductive performance, including increased days in milk at first service, increased services per conception, and increased days not pregnant (Schrick *et al.,* 2001; Radositis *et al.,* 2007).

The importance of bovine mastitis is not only the economic losses in the dairy industry but also the importance of mastitis in public health (Radositis *et al.,* 2007). Most dairy farms require the use of antimicrobial drugs for treatment of sick dairy cattle, and mastitis is the most common disease of lactating dairy cattle to be treated with antimicrobial drugs (Zwald *et al.,* 2006; Pol and Ruegg, 2007; Sawant *et al.,* 2009). The extensive use of antimicrobial drugs for treatment and control of mastitis has possible implications for human health through an increasing risk of antimicrobial residues in milk products (Ruegg and Tabone, 2000). Moreover, antimicrobial drugs usage may exert selective pressure for antimicrobial resistant strains of bacteria that may enter the food chain (Aarestrup, 2005). The food safety issues, the spread of zoonotic organisms and antimicrobial resistant strains of bacteria via milk and milk products are of greater concern especially in the countries that produce raw milk cheese and have a niche market for unpasteurized dairy products (Ruegg and Tabone, 2000).

2.3.4.Occurrence of mastitis pathogens in Ethiopia

Mastitis is economically one of the most important diseases in dairy cows worldwide (Seegers *et al.,* 2003; Halasa *et al.,* 2007). Subclinical mastitis (SCM), as compared to clinical mastitis (CM), is considered to account for a considerable proportion of the economic losses due to production losses and increased risk of CM on dairy farms (Petrovski *et al.,* 2006; Abrahmsén *et al.,* 2014). Subclinical mastitis can also result in decreased milk quality such as reduced shelf life of fresh milk (Busato *et al.,* 2000; Seegers *et al.,* 2003). Also in Ethiopia subclinical mastitis is reported to be highly

prevalent, with over 60% of the cows being infected (Dego and Tareke, 2003; Duguma *et al.,* 2014)and to cause significant economic loss (Lakew *et al.,* 2009; Tesfaye *et al.,* 2010). Dairy farmers in Ethiopia, however, generally do not seem to recognize subclinical mastitis as an important problem (Almaw *et al.,* 2009). Mastitis can be caused by many different bacterial species, subspecies and serovars (Radositis *et al.,* 2007).

In Ethiopia, over the years, several studies have been carried out in which mastitis pathogens were identified from different types of milk samples. Staphylococci are worldwide described as an important cause of mastitis (Pyörälä and Taponen, 2009)and have been found to be the most common mastitis pathogen in Ethiopia (Mekonnen *et al.,* 2005; Almaw *et al.,* 2009; Lakew *et al.,* 2009; Haftu *et al.,* 2012). *Staphylococcus aureus* is cultured from milk samples from both clinical mastitis and subclinical mastitis cases, whereas coagulase negative *S.aureus* is predominantly cultured from SCM cases (Mekonnen *et al.,* 2005; Lakew *et al.,* 2009; Haftu *et al.,* 2012).

2.4.Pathogenesis

Staphylococcus 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 (Patti *et al.,* 1994), allowing bacterial anchorage in normal and inflammed tissues (Foster and Höök, 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 (Paape *et al.,* 2000).*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 (Gill, 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).

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 (Gill, 2006). The presence of coagulase and deoxyribonuclease correlates positively with the virulence of the bacteria and is used for identification purpose (Quinn *et al.,* 2011). 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 masti tis, which can be fatal to the cow (Quinn *et al.,* 2011)

2.5.Diagnosis of bovine mastitis

Early diagnosis is very imperative as a result of increased economic costs of mastitis. Some of the assays that are frequently employed to examine the milk quality through detection of the swelling of mammary gland and diagnosis of the infection and its causative pathogens include California mastitis test, flow cytometry, culture test and Polymerase Chain Reaction (Pyörälä, 2003). Mastitis-causing pathogens usually are identified by culturing techniques, but their disadvantage lies with prolonged periods of laboratory work and cost effective materials (Awale *et al.,* 2012).

2.5.1.California Mastitis Test

The California Mastitis Test(CMT) is a cow-side test that is performed on dairy farms using a detergent to identify subclinical mastitis by an indirect estimation of the somatic

cell count in milk. The detergent (bromocresol purple) breaks down the cell membrane of somatic cells and followed by the liberation and combination of nucleic acid which then generates a gel-like medium with thickness that is relatively equal to the number of leukocytes (Viguier *et al.,* 2009). For reliable results, tests should be conducted just before milking after stimulating milk let down and discarding the foremilk. Managers of herds with high somatic cell count may have to cull heavier for mastitis, increase treatments for intramammary infections, increase efforts to avoid antibiotic residues in milk and cull animals, increase cost on facilities or milking equipment, and improve management to reduce the spread of new infections. Thus, emphasis should be on proper milking techniques, improved sanitation, effective use of teat dipping and dry period therapy and maintenance of milking equipment. Lower somatic cell count should result in higher milk yields and better milk quality (Quinn *et al.,*2004).

2.5.2.Culture tests

The surest way of diagnosing mastitis is by directly isolating and identifying any pathogenic microorganisms which may be present in the milk. This can be achieved by cultural methods and a number of additional determinative tests. To obtain correct results and avoid contamination and hence bias, it is important to work as securely and as accurately as possible under the circumstances. Similarly, the procedure of routine mastitis testing should be standardized and work protocols instituted (Viguier *et al.,* 2009).

2.5.3.Molecular biological methods used for mastitis diagnosis

Molecular biological methods used for mastitis diagnosis Various DNA-based identification assays can be used for the characterization of pathogens at different phylogenetic levels according to the aim of the test and primer design. These methods can detect either DNA or RNA. While the extraction or detection of DNA is more common and often technically easier than that of RNA due to the higher stability of DNA than RNA. For this reason, the DNA-based detection assays can detect non-viable and/or inactivated pathogens in opposite to those assays targeting the mRNA which is less stable and therefore can detect only viable pathogen. On the other hand, the detection of the genes encoding antibiotic resistance does not necessary mean that the bacteria are

resistant against antibiotics, but the detection of mRNA resulting from gene expression will deliver more accurate results (El-Sayed *et al.,* 2017).

2.5.4.Molecular markers of infectious mastitis inducers: Identification and genotyping

A Deoxyribonucleic acid signature means the identification of unique DNA sequences in the genome of a particular organism, which is absent in all other, even the closely related, microbes. These Phylogenetic markers help in bacterial characterization such as the *16SrRNA or 23S rRNA* genes (El-Sayed *et al.,* 2017). Such highly conserved genetic sequences are usually the first choice for primer design. In opposite to those highly conserved markers, it is not common to depend on virulence genes alone for bacterial identification as they are highly dynamic among related bacterial species/subspecies due to their location on mobile genetic elements which can even be transmitted from one species to another. However, if the planned reaction aims to differentiate among different genotypes of the invading microbe, certain genomic hotspots, polymorphic sequences, intergenic spacers and accessory/virulent genes can be selected for this purpose (Almeida, 2012).

2.5.5.Polymerase chain reaction (PCR)

Polymerase chain reaction is an *in vitro* amplification of unique organism specific target DNA sequences using sequence specific oligonucleotide primers and heat stable polymerase. The selected primers must have an exclusive sequence that bind specifically and selectively to previously defined DNA target sequence. The primers may either be designed to differentiate among members of the same species or to identify the organisms at subspecies level. By so doing, the primers allow the amplification and quantification of certain sequences. For the diagnosis of present pathogens, the primer target sequence must be highly conserved within all strains of the suspected species to avoid false negative results but variable among other species to avoid cross reaction resulting in nonspecific annealing leading to false positive results (Duarte *et al.,* 2015).

Different PCR systems were developed to offer a rapid, accurate and economic diagnosis of causative agents of mastitis. PCR can be applied on quarter -, pooled - and bulk milk samples. The sensitivity of detection limit decreases with the more dilution (pooling/ mixing) of infected milk with healthy milk. The quarter milk samples deliver the most

accurate data about the predominant pathogen in the farm with a clear higher level of sensitivity and specificity in comparison to pooled or bulk milk samples. On the other hand, the application of modern molecular tools in investigating pooled or bulk milk samples can deliver accurate data comparable with that data delivered when using quarter samples (Duarte *et al.,* 2015).

2.6.Treatment of bovine mastitis

The success of bovine mastitis therapy depends on the etiology, clinical presentation, and antimicrobial susceptibility of the etiological agent among other factors (Miltenburg *et al.,* 1996). Therapy failure in the management of mastitis could result from pathological changes that occur in the udder, etiology related factors, pharmacokinetic properties of the antimicrobial drugs, poor animal husbandry and inadequate veterinary services. However, the control of mastitis has been successfully achieved through the establishment of effective herd health control programs (Erskine *et al.,* 2002). Antimicrobial agents are the main therapeutic tools for the treatment and control of mastitis. Among the main reasons of low efficacy of antibiotic treatment of mastitis cases is the resistance of the bacteria to antimicrobials. Recently, several studies have been conducted to determine the antibacterial susceptibility patterns of mastitis pathogens isolated from clinical studies or submitted to diagnostic laboratories (Brown and Scasserra, 1990; Gitau *et al.,* 2011).

Antimicrobial agents are widely used for the treatment of bovine mastitis, respiratory tract infections and diarrhea in cattle. During acute infections and outbreaks of infectious disease in groups or herds it is important to use an effective antimicrobial treatment as early as possible. This empirical treatment is generally based on knowledge of the resistance pattern of the different bacterial pathogens to antimicrobial agents used in the particular animal species. Antimicrobial resistance is an increasingly important problem among several bacterial species causing infection in animals and humans in recent year. The problem for some bacterial species is so critical that there is few treatment options left (Levy, 2001; Aarestrup, 2005). The initial treatment of animals is commonly based on the experience regarding the expected resistance of the infectious agent. The fact that occurrence of antimicrobial resistance varies between countries and regions has the

potential to complicate that matter. Furthermore, knowledge of expected resistance is limited by the small proportion of different bacterial pathogens from infected animals that actually are investigated for their antimicrobial resistance pattern (Poole, 2002).

2.7.Control and prevention

A successful *S.aureus* control program will eliminate existing infections, prevalent new intra-mammary infections, and have a system for on-going monitoring of the infection status of the herd (Owens *et al.,* 1997; Radositis *et al.,* 2007). 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 *et al.,* 2002). Culling of cows with chronic mastitis is one of the cornerstone recommendations of the original point of mastitis control programs and it achieves, both a reduction in herd prevalence and a reduction in the risk subsequent spread of infection (Radositis *et al.,* 2007).

The use of long-acting intramammary treatment at the time drying-off is another cornerstone of recommended mastitis control programs (Dingwell *et al.,* 2003). 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).

Programs involving the selective treatment of dry cows have been attempted for several reasons such as the reduction of treatment costs, the preservation of protective minor infections, and to prevent the development of resistant bacteria. Until more sensitive and specific diagnostic indicators are developed, as well as better methods to prevent new dry period infections, blanket dry cow therapy is recommended (Schukken *et al.,* 2003).

2.8.Antimicrobial resistance test

Antimicrobial resistance testing determination is not only essential to ensure optimum antimicrobial therapy amongst patients but also for monitoring the spread of resistant

bacteria pathogens and resistant determinants (Acar and Rostel, 2001). Several tests are available for the detection of antimicrobial resistance and susceptibility in bacteria. These include the standard disk diffusion method that is widely utilized (Bauer *et al.,* 1966) as well as the micro broth dilution assay (Reller *et al.,* 2009).Other susceptibility testing methods include molecular assays such as PCR and DNA chips which provide an opportunity to monitor resistance in bacteria isolates. PCR assays have been used as standard methods for effective detection of resistance genes especially in bacterial strains with non-functional and non-expressed genes. The presence of such resistance genes is generally considered as an indication of a potential for exhibiting resistance to a particular antibiotic (Acar and Rostel, 2001). Although the use of molecular techniques for the detection of antibiotic resistance is becoming more popular, phenotypic assays remain the method of choice for most resistance determinants especially in settings that do not have adequate finance, equipment and highly trained personnels (Ambler and Stone, 2012).

