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ISOLATION AND IDENTIFICATION OF METHICILIN RESISTANT *S. aureus* FROM BOVINE MASTITIC MILK IN AND AROUND WOLAITA SODO, SOUTHERN ETHIOPIA.

MSc THESIS

BY

BINIAM TADESSE

DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND VETERINARY PUBLIC HEALTH

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COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE, BISHOFTU

ISOLATION AND IDENTIFICATION OF METHICILIN RESISTANT *S. aureus* FROM BOVINE MASTITIC MILK IN AND AROUND WOLAITA SODO, SOUTHERN ETHIOPIA.

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 Science in Microbiology

By

Biniam Tadesse

June, 2014

College of Veterinary Medicine and Agriculture, Bishoftu

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Microbiology, Immunology and Veterinary Public Health

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As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the Thesis prepared by: **Biniam Tadesse** Entitled**: Isolation and Identification of Methicilin resistant *S. aureus* from Bovine Mastitic Milk in and around Wolaita Sodo, Southern Ethiopia** and recommend that it be accepted as fulfilling the thesis requirement for the degree of **Masters of science in Veterinary Microbiology.**

Dr. Reta Duguma	________________	________________
Chairman	Signature	Date
Dr. Eshetu Lemma	________________	________________
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Dr. Takele Abayneh	________________	_______________
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Major Advisor	Signature	Date
Dr. Tesfaye Sisay	________________	________________
Co Advisor	Signature	Date
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Department chairperson	Signature	Date

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STATEMENT OF AUTHOR

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College of Veterinary Medicine and Agriculture, Bishoftu

Date of Submission: _________________________

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LIST OF ABBREVATIONS

agr	accessory gene regulator
CLSI	Clinical Laboratory Standard Institute
CMT	California Mastitis Test
CNS	Coagulase Negative Staphylococci
DNA	Deoxyribo Nucleic Acid
EFSA	European Food Safety Authority
MAR	Multiple Antibiotic Resistant
MIC	Minimum Inhibitory Concentration
MRSA	Methicilin Resistant *Staphylococcus aureus*
MSCRAMMs	Microbial Surface Components Recognizing Adhesive Matrix Molecules
NCCLS	National Committee for Clinical Laboratory Standards
NMC	National Mastitis Committee
OR	Odds Ratio
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
SCC	Staphylococcus Chromosome Cassette
SE	Staphylococcus Enterotoxin
SXT	Sulphamethoxazole-Trimethoprim

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ABSTRACT

*A cross sectional study was conducted from November 2013 to May 2014 in and around Wolaita Sodo town, Southern Ethiopia, to isolate and identify methicilin resistant S. aureus and their resistance to different antimicrobials and also identify risk factors associated with occurrence of dairy cow mastitis. A total of 257 dairy cows were included during the study period. Total of 1020 quarters were examined to detect clinical and subclinical mastitis by physical examinations of udder and milk and California mastitis Test (CMT), respectively. The prevalence of mastitis was 40.9%. Out of this, 4.66% and 36.18% were clinical and subclinical respectively. The univariate logistic regression showed that among potential risk factors considered from the farm attributes, age, milking hygiene, parity, lactation period, farm floor and previous mastitis treatment had significant (p=<0.05) effect on the prevalence of mastitis. However, breed was not a significant potential risk factor. 39 (15.1%) S. aureus was isolated and out of this, 8(3.11%) and 31(12.06%) were from clinical mastitis and subclinical mastitis respectively. The result showed a significant association of S. aureus with mastitis (p=*0.025*). The present study showed the resistance of S. aureus to penicillin G (100%), cefoxtin (71.8%), amoxicillin-clavulinic acid (61.5%), tetracycline (69.2%), streptomycin (66.7%), chloramphenicol (35.9%), sulphamethoxazole-trimethoprim (43.6%) and vancomycin (56.4%). In the present observation, 28 (71.8 %) S. aureus isolates not only showed MRSA but also multidrug resistance (MRSA) primarily to penicillin G, cefoxtin, tetracycline and streptomycin because of resistance to β-lactams and frequent use. Association of MRSA with age of cows was also highly significant (*p = 0.000) *and all MRSA were isolated from adult and old cows. 92.8% of MRSA isolates was found in previously mastitis treated animal showed significant association (p=0.001). The development of antimicrobial resistance is nearly always as a result of repeated therapeutic and/or indiscriminate use of them. Regular antimicrobial sensitivity testing helps to select effective antibiotics and to reduce the problems of drug resistance development towards commonly used antibiotics.*

Key words: antibmicrobials, association, cross sectional, dairy cows, MRSA, quarters, resistance, risk factors, S. aureus, significant, therapeutic.

1. INTRODUCTION

Staphylococci are Gram-positive bacteria, with diameters of 0.5 – 1.5 μm and characterized by individual cocci, which divide in more than one plane to form grape-like clusters. The staphylococci are non-motile, non-spore forming facultative anaerobes that grow by aerobic respiration or by fermentation. Staphylococci are tolerant to high concentrations of salt and show resistance to heat. Pathogenic staphylococci are commonly identified by their ability to produce coagulase, and thus clot blood .This distinguishes the coagulase positive strains, *S. aureus,* S. *intermedius* and *S. hyicus* from the other staphylococcal species such as *S. epidermidis*, that are coagulase-negative (Harris *et al*., 2002). *S. aureus* is both commensal and pathogen. It is found as a commensal associated with skin, skin glands and mucous membranes. *S. aureus* affects skin, soft tissues, bloodstream and lower respiratory tract. It also causes severe deep-seated infections like endocarditis and osteolmyelitis (Schito, 2006).

S. aureus plays its most significant animal pathogenic role as cause of intramammary infections in cattle and small ruminants leading to considerable economic losses in cattle farming. The pathogen is frequent causative agent of clinical or subclinical mastitis in cattle (Asperger and Zangerl, 2003). Presence of *S. aureus* on the skin and mucosae of food producing animals, such as ruminants, and the frequent association of the pathogen with mastitis, often leads to contamination of milk (Jablonski and Bohach, 1997). Contamination of milk can also occur from environmental sources during handling and processing (Peles *et al*., 2007). Milk is a good substrate for *S. aureus* growth and dairy products are common sources of staphylococcal food-poisoning (Morandi *et al*., 2007). *S. aureus* also causes severe animal diseases, such as suppurative disease, arthritis and urinary tract infections (Lowy, 1998).

In recent years, there has been increased concern about antibiotic resistant strains of *S. aureus*. Development of resistance has been attributed to the extensive therapeutic use of antimicrobials or to their administration as growth promoters in food animal production (Normanno *et al*., 2007). Isolates of *S. aureus* are frequently resistant to methicillin and essentially all other β-lactam antibiotics. The resistance to methicillin in staphylococci is mediated by the *mecA* gene that encodes a modified penicillin-binding protein (PBP), the PBP2a or 2’, which shows reduced affinity to penicillins, such as methicillin and oxacillin and for all other beta-lactam antibiotics. An organism with this type of resistance is referred to as methicillin-resistant *S. aureus* (MRSA). The *mecA* gene resides on a staphylococcal chromosomal cassette (SCCmec) (Kwon *et al*., 2006)

MRSA was initially reported as a nosocomial pathogen in human hospitals (hospital-associated MRSA) and was isolated from patients with compromised immune systems undergoing medical procedures. MRSA accounts for 30 to 40% of all hospital-acquired infections and for 40% to 70% of *S. aureus* infections in intensive care units (Gordon and Lowy, 2008). In the 1990s, a major change in the epidemiology of MRSA has been observed, with the appearance of cases affecting people with no epidemiological connection to hospitals; strains that cause such infections are referred to as community-acquired or community associated MRSA (EFSA, 2009).

Until recently, such strains were susceptible to many antibiotics other than β-lactams; however, resistance seems to be increasing, and multiple antibiotic resistant strains have started to emerge (Otter and French, 2010). There is now increasing concern about the public health impact of MRSA associated with food producing animals, because MRSA and, consequently, their resistance genes can spread from animals to humans by direct contact or through the food chain (Kluytmans, 2010). MRSA strains have been isolated in many countries from cows’ or small ruminants’ milk and various dairy products (Ünal *et al*., 2012).

There are also some studies on MRSA in some part of Ethiopia such as in Hawasa by (Daka *et al*., 2012), in Adama by (Abera *et al*., 2013), in and around Addis Ababa (Abebe *et al*., 2013). Concerning the study area (Wolaita Sodo), MRSA is poorly studied. Knowledge of MRSA is necessary to make decisions regarding antibiotic treatment and prerequisite for establishing control strategies. Without such local knowledge however, treatment measures fail and designing control programmes may be at best only guess-work and identification of MRSA data is needed to get some insight on their overall negative economic implications.

Therefore, the present study was contemplated with the following

Thus, the objectives of this study were

- Isolation and identification of *Staphylococcus aureus* from mastitic cow's milk.
- To estimate the occurrence of *MRSA strains* using antibiotic sensitivity test.
- To asses associated risk factors leading to MRSA mastitis infection.

2. LITERATURE REVIEW

In recent years, there has been increased concern about antibiotic resistant strains of *S. aureus*. Development of resistance has been attributed to the extensive therapeutic use of antimicrobials or to their administration as growth promoters in food animal production (Normanno *et al*., 2007). Isolates of *S. aureus* are frequently resistant to methicillin and essentially all other β-lactam antibiotics. The term 'MRSA' is used to describe strains of *S. aureus* resistant to semi-synthetic, penicillinase resistant, β-lactams such as methicillin, oxacillin or cloxacillin. MRSA strains are resistant to all cephalosporins, cephems and other β-lactams, such as ampicillin-sulbactam, amoxicillin-clavulanic acid, ticarcillin-clavulanic acid, piperacillin-tazobactam and the carbapenems (Lee, 2003).

2.1. History of MRSA in animals

The first report of MRSA in animals was in milk from Belgium cows with mastitis (Morgan, 2008). Until 2000, MRSA had been isolated sporadically from animals, in particular cows, small companion animals, and horses. With exception of some equine isolates, the nature of these cases suggested a human origin and no epidemics have been reported (Catry *et al*., 2010). In this respect, until the end of 20th century, both the scientific community and policy makers were convinced that animal husbandry was of little relevance for MRSA causing diseases in humans, but was particularly a problem based on antimicrobial use in human medicine. The situation has changed with a growing number of reports of MRSA in livestock, especially pigs and veal calves. MRSA has also been reported in companion animals and horses, as well as transmission between humans and animals (Catry *et al*., 2010).

Calling attention to this dramatic increase of MRSA in animals, van at the Veterinary Microbiological Diagnostic Center, in the Netherlands, reported 0% MRSA in isolates from equine clinical samples in 2002 and then 37% in 2008 (Van Duijkeren *et al*., 2010)

2.2. Nomenclature of MRSA strains

2.2.1. Hospital associated MRSA

MRSA first emerged in the 1960's but became increasingly problematic in the 1990's especially in intensive care unit settings where it became a major cause of nosocomial infections. Approximately 20 to 60% of humans are permanent or intermittent carriers of *S. aureus* and relevant sites include the anterior nares, axillae, perineum and vagina (Kluytmans, 2010). Clinical signs range from minor skin conditions (e.g., pimples, boils and impetigo) to severe disease, such as cellulitis and postoperative wound infections. In humans, *S. aureus* can also cause pneumonia, bacteraemia, meningitis, sepsis and pericarditis (Schito, 2006).

