

THESIS REF. No. \_\_\_\_\_

**ISOLATION, IDENTIFICATION AND DRUG RESISTANCE PATTERNS OF  
METHICILIN RESISTANT *STAPHYLOCOCCUS AUREUS* FROM MASTITIC  
COW'S MILK FROM SELECTED DAIRY FARMS IN AND AROUND  
KOMBOLCHA**

**M.Sc. THESIS**



**BY**

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PUBLIC HEALTH**

**JUNE, 2015**

**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 Science in Veterinary Microbiology**

**By**

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**June, 2015**

**Bishoftu, Ethiopia**

**Addis Ababa University**

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As members of the Examining Board of the final M.Sc. open defense, we certify that we have read and evaluated the Thesis prepared by: **Mesfin Negash** Entitled: **Isolation, Identification and drug resistance patterns of methicilin resistant *staphylococcus aureus* from mastitic cow's milk from selected dairy farms in and around kombolcha** and recommend that it be accepted as fulfilling the thesis requirement for the degree of **Masters of science in Veterinary Microbiology.**

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## STATEMENT OF THE AUTHOR

First, I declare that this thesis is my *genuine* 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 M.Sc. degree at Addis Ababa University, College of Veterinary Medicine and Agriculture 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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## **ACKNOWLEDGEMENTS**

I would like to express my deepest gratitude to my academic major advisor Dr. Asmelash Tassew (DVM, M.Sc., Assist. Prof.) for his overall guidance and advices, sharing his knowledge from the beginning to the end of my thesis paper.

My great thanks go to my co-advisors Dr. Biniam Tadesse (DVM, M.Sc.) and Dr. Biruk Tesfaye (DVM, M.Sc., Assist. Prof.), for their interesting supports and advices on this paper.

I am highly indebted to kombolcha Regional Veterinary Laboratory members, especially to W/ro Zewudie Abebe and W/ro Mestawet Dinku for their kind reception, preparing equipments and materials for the work and good willing to use their laboratory.

Much of the acknowledgement goes to my staff members of Artuma Fursi Werdeda agricultural development office, especially to Mr. Dagne Abebe for their encouragement and positive energy shown throughout my M.Sc. study.

Finally I would like to sincerely thank to my family for their consistent love, moral and financial support.

## LIST OF ABBREVIATIONS

|          |  |
|----------|--|
| Agr      | Accessory gene regulator   |
| AVMA     | American Veterinarian Medical Association                          |
| CA-MRSA  | Community Associated MRSA  |
| CLSI     | Clinical Laboratory Standard Institute                             |
| CMT      | California Mastitis Test   |
| CNS      | Coagulase Negative Staphylococci                                   |
| df       | degree of freedom  |
| DNA      | Deoxyribo Nucleic Acid   |
| EFSA     | European Food Safety Authority                                     |
| HA-MRSA  | Human Associated MRSA  |
| LA-MRSA  | Livestock Associated MRSA  |
| Masl     | Meter above sea level  |
| MIC      | Minimum Inhibitory Concentration                                   |
| MRSA     | Methicilin Resistant <i>Staphylococcus aureus</i>                  |
| MSSA     | Methicillin susceptible <i>Staphylococcus aureus</i>               |
| 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                   |
| PVL        | Panton-Valentine leukocidin                 |
| SCC        | Staphylococcus Chromosome Cassette          |
| SE         | Staphylococcus Enterotoxin                  |
| <i>Spa</i> | <i>Staphylococcus aureus</i> protein A gene |
| SSI        | Surgical site infection                     |
| SSTI       | Skin and soft tissue infection              |
| TSB        | Treptone soya broth                         |

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## **Isolation, Identification and Drug Resistance Patterns of Methicilin Resistant *S. Aureus* from Mastitic Cow's Milk from selected dairy farms in and around Kombolcha**