The disc diffusion susceptibility method is simple and practical and has been well standardized (Bauer *et al.,* 1966; CLSI, 2012). Disc diffusion refers to the diffusion of an antimicrobial agent of a specified concentration from discs, tablets or strips, into the solid culture medium that has been seeded with the selected inoculums isolated in a pure culture (Bauer *et al.,* 1966). Disc diffusion is based on the determination of an inhibition zone proportional to the bacterial susceptibility to the antimicrobial present in the disc. The test is performed by applying a bacterial inoculums of approximately $1–2\times10^8$ CFU/ml to the surface of a large (150 mm diameter) Mueller-Hinton agar plate. Up to 12 commercially-prepared, fixed concentration, paper antibiotic discs are placed on the inoculated agar surface. Plates are incubated for 16–24 hr at 35°C prior to determination of results(CLSI, 2012). The zones of growth inhibition around each of the antibiotic discs are measured to the nearest millimeter. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium. The zone diameters of each drug are interpreted using the criteria published by the Clinical and Laboratory Standards Institute (CLSI, 2012). The results of the disc diffusion test are "qualitative," in that a category of susceptibility (susceptible, intermediate or resistant). The advantages of the disc method are the test simplicity that does

not require any special equipment, the provision of categorical results easily interpreted by all clinicians, and flexibility in selection of discs for testing (Bauer *et al.,* 1966).

2.9.Antimicrobial resistance of *S.aureus*

Antimicrobial resistance has been an important public health concern. The problem with *S.aureus* became more complicated when it was found that it quickly developed resistance and was capable of producing many antibiotic resistant strains (Kitara *et al.,* 2011). The number of *S.aureus* strains that exhibited antimicrobial resistance properties has increased, together with the potential risk of inducing infections difficult to be treated or transmitting the same properties to the human microflora via foods. Multiple antibiotic resistant *S.aureus* strains have been isolated from milk obtained from cattle, beef and human samples in many part of the world (Shitandi and Mwangi, 2004; Pesavento *et al.,* 2007). Antibiotic resistant isolates might be transmitted to humans by the consumption of food products containing such resistant and multi-resistant bacteria and that the use of antibiotics as growth promoters in animal husbandry, especially of those commonly used for both human and animal care (Pereira *et al.,* 2009). Infection with such resistant strains is likely to be more severe and require longer hospitalization with incumbent increased costs, than infection with susceptible strains (Baron, 1992). The indiscriminate use of antibiotics for prophylactic and other therapeutic purpose could be the reason for increased antimicrobial resistance of *S.aureus*. Antibiotic resistant strains have been isolated from many food samples of animal origin (Thakkar *et al.,* 2014).

Globally, the widely accepted cause of antibiotic resistance is the overuse and misuse of antibiotics. In developing countries the situation is escalating in that besides the increasing use of antibiotics and their ready availability without prescription, poor sanitation condition around premises aid the spread of resistant strains (Barza and Travers, 2002). One of the biggest issues in antimicrobial resistance is the use of antibiotics in animals. There is a continuous debate on the association between antimicrobial use in the production of food animals and the emergence of resistant organisms in humans. In veterinary medicine, antibiotics are given for treatment of contagious and infectious animal diseases including clinical and subclinical mastitis at dairy farm level. However, besides human and veterinary use, antibiotics are widely used

in animal husbandry and other agricultural practices and this has significantly exaggerated the antibiotic resistance problem globally (Aarestrup, 2005). Of these practices, the most serious is the continuous administration of sub-therapeutic doses of antibiotics as growth promoters for food animals. This practice favours emergence and propagation of a large number of resistance genes (Levy and Marshall, 2004). Commensal bacteria could constitute a risk by being a reservoir of resistance genes. Resistant commensal bacteria of food animals may pass on their resistance to zoonotic bacteria and reach the intestine tract of humans (van den Bogaard and Stobberingh, 2000).

2.10. Classes of antibiotics and mode of action

The action of antibiotics can be classified into two as bacteriostatic agents and as bactericidal agents. Bacteriostatic types of antimicrobial agents such as tetracycline inhibit the multiplication and growth of the microorganisms. When the antimicrobial agent is removed, the microorganisms recommence multiplication. Bactericidal agents such as fluoroquonoline disintegrate and kill the microorganisms and the action is irreversible (Coyle, 2005). There are at least six mechanisms of action by which antimicrobials exert their action to the target microorganisms. These include inhibition of cell wall synthesis, inhibition of proteins synthesis either the 30S ribosomal subunit or the 50S ribosomal subunit proteins, inhibition of DNA synthesis, competitive inhibition of folic acid synthesis, inhibition of RNA synthesis and the use of other DNA inhibitors (Walsh, 2003). The major classes of antimicrobial agents with their mode of action are presented in figure 3 below.

2.11. Mechanism of antimicrobial drug resistance

Microorganisms develop antimicrobial resistance characteristics through different mechanisms (Figure 3). Some produce an enzyme that destroys the antibiotics. Enzymes such as β-lactamase disintegrate penicillin and cephalosporin. Other enzymes also modify the structure of the antibiotics so that it will no longer be effective. Such resistance mechanisms are observed against chloramphenicol and aminoglycoside antibiotics. Some resistance mechanisms also interfere with the transportation of the antibiotics inside the cell by pumping out the antibiotics from the cell to the outside. Such mechanisms of

resistance has emerged for tetracycline, chloramphenicol and fluoroquinolones (Levy and Marshall, 2004). One type of resistance mechanism can be expressed by many types of genes and also for one type of antibiotic more than one type of resistance mechanisms can be expressed (Levy and Marshall, 2004).

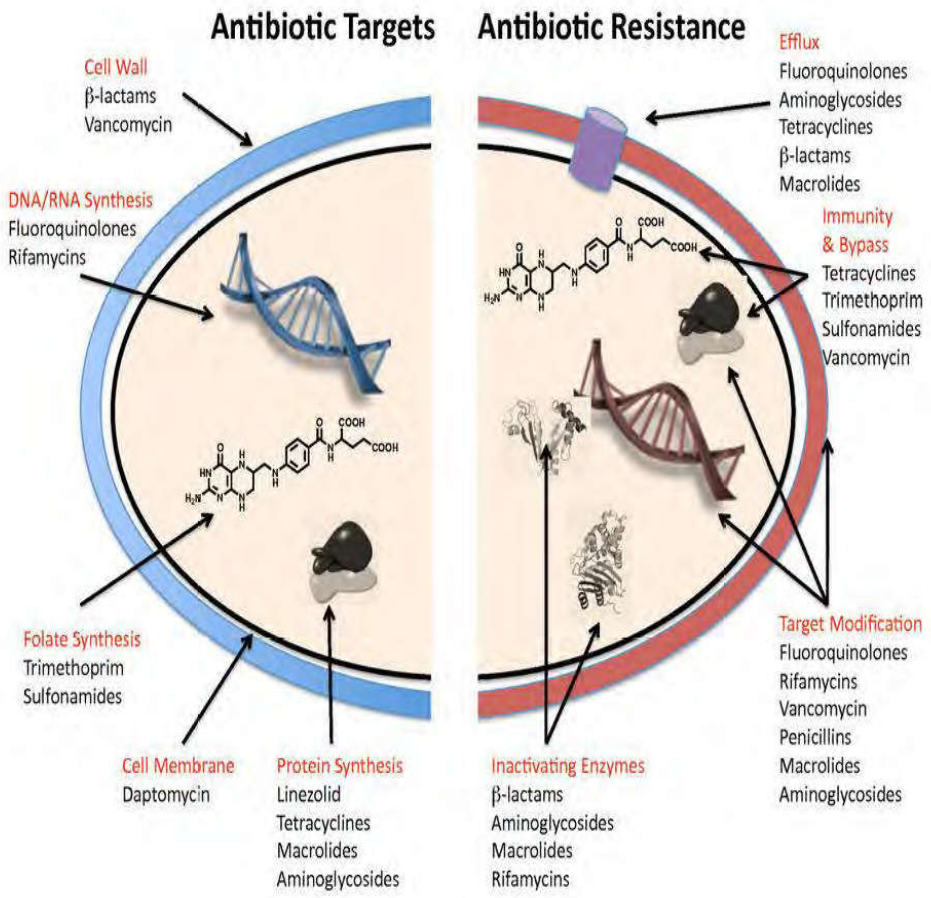

Figure 3: Mechanisms used by common antibiotics to deal with bacteria and ways by which bacteria become resistant to antibiotics

Source: (Wright, 2010).

3.MATERIALS AND METHODS

3.1.Study area

This study was conducted in selected areas of central Ethiopia including Adaberga, Ambo, Bishoftu and Holeta as indicated in figure 4. The study areas were purposively selected based on the abundance of dairy farms that constituting the known milk sheds to the Addis Ababa and based on their agro-ecological differences. The descriptions of each area was summarized below:

Adaberga is located in Oromia regional State, West Shewa zone which is about 70 km from the capital city of the country; Addis Ababa. The total area of the woreda is 798.35 km^2. It is located at 9º12' to 9º 37' latitude and 38º 17' to 38º 36' longitude. The altitude of the area ranges from 1400 to 3500 meters above sea level. It is characterized by agro ecologies like low land, middle and high lands which covers 37, 34 and 29% of the area respectively. An annual average of rainfall ranges from 918to 1368mm while the minimum and maximum temperature reaches10 and 25°C, respectively. The primary wet season extends from June through October. July and August are the wettest months. The farming system of the area is mixed type where crop production and livestock rearing are done side by side. Exotic zebu cross breed dairy cows are the most dominant breed when compare indigenous breed and they practiced semi intensive production system (CSA, 2016).

Ambo is located Western Shewa zone of Oromia regional state, 115 km far from Addis Ababa. The area is found at a longitude of 37º32' to 38º 3' E, and latitude of 8º 47' to 9º 20' N and the altitude range is from 1900 to 2275 meters above sea level. The climatic condition of the area is 23% highland, 60% mid altitude, and 17% lowland. It has an annual rainfall and temperature ranging from 800 -1000 mm and 20 -29°C, respectively. The rainfall is bi-modal with the short rainy season from February to May and long rainy

season from June to September. Agriculture is the main occupation of the population of the area. The agricultural activities are mainly mixed type with cattle rearing and crop production under taken side by side. Semi-extensive system of livestock management predominate in this area. Exotic zebu cross breed dairy cows are the most dominant breed when compare indigenous breed and they practiced semi intensive production system (CSA, 2016).

Bishoftu is located in Oromia National Regional State about 45 km South-east of Addis Ababa. It is found at 90°N latitude and 40°E longitude and at an altitude of 1850 meters above sea level in the central Ethiopia. It has a human population of about 95,000. It experiences a bimodal pattern of rainfall with the main rainy season extending from June to September (of which 84% of rain is expected) and a short rainy season from March to May with an average annual rainfall of 800mm. The mean annual minimum and maximum temperatures are 12.3 and 27.7°C, respectively, with an overall average of 18.7°C. The highest temperatures are recorded in May and the mean relative humidity is 61.3%. It is the center of Ada'aliben woreda and it has a total land area of about 1610.56 km^2 and is divided in to three agro-ecological zones namely midland (94%) highland (3%) and lowland (3%) (CSA, 2016).