HA-MRSA harbors large staphylococcal chromosome cassettes (SCC*mec* types I-III), which encode one (SCC*mec*type I) or multiple antibiotic resistance genes (SCC*me*c type II and III). Resistance to antibiotics may have allowed the bacterium to survive an environment where antibiotic use is frequent (Klevens *et al*., 2006). It has therefore been suggested that HA-MRSA represents less robust strains of *S. aureus* that could only survive environments where bacterial competition is limited by antibiotic pressure. In support of this viewpoint, HA-MRSA shows a longer generation time compared to methicillin sensitive *S. aureus* (MSSA) (30 minutes for HA-MRSA versus 23 minutes for MSSA) (Wang *et al*., 2007).

Consistent with the last finding, many clinical HA-MRSA isolates exhibit a *agr*$^{-}$ or a mixed *agr*$^{+}$ and *agr*$^{-}$ genotype. Though these genotypes could explain the relative nonpathogenic nature of HA-MRSA toward immunocompetent hosts, it is possible that an *agr*$^{-}$ or a mixed *agr*$^{+}$ and *agr*$^{-}$ genotype could be beneficial for HA-MRSA survival in the healthcare setting; *agr*$^{-}$ genotype could for example facilitate biofilm formation and proliferation on plastic tubings (Shopsin *et al*., 2008).

As physicians attempt to grapple with the antibiotic resistance problem posed by HA-MRSA, increasingly there are reports of the more virulent CA-MRSA infiltrating the healthcare setting. The impact of this migration bears more careful monitoring as it may demand more aggressive and different control and treatment strategies (Liu *et al*., 2008).

2.2.2. Community associated MRSA

Until the late 1990's MRSA infections were largely confined to immune compromised individuals or individuals with healthcare exposure. In 1997, death of four healthy children from MRSA pneumonia and sepsis heralded the arrival of a new type of MRSA. Soon thereafter, MRSA cases burgeoned across continents; the majority of cases were confined to few clonal lineages that were markedly different from HA-MRSA, shared a small sized Type IV SCC*mec* cassette, and encoded the genes for the Panton-Valentine Leukocidin (PVL) (Vandenesch *et al*., 2003).

2.2.3. Livestock Associated MRSA (LA-MRSA)

Livestock associated refers mainly to the clonal spread of a certain MRSA strain (ST398) that colonise different food animal species (including horses) and may cause infections in humans. Companion animals and horses may be colonised with a variety of strains due to their close contact with humans. Thus these species may act as carriers of MRSA originating from humans (a so called "humanosis") (Morgan, 2008). During the period 1970-2000, MRSA has been sporadically isolated from animals, in particular cows, small companion animals, and horses. With the exception of some equine isolates, the nature of these cases suggested a human origin and no epidemics have been reported (Leonard and Markey, 2008).

Thus, until the end of the 20th century both the scientific community and policy makers were convinced that MRSA in human medicine had nothing to do with animal husbandry but was a problem solely based on the antimicrobial use in human medicine. The situation has now changed, with an increased number of reports on LA-MRSA in livestock, especially swine and veal calves. MRSA has also been reported in companion animals and horses, as well as transmission between humans and animals (Leonard and Markey, 2008). Sometimes distinct animal specific-lineages such as LA-MRSA have been involved (Cuny *et al*., 2006) but in many occasions human associated MRSA genotypes have been isolated (Weese, 2008).

Livestock Associated Methicillin-resistant *S. aureus* (LAMRSA) belonging to the clonal complex 398 (LA-MRSA CC 398) is considered to be zoonotically important because of its capacity to colonize a wide range of hosts (Paterson *et al*., 2012). Bovine and human MRSA strains indistinguishable by phenotyping and genotyping methods have been found providing evidence for MRSA transmission between human and cattle (Hata *et al*., 2010).

2.2. MRSA in food-producing animals

Food producing animals pose a potential risk of infection to humans both through direct contact and through food products if not handled correctly and processed. Since 2003, with the first isolation of a novel pig-associated MRSA strain (ST398) in the Netherlands, in dairy cows, *S. aureus* is a commonly isolated mastitis causing pathogen. Antimicrobials are widely used in the dairy industry for the prevention and treatment of bovine mastitis and other infectious diseases (Hendriksen *et al*., 2008)

S. aureus is also an important food-borne pathogen. Staphylococcal food poisoning is caused by ingestion of food containing one or more preformed enterotoxins (SEs) produced by *S. aureus*. Staphylococcal food poisoning ranks third among reported food-borne diseases in the world (Boerema *et al*., 2006). In 2006, *S. aureus* toxins were responsible for 49% of 482 human food-borne outbreaks caused by bacterial toxins and 4% of all reported outbreaks reported by EU Member States (EFSA, 2007). Symptoms have a rapid onset and include nausea, vomiting and diarrhoea (Jablonski and Bohach, 1997; Kluytmans *et al*., 1995).

There are five classical enterotoxins (SEA, SEB, SEC, SED and SEE), six new types of enterotoxins (SEG, SEH, SEI, SER, SES, and SET) and ten staphylococcal-like (Se*l*, designated as SE*l*J to SE*l*V) proteins. It is known that about 95% of staphylococcal food poisoning cases are caused by the classical enterotoxin types. The remaining 5% of outbreaks may therefore be associated with other enterotoxins (Bergdoll and Wong, 2006). SEH have clearly been involved in food poisoning outbreaks (Jorgensen *et al*., 2005) whereas SEG and SEI (Omoe *et al*., 2002) and SER, SES, and SET (Ono *et al*., 2008) were proved to be more or less emetic, with a possible incidence in food safety.

Cows with mastitis have been the most likely to harbor MRSA, and they may be related to horizontal transfer via wet hands of colonized or infected dairy farm workers, and selection by the use of antibiotics to treat mastitis (Morgan, 2008). MRSA strains isolated from cows with subclinical mastitis were phenotypically and genotypically indistinguishable from the strain from the person who worked with these animals. These strains were determined as ST1, *spa* type t127, SCC*mec*IVa. The authors considered these strains epidemiologically related, indicating transmission from cow to human or from human to cow (Juhász-Kaszanyitzky *et al*., 2007). Twenty five MRSA ST398 isolates from cases of bovine clinical mastitis and two isolates from farm workers originating from 17 dairy farms were studied in Germany, evaluating the genetic relatedness, antimicrobial resistance and virulence properties (Feβler *et al*., 2010).

2.4. Zoonotic Implications of Bovine MRSA

MRSA infected cattle acts as a reservoir and later transmit the infections to other animals and humans (Spoor *et al*., 2013). MRSA colonization in cattle may be an occupational risk to the people in close contact with MRSA infected cattle such as veterinarians, farmers, milkers and people working at slaughterhouses (Paterson *et al*., 2012). Transmission of animal MRSA to veterinary personnel has been found and it is more common for large animal personnel than small animal personnel (Wulf *et al*., 2008).

Although, MRSA has been reported as transmissible diseases of zoonotic as well as humanotic importance, the direction and routes of transmission are superficially understood. Some authors have reported bidirectional transmission of MRSA (AVMA, 2014). Animal to human transmission occurs through direct contact, environmental contamination and through handling of infected animal's product (Nunang and Young, 2007) whereas human to animal transmission is still unclear (Weese, 2010).

2.5. Virulence factors and disease

The armamentarium of virulence factors of *S. aureus* is extensive, with both structural and secreted products playing a role in the pathogenesis of infection. Two remarkable features of staphylococci are that a virulence factor may have several functions in pathogenesis and that multiple virulence factors may perform the same function. In establishing an infection, *S. aureus* has numerous surface proteins, called “microbial surface components recognizing adhesive matrix molecules” (MSCRAMMs) that mediate adherence to host tissues. MSCRAMMs bind molecules such as collagen, fibronectin, and fibrinogen, and different MSCRAMMs may adhere to the same host-tissue component. MSCRAMMs appear to play a key role in initiation of endovascular infections, bone and joint infections, and prosthetic-device infections. Different *S. aureus* strains may have different constellations of MSCRAMMs and so may be predisposed to causing certain kinds of infections (Menzies, 2003)

Once *S. aureus* adheres to host tissues or prosthetic materials, it is able to grow and persist in various ways. *S. aureus* can form biofilms (slime) on host and prosthetic surfaces, enabling it to persist by evading host defenses and antimicrobials (Donala and Costerton, 2002). The ability to form and reside in biofilms is one reason why prosthetic-device infections, for example, can be so difficult to eradicate without removal of the device. *S. aureus* is also able to form small-colony variants (SCVs), which may contribute to persistent and recurrent infection. SCVs are able to “hide” in host cells without causing significant host-cell damage and are relatively protected from antibiotics and host defenses. They can later revert to the more virulent wild-type phenotype, possibly resulting in recurrent infection (Proctor and Peters, 1998)

S. aureus has many other characteristics that help it evade the host immune system during an infection. Its main defense is production of an anti phagocytic microcapsule (most clinical isolates produce type 5 or 8). The zwitter ionic capsule (both positively and negatively charged) can also induce abscess formation (O'Riordan and Lee, 2004). The MSCRAMM protein A binds the Fc portion of immunoglobulin and, as a result, may prevent opsonization. *S. aureus* may also secrete chemotaxis inhibitory protein of staphylococci or the extracellular adherence protein, which interfere with neutrophil extravasation and chemotaxis to the site of infection. In addition, *S. aureus* produces leukocidins that cause leukocyte destruction by the formation of pores in the cell membrane (Foster, 2005)

During infection, *S. aureus* produces numerous enzymes, such as proteases, lipases, and elastases that enable it to invade and destroy host tissues and metastasize to other sites. *S. aureus* is also capable of producing septic shock. It does this by interacting with and activating the host immune system and coagulation pathways. Peptidoglycan, lipoteichoic acid, and α-toxin may all play a role. In addition to causing septic shock, some *S. aureus* strains produce super antigens, resulting in various toxinoses, such as food poisoning and toxic shock syndrome (McCormic *et al*., 2001). Unlike the structural components noted earlier, these super antigens can produce a sepsis-like syndrome by initiating a “cytokine storm.” Some strains also produce epidermolysins or exfoliative toxins capable of causing scalded skin syndrome or bullous impetigo (Prevost *et al*., 2003)

Regulation of expression of staphylococcal virulence factors plays a central role in pathogenesis. To reduce undue metabolic demands, expression occurs in a coordinated fashion only when required by the bacterium. Expression of MSCRAMMs generally occurs during logarithmic growth (replication), whereas secreted proteins, such as toxins, are produced during the stationary phase. During infection, the early expression of the MSCRAMM proteins facilitates initial colonization of tissue sites, whereas the later elaboration of toxins facilitates spread. The accessory gene regulator (*agr*) is a quorum sensing system that plays a critical role in the regulation of staphylococcal virulence (Novick, 2003).

2.6. Antibiotic resistance

The term ‘MRSA’ is used to describe strains of *S. aureus* resistant to semi-synthetic, penicillinase resistant, β-lactams such as methicillin, oxacillin or cloxacillin. MRSA strains are resistant to all cephalosporins, cephems and other β-lactams, such as ampicillin-sulbactam, amoxicillin-clavulanic acid, ticarcillin-clavulanic acid, piperacillin-tazobactam and the carbapenems. This group of organisms is also frequently resistant to most of the commonly used antimicrobial agents, including the aminoglycosides, macrolides, chloramphenicol, tetracycline and fluoroquinolones (Lee, 2003).

β-lactam antibiotics damage bacteria by inactivating penicillin-binding proteins, enzymes that are essential in the assembly of the bacterial cell wall (Pinho *et al*., 2001). These antibiotics inactivate the four native penicillin-binding proteins found in staphylococci. As a result of the weakened cell wall, treated bacteria become osmotically fragile and are easily lysed. The staphylococcal β-lactamase protein, which cleaves the β-lactam ring structure, confers resistance to penicillin, but not to semi-synthetic penicillins. In MRSA, resistance to all β-lactam antibiotics, including the semi-synthetic penicillins, is conferred by the penicillin-binding protein PBP2’ (or PBP2a) that has a very low affinity for β-lactam antibiotics and is thought to aid cell wall assembly when normal penicillin-binding proteins are inactivated. PBP2a is encoded by the *mecA* gene, which is located in the staphylococcal cassette chromosome (SCC*mec*) (Katayama *et al*., 2000).