### **ABSTRACT**

A cross sectional study was conducted from January 2015 to May 2015 in and around kombolcha town, Eastern Amhara, to isolate and identify methicilin resistant *S. aureus* and their resistance to different antimicrobials and also identify risk factors associated with the occurrence of dairy cow mastitis. A total of 150 dairy cows were included during the study period. A total of 600 quarters were examined to detect clinical and subclinical mastitis by physical examinations of udder and milk and California mastitis Test, respectively. The prevalence of mastitis was 56%. Out of this, 10% and 46% were clinical and subclinical respectively. Among potential risk factors considered from the farm attributes, age, milking hygiene, parity, and floor system had significant ( $p < 0.05$ ) effect on the prevalence of mastitis. However, breed, previous treatment and lactation period have not been a significant potential risk factor on the occurrence of mastitis. *Staphylococcus aureus* was isolated at a rate of 11 (73.3%) and 29 (42%) in clinical and subclinical mastitis, respectively. The result showed that the occurrence of *Staphylococcus aureus* in clinical mastitis were found to be significantly higher than subclinical mastitis ( $p = 0.028$ ). The present study showed that *S. aureus* was resistant to penicillin G (100%), Amoxicillin (100%), cefoxitin (42.7%) and tetracycline (77.4%) however all the isolates were found to be totally (100%) susceptible to the gentamycin disc. Out of the resistance *S. aureus* isolates, 24 (45.3%) were found to be multidrug resistant against 4 antibiotic discs primarily to penicillin G, ampicillin, cefoxitin, and erythromycin. 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. In conclusion, among different risk factors, age of the animal, milking hygiene, parity and floor type were critical for the occurrence of mastitis. Mastitis caused by *S. aureus* is one of the major problems of dairy cows in milk production in the study area.

**Key words:** antimicrobials, mastitic cows, MRSA, prevalence resistance, risk factors.

## 1. INTRODUCTION

Mastitis, inflammation of the mammary gland, is a costly production disease affecting the dairy cattle industry worldwide. Mastitis may be caused by either infectious or non-infectious agents. Infectious mastitis results from bacterial, mycotic or algal pathogens. Non-infectious mastitis is the result of injury, chilling, bruising or rough or improper milking. But it is almost due to the effect of infection by bacteria or mycotic pathogens. A total of about 140 microbial species, subspecies and serovars have been isolated from the bovine mammary gland. Pathogens causing mastitis in cattle are divided into major pathogens (those that cause clinical mastitis) and minor pathogens (those that normally cause subclinical mastitis and less frequently clinical mastitis (Firaol *et al.*, 2013).

*Staphylococcus aureus* is a versatile pathogen of humans and animals that causes a wide variety of the disease (Abebe *et al.*, 2013). The bacterium is a colonizer of the skin and mucosae from which it can invade multiple organs. In livestock *Staphylococcus aureus* is an important cause of mastitis, skin and soft tissue infections (SSTI) and to lesser extent infections of the locomotory system. Surgical site infections (SSI) in which *S. aureus* is isolated have been increasingly reported in small companion animals and horses (Normanno *et al.*, 2007).

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  $\beta$ -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  $\beta$ -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).

Milk-secreting tissues and various ducts throughout the udder can be damaged by bacterial toxins, and sometimes permanent damage to the udder occurs. Severe acute cases can be fatal, but even in cows that recover there may be consequences for the rest of the lactation and subsequent lactations. The illness is in most respects a very complex disease, affected by a variety of factors: it can be present in a herd subclinically, where few, if any, symptoms are present in most cows. Practices such as close attention to milking hygiene, the culling of chronically-infected cows, good housing management and effective dairy cattle nutrition to promote good cow health are essential in helping to control herd mastitis levels (Ricardo, 2011).

There are also some studies on MRSA in some part of Ethiopia such as in Hawasa (Daka *et al.*, 2012), in Adama (Abera *et al.*, 2013), in and around Addis Ababa (Abebe *et al.*, 2013). Concerning the study area south wollo, in and around kombolcha, MRSA is not studied. Knowledge of MRSA is necessary to make decisions regarding antibiotic treatment and prerequisite for establishing control strategies in the area.