Holeta is located at 40km west of Addis Ababa and at an elevation of 2400meter above sea level in the central Ethiopia (9°3'N and 38°30'E). The area is characterized by mild subtropical weather, with average minimum and maximum annual temperatures of 6.3 and 22.1°C, respectively. The area also experience bimodal rain fall pattern with a long rainy season extending from July to September while the short rainy season extends from March to April. Agriculture is the main occupation of the population of the area. The agricultural activities are mainly mixed type with cattle rearing and crop production under taken side by side. Semi-extensive system of livestock management predominate in this area. Exotic zebu cross breed dairy cows are the most dominant breed when compare indigenous breed and they practiced semi intensive production system (CSA, 2016).

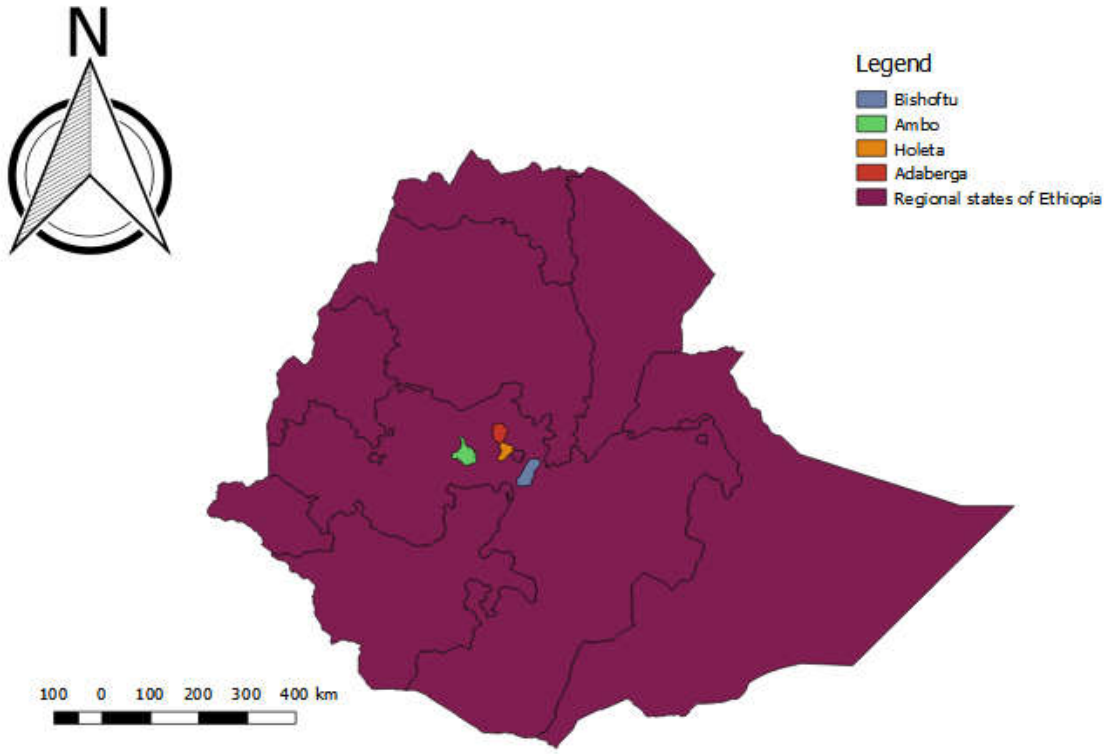

Figure 4: Map of Ethiopia indicating study areas of the research. Specific sampling areas are indicated in different colors that are explained in the legend.

3.2.Study design

A cross sectional study design was conducted from November 2018 to May 2019 with the objectives to determine prevalence bovine subclinical mastitis, *S.aureus* and antimicrobial resistance profile and detect antimicrobial resistance and virulence genes in *S.aureus* isolated from milk with bovine subclinical mastitis in central Ethiopia.

3.3.Study population

The study populations were lactating exotic zebu cross breed dairy cow which have not treated for mastitis either intramammary or systemic route during the study period. All dairy farms with herd size ranging from 20-110 cows, which were managed under semi intensive or intensive management system were included as study population.

3.4.Sample size determination and sampling method

Sample size of the study was determined based on sample size determination method as described by (Thrusfield, 2005) with a 95% confidence interval and 5% desired precision using the following formula.

$$N = \frac{(1.96)^2 \times Pexp(1 - Pexp)}{d^2}$$

Where:-

1.96 =the value of Z at 95% confidence interval

N= number of sample size

P_{exp}= expected prevalence = 79%;

d^2= absolute precision = 5%

CI= confidence interval (95%)

The sample size required for this study was based on the expected prevalence of bovine subclinical mastitis according to Thrusfield, (2005. The previous study on prevalence of bovine subclinical mastitis reported different prevalence at different time by different authors between 62.9 and 95% prevalence rate that has been reported by (Dego and Tareke, 2003; Argaw and Tolosa, 2008; Mekibib *et al.,* 2010; Bedada and Hiko, 2011; Tolosa *et al.,* 2013; Duguma *et al.,* 2014) in central Ethiopia. Therefore, the average prevalence value was equal to 79%, this is value was considered as expected prevalence value for this study. Therefore, using the 79% expected prevalence, 95% Confidence interval and 5% type 1 error, the number of lactating exotic zebu cross breed dairy cow required to determine the prevalence of bovine subclinical mastitis was estimated to be at least 255. But to increase the precision 265 lactating dairy cows were screened. Simple random sampling method using lottery method was employed to collect the mastitis milk samples. Therefore, lactating dairy cows were selected randomly using tag number (Identification number) if more than 30 lactating dairy cows in farm, otherwise all lactating dairy cows were screened.

3.5.Methodology

3.5.1.California Mastitis Test (CMT)

The California mastitis test was conducted to diagnose the presence of bovine subclinical mastitis and it was carried out according to previously established method (Quinn *et al.,* 2004). Briefly, about 2 ml 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 reagent was added. A gentle circular motion was applied in a horizontal plane for 15sec. The result was scored based on the gel formation and categorized as negative if there was no gel formation, or positive if there was gel formation at least in one quarter ranging from Trace (T) to +3, based on gel formation (Appendix III). Hence, a cow was considered mastitis positive, if one or more quarters were CMT positive. Then we proceed to collect samples from these CMT positive cows.

3.5.2.Sample collection and transportation

The mastitis milk samples were taken from CMT positive dairy cows and collected according to earlier protocol (Quinn *et al.,* 2004). Briefly, quarters were washed with tap water and dried with clean towel. The teat ends were then cleaned with cotton soaked with 70% ethyl alcohol. Then, after discarding the first three streams of milk, 5–10 ml milk was collected aseptically into a sterile screw-cupped, pre-labeled test tube, by holding it in inclined position, so that, the pathogen that going to be recovered come from mammary gland. Finally, milk sample was held in an ice box for transportation to animal biotechnology research laboratory at Holeta National Agricultural Biotechnology Research Center under Ethiopian Institute of Agricultural Research (EIAR) for isolation and identification of bacteria from milk samples and further molecular work. The samples were immediately cultured or stored at 4°C for a maximum of 24hr until cultured on standard bacteriological media.

3.5.3.Isolation and identification of S.aureus

The organisms were isolated and identified as described by (Quinn *et al.,* 2004). Briefly, each milk sample was swabbed on nutrient agar medium and then incubated overnight at 37°C. Next day different bacterial colonies were closely observed for their morphology,

color and consistency. Gram staining was used as primary identification test and suspected colonies were streaked on Mannitol salt agar in primary, secondary and tertiary streak and incubated for 24 hr at 37°C. The smears prepared from each of the sub-cultured colony were fixed by gentle heat, stained using Gram's staining method and examined under oil immersion in order to check the purity of the cultures. After ascertaining the pure growth, the bacterial isolates were allocated code numbers accordingly and the colonies were transferred to paired nutrient slants and were kept under refrigeration at 4°C.

3.5.4.Phenotypic characterization of S.aureus isolates

Catalase test

Catalase test was performed according to protocol described by(Quinn *et al.,*2004). Briefly, Two to three drops of 3% hydrogen peroxide was taken on microscopic slide. Bacterial colonies from *S.aureus* were picked up with inoculation loop and transferred on to hydrogen peroxide on glass slide, to test catalase activity. The production of gas bubbles constitutes positive reaction.

Mannitol salt agar (Mannitol fermentation)

Mannitol fermentation test was performed based on previously described protocol (Quinn *et al.,*2004). The colonies that were confirmed by staining reaction and catalase test were streaked on mannitol salt agar plate, incubated at 37°C, and examined after 24-48hr for growth and fermentation. The presence of growth and change of pH in the media (red to yellow color) regarded as presumptive identification of *S.aureus* or coagulase-positive *Staphylococcus (*CPS*).*

Coagulase production

Coagulase test was determined by the method described by Quinn *et al.* (2004). This test was performed as a tube coagulase test. The selected *Staphylococcus* was sub-cultured into brain heart infusion broth and incubated at 37°C for 24hr. Then, 0.5 ml of broth culture and 0.5 ml of sterile rabbit plasma (NVI, Bishoftu, Ethiopia) were put into a narrow sterile tube along with a control tube containing a mixture of 0.5 ml of sterile

brain heart infusion broth and 0.5 ml of rabbit plasma were incubated at 37°C and examined after 4 and 24hrs of incubation and observed for the clot formation. Any coagulation of plasma regarded as positive at either of the readings when compared to the control.

Maltose fermentation test

This test was carried out according to Quinn *et al.(*2004)protocol and using commercially available purple agar base (Difco) with the additional one percent maltose to differentiate the pathogenic *S.aureus*. The suspected culture was inoculated on purple agar base media plate with 1% of maltose and incubated at 37 °C for 24hrs. Rapid fermentation of maltose by *S.aureus* caused yellow discoloration of the medium due to change in pH.

3.5.5.Antimicrobial resistance test

The antimicrobial resistance testing was carried out in animal biotechnology research laboratory ,Holeta National Agricultural Biotechnology Research Center (EIAR). Antimicrobial resistance of the biochemically confirmed *S.aureus* isolates were tested against nine antimicrobial agents (Abetek, Liverpool, UK) using the Kirby-Bauer disc diffusion method on Mueller Hinton agar (HiMedia, Mumbai, India) following the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2012).The isolates were classified in accordance with the guideline of the National Committee for Clinical Laboratory Standards (CLSI, 2012) as susceptible, intermediate or resistance for each antimicrobials tested according to the manufacturer's instructions by measuring the zone of inhibition around the antimicrobials disc. The list of a panel of antimicrobial agents utilized, their symbols, concentration and break points are shown in table 4 below.

Table 4:CLSI breakpoints for *S.aureus* available for these antimicrobial agents used

Antimicrobial agent (disk code)	Content (µg/disk)	Inhibition zone diameter interpretive criteria (nearest whole mm)		
		Susceptible	Intermediate	Resistant
Ampicillin(AMP)	10µg	≥15	12-14	≤11
Chloramphenicol (CHL)	30µg	≥18	13-17	≤12
Ciprofloxacin(CPR)	5µg	≥21	16-20	≤15
Cefoxitin(CXT)	30µg	≥22	-	≤21
Erythromycin (ERY)	15µg	≥23	14-22	≤13
Gentamicin (GEN)	10µg	≥15	13-14	≤15
Penicillin (PEN)	10 units	≥29	21-28	≤20
Streptomycin (STR)	10 µg	≥15	12-14	≤11
Tetracycline (TET)	30µg	≥19	15-18	≤14

Source: (CLSI, 2012)

3.5.6.*Molecular confirmation of S.aureus isolates*

Bacterial genomic DNA extraction

Bacterial genomic DNA of all *S.aureus* isolates was extracted from culture using Quick-DNA^{TM}-96 plus kit (Zymo Research Corp., Irvine, USA) following the manufacturer's instructions (Appendix IX). After genomic DNA extraction, the quality and quantity of the extracted genomic DNA was determined using a Nanodrop 2000Spectrophotometer (Thermo Scientific, USA) and diluted to a working concentration of 50 ng/µl. The extracted genomic DNA was stored at -20°C until used for molecular confirmation of *S.aureus* and characterization of antimicrobial resistance and virulence genes.