The confirmation of the presence of the *mecA* gene, has until recently been the ‘golden standard’ for detection of MRSA worldwide. However, a novel *mecA* homologue (with approximately 70% similarity to the *mecA* gene) that also confers methicillin resistance was identified in *S. aureus* isolates from dairy cattle and humans. This gene, previously denoted as *mecALGA251*, has been designated *mecC* (Laurent *et al*., 2012; Petersen *et al*., 2012). Additional genes, which are also found in susceptible isolates, can affect the methicillin resistance phenotype in *S. aureus*, resulting in heterogeneity of resistance and making detection of resistance difficult (de Lencastre and Tomasz, 1994). Some strains of *S. aureus* possess an alternative resistance mechanism, attributable to the hyper-production of the *S. aureus* β-lactamase enzyme, which inactivates the antibiotic agents by hydrolysing the β-lactam ring of penicillin and cephalosporin compounds (Brown *et al*., 2005). Vancomycin was the only antibiotic available for treating MRSA infections. However, vancomycin resistant MRSA strains, including some community acquired-MRSA strains, have increasingly been reported, thereby causing public health concern (Tenover and Goering, 2009).

2.7. Identification and typing of MRSA

MRSA can be identified using phenotypic (antimicrobial susceptibility testing) or genotypic methods. In general, the phenotypic methods are easier to perform, easier to interpret, cost-effective and widely available; however they are less discriminatory. The genotypic methods are more discriminatory, but are expensive and technically demanding (Mehndiratta and Bhalla, 2012).

Measurement of the Minimum Inhibitory Concentration (MIC) by using the dilution method has traditionally been the reference method for primary diagnosis of methicillin resistance. This method, performed on broth or agar, aims to measure the lowest concentration of the assayed antimicrobial agent (oxacillin) that, under defined test conditions, results in visible growth inhibition of the bacterium (Wiegand *et al*., 2008).

Another method commonly used for the detection of MRSA is the disk diffusion test. This test is performed by applying the bacterial inoculum onto the surface of Mueller-Hinton agar plates. Commercially-prepared, fixed-concentration, antibiotic-impregnated paper disks are placed on the inoculated agar surface. After appropriate incubation, the zones of growth inhibition around the antibiotic disks are recorded and resistance is evaluated according to the Clinical Laboratory Standards Institute (2009).

The results of the disk diffusion test are influenced by a range of factors, including the growth medium, the NaCl concentration and temperature. Commercial MIC tests and automated antimicrobial susceptibility testing systems are widely used for MRSA detection (Reller *et al.*, 2009). A commercial agglutination test based on the detection of PBP2a is also available for screening of methicillin resistance (Kluytmans *et al.*, 2002).

Definitive identification of MRSA is achieved upon detection of the *mecA* gene by Polymerase Chain Reaction (PCR). The definition of MRSA relying only on susceptibility tests can overestimate methicillin resistance; isolates that do not carry *mecA* can appear to be phenotypically resistant to methicillin (Lee *et al.*, 2004). In order to harmonize monitoring of MRSA in animals and foods in the EU, EFSA proposed that the MRSA definition should be made by the examination for the presence of *mecA* or the recently described *mecC* using multiplex PCR or, in isolates negative for these genes, by phenotypical tests for resistance to cefoxitin (EFSA, 2012).

The *S. aureus* population, including MRSA, consists of different clonal lineages, also called clonal complexes. Clones or strains of MRSA are differentiated using genetic typing tests, such as *spa* typing, Multi Locus Sequence Typing, Pulsed-Field Gel Electrophoresis, SCC*mec* typing and other tests (Catry *et al.,* 2010). These techniques are mainly useful for epidemiological studies and more than one method may be necessary to identify a given strain. At present, the best single method for determining the MRSA lineage is *spa* typing, which involves DNA sequencing of short nucleotide repeats in the polymorphic X region of the *S. aureus* protein A gene (*spa*). Different *spa* repeats are assigned an α-numerical code (r01, r02, etc.) and the repeat succession determines the *spa* type (e.g., t001, t002, etc.) by submission of the results to the RIDOM StaphType Database (www.spaserver.ridom.de) (EFSA, 2012).

In general, isolates with a similar succession of *spa* sequences belong to closely related sequence types, which can be assigned to the same CC. *spa* typing, can help distinguish isolates that are indistinguishable by Multi Locus Sequence Typing or Pulsed-Field Gel Electrophoresis (Enright *et al*., 2000).

Multi Locus Sequence Typing enables the assignment of sequence types to MRSA isolates. However, this technique is not suitable for routine surveillance of MRSA, because of the high cost involved and the requirement for high-throughput DNA sequencing (Harmsen *et al*., 2003). SCC*mec* typing classifies SCC*mec* elements into types and subtypes on the basis of their structural differences. Most of the used methods rely on PCR mapping of cassette elements, such as the *mec* complex, the *ccr* complex and the J region (Mehndiratta and Bhalla, 2012). Until recently, eight main types of SCC*mec* (type I to type VIII) along with many subtypes had been distinguished among MRSA isolates. Some of these types were more common than others. In the last few years, new types of SCC*mec* (IV to XI) were identified and additional subtypes and different variants of already existing ones were discovered (Turlej *et al*., 2011). SCC*mec* type XI carries the recently described *mecC* gene (EFSA, 2012).

Pulsed-Field Gel Electrophoresis of DNA fragments restricted with the *Sma*I enzyme is considered to be the gold standard for typing MRSA isolates (Moussa *et al*., 2011). However, it is important to follow uniform standard protocols to achieve types of SCC*mec* internationally comparable results. There is no consensus regarding the best method for typing MRSA strains. Application of any typing method requires careful assessment of its suitability and an individual approach depending upon the purpose of the study (Mehndiratta and Bhalla, 2012).

2.8. Transmission

MRSA can be transmitted from person to person, as well as from animals to humans and *vice-versa*. Transmission usually occurs by direct contact, often via the hands, with colonized or infected people or animals (Ferreira *et al*., 2011). MRSA carriage rates in the general human population usually vary between geographic regions from <1% to 5% (Leonard and Markey, 2008). In human hospitals, colonized and infected patients are the main reservoirs of

MRSA, which is typically spread from patient to patient via hands of staff (EFSA, 2009). Transmission routes of MRSA are probably similar to those of other *S. aureus* strains, but there are likely to be differences in efficiency of host colonization following exposure (Kawada *et al*., 2003). Whether a person becomes a persistent nasal carrier or not depends on various factors that are still poorly understood (Peacock *et al*., 2001).

Carrier animals serve as reservoirs of MRSA and they may transmit the pathogens to other animals or humans (Cuny *et al*., 2010). Some MRSA lineages tend to predominate in specific geographical regions and show host specificities; therefore, they tend to be associated with animals more than with humans and *vice-versa* (Sung *et al*., 2008). CC398 is the MRSA lineage most often associated with asymptomatic carriage in intensively reared food-producing animals, primarily in pigs, but also in cattle and perhaps in poultry (EFSA, 2009). Although this strain is mainly found to colonize animals without causing clinical diseases, in a few isolated cases, it caused clinical infections in animals. Colonization with livestock associated MRSA, especially CC398, has been reported frequently in people who work with such animals, i.e. farmers, veterinarians and their family members (Cuny *et al*., 2009).

MRSA isolates can be also shared between personnel and animals, including dogs, cats and horses, in veterinary hospitals and between companion animals and their owners in households (Weese *et al*., 2006). Indeed, in a few cases companion animals have been implicated as sources of human infections (Faires *et al*., 2009; Ferreira *et al*., 2011). Food may be contaminated with MRSA; handling or eating contaminated food is also a potential means of transmission. In hospital outbreaks, contaminated food can disseminate the organism to patients as well as to healthcare workers (EFSA, 2009).

Considering the increasing evidence of MRSA presence in food-producing animals, the concerns regarding MRSA contamination of food of animal origin, may be reasonable (Weese, 2010). However, and despite the reported increases in both the MRSA food contamination and in the incidence of human community acquired-MRSA infections, there are no reports of a direct link between them (EFSA, 2009). Further investigations are needed to determine the true role of food of animal origin in transmission of MRSA from animals to humans. Another troubling aspect of food-associated MRSA is that MRSA frequently contain staphylococcal enterotoxin genes, including genes encoding for enterotoxins most often associated with food poisoning (SEA, SEB, SEC, SED) (EFSA, 2008).

Different combinations of staphylococcal enterotoxin genes are associated with different MRSA clones, but the reasons of this association remain unclear (Ferry *et al*., 2006; Tristan *et al*., 2007). Clinically, food poisoning caused by MRSA should be no different than that caused by other *S. aureus* strains (Weese, 2010). To date, only a small staphylococcal food poisoning outbreak due to MRSA has been reported in Tennessee, USA. Three family members who consumed a meal of shredded pork barbeque and coleslaw salad became ill with nausea, vomiting and stomach cramps. The same strain of MRSA was isolated from the three family members, the coleslaw salad and a food handler at the convenience market where the food was purchased. This outbreak strain was most likely of human origin (Jones *et al*., 2002). Increased prevalence of MRSA amongst *S. aureus* strains could lead to a higher prevalence of toxinogenic *S. aureus* (EFSA, 2008).

2.9. General preventive and control measures

Good hygiene is an important general preventive and control measure, both in homes and human and animal healthcare environments, because environmental contamination with MRSA acts as a reservoir for infection. Known MRSA-positive animals should be nursed apart from other animals, with strict washing of the hands, gloves and gowns if in close contact. Recording the history of contact with human or animal MRSA, as well as an early culture of a wound non-responsive to first-line therapy allows for earlier recognition of MRSA and its appropriate management. Furthermore, when faced with repeated and inexplicable failure of human decolonization, clinicians can investigate nearby exposure to animals and birds that could be the reservoirs (Morgan, 2008).

Below, are some pre colonized specific measures cited from (Catry *et al*., 2010).

Specific measures for livestock animals

- ✓ Reduction of antimicrobial selective pressure in livestock by avoiding routine mass medication
- ✓ Prevention of transmission of MRSA between and within the farms with sanitary measures of control between herds and during transportation
- ✓ Identification and isolation of animals to minimize the risk for zoonotic infection
- ✓ Use of contact precautions such as protective outerwear, overalls, aprons or coats and boots or overshoes that are not worn elsewhere
- ✓ Protective outerwear and all the items handled during the treatment of MRSA-positive animals should be considered potentially contaminated
- ✓ Hands can be hygienically cleaned with alcohol gel pouches, which are essential but need to be used correctly.
- ✓ Proper cleaning and disinfection of contaminated environments, including transport vehicles. Special attention should be paid to dust in stables
- ✓ Animal owners should be informed about the risks and necessary precautions.

Reducing carriers on MRSA positive livestock farms

- ✓ Control and/or treatment of colonized and infected animals with or without anti microbial is necessary for the reduction of carriers.
- ✓ The affected animals need to be immediately separated from healthy animals. In extreme cases culling of infected animals is a further option. Milk of animals with mastitis by MRSA must be destroyed.
- ✓ The choice of antimicrobials should always be based on a susceptibility test, and all precautions should be taken that the drug reaches the infected site with appropriate concentrations.