Therefore, this study was designed with the following objectives:

- Isolation and identification of methicillin resistant *Staphylococcus aureus* (MRSA) from mastitic cow's milk.
- Determining the occurrence of bovine mastitis in the selected dairy cows in the study area
- Assessment of potential risk factors associated with the disease
- Determining the antimicrobial resistance patterns of the isolates to the selected antibiotic discs

# 1. LITERATURE REVIEW

## 2.1 MRSA reservoirs and host specificity

MRSA clones have originated in at least three separate settings: human hospitals, human carriers outside of hospitals (community), and livestock animals. This has occurred at different times and in different geographical locations. The subsequent spread of these MRSA clones over time has led to some hospital isolates that are now found in the community and vice versa, and livestock strains that are increasingly found in humans. However, the reservoirs, distribution patterns and strategies for dealing with MRSA in each MRSA strains are different (EFSA, 2009).

### 2.1.1. Hospital associated MRSA (HA-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*mec* 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*<sup>-</sup> or a mixed *agr*<sup>+</sup> and *agr*<sup>-</sup> genotype. Though these genotypes could explain the relative nonpathogenic nature of HA-MRSA toward immunocompetent hosts, it is possible that an *agr*<sup>-</sup> or a mixed *agr*<sup>+</sup> and *agr*<sup>-</sup> genotype could be beneficial for HA-MRSA survival in the healthcare setting; *agr*<sup>-</sup> 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).

### *2.1.2. Community associated MRSA (CA-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).

### *2.1.3. 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”). 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 (Morgan, 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).

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. 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 (*SeI*, designated as SE/J to SE/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*mecIVa*. 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 (Feßler *et al.*, 2010).

### **2.3. 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).

### **2.4. 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  $\alpha$ -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).

## **2.5. Antibiotic resistance**

The term ‘MRSA’ is used to describe strains of *S. aureus* resistant to semi-synthetic, penicillinase resistant,  $\beta$ -lactams such as methicillin, oxacillin or cloxacillin. MRSA strains are resistant to all cephalosporins, cephems and other  $\beta$ -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).

$\beta$ -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  $\beta$ -lactamase protein, which cleaves the  $\beta$ -lactam ring structure, confers resistance to penicillin, but not to semi-synthetic penicillins. In MRSA, resistance to all  $\beta$ -lactam antibiotics, including the semi-synthetic penicillins, is conferred by the penicillin-binding protein PBP2' (or PBP2a) that has a very low affinity for  $\beta$ -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*  $\beta$ -lactamase enzyme, which inactivates the antibiotic agents by hydrolysing the  $\beta$ -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).

## **2.6. 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 sodium chloride 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 European Union, 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  $\alpha$ -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](http://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 *SmaI* 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).

## 2.7. 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).

## **2.8. 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).

*Specific measures include*

- ✓ 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 (Catry *et al.*, 2010).