PCR amplification of 16S rRNA gene

Polymerase chain reaction (PCR) was used to amplify the *16S rRNA* gene fragment for the confirmation of *S.aureus* isolates according to previously described protocol (Riffon *et al.,* 2001) using EdvoCycler™ PCR machine (Edvotek, Inc, Bethesda). Primers used for the PCR amplification were synthesized by Sigma-Aldrich (Bonn, Germany) and master mix synthesized by Bio-Basic company (Bio-Basic Inc, Canada). Details of primer sequences, their specific targets and expected amplicon sizes were summarized in table 5. Lyophilized primers for the target genes were reconstituted using DNase-RNase free sterile water to obtain 100 µM stock solution. All primers were stored at -20°C and then finally diluted to working concentration of 10 µM. PCR was carried out in a total volume of 25µl containing 12.5µl of 1X *Taq* PCR Master Mix (Bio-Basic Inc, Canada), 1µl of *16S rRNA* primer each (forward and reverse), 3µl of DNA template and 7.5µl sterile nuclease free water. The cyclic conditions were standardized as follows 4 min of initial denaturation at 94°C, followed by 35 cycles of amplification and each cycle consisted of denaturation at 94°C for 30sec, annealing at 55°C for 30sec and elongation at 72°C for 45sec and final elongation at 72°C for 10min followed by a holding step at 4°C. PCR products were run on a 1.5% agarose (w/v) gel using electrophoresis, stained with gel red at 120 volts for 1hr and visualized under UV light using a BioDoc-it™ imaging system (Cambridge, UK). We used nuclease free water as negative control and 100bp DNA ladder (Bioneer) as molecular ruler.

3.5.7.*Screening for antimicrobial resistance and virulence genes of S.aureus*

Molecular detection of antimicrobial resistance genes

PCR amplifications of four antimicrobial resistance genes which included methicillin resistant gene (*mecA*), erythromycin resistance genes (*erm*A, *erm*C and *msr*A*)* were carried out with a pair of specific primers (Table 5) and using previously described protocol (Sawant *et al.,* 2009; Melo *et al.,* 2014). Details of primer sequences, their specific targets and expected amplicon sizes were summarized in table 5. The reactions were performed in a final volume of 25 µl each made by 12.5µl of 1X*Taq* PCR Master Mix, 1µl of 10 µM primer (each forward and reverse), 3µl of DNA template and 7.5µl sterile nuclease free water. PCR

conditions described by the original designers of primers (Table 5). Following completion of reactions, PCR products were run on a 1.5% agarose gel using electrophoresis, stained with gel red at 120 volts for 45min and visualized under UV light using a BioDoc-it^{TM}imaging system (Cambridge, UK). We use nuclease free water as negative control and 100bp DNA marker as molecular marker.

Molecular detection of virulence genes in S.aureus

Similarly, PCR assays were also used to detect the following six staphylococcal virulence genes including the staphylococcal enterotoxin A(*sea*), exfoliative toxin A(*eta*), beta-hemolysin toxin (*hlb*), clamping factor A (*clfa*), intercellular adhesion D (*icaD*) and toxic shock syndrome toxin-1 (*tst-1*) according to previously described protocol (Mehrotra *et al.,* 2000; Greco *et al.,* 2008; Kumar *et al.,* 2009; Li *et al.,* 2018a). Molecular detection of virulence genes were carried out using PCR with previously described oligonucleotide primers in table 5. The reactions were performed in a final volume of 25µl each made by 12.5µl of 1X *Taq* PCR master mix (BioBasicinc, Canada), 2µl of a couple of primers (10µM), 3µl of template DNA and 7.5µl of nuclease free water. PCR conditions described by the original designers of primers were used (Table 5). PCR products were run on a 1.5% agarose (1.5%) gel using electrophoresis, stained with gel red at 120 volts for 1hr and visualized under UV light using a BioDoc-it^{TM}imaging system (Cambridge, UK).A 100 bp DNA Ladders (Qiagen, Germany) was used to determine the fragment sizes.

Table 5: Primers used in this study and the PCR conditions for amplifying species specific, antimicrobial and virulence genes

Target gene	Primer name and its sequence (5' ——▶3')	Product size (in bp)	Amplification conditions	References
S.aureus specific *(16S rRNA)*	16S rRNA_F: CGA TTC CCT TAG TAG CGG CG 16S rRNA_R: CCA ATC GCA CGC TTC GCC TA	1267	94°C for 30 sec 55 °C for 30 sec 72°C for 45 sec	(Riffon *et al.,* 2001)
Methicillin resistance *(mec*A)	MECA_F: GGCTATCGTGTCACAATCGTT MECA_R: TCACCTTGTCCGTAACCTGA	689	95 °C for 45 sec 55 °C for 30 sec 72 °C for 45 sec	(Melo *et al.,* 2014)
Intracellular adhesive toxin D (*icaD*)	ICAD_F: AAGCCAGACAGAGGCAATATCCA ICAD_R: AGTACAAACAAACTCATCCATCCGA	249	94°C for 30 sec 53.5°C for 30 sec 72°C for 30 sec	(Greco *et al.,* 2008)
Erythromycin resistance A *(erm*A)	ERMA_F: ATCGGATCAGGAAAAGGACA ERMA_R: CACGATATTCACGTTTTACCC	486	94°C for 1 min 49°C for 30 sec 72°C for 30 sec	(Sawant *et al.,* 2009)
Erythromycin resistanceC *(ermC)*	ERMC_F: TGAAATCGGCTCAGGAAAAG ERMC_R: CAAACCCGTATTCCACGATT	272	94°C for 1 min 52°C for 30 sec 72°C for 30 sec	(Sawant *et al.,* 2009)
Macrolide resistance A *msrA*	MSRA_F: TGGTACTGGCAAAACCACAT MSRA_R: AAACGTCACGCATGTCTTCA	1000	94°C for 30 sec 52°C for 30 sec 72°C for 30 sec	(Sawant *et al.,* 2009)
Toxic shock syndrome toxin-1 (*tst-1)*	TST_ F : ATGGCAGCATCAGCTTGATA TST_R : TTTCCAATAACCACCCGTTT	350	94°C for 2 min 55°C for 2 min	(Mehrotra *et al.,*

			72°C for 1 min	2000)
Staphylococcus aureus enterotoxin A*(sea)*	SEA-F : TTGGAAACGGTTAAAACGAA SEA-R : GAACCTTCCCATCAAAAACA	120	94°C for 2 min 55°C for 2 min 72°C for 1 min	(Mehrotra *et al.,* 2000)
Exfoliative toxin A (*eta*)	ETA-F : CGCTGCGGACATTCCTACATGG ETA-R : TACATGCCCGCCACTTGCTTGT	676	94 °C for 30 sec 57°C for 30 sec 72°C for 45 sec	(Li *et al.,* 2018a)
Beta-hemolysin toxin (*hlb)*	HLB-F :GTGCACTTACTGACAATAGTGC HLB-R : GTTGATGAGTAGCTACCTTCAGT	309	94 °C for 30 sec 58°C for 30 sec 72°C for 20 sec	(Li *et al.,* 2018a)
clumping factorA *(ClfA*)	CLFA-F GCAAAATCCAGCACAACAGGAAACGA CLFA-R : CTTGATCTCCAGCCATAATTG GTGG	638	94 °C for 45 sec 55°C for 60 sec 72°C for 60 sec	(Kumar *et al.,* 2009)

3.5.8. Data management and analysis

The data generated from the study was arranged, coded and entered to excel spread sheet (Microsoft® office excels 2007) and subjected to descriptive statistics. The Chi-square test was applied to determine existence of any association between sampling areas and CMT results using STATA program *version*13 software (STATA Co. Texas, USA). The prevalence of mastitis was calculated by dividing the number of positive animals for CMT to the total number of animals examined times 100% (Thrusfield, 2005). The significance level was set at P-value (0.05) and 95% confidence level. In all cases, 95% confidence level and p-value less than 0.05 was consider as statistical significance.

4.RESULTS

4.1.Prevalence of bovine subclinical mastitis and *S.aureus* isolates

A total of 265 lactating dairy cows from various dairy farms from central Ethiopia were screened by using California mastitis test (CMT) for bovine subclinical mastitis. The number of cows screened from each study area was summarized in table 6. Out of 265 lactating dairy cows screened, 130 (49.06%, 95% CI: 43.08-55.06) of them were found positive by CMT for either of the four quarters. California mastitis test (CMT) positive milk samples were obtained from different sampling areas, which included 54 (61.36%, 95%CI: 38.09-80.39) from the Adaberga, 16 (69.57%, 95% CI: 37.40-89.74) from Ambo and 16 (40%, 95%CI: 17.96-67.00) from Bishoftu and 44 (38.60%, 95% CI: 30.12-47.82) from Holeta. In the present study, the highest and lowest prevalence of bovine subclinical mastitis was recorded samples were collected from Ambo and Holeta with prevalence of 69.57 and 38.60%, respectively. The prevalence of bovine subclinical mastitis was statistically significant between different sampling areas (X^2=15.70; p=0.0013) as indicated in table 6.

On the other hand, 100 (76.92%, 95% CI: 68.79-83.44) milk samples from 130 CMT positive milk samples were tested positive for *S.aureus* based on phenotypic characterization bacterial isolates recovered from mastitis milk samples. Phenotypically positive *S.aureus* isolates were obtained from different sampling areas, which included 37 (68.52%, 95% CI: 54.80-79.62) from Adaberga, 12 (75%, 95% CI: 47.97-90.71) from Ambo and 16 (100%) from Bishoftu and 35 (79.55%, 95% CI: 64.80-89.15) from Holeta as represented in table 7. Regarding the sampling areas, the highest and lowest recovery rate of *S.aureus* isolates were observed in Bishoftu and Adaberga from CMT positive milk samples, respectively.

Table 6: Prevalence of bovine subclinical mastitis and its association between different sampling areas in this study

Sampling area	No. of cow screened	No. of CMT positive cows	Prevalence (95%CI)	X^2	P-value
Adaberga	88	54	61.36 (38.09-80.39)		
Ambo	23	16	69.57 (37.40-89.74)		
Bishoftu	40	16	40.00 (17.96-67.00)	**15.70**	**0.0013**
Holeta	114	44	38.60 (30.12-47.82)		
Total	**265**	**130**	**49.06 (43.08 – 55.06)**		

Keys: CMT-California Mastitis Test, Cl-Confidence Interval, X^2-Chi-square

Table 7: Prevalence of *Staphylococcus aureus* isolated from milk with bovine subclinical mastitis in the study area

Sampling area	No. of CMT positive cows screened	No. of phenotypic positive *S.aureus*	Prevalence (95% CI)
Adaberga	54	37	68.52(54.80 – 79.62)
Ambo	16	12	75.00(47.97 – 90.71)
Bishoftu	16	16	100
Holeta	44	35	79.55(64.80 – 89.15)
Total	**130**	**100**	**76.92(68.79– 83.44)**

4.2.Antimicrobial resistance test

A total of 100 phenotypically positive *S.aureus* isolates were tested to evaluate their resistance against a panel of 9 antimicrobials agents. In the present study, *S.aureus* isolates were found variably resistant to the antimicrobials tested. Data depicting the susceptibilities of the isolates were presented as percentages and are shown in figure 5. A large proportion (50% to 94.6%) of the *S.aureus* isolates obtained from Adaberga, Ambo, Bishoftu and Holeta were resistant to ampicillin, cefoxitin, penicillin and streptomycin. Despite the fact that a relatively large proportion (75% to 83.3%) of the isolates from Ambo, Bishoftu and Holeta were resistant to streptomycin, on the contrary, only a small proportion (43.24%) of the isolates from Adaberga sampled were resistant to this antimicrobial agent as summarized in table 8. Also intermediate sensitivity of *S.aureus* isolates was highest towards Erythromycin (50%), Ciprofloxacillin (35%) and followed by Gentamycin (24%) and Streptomycin (14%). Moreover, isolates obtained from Ambo were relatively highly susceptible to ciprofloxacillin and gentamycin. Similar low level resistance was observed against chloramphenicol as summarized in table 8.