3. MATERIALS AND METHODS

3.1. Study Area

The study was conducted in and around Wolaita Soddo town, Southern Ethiopia. Wolaita zone is located about 329 km south of Addis Ababa at an altitude of 700-2950 m above sea level with a total land area of 4537.5 square kilometers, is located between 6º4´N to 7º1´N and 37º4´E to 38º2´E (Figure 1). It has got an average annual rain fall ranging from 450-1446 mm. The rain fall over much of the areas is typically bimodal with the major rainy season extending from June-September and the short rainy season occurs from February-April. The mean annual maximum and minimum temperature of the area is 34.12 and 11.4°C, respectively. The area is divided into three ecological zones: *Kola (*lowland < 1500masl), wo*inadega* (mid altitude 2300 masl) and *Dega* (highland >2300masl). Most of the area lies within the mid altitude. The area has 51.7% cultivated land, 6.4% cultivable land, 11.9% grazing land and 30% others. The average crude population density is 342 Person/Km^2. The population of Wolaita zone is about 1,501,112 of which 11.7% lives in towns and the rest 88.3% live in rural areas (CSA, 2007). The predominant farming system is a mixed crop-livestock production. The livestock population of Wolaita zone is estimated to be 886,242 bovine, 117,274 ovine, 99,817 caprine, 41,603 equines and 442,428 poultry. The zone consists of twelve Woredas (Districts) (Wzfedd, 2005).

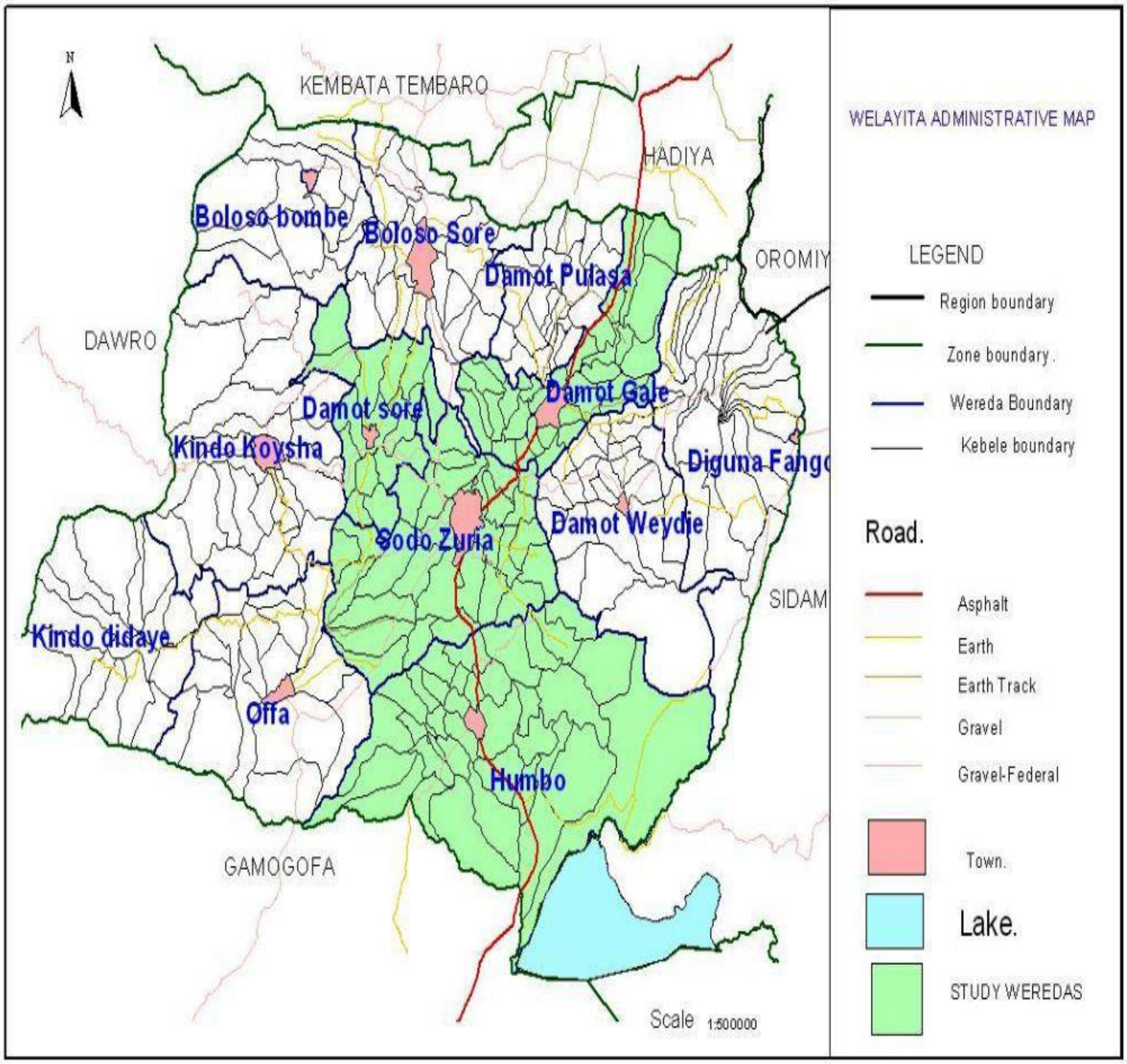

Figure 1**.** Map of Wolaita zone depicting the study areas (Wzfedd, 2005).

3.2. Study design

A cross sectional type of study was conducted from November 2013 to May 2014 to isolate and identify methicillin resistance *Staphylococcus aureus.*

3.3. Study population

The study animals were dairy cattle in the study areas. Four breeds of cattle (Cross breed, Holstein-Friesian, Jersey and Zebu) were included in the study.

3.4. Sample size

Purposive sampling technique was applied on all available dairy cows in the study area. A total of 257 dairy cows from 29 dairy farms in and around Wolaita Sodo namely (Arada, Wadu, Damot Gale, Damot Waja, Larena, Mehal and Merkato) were selected conveniently based on the availability of dairy cows and the willingness of the owners to cooperate in the farms.

3.5. Study methodology

3.5.1. Questionnaire survey

A semi-structured questionnaire was developed and pre-tested, and all information relating to the study objectives was recorded. Data were collected on potential risk factors for the occurrence of mastitis in dairy cows based on observation and by interviewing the farm owners. The animal level factors such as herd size, presence of teat lesion, teat blindness, body condition, parity, lactation stage, breed and age difference was recorded. The farm level factors such as housing types, farm hygiene, previous history of treatment of mastitis, barn floor status, type of milking method, use of towels, milking sequences and hygiene was recorded. Udder and milk abnormalities (injuries, blindness, tick infestation and indurations, swelling, milk clots, abnormal secretion, etc.) were also recorded.

Clinical inspection of the udder

Udders of the cows were examined by visual inspection and palpation for the presence of any lesion, pain, heat and swelling. In addition, milk from each quarter was withdrawn and checked for any change in color and consistency.

California mastitis test (CMT)

The California mastitis test was conducted to diagnose the presence of subclinical mastitis and it was carried out according to standard procedures. A squirt of milk from each quarter of the udder was placed in each of four shallow cups in the CMT paddle and an equal amount of the reagent was added. A gentle circular motion was applied in a horizontal plane. Positive samples show gel formation within a few seconds. 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 ranging from +1 to +3 (Appendix II). If at least one quarter was positive by the CMT then the cow was considered positive (Quinn *et al*. 1994).

3.6. Sampling method

Strict aseptic procedure was followed when collecting milk samples in order to prevent contamination with microorganisms present on the skin udder and teats, on the hands of samplers and on the barn environment. Teat ends were cleaned and disinfected with ethanol (70%) before sampling. Strict foremilk (first jets) were discharged to reduce the number of contamination of teat canal (Quinn *et al.,*. 1999). Sterile universal bottle with tight fitting cups were used. The universal bottle was labeled with permanent marker before sampling. To reduce contamination of teat ends during sample collection, the near teats were sampled first and then followed by the far ones (Quinn *et al.,*. 1999).

Milk samples were collected from each of clinically and sub clinically mastitic non-blind quarters of the selected cows for bacterial isolation. About 10ml of milk was aseptically collected from each mastitis positive quarter using sterile universal bottle. Then, samples were transported in an ice box to Wolaita Sodo University Microbiology laboratory for microbiological examination. If immediate inoculation is not convenient, samples were kept at 4°C until cultured for isolation.

3.7. Laboratory work

3.7.1. Culturing and Biochemical tests

A loop of milk sample was streaked on 5% sheep blood agar and the plates were incubated aerobically at 37 ^{O}C and examined after 24hrs of incubation for growth. The colonies were provisionally identified on the basis of staining reaction with Gram's stain, cellular morphology and hemolytic pattern on blood agar. The representative colonies were sub cultured on blood agar plate and on nutrient slants and incubated at 37 ^{O}C. The slants were preserved and maintained for characterizing the isolates. Catalase test, oxidative-fermentative test, slide coagulase test, growth on manitol salt agar and on purple agar base were performed and *S. aureus* were isolated for further test

3.7.2. Anti-microbial susceptibility testing

The *Staphylococcus aureus* isolates were tested for anti-microbial susceptibility by disc diffusion method (Quinn *et al.*, 1999). It is stated in the absence of methicilin the best alternative is to use cefoxitin for MRSA identification (National Comitte for Clinical Laboratory Standard, 2012). The following antibiotics were used for testing: cefoxtin (30µg), vancomycin (30µg), penicillin G (10µg), tetracycline (30µg), streptomycin (1ug), chloramphenicol (30µg), sulphamethoxazole trimethoprim (30µg) and amoxacilin (30µg) Oxoid Company (Hampshire, England). Colonies isolated from pure culture were transferred into a test tube of 5 ml pepton and suspension was made and incubated at 37^{o}c for 8 hr. The turbidity of the suspension was adjusted comparing with that of 0.5 McFarland standards. Muller-Hinton Agar plate was prepared and a sterile cotton swab was dipped into the suspension and swabbed on the surfaces of Muller-Hinton Agar plate. Then, the antibiotic discs were placed on the agar plate using sterile forceps and pressed gently to ensure the complete contact with the agar surface. The plates were read after 24hrs of incubation at 35^{O}C under aerobic condition. The isolates were classified in accordance with the guideline of the National Committee for Clinical Laboratory Standards (CLSI, 2006) as susceptible, intermediate or resistance for each antibiotic tested according to the manufacturer's instructions by measuring the zone of inhibition around the antibiotic disc. Intermediate results were considered resistant (Huber *et al*., 2011). Multiple antibiotic resistant (MAR) phenotypes were recorded for isolates showing resistance to three and more antibiotics (Rota *et al*., 1996).

3.8. Data management and statistical analysis

Processing of data was done by computer software. Data was coded and entered to MS Excel spreadsheet and checked for accuracy. After validation, it was transferred and processed using computer software SPSS version 20 for analysis.

Pearson's chi-square tests were used when appropriate to analyze the proportions of categorical data. Odd ratio and 95% CI were computed and the 95% confidence level was used and results were considered significant at $P < 0.05$

4. RESULTS

4.1. Occurrence

Of the total 257 lactating cows examined during the study period 105 (40.9%) had mastitis, of which 4.66% (12/257) and 36.18% (93/257) showed clinical and subclinical mastitis, respectively (Table 1).

Table 1. Occurrence of clinical and subclinical mastitis

Type of mastitis	N (257)	positive	percent
Clinical		12	4.66 %
Subclinical		93	36.18 %

Out of the 1010 quarters examined, the quarter level occurrence shows was 21% (212); from which 7.33 % (74) and 13.66 % (138) were found in front quarters and hind quarters, respectively. From the total examined quarters (1028), 18 quarters were found blind.