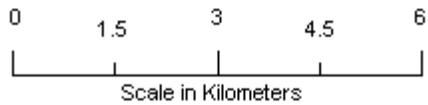
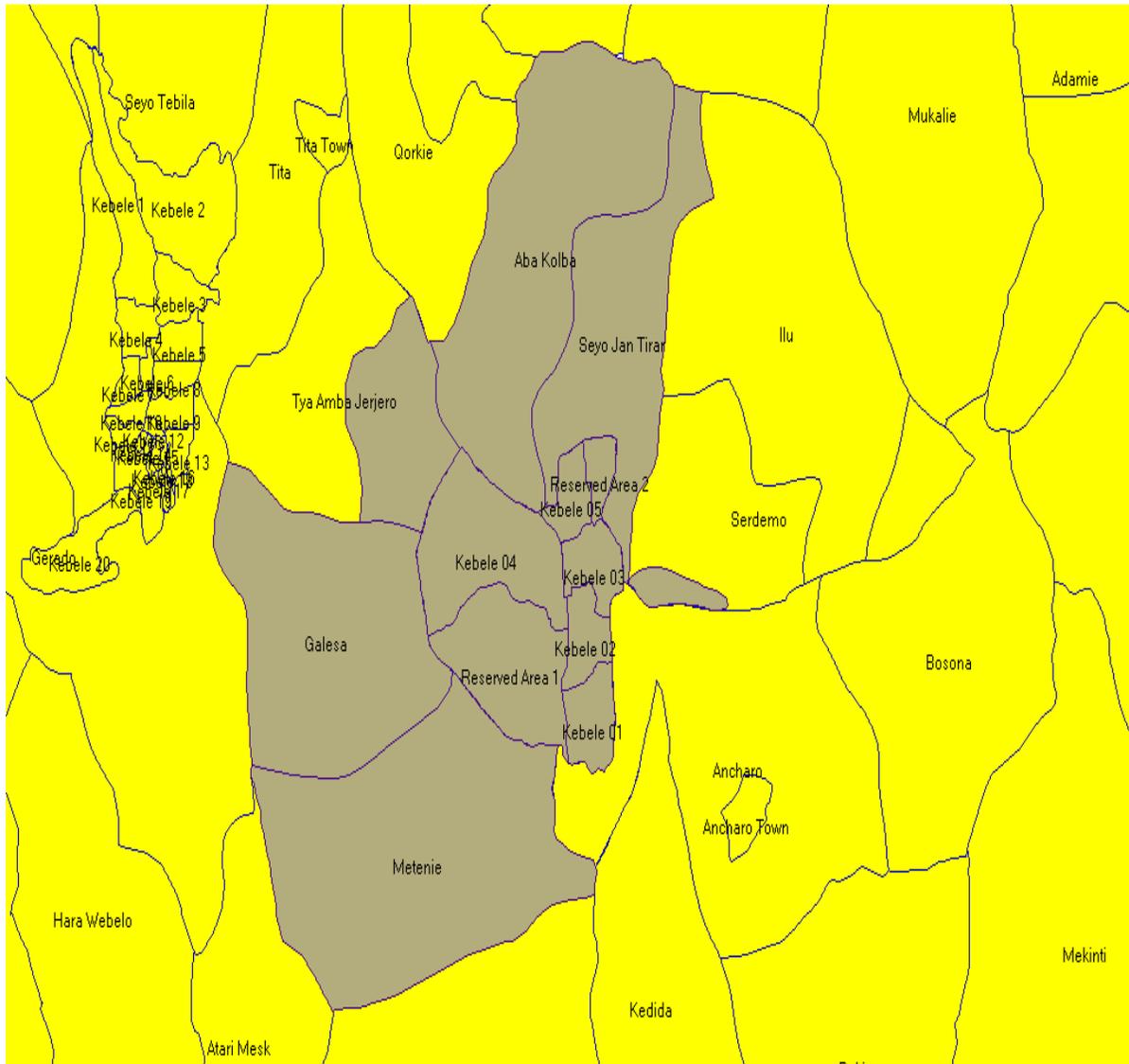
### **3. MATERIALS AND METHODS**

#### **3.1. Study Area**

The study was conducted at Kombolcha Town, which is found in South Wollo Administrative Zone of Amhara National Regional State in North Eastern Ethiopia. The study area is located 376km north of Addis Ababa with  $11^{\circ}08'49''$  N latitude and  $039^{\circ}07'37''$  E longitude at an altitude of 1840 meter above sea level (masl).

Based on the central statistical agency 2005, kombolcha town has an estimated total human population of 68,766 of which 36,102 are males and 32,664 females. The woreda has an estimated area of 8.66 square kilometers, which gives kombolcha a density of 7940.60 people per square kilometer. Animal population includes 22,455 cattle, 9,537 sheep and 15,310 goats.