Similarly, multi-drug resistance (MDR) phenotypes were generated from 100 *S.aureus* isolates showing resistance to three or more antibiotics according to (Coyle, 2005). The MDR phenotype PEN-AMP-CXT-TET-GEN was observed in 45% of the isolates from Adaberga and in 42% of Ambo isolates. The MDR phenotypes PEN-AMP-CXT was dominant among 27, 22 and 26% of Adaberga and Bishoftu and Holeta samples, respectively. The predominant MDR phenotypes for isolates from Bishoftu and Holeta were PEN-AMP-CXT-STR-TET and PEN-AMP-CXT-STR were obtained at 33 and 24%, respectively. These results indicate that in the present study, MDR *S.aureus* were isolated from milk samples.

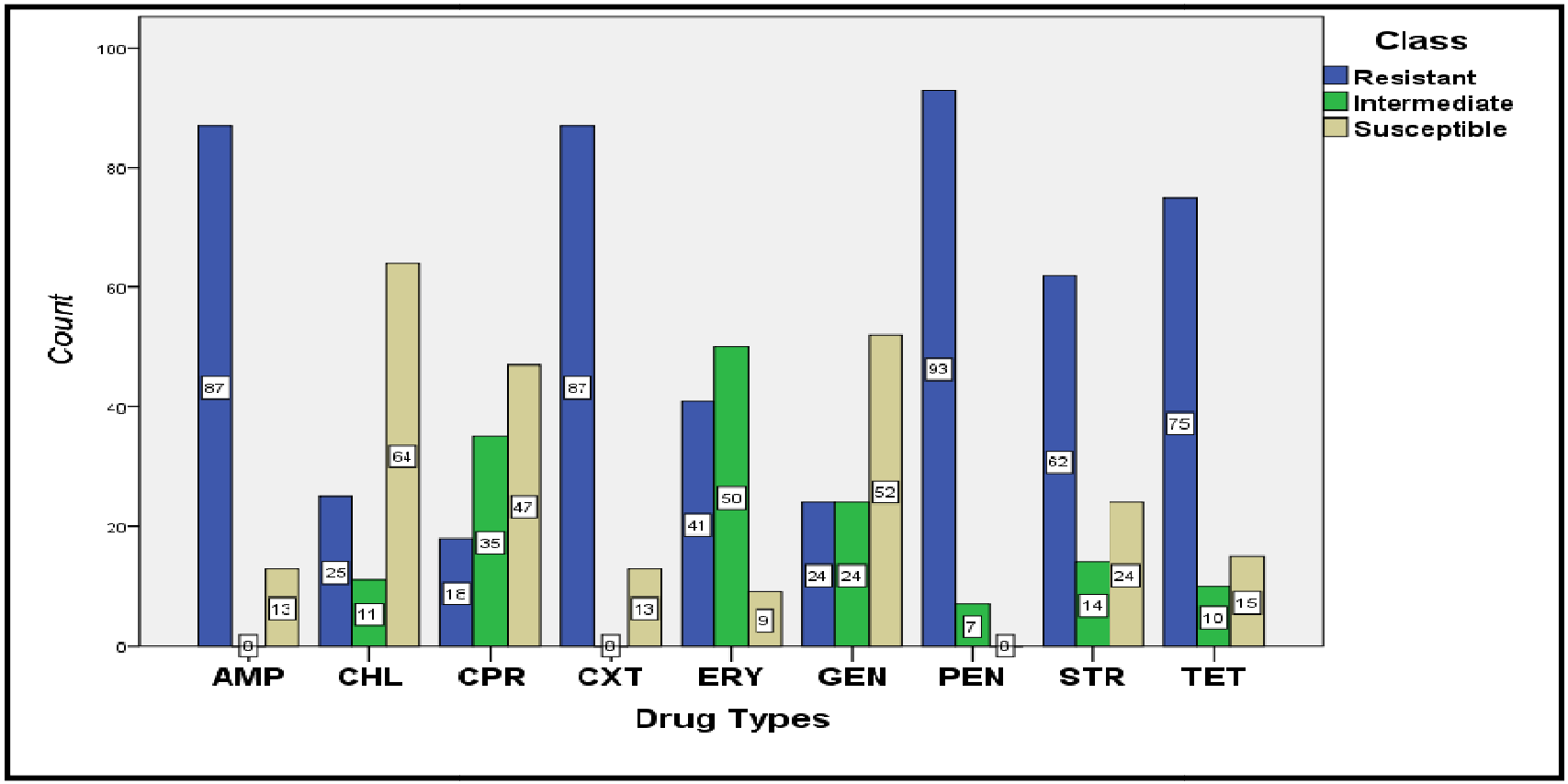

Figure 5: Antimicrobial resistance profiles

Key: AMP = Ampicillin; CHL = Chloramphenicol; CPR = Ciprofloxacillin; CXT = Cefoxitin; ERY = Erythromycin;GEN = Gentamycin; PEN = Penicillin; STR = Streptomycin; TET = Tetracycline

Table 8: The number and percentages of *S.aureus* isolates from various sampling sites that were resistant to different antimicrobials

Sampling area		AMP	CHL	CPR	CXT	ERY	GEN	PEN	STR	TET
Adaberga	**NR**	33	9	7	33	16	8	35	24	16
NT=37	**%**	89.20	24.32	18.92	89.20	43.24	21.62	94.60	64.86	43.24
Ambo	**NR**	11	4	1	11	5	1	11	6	10
NT=12	**%**	91.67	33.33	8.33	91.67	41.67	8.33	61.67	50	83.33
Bishoftu	**NR**	13	4	4	13	5	6	15	10	12
NT=16	**%**	81.25	25	25	81.25	31.25	37.5	93.75	62.5	75
Holeta	**NR**	30	8	6	30	15	9	32	22	27
NT=35	**%**	85.71	22.85	17.14	85.71	14.28	25.71	91.42	62.85	77.14

Key: NT = Number tested; NR = number resistant

Table 9: Predominant multi-drug resistance phenotypes for *S.aureus* isolates

Sampling area	phenotypes	No. observed	Percentage (%) observed
Adaberga(N=11)	PEN-AMP-CXT	3	27
	PEN-AMP-CXT-TET	1	9
	PEN-AMP-CXT-TET-GEN	5	45
Ambo(N=7)	PEN-AMP-CXT-TET-GEN	3	42
	PEN-AMP-TET-STR	1	14
Bishoftu(N= 9)	PEN-AMP-CXT	2	22
	PEN-AMP-CXT-STR	1	11
	PEN-AMP-CXT-STR-TET	3	33
Holeta(N=15)	PEN-AMP-CXT	4	26
	PEN-AMP-CXT-STR	6	24
	PEN-AMP-CXT-STR-TET	3	20
	PEN-AMP-CXT-TET	1	6

4.3.Molecular confirmation of *S.aureus* isolates

Though *S.aureus* could be identified by its various phenotypic characteristics in the present investigation but the genotypic confirmation was also carried out with a PCR based method involving *S.aureus* species specific primer targeted against *16S rRNA* gene. From a total of 100 biochemically positive *S.aureus* isolates, 68% (68/100) of them were confirmed *S.aureu s*isolatesbyusing PCR amplification. These molecular confirmed *S.aureus* isolates were obtained from different sampling areas, which included40% (27/68) from the Adaberga, 7.35% (5/68) from Ambo, and 10.30% (7/68) from Bishoftu and 43% (29/68) from Holeta. Figure 6 shows a 1.5% agarose (w/v) gel depicting the *16S rRNA* gene fragments that were amplified by PCR using genomic DNA extracted from *S.aureus* isolates. The *16S rRNA* gene fragments with the expected PCR product size of

1267 base pair was obtained. Prevalence rate of *S.aureus* isolates that were positive for *16S rRNA gene* was summarized in figure 7.

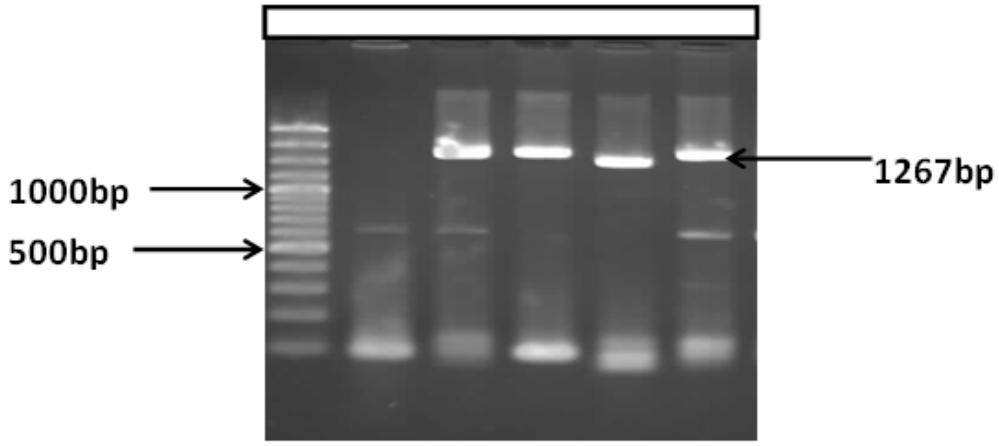

Figure 6: Agarose gel electrophoresis analysis for the *16S rRNA* gene in selected *S.aureus* isolates

Lane M = 100kb DNA marker, lane 1 = negative control, lanes 2-5 = test samples

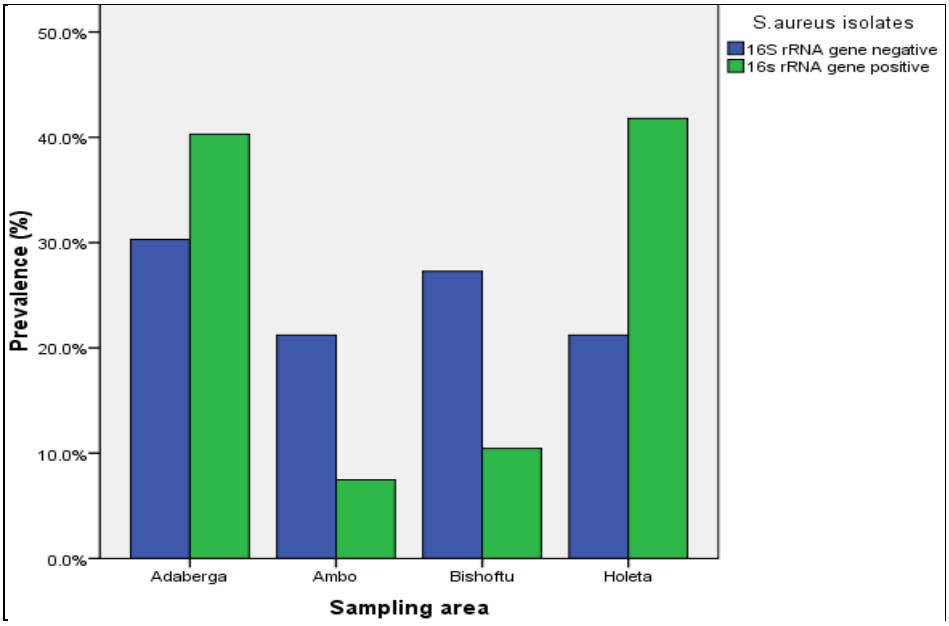

Figure 7: Prevalence rate of *S.aureus* isolates that were positive for *16S rRNA gene*

4.4.Prevalence of antimicrobial resistance and virulence genes of *S.aureus*

4.4.1. Molecular detection of antimicrobial resistance genes

Antimicrobial resistance genes were detected in all 68 molecular confirmed *S.aureus* isolates, regardless of antimicrobial susceptibility phenotypes. Results obtained are shown in table 10. Only the methicillin resistance A gene (*mec*A) was amplified from the isolates and other antimicrobial resistance genes, including *erm*A, *erm*C *and msr*A were not detected in any of the isolates in the present study. Out of 68 isolate of *S.aureus* isolates confirmed by PCR, 17.64% (12/68) of the isolates possessed the *mec*A gene and a large proportion of these isolates were obtained from Holeta 58.34% (7/12). None of the isolates from Adaberga possessed the methicillin resistance (*mec*A) gene. Figure 8 shows a 1.5% agarose (w/v) gel depicting the *mec*A gene fragments that were amplified by PCR using genomic DNA extracted from *S.aureus* isolates with expected amplicon size (689 bp).