Table 2. Risk factors for quarter level mastitis

Factors	Categories	Positive quarters				
		Front quarter	Hind quarter	x^2	df	p- value
Breed	HF	35	53	10.311[a]	3	0.016
	Jersey	5	33			
	Cross	28	46			
	Zebu	6	6			
Lactation Period	Early	38	82	19.663[a]	2	0.000
	Mid	18	6			
	Late	18	50			
Floor system	Cement	26	27	6.228[a]	1	0.013
	Mud	48	111			

4.2. Risk factors associated with mastitis

The questionnaire survey and observation data result shows age, parity, milking hygiene and floor are among the potential risk factors which are associated with mastitis (Table 3)

Table 3. Result of univariate logistic regression of farm attribute risk factors with mastitis

Factors	Categories	Total No	No (%) Positives	OR	P-Value	CI
Age	Young (<4)	86	17(19.76%)	43.086	0.000	-
	Adult (4-7)	153	71(46.4%)			
	Old (>7)	18	17(94.44%)			
Breed	Zebu	18	9(50%)	1.082	0.779	-
	Cross	149	59(39.59%)			
	HF	60	26(43.33%)			
	Jersey	30	11(36.66%)			
Parity	Few (≤ 2)	160	34(21.25%)	82.013	0.000	-
	Moderate (3&4)	79	53(67.08%)			
	Many (>4)	18	18(100%)			
Lactation Period	Early	104	52(50%)	7.582	0.024	-
	Mid	41	11(26.82%)			
	Late	112	42(37.5%)			
Previous Mastitis Rx	Yes	85	46(54.11%)	9.177	0.002	.002- .003
	No	172	59(34.3%)			
Floor	Cement	107	31(28.97%)	10.91	0.001	.001- .001
	Mud	150	60(49.33%)			
Milking Hygiene	Good	137	43(31.38%)	10.94	0.001	.001- .001
	Poor	120	62(51.66%)			

4.3. Percentage of *S. aureus* isolates

From 105 animals, a total of 212 samples (12 clinical cows i.e. 32 teats and 93 CMT-positive subclinical cows i.e. 180 teats) were collected and cultured. The isolates of *Staphylococcus aureus* in Subclinical mastitis are significantly higher than clinical mastitis. *S. aureus* was isolated at a rate of 12.06% (31) and 3.11% (8) in subclinical and clinical mastitis infections, respectively (Table 4)

Table 4. *Staphylococcus aureus* isolates by the mastitis type (n= 257).

Bacteria isolated	Examined animals (N=257)	Type of mastitis	
		Subclinical (n=93)	Clinical (n=12)
Staphylococcus aureus	39(15.1%)	31(12.06%)	8(3.11%)

$X^2 = 5.058^a$, OR= 4.871, df=1, p-value = 0.025

4.4. Invitro antibiotic sensitivity test

Antibiotics of veterinary and human health relevance were considered in this study has demonstrated the existence of alarming levels of resistance of *S. aureus* to commonly used antimicrobial agents in the study farms. The present study has demonstrated the existence of alarming levels of resistance of *S. aureus* to commonly used antimicrobial agents in the study farms. 71.8% of the S. aureus were found to be resistance to cefoxtine which shows the prevalence of MRSA. The resistance profile of penicillin and tetracycline also 100% and 69.2% respectively (Table 5).

Table 5. Antibiotic-resistance profiles of *Staphylococcus aureus* isolated from mastitis milk (n = 39).

Antibiotics tested	Susceptible (%)	Intermediate (%)	Resistance (%)
SXT	56.4	-	43.6
Chloramphenicol	53.8	10.3	35.9
Tetracycline	20.5	10.3	69.2
Cefoxitin	28.2	-	71.8
Streptomycin	20.5	12.8	66.7
Vancomycin	28.2	15.4	56.4
Penicillin G	-	-	100
Amoxacillin	30.8	7.7	61.5

SXT- Sulphamethoxazole Trimethoprim

4.5. Association of MRSA with age of cows and previous treatment

Older cows were more often multi drug resistant *S. aureus* positive than cows of younger age. All of the animals under old age category group are multi drug resistant and all unidrug resistant isolates are from young age category (Table 6). MRSA was found to be associated with previous treatment history of the animal. It shows that 26 (66.6%) of the isolate had previous history of treatment (Table 7). All of the isolated MRSA were from adult and old age category and no susceptibility is recorded in old age (Figure 2).

Table 6. Association of drug resistance pattern of *S. aureus* and age of cows

Age of cows	Resistance pattern			
	One drug	two drug	Multi drug	
Young	3(75%)	1(25%)	0(0%)	
Adult	0(0%)	7(23.3%)	23(76.7%)	
Old	0(0%)	0(0%)	5(100%)	
Total	3	8	28	39

X^2 = 30.956[a] df = 4 p-value = 0.000

Table 7. Association of cefoxitin resistance pattern with previous treatment

Cefoxtin resistance	Previous mastitis treatment	
	Yes	No
Positive	26	2
Negative	5	6

X^2=.10.883[a] df = 1 p-value = 0.001 OR= 10.01

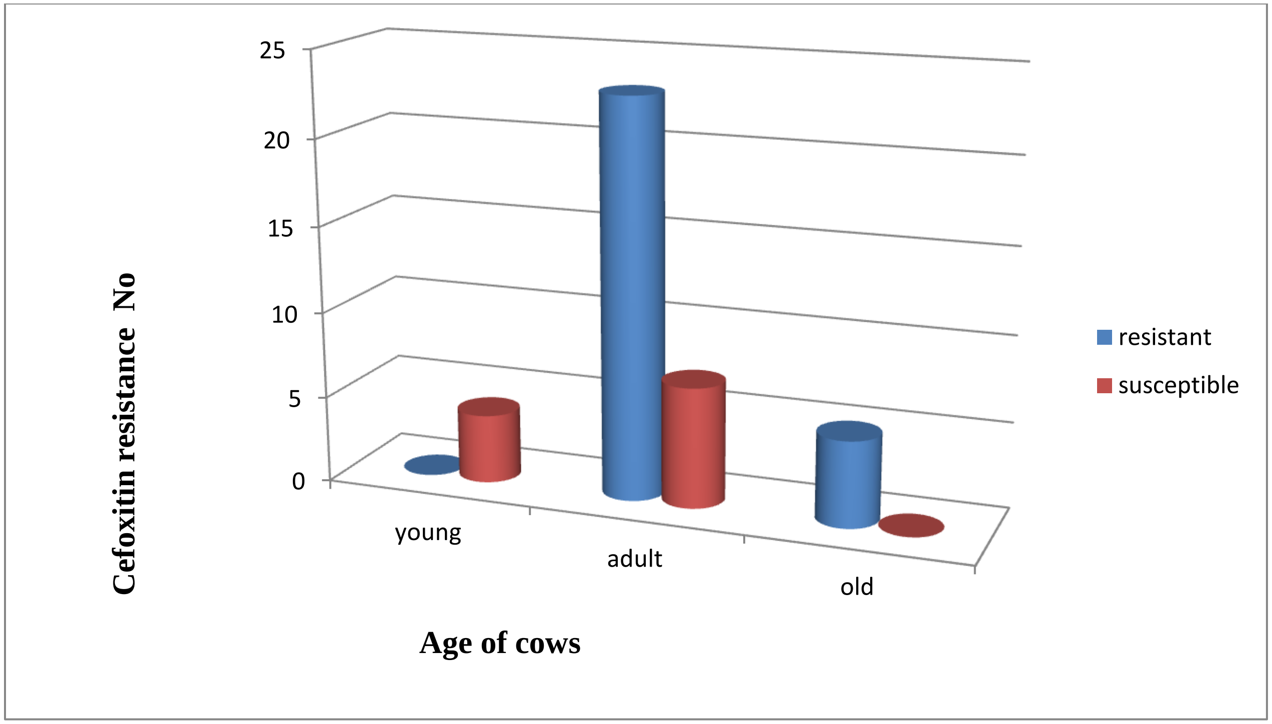

Figure 2. Association of number cefoxitin resistant *S. aureus* with age of cows

5. DISCUSSION

The study was conducted on bovine mastitis in Wolaita Sodo and its surroundings to determine the occurrence and major risk factors associated with MRSA in mastitis infection. The result revealed an overall occurrence of mastitis 40.9% and 21% was quarter level in the study area. This result agrees with the previous report by Kerro and Tareke, (2003) who recorded a finding of 40% in cows and 19 % in quarters in Southern Ethiopia. This report was also in agreement with the assertion by Radostits *et al*., (2000) that, in most countries and irrespective of the cause, the prevalence of mastitis is about 50% in cows and 25% in quarters. The infection rate in cows was similar to the findings of Abdelrahim *et al*., (1990), who found a prevalence of 45.8% in Sudan. The current finding of the study is higher than that of Bitew *et al*., (2010) and Mulugeta and Wassie, (2013), who reported an overall prevalence of 28.8% at Bahir Dar and 29.5% around Wolaita Sodo, respectively. On the other hand, it is comparably low when compared with the work of Sori *et al*., (2005) and Lakew *et al*., (2009) who reported a prevalence of 52.78% in and around Sebeta and 64.4% in Asella, respectively. Since mastitis is a complex disease and the difference in results could be due to difference in management system of the farm, the breeds of cattle considered and the geographical locations of the studies.

The occurrence of clinical mastitis recorded in the present study is 4.66 % and that of subclinical mastitis 36.18%. The result is in agreement with 36.7% sub clinical mastitis prevalence reported by Abera *et al*., (2013). In the current study the rate of sub-clinical mastitis (36.18%) was higher than that of the clinical mastitis (4.66 %) as (36.7 versus 10 %) was reported by Abera *et al*., (2013) in Adama town. In most reports including the present study, clinical mastitis is far lower than subclinical mastitis. This could be attributed to little attention given to subclinical mastitis, as the infected animal shows no obvious symptoms and secrets apparently normal milk and farmers, especially small holders, are not well informed about invisible loss from sub clinical mastitis. In Ethiopia, the subclinical forms of mastitis received little attention and efforts have been concentrated on the treatment of clinical cases (Almaw *et al*., 2008).

The increasing occurrence of mastitis with increasing age was in agreement with the findings by Kerro and Tareke, (2003) who found that, the risk of clinical and subclinical mastitis increase significantly with the advancing age of the cow. This might be due to the increased opportunity of infection with time and the prolonged duration of infection, especially in a herd without mastitis control program (Radostits *et al*., 2007).

The study showed that breed was not significantly influenced the occurrence of mastitis. Almaw *et al*., (2009) in Gondar town and its surroundings, Sori *et al*., (2005) in and around Sebeta showed that breed significantly influenced the occurrence of mastitis. Mastitis occurrence among breeds might reflect the differences in management rather than a true genetic difference (Radostits *et al*., 2007).

In this study mastitis occurrence was associated with parity of the animal evaluated and found statistically significant. The increased occurrence of mastitis with parity in the current study is comparable with the previous reports of Mulugeta and Wassie, (2013) in Wolaita Sodo town, Mekibib *et al*., (2010) in Holota town and Haftu *et al*., (2012) in northern Ethiopia. The association might be due to the increased opportunity of infection with time and the prolonged duration of infection, especially in a herd without mastitis control program (Radostits *et al*., 2007).

The observed higher occurrence of mastitis during early lactation as compared to mid and late lactation stages was significant. The finding of higher infection in cows in early lactation stage followed by late and medium lactation stages in the study concurs with previous reports of Mulugeta and Wassie, (2013); Biffa *et al*., (2005) and Tamirat, (2007). In cows most new infections occur during the early part of the dry period and in the first two months of lactation (Radostits *et al*., 2007). This may be due to an absence of dry period therapy and birth related influences. Radostits *et al*., (2000) suggested that, the mammary gland is more susceptible to new infection during the early and late dry period, which may be due to the absence of udder washing and teat dipping, which in turn may have increased the presence of potential pathogens on the skin of the teat. Moreover, during a dry period due to the low bactericidal and bacteriostatic qualities of milk, the pathogens can easily penetrate into the teat canal and multiply; this can be carried over into the post parturient period and ultimately develop into mastitis.