The kombolcha town experiences a bi-modal rainfall, the short rainy season occurs usually from March 15 to May. The minimum and maximum mean annual rainfall in and around kombolcha ranges from 750 to 900mm. The average minimum and maximum daily temperature during short and long rains are  $23.9^{\circ}\text{C}$  and  $11.7^{\circ}\text{C}$  respectively and the relative humidity of the area varies from 23.9% to 79%.(kombolcha town agricultural Office)



**Source:** south wollo administrative zone

### 3.2. Study design

A cross sectional type of study was conducted from January 2015 to May 2015 to isolate and identify methicillin resistance *Staphylococcus aureus*.

### **3.3. Study population**

The study animals were dairy cattle in and around kombolcha town. Four breeds of cattle (Holstein-Friesian, Jersey, Cross breed and local (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 150 dairy cows from 18 selected dairy farms in and around kombolcha were selected conveniently based on the availability of dairy cows.

### **3.5. Study methodology**

#### *3.5.1. Questionnaire survey*

A questionnaire was developed 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 or handlers. 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, swelling, milk clots, abnormal secretion, etc.) were also recorded.

#### *3.5.2. Clinical examination of the udder*

Udders of the selected dairy 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.

#### *3.5.3. California mastitis test (CMT)*

The California mastitis test (CMT) 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 CMT paddle and an equal amount of the reagent, approximately 4-5 drop 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 and record as 0 (zero), or positive if there was gel formation ranging from trace(T) to +3 (Appendix II). If at least one quarter was positive by the CMT then the cow was considered as 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 test tubes with tight fitting cups were used. The test tube 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 test tube. Then, samples were transported in an ice box to Kombolcha Regional 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 full of milk sample was streaked on 5% sheep blood agar and the plates were incubated aerobically at 37 °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 nutrient agar and incubated at 37 °c for 24 hrs. The isolated colonies from nutrient agar were exposed to Catalase test, slide or tube coagulase. coagulase positive colonies were grown on manitol salt agar and *S. aureus* were isolated for Anti-microbial susceptibility testing (Quinn *et al.*, 1994).

### 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). Drugs like cefoxitin (30µg), gentamycin (10µg), erythromycin (15µg), streptomycin (10µg), tetracycline (30µg) ampicillin (10µg) and penicillin G (10 units) were used for Anti-microbial susceptibility testing. Approximately 3-5 Colonies isolated from pure culture were transferred into a test tube of 1ml tryptone soya broth (TSB) and incubated at 37°C for 24hrs. The turbidity of the suspension was adjusted by adding 9ml saline water. 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 disc dispenser. The plates were read after 24hrs of incubation at 37°C under aerobic condition. However cefoxitin discs were incubated at 35°C for 24 hrs. 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).

### 3.8. Data management and analysis

Collected 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. Odds ratio and 95% CI were computed and the results were considered significant at  $P < 0.05$

### *Quality assurance*

Confidence in the reliability of test results is increased by following adequate quality assurance procedures, and the routine use of control strains, *S. aureus* ATCC25923 as a positive control and *E. coli* ATCC-25922 as a negative control (for culture on MSA) were taken as an important part of quality control for culture and antimicrobial susceptibility test. Thus, quality control microorganisms yielded values within the established ranges, indicating that the test was performed in a satisfactory manner.

## **4. RESULTS**

### **4.1. Prevalence of mastitis**

From the total 150 lactating cows examined during the study period 84 (56%) cows had mastitis, of which 15 (10%) and 69 (46%) showed clinical and subclinical mastitis respectively (Table 1).

Table 1. Clinical and subclinical mastitis by animal (n=150)

| Type of mastitis | Positive | Percent (%) |
|------------------|----------|-------------|
| Clinical         | 15       | 10%         |
| Sub clinical     | 69       | 46%         |

Out of the 600 quarters examined, the quarter level occurrence of mastitis showed 202 (33.7%); from which 70 (11.7%) were found in front quarters and 132 (22%) in hind quarters. From the total examined quarters (600), 6 quarters were found blind (table 2 and 3).