Table 10: Number of *S.aureus* isolates that were positive for the targeted genes

Sampling area	**No. of isolates tested**	**No. of isolate positive for target genes**			
		***mec*A**	***erm*A**	***erm*C**	***msr*A**
Adaberga	27	0	0	0	0
Ambo	5	2	0	0	0
Bishoftu	7	3	0	0	0
Holeta	29	7	0	0	0
Total	**68**	**12**	**0**	**0**	**0**

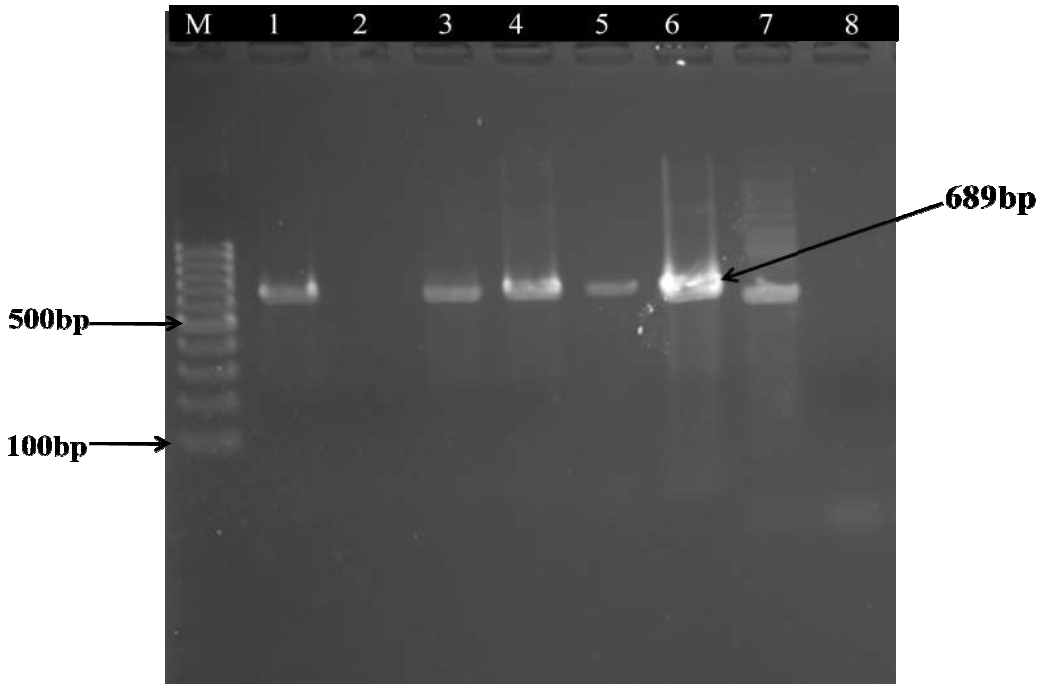

Figure 8: Agarose gel electrophoresis analysis for the *mecA* gene in *S.aureus* isolates

Lane M = l00bp DNA marker, Lanes 1- 7 = test samples, lane 8 =negative control

4.4.2.Molecular detection of virulence genes in S.aureus

The isolates for the current study were obtained from bovine milk representing four geographical locations in the central highlands of Ethiopia. The distribution of virulence genes of *S.aureus* isolates from these regions is shown in Table 10. In present study the 68 isolates of *S.aureus* were tested for six virulence genes including *tst*,*hlb, eta*, *sea, clf*A and *ica*D using PCR amplification techniques. Only the *clf*A and *eta* genes were positive from the isolates that were screened but *sea, tst-1, hlb* and *icaD* genes were negative in any of the isolates. Out of 68 isolates,25%(17/68) and 22.05% (15/68) were possessed the *eta and clf*A genes, respectively and a large proportion of these isolates were obtained from Holeta 46.67% (7/15) and Adaberga 52.94% (5/17) for *eta and clf*A genes, respectively. Figure 8 shows a 1.5 % agarose (w/v) gel depicting the *eta* and *clf*A genes fragments that were amplified by PCR using genomic DNA extracted from *S.aureus* isolates. The expected PCR product sizes obtained from these PCR products were 638 and 676bp for *clf*A and *eta*, respectively.

Table 11: Distribution of virulence genes in the study area

Sampling area	No. of isolates tested	No. of isolate positive for target genes					
		tst	***hlb***	***eta***	***sea***	***ica*D**	***clfA***
Adaberga	27	0	0	4	0	0	9
Ambo	5	0	0	1	0	0	1
Bishoftu	7	0	0	3	0	0	2
Holeta	29	0	0	7	0	0	5
Total	**68**	**0**	**0**	**15**	**0**	**0**	**17**

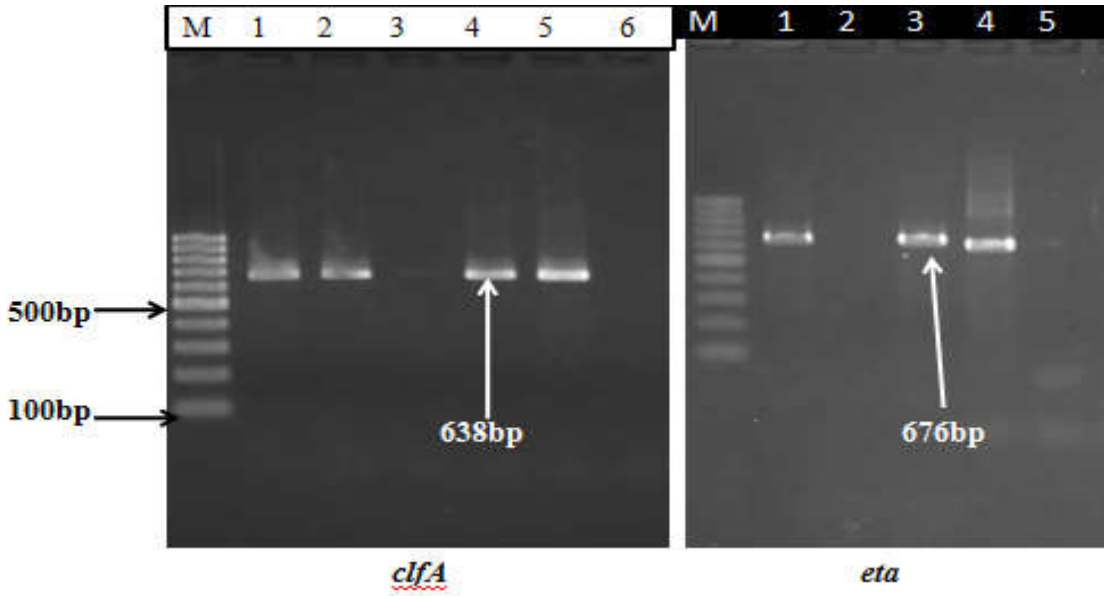

Figure 9: Agarose gel electrophoresis of amplicons of *clfA* and *eta* genes of *S.aureus isolates*

Left side: lane M= l00bp DNA maker, lanes 1-5= test samples, lane 6 = negative control

Right side: lane M= l00bp DNA maker, lanes 1-4 = test samples, lane 5 = negative control

5.DISCUSSION

5.1. Prevalence of bovine mastitis and *Staphylococcus aureus*

Bovine mastitis remains a serious and common disease in animals with significant economic losses in dairy industry worldwide (Momtaz *et al.,* 2012) as well as in Ethiopia (Mungube *et al.,* 2004); therefore, identifying mastitis causing bacteria with their antimicrobial resistance profile, and investigating their virulent determinants using molecular methods has a paramount importance to control the intra-mammary infection (Murakami *et al.,* 1991). A wide variety of pathogens are responsible for mastitis in animals. Among several bacterial pathogens that can cause mastitis, *Staphylococcus aureus* being the most important agent because it causes chronic and deep infection in the mammary glands that is extremely difficult to be cured (Momtaz *et al.,* 2012).

The overall cow-level prevalence of subclinical mastitis in central Ethiopia was 49.05%. This result was almost in agreement with findings from similar studies by Ayana et al. (2017) from Bishoftu, Arga et al. (2012) from Ambo, Dego and Tareke (2003) from southern Ethiopia and Mungube et al. (2004) from Addis Ababa, which reported prevalence rates of 46.09%, 58.82%, 40.4% and 39.8%, respectively. However, the result of the present study was lower than the reports of Duguma et al. (2014) who reported a prevalence of mastitis 81% in Holeta, central highland of Ethiopia, Bishi (1998) who reported 69.8% in dairy farms of Addis Ababa and its vicinity and Mekibib et al. (2010) who reported 74.7% around Addis Ababa. Moreover, the present finding was higher than previous reports by Workineh et al. (2002) who reported a prevalence of 25.1% in Addis Ababa and Delesse (2010) who reported a 10.3% with prevalence of mastitis in dairy farms around Holeta town, Ethiopia. As mastitis is a complex disease, the difference of prevalence observed from the current study could be due to interactions of various factors such as dairy cow management system and husbandry practice, environmental conditions, animal risk factors and virulence factors of the circulating causative agents (Radositis *et al.,* 2007) and also difference in study methods used, period of investigation and specific farm-level intervention might contribute in the variation of the prevalence of mastitis.

According to the bacteriological finding of this study,79.92% (100/130) *S.aureus* isolates were recovered from 130 CMT positive milk samples. The finding of the current study was higher than the previous findings which were done around Sebeta (44.03%) by Sori et al. (2005), in Holeta agricultural research centre (43.3%) by Duguma et al. (2014), in and around Holeta town (47.1%) by Mekibib et al. (2010) and in Bishoftu area (39.5%) by Asrat et al. (2013). This high rate of *S.aureus* in this study might suggest the high rate transmission of *S.aureus* infection which might occurred because of poor hygienic conditions during milking process in which contamination with *S.aureus* might occurred from contaminated milker's hands and milking equipment (Radositis *et al.,* 2007). Apart from Ethiopia, *S aureus* has also been reported as the chief etiological agent of mastitis in cattle by many studies from African and Asian countries (Abebe *et al.,* 2016). Though direct comparisons among studies might be difficult, but in general, the variation in the prevalence between the present and previous studies might be due to differences in detection methods, geographical location of the study sites, and differences in farm management practices in each studied farms. .