In agreement with Abera *et al*., (2013) in Adama town and Fekadu *et al*., (2005) in southern Ethiopia, the finding of a high prevalence of mastitis in farms with muddy floors when compared with concrete floor types shows the occurence of mastitis is significantly associated with the housing (bedding) type or condition of the farm. This is due to association with poor sanitation and cows which were maintained in dirty and muddy common barns with bedding materials that favor the proliferation and transmission of mastitis pathogens.

Occurrence of mastitis was significantly associated with milking hygienic practice. Cows at farms with poor milking hygiene standard are severely affected than those with good milking hygiene practices (Mulugeta and Wassie, 2013; Lakew *et al*., 2009; Sori *et al*., 2005). This might be due to absence of udder washing, milking of cows with common milkers' and using of common udder cloths, which could be vectors of spread especially for contagious mastitis (Radostitis *et al*., 1994).

The quarter level mastitis occurrence recorded in the current study is comparable with the finding of 19% in quarters by Kerro and Tareke, (2003). The hind quarters were affected higher than the front quarters. This is due to the fact that the hind quarters are highly predisposed for contamination with dirt. In addition to this, large amount of milk is produced from hind quarters and as a result the pressure on the teat canal forces the canals to be opened widely which allows entrance of microbes. The observation of blind quarters in this study might be an indication of a serious mastitis problem on the farms and of the absence of culling that should have served to remove a source of mammary pathogens for the cows.

Microbiological examination of milk from lactating dairy cows shows the presence for *S. aureus* in the present study. The present finding is in line with the findings of Abebe *et al*., (2013) who reported 15.5% at Addis Ababa. It also shows similarity with the findings observed in Egypt (17.2%) (Seedy *et al*., 2010). This is a low result when it is compared with that of Bedada and Hiko, (2011), Workineh *et al*., (2002) and kerro and Tareke, (2003) who reported 39.1%, 39.2% and 40.3% *S. aureus* isolates at Assela, Addis Ababa and Southern Ethiopia, respectively. The possible explanation for the variation might be that *S. aureus* is a contagious pathogen transmitted from one cow to another or individual by contact with animals during unhygienic milking procedures (Rowe, 1999).

The presence of isolates *S. aureu* in subclinical mastitis was significantly higher than that of clinical mastitis and this is due to *S. aureus* has adapted to survive in the udder and establish chronic and subclinical infections. From there it shed into the milk, which serves as a source of infection for healthy cows during the milking process (Radostitis *et al*., 1994).

The present study showed the resistance of *S. aureus* to penicillin G, cefoxtin, amoxicillin-clavulinic acid, tetracycline, streptomycin, chloramphenicol, sulphamethoxazole-trimethoprim and vancomycin. This is in accordance with the findings of Abebe *et al*., (2013) who reported resistance of *S. aureus* to penicillin (94%), tetracyclin (73.8%) around Addis Ababa. In line with the current finding, 94.4% and 58.3% resistance were also recorded around Adama (Abera *et al*., (2013) to penicillin and sulphamethoxazole trimethoprim respectively. Moreover, the present study has demonstrated the existence of alarming level of resistance of *S. aureus* to commonly used antimicrobials (pencillin G and tetracycline) in the study farms for dairy cows. The results were in accordance with reports from earlier studies in other countries (Jakee *et al*., 2008; Edward *et al*., 2002; Gentilini *et al*., 2002) suggesting a possible development of resistance from prolonged and indiscriminate usage of some antimicrobials.

Absolute resistance to Penicillin G must be of a great concern; since this antibiotic represents the main antibiotic group recommended for Staphylococcal mastitis treatment and regular use of antibiotics for the treatment of cows may result in the spread of resistant strains. Antibiotic resistance is carried on plasmids and transposons which can pass from one Staphylococcal species to another (Hulya *et al*., 2006). This is not surprising because penicillin G and tetracycline are the most commonly used antimicrobials for the treatment of infection or mastitis in veterinary practice in Ethiopia. The resistance of *S. aureus* to penicillin and cefoxtin may be attributed to the production of beta lactamase, an enzyme that inactivates penicillin and closely related antibiotics. It is believed that around 50% of mastitis causing *S. aureus* strains produces beta-lactamase (Green and Bradely, 2004).

Similar with the finding of Abebe *et al*., (2013) in Adama town, lower resistance was recorded for chloramphenicol and sulphamethoxazole trimethoprim. The reason why chloramphenicol and sulphamethoxazole-trimethoprim were less resistant might be that they are not frequently used in the study area in veterinary services, and perhaps in human medicine. Similar suggestion was given by Jaims *et al*., (2002) that the development of antimicrobial resistance is nearly always as a result of repeated therapeutic and/or indiscriminate use of them.

The resistance of *S. aureus* isolates to beta-lactams such as penicillin, cefoxitin, ampicillin and tetracycline was evident. High percentage of *S. aureus* was resistant to penicillin, cefoxtin, ampicillin, tetracycline and streptomycine. In agreement with the finding of by (Derese *et al*., (2012), the study showed cefoxitin resistant isolates was obtained from the milk in this study area. All cefoxitin resistant *S. aureus* were also resistanct to penicillin G. Out of the 28 cefoxitin resistant *S. aureus* isolates, 85.71% of were also resistant to amoxicillin. This is an indicator of MRSA (Daka *et al*., 2012).

MRSA strains have developed multidrug resistance worldwide with broad diversity in prevalence rate in different regions (Normanno *et al*., 2007). In the present observation, *S. aureus* isolates showed multidrug resistance primarily to penicillin G, cefoxtin, tetracycline and streptomycin because of resistance to β-lactams and frequent usage of the drugs. This is comparable with findings of (Sharma *et al*., 2011) who reported a 70% prevalence of multidrug resistant *S. aureus* from raw milk of dairy cattle in India.

The study shows most of the MRSA isolates were from previously treated animals. This showed a strong association between previous treatment and occurrence of MRSA. A causal relationship between the use of antimicrobial drugs and MRSA has been demonstrated in LA-MRSA and often co-resistant to several other antimicrobial agents (Tacconelli *et al*., 2008).

The association between MRSA and age of cows was significant. All of the isolated MRSA were from adult and old age category. The possible explanation for this may be as the age of cow's advances, the chance of having treatment with antibiotic increases.

Though vancomycin is the drug of choice for the treatment of MRSA, vancomycin resistance recorded in the study is in agreement with the finding of 57.1% by Daka *et al*., (2012) in Hawasa town. However, the result is a very high finding when it is compared with Tariku *et al*., (2011) who reported resistance of *S. aureus* to vancomycin as 3% in dairy farms in Jimma town. It has also been documented that MRSA isolates that are resistant to beta-lactam antibiotics may develop induced resistance to Vancomycin (Gündoğan *et al*., 2005).

.

6. CONCLUSION AND RECOMMENDATIONS

Different risk factors are associated with mastitis in the study area. Among these, miliking hygiene, floor type and age of the animal were critical. Mastitis caused by *S. aureus* is one of the major problems of dairy cows in milk production. It was found that the majority of the tested isolates were resistant to the various antimicrobial agents especially penicillin G, cefoxitin, tetracycline, streptomycin and amoxicillin. It was also observed that large proportions of the isolates were susceptible to sulphamethoxazole-trimethoprim and chloramphenicol. In the present observation, *S. aureus* isolates showed multidrug resistance primarily to penicillin G, cefoxtin, tetracycline and streptomycin because of resistance to β-lactams and frequent use. The findings suggest that multi drug resistances *S. aureus* are present in higher concentrations in dairy farms of the study area which indicated that MRSA spread within the farm. The possible explanations for the high record of multi drug resistant *S. aureus* in dairy farms may be due to the unrestrictive and uncontrolled use of antibiotics in dairy farms.

Based on the above conclusion the following points are forwarded

- *Staphylococcus aureus* mastitis control strategy should be initiated and promoted in the study area;
- Awareness should be created among dairy farm owners and dairy workers on the effect of MRSA;
- Veterinarians should be aware of the concerns regarding MRSA and should educate the owners/ handlers the risks;
- There should be regular antimicrobial sensitivity testing to select effective antibiotics and also help to reduce the problems of drug resistance development towards commonly used antibiotics;
- Risk factor controlling mechanism should be implemented
- Furthermore, impacts and dynamics of genetic antibiotic determinants should also be investigated using molecular methods.

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8. APPENDICES

Appendix I. Questionnaire format

Owners Name: ________________Address_______________

Date of sample Collection____________________

1. History of cow:

Breed_____

Body condition: Good ________bad________

Is your cow bought from or farm raised? Bought______ farm raised_______

Tick infestation: present _______absent_____

Teat Lesion: present_______ absent_____

Edema of udder and teat: present_________ absent__________

Blindness of teat canal: Blind __________ not blind__________

Herd size: 1-3__________above 3__________

Gross milk quality: watery____ blood tinged____ clots/flakes___ normal___

Sample collected from: HR___HL____FR___FL_____

CMT score: HR______HL______FR______FL_______

2. **Milking practice**

Do you wash your hand before and in between milking? yes____ no____

Do you wash your hand in between milking? yes_______ no______

Do you wash udder before milking? yes_____ no_____

Are separate towels used for each cow? yes____ no____

When do you milk cows with mastitis? first_______ last______ any time

3. **Housing**

Floor: concrete_________________ mud _________

Manure removal: daily________ weekly__________ monthly____ other (specify)______

General hygiene: Good ________bad_________

4. **Mastitis situation**

Previous mastitis problem in the farm? Yes_______ no_______

Can you differentiate healthy udder from diseased udder? Yes_______ no______

Do you treat mastitis case as they occur? yes_________ no_________

Person treating mastitis? Vet professional________ myself________

5. **Drug usage**

Mention any drug you know used for treatment of any disease

Name those used for mastitis treatment ___________________

Is there problem of cure after therapy? ___________________

Do you have knowledge about dry cow therapy? Yes________ no________

Do you practice dry cow therapy? yes_________________ no__________

Appendix II. Check list format used for recording data

Appendix table 1. Farm visit data collection format

Q	FR		FL		HR		HL	
M.T	Cli.	Subcli	Cli.	Subcli.	Cli.	Subcli.	Cli.	Subcli.