Table 2. Mastitis positive quarter by breed

| Breed       | mastitis positive quarter |      | Total |
|-------------|---------------------------|------|-------|
|             | Front                     | Hind |       |
| HF          | 49                        | 85   | 134   |
| Jersey      | 3                         | 14   | 17    |
| Cross       | 6                         | 15   | 21    |
| Local(zebu) | 12                        | 18   | 30    |
| Total       | 70                        | 132  | 202   |

$X^2=3.110^a$ , OR=3.367, P-value=.375

Table 3. Mastitis positive quarter by hygiene

| Hygiene | mastitis positive quarter |      | Total |
|---------|---------------------------|------|-------|
|         | Front                     | Hind |       |
| Good    | 46                        | 0    | 46    |
| Poor    | 24                        | 132  | 156   |
| Total   | 70                        | 132  | 202   |

$X^2=112.321^a$ , OR=126.742, P-value=.000

#### 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 4)

Table 4. Result of risk factors with mastitis

| Risk factors     | Catagories        | N   | Positive (%) | OR     | P-value |
|------------------|-------------------|-----|--------------|--------|---------|
| Age              | Young (<5)        | 40  | 6 (15)       | 61.288 | .000    |
|                  | Adult (5-8)       | 83  | 51 (61.4)    |        |         |
|                  | Old (>8)          | 27  | 27 (100)     |        |         |
| Breed            | HF                | 86  | 52 (60.5)    | 2.290  | .513    |
|                  | Jersey            | 21  | 12 (57.1)    |        |         |
|                  | Cross             | 22  | 10 (45.5)    |        |         |
|                  | Local (zebu)      | 21  | 13 (61.9)    |        |         |
| Parity           | Few (2)           | 46  | 14 (30.4)    | 17.817 | .000    |
|                  | Moderate (3-4)    | 57  | 38 (66.7)    |        |         |
|                  | Many (>4)         | 47  | 32 (68.1)    |        |         |
| Lactation period | Early             | 79  | 43 (54.4)    | .167   | .683    |
|                  | Late              | 71  | 41 (57.7)    |        |         |
| Previous Rx      | Yes               | 124 | 72 (58.1)    | 1.229  | .266    |
|                  | No                | 26  | 12 (46.2)    |        |         |
| Floor system     | Concrete (cement) | 74  | 32 (43.2)    | 9.753  | .002    |
|                  | Mud (soil)        | 76  | 52 (68.4)    |        |         |
| Milking Hygiene  | Good              | 51  | 20 (39.2)    | 8.847  | .003    |
|                  | Poor              | 99  | 64 (64.6)    |        |         |

#### 4.3. Percentage of *S. aureus* isolates

Milk samples collected from 84 mastitis positive cows (15 clinical cows and 69 CMT-positive subclinical cows) or from 202 teats were cultured on blood agar. coagulase positive *Staphylococcus species* were isolated from 40 cows (53quarters) from 54 cows (67 quarters) colonies cultured on manitol salt agar. *Staphylococcus aureus* was isolated at a rate of 11 (73.3%) and 29 (42%) in clinical and subclinical mastitis, respectively. The isolates of *Staphylococcus aureus* in clinical mastitis are found to be significantly higher than subclinical mastitis (Table 5).

Table 5. *Staphylococcus aureus* isolates by mastitis type (n= 150).

| Bacteria isolated | Total Examined<br>Animals(150) | Types of mastitis  |                 |
|-------------------|--------------------------------|--------------------|-----------------|
|                   |                                | Subclinical (n=69) | Clinical (n=15) |
| <i>S. aureus</i>  | 40                             | 29 (42%)           | 11 (73.3%)      |

$X^2= 4.841^a$ , OR=4.968, df= 1, p-value= 0.028

#### 4.4. Antimicrobial sensitivity test

Antibiotics of veterinary and human health relevance were considered in this study has demonstrated the existence of alarming levels of resistance of *Staphylococcus aureus* to commonly used antimicrobial agents in the study area (farms). The present study has demonstrated the existence of alarming levels of resistance of *Staphylococcus aureus* to commonly used antimicrobial agents in the study area (farms). From 53 *Staphylococcus aureus* grown on muller-hinton agar 25 (47.2%) *Staphylococcus aureus* were found to be resistant to cefoxitin which shows the prevalence of MRSA. The resistance pattern of both penicillin G, and amoxicillin and tetracycline is 53 (100%) and 41 (77.4%) respectively (Table 6).