5.2.Antimicrobial resistance test

Previous studies have revealed that an increasing trend towards the occurrence of *S.aureu s*isolates that portray multiple antimicrobials resistance phenotypes (Normanno *et al.,* 2007; Pesavento *et al.,* 2007) ; hence, *S.aureus* isolates that harbour multiple antimicrobials resistant traits have been reported to negatively impact on the treatment of staphylococcal infections. The present study showed a higher level of resistance of *S.aureus* to penicillin (93%), ampicillin (87%), tetracycline (75%) and cefoxitin (87%). The current finding was in line with the findings of Abebe et al. (2016) who reported resistance of *S.aureus* to penicillin (94%), tetracycline (73.8%) around Addis Ababa and (Abera *et al.,* 2013) who recorded 94.4% to penicillin around Adama and Sori et al. (2005) who recorded 87.2% *S.aureus* isolates were found to be resistant to penicillin. In general, the current study able to show that susceptibility of *S.aureus* to commonly used antimicrobials, penicillin, ampicillin, cefoxtin and tetracycline in study area was very low. The possible justification for this could be the development of alarming level of resistance of *S.aureus* due to the regular use of these commonly used antibiotics for the treatment of cows that might resulted in the spread of resistant strain in study area. This

result was in accordance with reports from earlier studies in other countries, suggesting a possible development of resistance from prolonged and indiscriminate usage of some antimicrobials (Enright *et al.,* 2002).Previous report indicated that the prevalence of antimicrobials resistance in *S.aureus* isolates become a serious problem in a dairy herds (WANG *et al.,* 2009).The over-use of antibiotics in dairy farms is one of the major factors responsible for the emergence of drug resistant bacteria. Furthermore, isolates that are resistant to ampicillin may cross select for resistance to other beta-lactams including penicillin, therefore, resistance to ampicillin is an indication of the resistance of the isolates to other beta-lactam antibiotics (WANG *et al.,* 2009). Methicillin-resistant *Staphylococcus aureus* (MRSA) is currently a major burden in veterinary and human medicine (Tangka *et al.,* 2002). This type of resistance is considered to be one of the most important and has been implicated in many animal and human illnesses that have resulted in high mortality. It is therefore, very important to implement a systemic application of an in vitro antibiotic susceptibility test prior to the use of antibiotics in both treatment and prevention of intra-mammary infections.

5.3.Molecular confirmation of *S.aureus*

Due to the limitations of bacteriological cultural methods, the development of polymerase chain reaction (PCR) based methods can be used as a rapid and sensitive diagnostic tool to detect the presence of *S.aureus* using specific primer targeted against *16S rRNA* gene, provide a promising option for the rapid identification made in hours, rather than days consumed by conventional cultural methods(Francois *et al.,* 2003)**.**In present study, out of 100 phenotypically confirmed *S.aureus* isolates, 68% of them were confirmed *S.aureus* isolates by using PCR amplification. The finding of this study was in agreement with (Li *et al.,* 2018b). Regardless of the isolation and identification techniques employed, the confirmation of *S.aureus* in milk using molecular highlights the need for both strict farm management practices and proper sanitary procedures to be implemented during milking operations.

5.4.Prevalence of antimicrobial resistance and virulence genes of *S.aureus*

5.4.1.Molecular detection of antimicrobial resistance genes in S.aureus

In the present study, the isolates of *S.aureus* were tested for four antimicrobial resistance genes which included methicillin resistance gene (*mec*A), erythromycin resistance genes (*erm*A, *erm*C) and macrolide resistance A gene (*msr*A). Among four antimicrobial resistance genes screened, only *mec*Agene was amplified, 17.64% (12/68) of *S.aureus* isolates out of 68 molecular confirmed *S.aureus* isolates and other antimicrobial resistance genes which included *erm*A,*erm*C *and msr*A were not detected in any of the isolates in the present study.In this study, *mec*A positive *S.aureus* was detected in milk samples collected from different sampling areas, including Ambo 16.67% (2/12), Bishoftu 25% (3/12) and Holeta 53.33% (7/12) based on the presence of the *mecA* gene amplicon. However, the present study was disagree with the report from the Central highlands of Ethiopia by Seyoum et al. (2016) and Mekonnen *et al.* (2018) from North-West Ethiopia, who did not detect any *mec*A positive *S.aureus* in their study. Variation in the proportion of *mec*A positive *S.aureus* in comparison to other researcher might be due to the difference in sample size, antibiotic use in animal husbandry and hygiene practices among the dairy farms.

The *mec*A gene was detected in some phenotypically identified isolates that resist cefoxitin. The existence of *mec*A positive cefoxitin resistance *S.aureus* in milk has been reported in many previous studies (UGWU *et al.,* 2015). The presence of mecA-positive MRSA strains in bovine milk samples has been reported in many countries (Kreausukon*et al.,,* 2012). The presence of mecA negative MRSA strains in bovine milk samples has also been reported by (Kumar *et al.,* 2011). However, *mec*A-negative cefoxitin resistance *S.aureus* has been also recovered from bovine milk; the resistance revealed by *mec*A-negative cefoxitin resistance *S.aureus* isolates might be attributed to the presences of other beta-lactam resistance mechanisms (Malik *et al.,* 2007). This indicated the presence of incompatibility between the detection of methicillin resistance phenotypically using cefexitin discs and the absence of *mec*A gene in some MRSA isolates. This finding is in accordance with Garcia-Alvarazeet al. (2011) who identified phenotypic MRSA isolates without *mec*A gene. This may be attributed to the presence of

PCR inhibitors or other physical factors that may have compromised the sensitive of PCR in the detection of *mecA* gene.

In the present study, multiple drug resistant (MDR) *S.aureus* strains defined as isolates that were resistant to three or more antibiotics were obtained in 54.26% of some of the milk samples analyzed. Development of multiple antibiotic resistance among most of these isolates may be attributed to transmission of resistance (R-factor) which is a plasmid-mediated genetic determinants. *Staphylococcus aureus* often contain multiple plasmids that may contain various numbers of antibiotic resistant genes (Yamamoto *et al.,*2013). Given the fact that antibiotic resistance traits in bacteria species including *S.aureus* may occur either spontaneously by mutation and selection or by acquisition of new genetic material from other resistant organisms through transformation, transduction and conjugation it is usually not surprising that the antibiotic resistance profiles of isolates from the same region may vary considerably.

5.4.2.Molecular detection of virulence genes in S.aureus

The pathogenicity of *S.aureus* is closely related to presence of various virulence genes. In this study, six virulence factors of the pathogen were screened but only two of them were positive based on PCR amplifications. Our data showed that 15 out of 68 *S.aureus* isolates carried exfoliative toxin A (*eta*) (22.05%, 15/68) and 17 out of 68 *S.aureus* isolates contained *clfA* (25%, 17/68) genes. There has been no published information regarding clamping factor A (*clf*A) in the Ethiopian context. This is the first investigation regarding to *clf*A gene in Ethiopia and there is no other work on this virulence factors. There is no report on exfoliative molecular characterization. The presence of the clumping factor gene is considered as *Staphylococcus* species virulence gene in development and severity of mastitis in cows (Aarestrup *et al.,* 1995). The above results suggested that *S.aureus* isolates with different genetic background have different ability to acquire mobile genetic elements such as plasmids, phages and pathogenicity islands.

6.CONCLUSION AND RECOMMENDATIONS

The present study has revealed that bovine mastitis, is a widely prevalent disease in the dairy farms of central Ethiopia. The present investigation also explored that *S.aureus* is an important cause for bovine mastitis, which warns the higher public health risk due to consumption of raw milk and its products. The study also demonstrated that isolates are characterized by multiple drug resistance to commonly prescribed drugs in veterinary and human pharmacies. Dairy cows in the study area had high rates of infection by multi-drug resistant *S.aureus* isolates especially methicillin resistance A (*mecA)* gene possessing different virulent genes which, included clumping factor A (*clf*A) and exfoliative toxin A (*eta*) which may hold a serious threat to human and animal health. Though the development of antimicrobial resistant determinants in *S.aureus* is associated with the uncontrolled usage of antimicrobial agents in human and veterinary medicine, the incidence of drug-resistant *S.aureus* in bovine milk samples warrants closer monitoring. Therefore, careful monitoring for the resistance status is an utmost need since the transmission of this pathogen is dynamic and involves human, animals, and likely the farm production environment. Based on the above conclusion, the following recommendations were forwarded,

- Improve dairy farm workers' awareness on bovine mastitis would be helpful in mastitis transmission and its control measures
- Encouraging prudent and judicious use of antimicrobial drugs in veterinary and public health services
- Careful monitoring of the resistance status of *S.aureus* in dairy environment is needed, as *S.aureus* transmission is dynamic and involves animals and likely the farm production environment.
- Apply one health approach by participating multidisciplinary experts to control antimicrobial resistant and virulent strain of *S.aureus* transmission between animal and human is helpful.
- Furthermore, impacts and dynamics of antimicrobial and virulence genetic determinants need to be investigated using molecular methods.

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8. APPENDICES

Appendix I: Sample record sheet

S.No	Cow ID	Sample ID	CMT result	Types of mastitis	Breed	Age	Parity	Farm size	Other abnormalities
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									

Key:CMT- California Mastitis Test

Appendix II: Record format for laboratory isolation and identification of *S.aureus*

S.NO	Sample ID	Colony characterization on BAP	Haemolysis	Gram stain	Catalase test	Coagulase reaction	MSA	Bromocresol purple with 1% lactose	*Staphylococcus aureus*
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									

Keys: BAP-Blood agar plate; MSA-Mannitol salt agar

Appendix III: Bovine mastitis screening of lactating dairy cows using CMT reagent

Photo: Mastitis screening by CMT

Appendix IV: Interpretation for the California Mastitis Test

CMT Score	Interpretation	Visible reaction
0	Negative	Milk fluid is normal
T	Trace	Slightly precipitation
1	Weak positive	Distinct precipitation but not gel formation
2	Distinct positive	Mixture thickens with gel formation
3	Strong positive	Strong gel that is cohesive with a convex surface

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

AppendixV: Flow chart showing procedure for isolation and identification of *S.aureus* from mastitis milk samples

According to Quinn et al, 2004, the following flow chart was used for bacteriological analysis

Milk sample collection

↓

Streak a loop full of sample on blood agar plates

↓

Sub-culturing on nutrient agar

↓

Incubate at 37 °C for 24 hr under aerobic culture conditions

↓

Gram staining (positive, cocci resembling grapes)

↓

Catalase test-3 % H_2O_2 (positive)

↓

Coagulase test (Positive)

↓

Fermentation test on MSA and PAB (highly fermentative)

↓

If*S.aureus* confirmed phenotypically, it subjected to molecular work for molecular confirmation and further analysis

Appendix VI : Gram stain procedure

1. Make a thin bacterial colony smear and allow it to dry on the air
2. Fix the dried smear by passing through the Bunsen flame two to three times taking care not to overheat the smear
3. Flood the fixed smear with Gram's crystal violet (primary stain). Let stand for 60sec
4. Pour off the stain and gently wash with tape water.
5. Flood with Gram's iodine (mordant) solution. Allow it to remain for 60 seconds.
6. Pour off the iodine solution and gently wash with tape water.
7. Decolorize with Gram's decolorizer solution 95% acetone alcohol) for 15-20seconds until the blue dye no longer flows from the smear and gently wash thesmear with tape water.
8. Counterstain with Gram's safranin solution or carbolfuschin (counter stain) for 60sec.
9. Wash off the red safranin solution with water. Blot with bibulous paper to remove the excess water. Alternatively, the slide may shake to remove most of the water and air-dried.
10. Examine the finished slide under a microscope (oil immersion objective).
11. Interpretation: Bluish purple colour indicate Gram positive and pinkish colour indicate Gram negative bacteria

Appendix VII: Antimicrobials resistant testing and inhibition zone measurements

A. Preparation of inoculums

Inoculation of distinct colony in to 5ml nutrient broth incubated at 35-37°C for about 5 hours. Then the turbidity is compared with 0.5MacFarland standard. This standard is prepared by adding 0.5ml of 1 % (11.75g/liter) $BaCl_2.2H_2O$ to 99.5ml of 1% (0.36N) H_2SO_4.

B. Inoculation to Muller- Hinton Agar

Muller-Hinton Agar cooled to 50°C and poured into a sterile petri dish on level surface to a depth of 4mm. Then a sterile cotton swab on a wooden applicator stick is used to transfer the diluted bacterial suspension to a plate; excess fluid must be squeezed out by rotating the swab against the sides of the tube. The plate is seeded uniformly by rubbing the swab against the entire agar surface in three different planes roughly 60 degrees to each other's.