Q= Quarters, M.T= Mastitis type

Appendix table 2. CMT results and Interpretation

Score	Interpretation	Visible reaction
0	Negative	Milk fluid and normal
T	Trace	Slight precipitation
1	Weak positive	Distinct precipitation but no gel formation
2	Distinct positive	Mixture thickness with gel formation
3	strong positive	Viscosity greatly increased, strong gel i.e. cohesive with a convex surface

Source: (Quinn *et al.*, 1994)

Appendix III. Flow chart for isolation and identification of *S. aureus* from milk

Collection of milk sample

⇓

CMT

⇓

Culture CMT +ve milk sample

⇓

Inoculation on 5% sheep blood agar

⇓

Incubation at 37 ^{O}C for 24-48 hours

⇓

Observation of colony characteristics

⇓

Sub culturing in blood agar and nutrient agar

⇓

Incubation at 37 ^{O}C for 24 hours

⇓

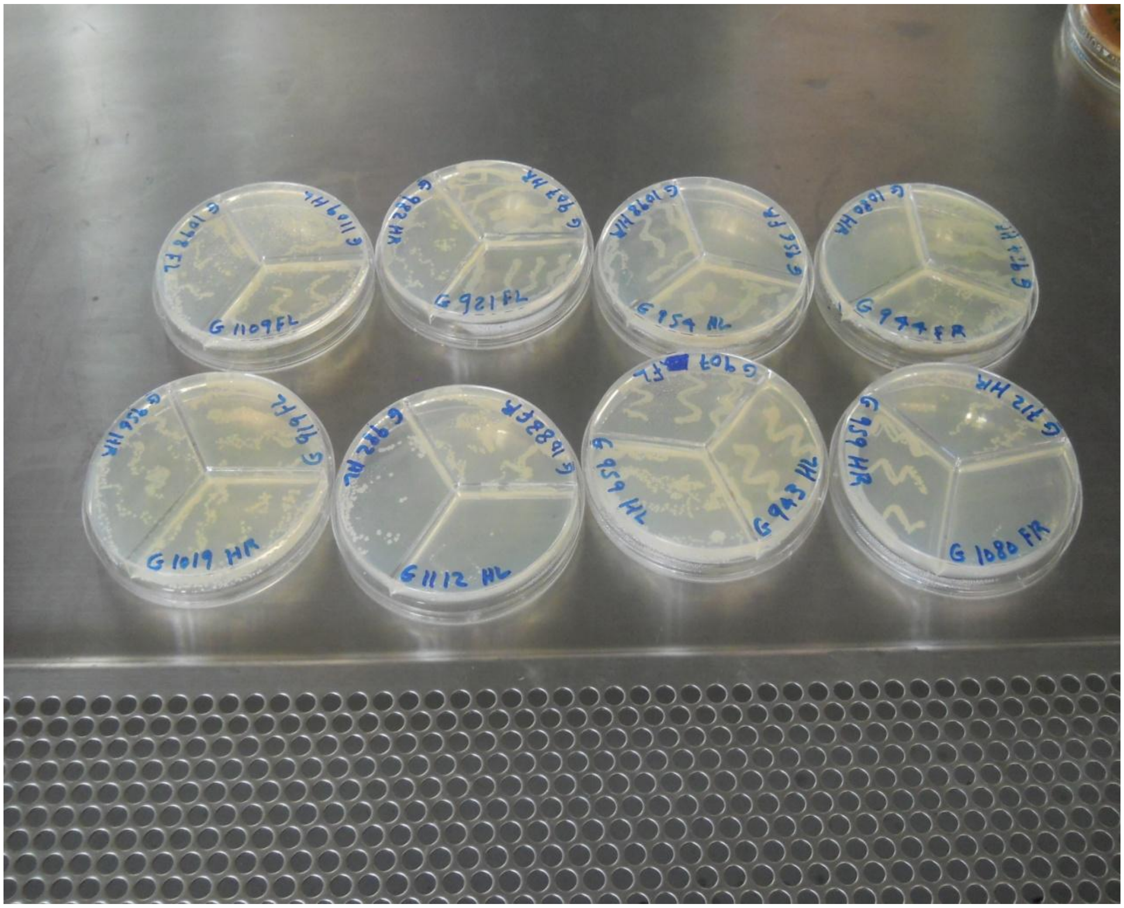
Primary identification

- ✓ Gram staining
- ✓ Catalase test
- ✓ O-F test

Secondary bio-chemical tests

- ✓ Growth on manitol salt agar
- ✓ Purple agar base
- ✓ Drug sensitivity

Appendix IV. Primary and secondary identification tests



Appendix figure 1. Colony growth on nutrient agar

Gram stain (carter, 1984)

Procedure:

- Make a thin smear or film.
- Allow the film to dry in air.
- Fix the film by passing through the Bunsen flame several times.
- Flood the slide with crystal violent for 30 to 60 seconds.

- Pour of the stain and wash the remaining stain with iodine solution.
- Wash off the iodine and shake the excess water from the slide.
- Decolorize with acetone alcohol.
- Counter stain with safranin for 30 to 60 seconds and wash with water.

KOH test (Quinn *et al.,* 1999)

A loop full of the culture is taken from a non – selective medium and mixed with an equal amount of 3% KOH on a clean microscopic slide. After through mixing the loop is lifted at intervals to see whether a gel is forming.

- Interpretation: If the bacterium is gram negative a viscous gel forms within 60 seconds while if no gel formed if the bacterium is gram positive.

Catalase test (Quinn *et al.,* 1999)

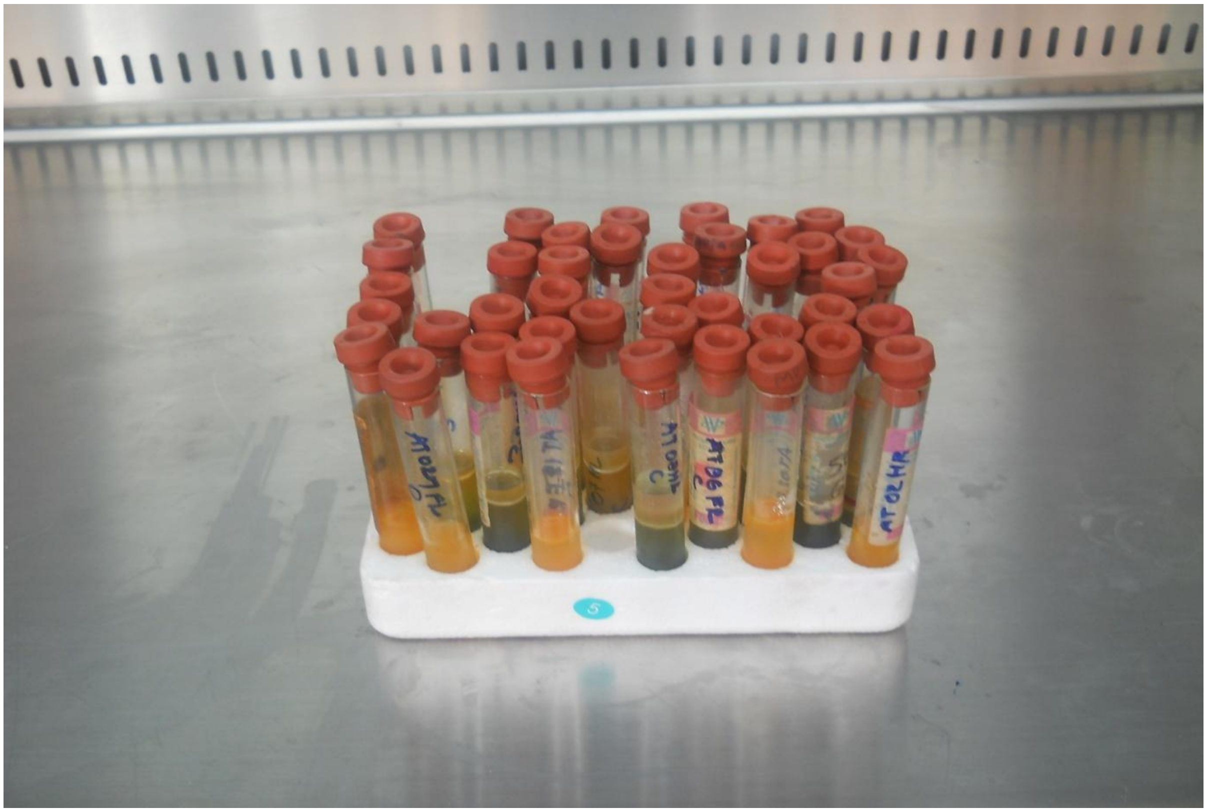
Principle: the breakdown of hydrogen peroxide into oxygen and water is mediated by the enzyme catalase.

Procedure: a loopful of the bacterial growth is taken from the top of the colonies avoiding the blood agar medium. The bacterial cells are placed on a clean microscope slide and a drop of 3% H2O2 is added. An effervescence of oxygen gas, within a few seconds, indicates a positive reaction.

O-F test (Oxidative and Fermentative test) (Quinn *et al.,* 1999)

Procedure: prepare O-F base medium and when the O-F base has cooled to 50^0c add 20ml of sterile glucose solution into 200ml of O-F base, for final concentration of 1% glucose and dispense in to tubes.

Two tubes of the O-F medium are heated in a breaker of boiling water immediately before use to drive off any dissolved oxygen and the tubes are then cooled rapidly under cold running water. Both tubes (sealed tube) to a depth of about 1cm and the tubes are incubated at 37^0c and examined in 24hrs and then daily for up to 14 days.



Appendix figure 2. OF test

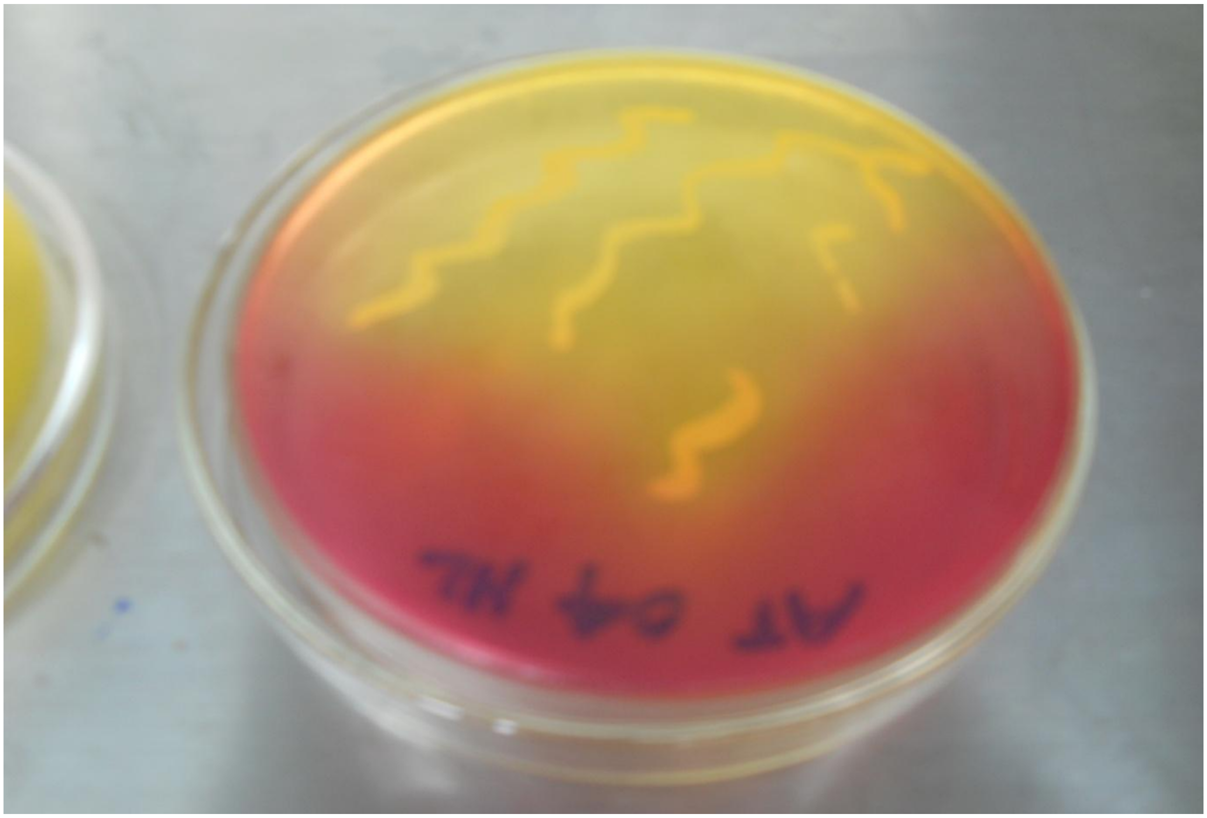
Appendix table 3. Differentiation of mastitis causing Staphylococcus species and micrococcus species.

test	*S.aureus*	CNS	micrococcus
Catalase	+	+	+
Coagulase	+	-	-
Hemolysis	+	-	-
Manitol (A)	+	-	-
Maltose (A)	+	v	-
Glucose (A)	+	+	-

+= Positive reaction, - = negative reaction, v = variable reaction, A= acid production

Mannitol Salt Agar (Quinn *et al.*, 1999)

The colonies that were confirmed by staining reaction, catalase test, OF test and coagulase test were streaked on mannitol salt agar plate and incubated at 37 ^{O}C and examined after 24-48 h for growth. The presence of growth and change of pH in the media (red to yellow color) regarded as presumptive identification for *S. aureus* (Quinn *et al.*, 2000).