C. Disc application

Within 15 minutes (time used to dry the inoculums) after the plates are inoculated, antimicrobial impregnated discs are applied to the surface of the inoculated plates by hand using a sterile forceps. All discs gently pressed down on to the agar with forceps to ensure complete contact with the agar surface. The disc should no closer than 1.5 cm to the edge of the plate and they should rest 24 mm apart from each other. The large Petridishes accommodate 6 discs in outer ring and three in the center, where as no more than 5 should be placed in small plates (10cm plates). Incubate the plates inverted aerobically for 24 hours at 37°C.

D. Zone of inhibition measurement chart for antimicrobials used in the study

Photo 2: Zone of inhibition measurement for antimicrobial resistance

Appendix VIII: Composition and preparation of media used for the study

A. **Nutrient agar medium:** The composition of nutrient agar usedwas

S. No.	Ingredients	g/l
1	Peptic digest of animal tissue	5.00
2	Sodium chloride	5.00
3	Beef extract	1.50
4	Yeast extract	1.50
5	Agar	15.00

Preparation:-The nutrient agar medium was prepared by adding 28.00 gramsof nutrient agar (Hi-Media) to one liter of distilled water, heated to boiling to dissolve medium completely. The medium was cooled to room temperature and pH adjusted to 7.4. Then sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

B. Nutrient broth: The composition of nutrient broth was:

S. No.	**Ingredients**	**g/l**
1	Peptic digest of animal tissue	5.00
2	Sodium chloride	5.00
3	Beef extract	1.50
4	Yeast extract	1.50

Preparation:-The nutrient broth (Hi-Media) was prepared by adding 13.00grams of nutrient broth in one liter of distilled water, heated to dissolve the medium completely. The broth was cooled at room temperature and pH of the medium was adjusted to 7.4. Then sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

C. Mannitol salt agar medium: The composition of Mannitol salt agar medium was :

S. No.	Ingredients	g/l
1	Peptic digest of animal tissue	5.00
2	Pancreatic digest of casein	5.00
3	Beef extract	1.00
4	Sodium chloride	75.00
5	D- Mannitol	10.00
6	Phenol red	0.025
7	Agar	15.00

Preparation:-The mannitol salt agar medium was prepared by adding 111.02grams of above medium (Hi-Media) in one liter of distilled water and dissolved by heating. The pH of the medium was adjusted to 7.4 after cooling at room temperature. Then sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

D. *Mueller-Hinton agar medium*: The composition of medium was :

S. No	Ingredients	g/l
1	Casein enzymatic hydrolysate	17.50
2	Agar	17.00
3	Beef extract	2.00
4	Starch soluble	1.50

Preparation:-The above medium was prepared by adding 39.00 grams of Mueller-Hinton agar (Hi-Media) in one liter of distilled water and was dissolved by gentle heat. The medium was brought at room temperature to adjust pH to 7.4. Then sterilization of medium was done by autoclaving at 15 lbs pressure (121°C) for 15 minutes and cooled at room temperature prior to dispense.

E. ***Blood agar medium***:

To prepare blood agar, above mentioned nutrient agar medium was prepared and autoclaved. The temperature of the medium was brought down to approx. 40°C then aseptically collected sheep blood was mixed well in the medium at the rate of 5% (vol./vol.). The medium was then poured in sterilized petri plates and left to solidify.

Bacteriological media preparation and sterilization

Photo 3: Media preparation and sterilization

Appendix IX: Conventional PCR reaction protocol and its master mix preparation

A. Genomic DNAextraction using Quick-DNA^{TM} 96 plus kitprotocol

Procedure

1. Add up to 50µl sample to each well of a **deep-well block** and add 50µl **bio-fluid and cell buffer(red**) and finally add 5µl **proteinase K**
2. Mix thoroughly, seal with film, and then incubate at 55°C for 20 minutes
3. Add 1 volume Genomic Binding Buffer to the digested samples, mix thoroughly. Example: add 105µl genomic binding buffer to the 105µl digested samples
4. Transfer the lysates to the well of the **Zymo-spin™ 1-96-xl plate** on a **collection plate.** Centrifuge(≥3,500xg) for 5 minutes. Discardthe flow-through
5. Add 200µl **DNA pre-wash buffer** to each well and centrifuge for 5 minutes. Discardthe flow-through
6. Add 500µl **g-DNA wash buffer** to each well and centrifuge for 5 minutes.Discardthe flow-through
7. Add 200µl **g-DNA wash buffer** to each well and centrifuge for 5 minutes.Discardthe flow-through
8. To elute the DNA, transfer to an **Elution plate**. Add ≥15µl DNA **Elution buffer**, incubate 3 minutes, and then centrifuge for 5 minutes
9. Close the elution plate, identify and store DNA on ice if to be used immediately, or at -20°C.

B. Prepare a reaction Master Mix using the following protocol:

1. Thaw the Master Mixture and other frozen reaction components on ice.

2. Mix thoroughly by inverting tubes a number of times, briefly centrifuge and then place on ice.

3. Prepare (on ice) enough master mix for **n**reactions (including one extra reaction to compensate for any pipetting errors) by adding all required components except DNA template.

Master mix preparation

Components	Final concentration	Volume for 1 reaction (µl)*	Volume for nreactions (µl)*
2X *Taq*PCR M aster Mix	1X	12.5	
Primer F (10 µM)	0.4µM	1	
Primer R (10 µM)	0.4µM	1	
Nuclease-free H_2O	-	7.5	
Total volume		**22**	

*Final volume of 23 µl for a single PCR reaction.

4. Mix master mix by inverting tubes a number of times before spinning down the mix.
5. Aliquot **22µl**of the master mix to individual strip tubes being careful to add the mix to the bottom of the tubes.
6. Add 3µl of extracted **DNA template**to each tube

1.Sample (unknown), ***n number of samples can be included***

2. Positive control

3. Negative template control (nuclease free water)

C. Perform most PCR reactions using the following cycling program:

Steps	Temperature	Time	No. of cycles
Initial denaturation	94°C	4min	1
Denaturation	94°C	30sec	
Annealing	45-68°C	30sec	35
Extension	72°C	1kb/min	
Final extension	72°C	10min	1
Hold	4	Forever	-

D. **Electrophoresis**

- At the end of the PCR program, load2µl of loading buffer with gel red for 5µl of amplified PCR product on 1.5% agarose gel (1.5g of agarose in 100 ml of 1X TAE)
- Load 4 µl of DNA molecular weight marker (DNA ladder) in to the first wells
- Run the electrophoresis at 120 volts for 45 min
- Amplified fragment size were**638, 676, 689 and 1267bp** for *Clf*A, *eta,mec*A and *16S rRNA* genes, respectively for this study.

E. Gel Capture

- Use the Gel documentation system to snap the gel.

Appendix X: Miscellaneous picturesduring my laboratory work for this study

A. Culture characteristics of the Staphylococcus organisms on mannitol salt agar(MSA)and purple agar base (PBA).

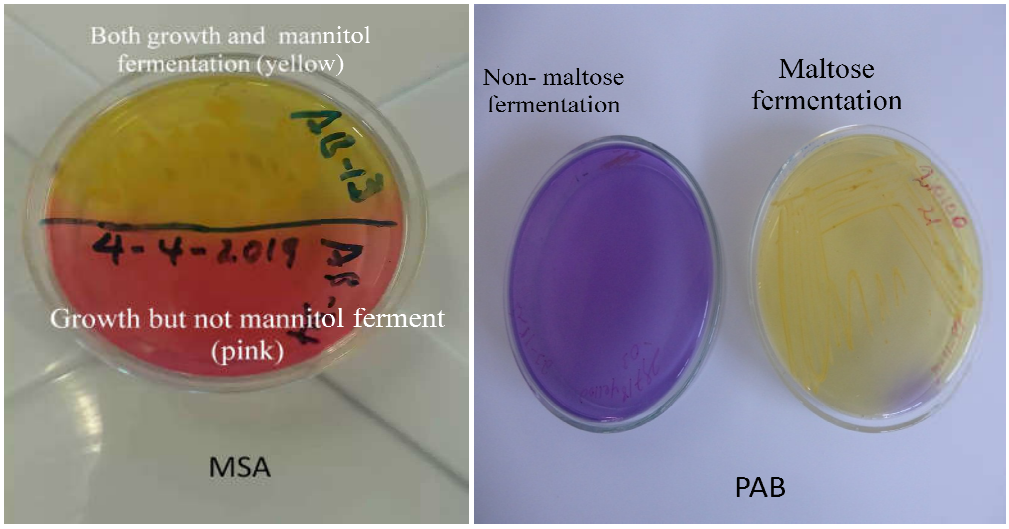

Photo 5: Mannitol salt agar and purple agar base

Photo 4: Tube Coagulase test

B. Work flow in molecular laboratory in this study

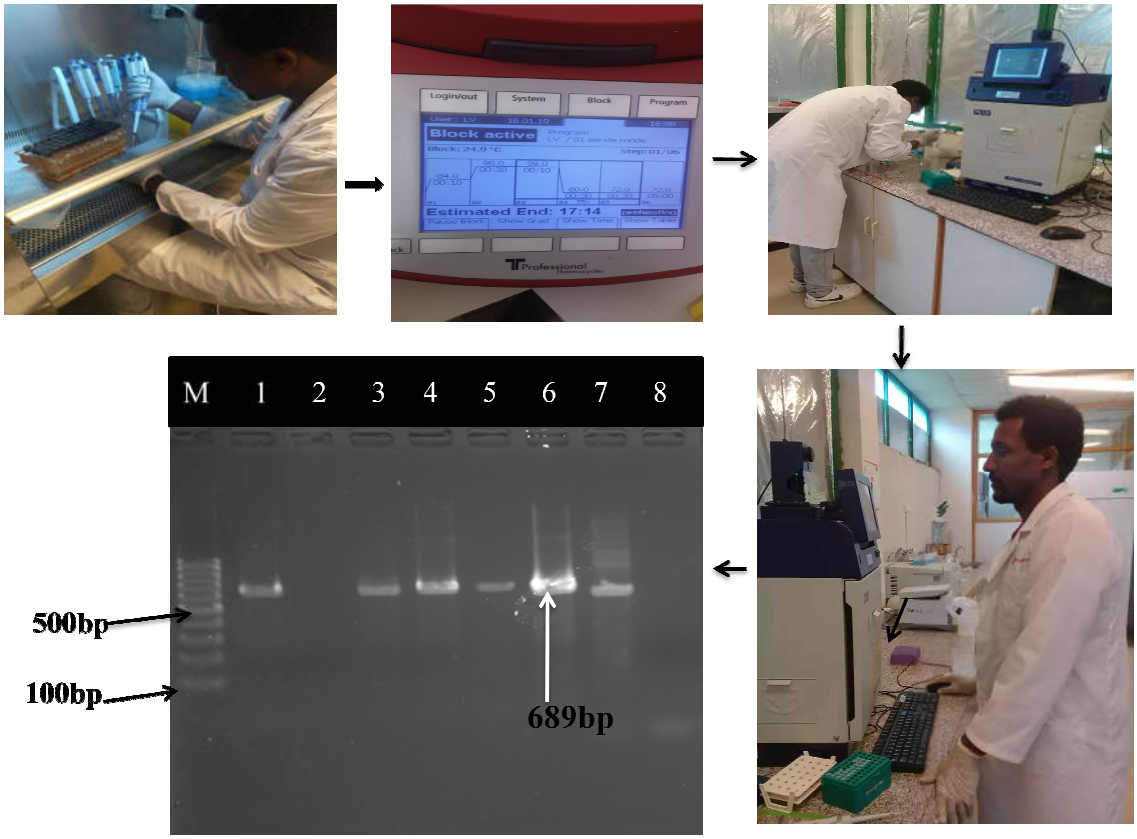

Photo 6: From bacterial genomic DNA extraction to post PCR analysis