Appendix figure 3. Growth on manitol salt agar

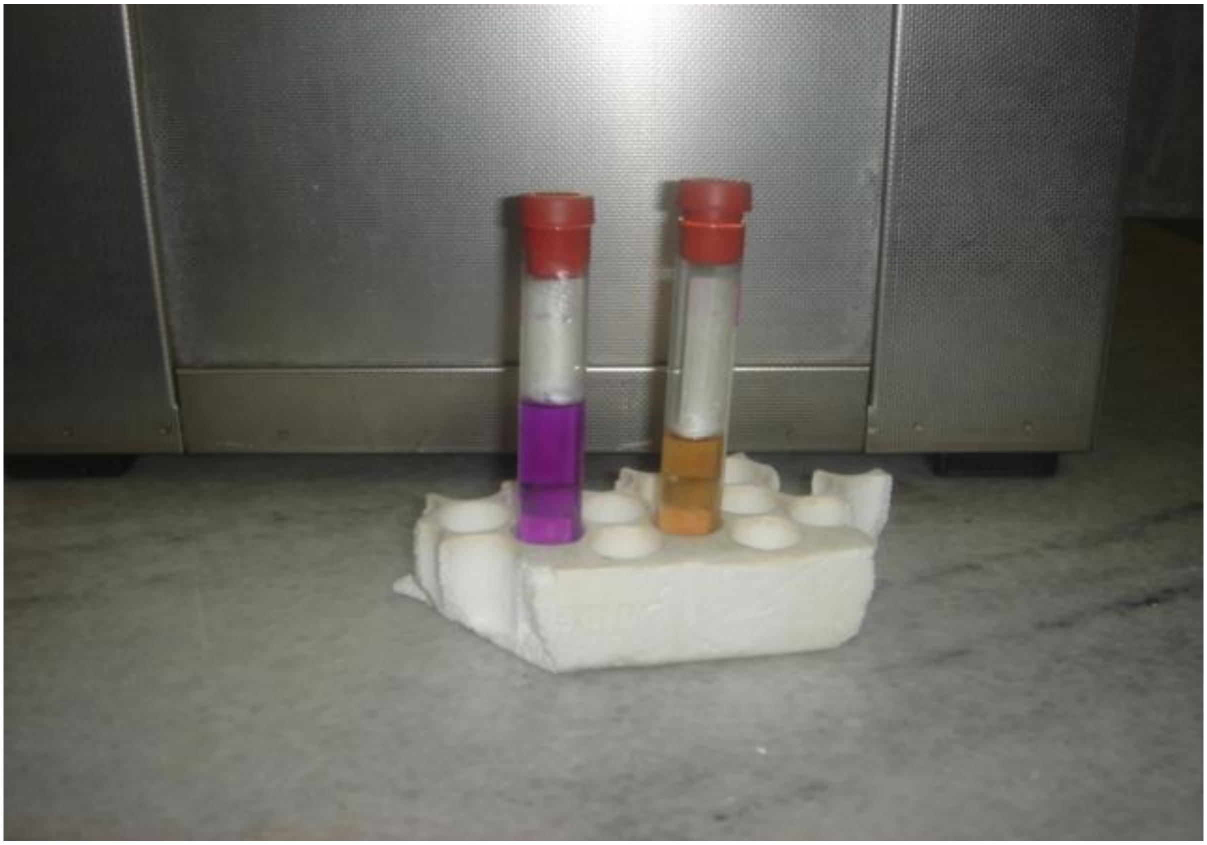
Purple base agar test

Principle: purple base agar contains maltose as a substrate and bromocresol purple indicator. If the bacterium ferments maltose, the indicator changes to yellow color.

Material used: sterilized purple agar base, sterile test tube, flask, digital balance, incubator, wire loop, autoclave, measuring cylinder, sterile water, aluminum foil.

Procedure

- ✓ A tube of purple base agar media was prepared.
- ✓ The tube was inoculated with a loopful of bacteria from blood agar.
- ✓ The test tubes were placed in incubator at 37^{o}c.



Appendix figure 4. A positives result on purple base agar. Right (*S. aureus*), left (negative control)

Appendix V. Antibiotic sensitivity test

- Preparation of inoculums

Inoculation of distinct colony in to 5ml nutrient b incubated at 35-37^{0}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) $Bacl_2$ $2H_20$ to 99.5ml of 1% (0.36N) H2SO4.

- Inoculation to Muller- Hinton Agar

Muller-Hinton Agar cooled to 50 ^{o}c and poured into a sterile petri dish on level surface to a depth of 4mm. this is equivalent to 60ml in 15cm plate and 25 ml in 10cm plate for slow growing bacteria 5 % defiberinated whole blood could be added. 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 others.

- Disc application

Within 15 minutes (time used to dry the inoculums) after the plates are inoculated, antibiotic 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 35^{0}c but not 37^{0}c.

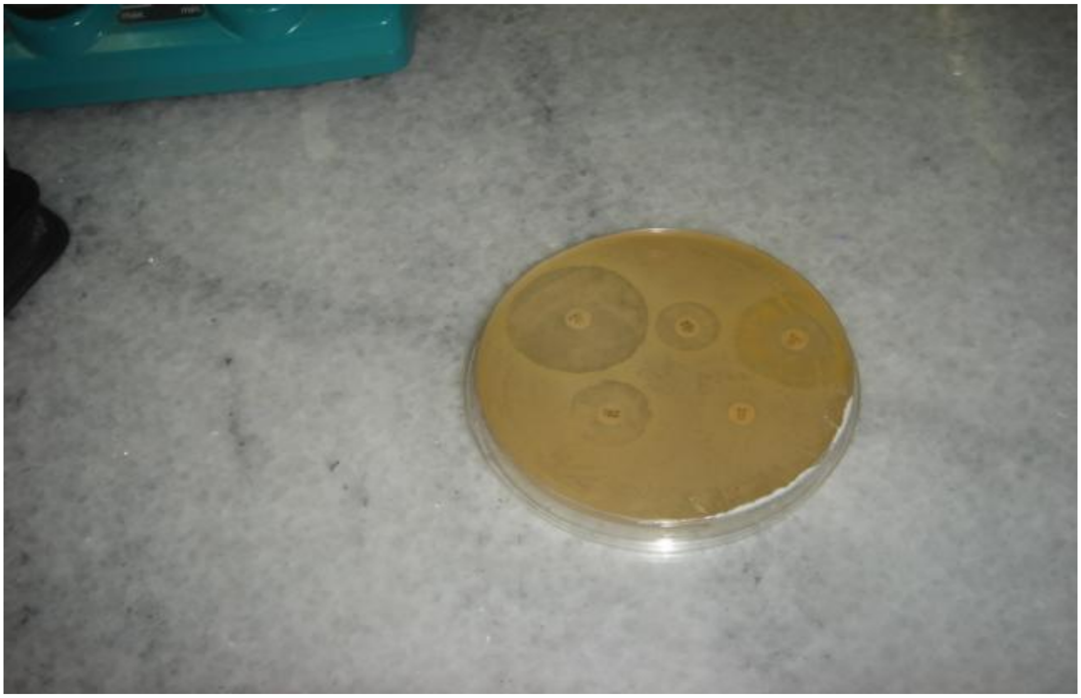
- Interpretation

Zone of inhibition is measured in millimeters using a transparent ruler on the under surface of the Petri dish. For measuring purpose the end point is taken as complete inhibition of growth as determined by naked eye. The result is interpreted according to the table presented below.

Appendix table 4. Zone of inhibition interpretation chart for Antimicrobials

		In mm (milliliter)		
Antimicrobial agents	disc potency	resistance	intermediate	susceptible
Streptomycin	10 µ	≤ 11	12-14	≥15
Tetracycline	30µ	≤ 14	15-18	≥19
Penicillin G	10 U	≤ 20	21-28	≥ 29
Vancomycin	30 µ	≤ 9	10-11	≥12
Cefoxitin	30 µ	≥ 22	-	≥ 21
Chloramphenicol	10 µ	≤ 15	16-20	≥ 21
Amoxacilin	30 µ	≤ 19	-	≥ 20
SXT	30 µ	≤ 10	11-15	≥ 16

Source (NCCL, 2011)



Appendix figure 5. Antibiotic sensitivity test

Appendix VI. Medias used for bacteriological examination

1. Nutrient agar (Oxoid, England)

Composition (g/l): Lab-Lemco powder1.0; Yeast extract 2.0 ; Peptone 5.0; Sodium chloride 5.0; Agar15 ; pH: 7.4 ± 0.2

Directions: Suspend 28 g in 1 liter of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121^0C for 15 minutes.

2. Mannitol salt agar (Oxoid, England)

Composition (g/l): Lab-Lemco' powder 1.0 Peptone 10.0 Mannitol 10.0 Sodium chloride 75.0 Phenol red 0.025 Agar 15.0 pH: 7.5 ± 0.

Directions: Suspend 111g in 1 liter of distilled water and bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes.

3. Gram's reagent

- Crystal violet
- Gram's iodine (mordant)
- Ethanol 95%
- Counter – stain (carbon fuchsine / safranin)

4. O-F Basal Medium (himedium,india)

Composition g/l: Sodium Chloride5; Casein enzymatic hydrolysate 2g ;Agar 2g ; Dipotassium phosphate 0.3g ; Bromothymol blue 0.08g ;

Direction : Dissolve 9.4 g in 1000ml distilled water. Gently heat to dissolve the medium completely. Dispense in 1000ml quantities and sterilized by autoclaving at 121^0 C for 15 minutes. To first 100 ml of sterile medium aseptically add 10 ml of sterile 10 % Dextrose solution. To second 100 ml add 10 ml sterile 10 % lactose solution. To third 100 ml add 10 ml sterile 10 % Sacoharose solution. Mix and dispense in 5ml amounts in sterile tubes in duplicate for aerobic and anaerobic fermentation solution.

5. Edwards medium, modified (oxoid, England)

Composition (g/l): "Lab-Lemco" powder 10; peptone; aesculin1.0; sodium chloride 5.0; crystal violet 0.0013; thallus sulphate 0.33; agar 15.

Direction: suspend 41g in 1 liter of distilled water. Bring to the boil to dissolve completely Sterilize by autoclaving at 115 for 20 minutes. Cool to 50^0c, add 5-7 % of sterile bovine or sheep blood. Mix well and pour plate

6. . Muller Hinton Agar (Oxoid, England)

Compodition (g/l): beef extracts 2; acid hydrolysate of casein 17.5; starch1.5; agar 17.

Direction: suspend 38 g of the powder in 1 liter of distilled water. Mix thoroughly, heat with frequent agitation and boil for 1 minute to completely dissolve the powder Autoclave at 121^oc for 15 minutes. Cool tubed medium in slanted position for

7. Blood agar (oxoid, England)

Composition (g/l) hear muscle, infusion from (solids) 2.0; pancreatic digest of casein13.0; Yeast extract 5.0;sodium chloride 5.0;agar 15.0

Direction: suspend 40 g of powder in 1 liter of distilled water. Mix thoroughly and heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Autoclave at 121^0c for 15 minutes. Cool the base to 45 - 50^0 c and add 5 % sterile defiberinated sheep blood.