Table 6. Summary of result of antimicrobial sensitivity test (n = 40 cows with 53 quarters).

| Antibiotics tested | Susceptible (%) | Intermediate (%) | Resistance (%) |
|--------------------|-----------------|------------------|----------------|
| Tetracycline       | 4 (7.5)         | 8 (15.1)         | 41 (77.4)      |
| Cefoxitin          | 28 (52.8)       | -                | 25 (47.2)      |
| Streptomycin       | 39 (73.6)       | 14 (26.4)        | -              |
| Penicillin G       | -               | -                | 53 (100)       |
| Erythromycin       | 43 (81.1)       | 10 (18.9)        | -              |
| Gentamycin         | 53 (100)        | -                | -              |
| Amoxicillin        | -               | -                | 53 (100)       |

Out of the resistance *S. aureus* isolates, 24 (45.3%) were found to be multidrug resistance against 4 antibiotic discs primarily to penicillin G, ampicillin, cefoxitin, and erythromycin.

#### 4.5. Cefoxitin resistance pattern with previous treatment

From a total of 18 (45%) cows which shows resistance to cefoxitin 14 (77.8%) cows were previously treated. From a total of 22 (55%) cows which shows susceptibility to cefoxitin 17 (77.3%) cows were cefoxitin susceptible without previous treatment. Therefore MRSA was found to be associated with previous treatment history of the animal with cefoxitin resistance.

Table 7. Cefoxitin resistance pattern with previous treatment

| cefoxitin resistance Pattern   | previous mastitis treatment |            | Total     |
|--------------------------------|-----------------------------|------------|-----------|
|                                | Yes                         | No         |           |
| susceptible pattern per animal | 5 (22.7%)                   | 17 (77.3)  | 22 (55%)  |
| Resistance pattern per animal  | 14 (77.8)                   | 4 (22.2%)  | 18 (45%)  |
| Total                          | 21 (52.5%)                  | 19 (47.5%) | 40 (100%) |

$X^2 = 12.031^a$ , OR=12.7000, df= 1, p-value=.001

#### 4.6. Cefoxitin resistance pattern with age of the animal

From all age groups of animal old age cows were more often cefoxitin resistant *S. aureus* positive than cows of adult and young aged cows. 8 (80%) of the cows under old age category group are cefoxitin resistant (Table 6).

Table 8 Cefoxitin resistance pattern with age of the animal

|                                | Young      | Adult      | Old       | Total     |
|--------------------------------|------------|------------|-----------|-----------|
| Susceptible pattern per animal | 8 (47.1%)  | 13 (72.2%) | 1 (20%)   | 22 (55%)  |
| Resistance pattern per animal  | 9 (52.9%)  | 5 (27.8)   | 4 (80%)   | 18 (45%)  |
| Total                          | 17 (42.5%) | 18 (45%)   | 5 (12.5%) | 40 (100%) |

$X^2= 13.051^a$ , OR=15.231, df= 2, p-value=.001

## 5. DISCUSSION

The study was conducted on small and middle sized dairy farms in and around kombocha town to determine the prevalence of mastitis and asses the major risk factors associated with MRSA. The result revealed that an overall prevalence of mastitis 84(56%) and 202 (33.7%) at quarter level. This result agrees with the previous researches conducted by 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. This report is also in consistent with the findings of Radostits *et al.* (2000) that, in most countries and irrespective of the cause, the prevalence of mastitis was about 50% at cow level and 25% at quarter level.

The current finding of the study is comparably higher than the work of Kerro and Tareke (2003) who recorded 40% in cows and 19 % in quarters in Southern Ethiopia. The infection rate in cows was also higher than that of Bitew *et al.* (2010) and Mulugeta and Wassie (2013), who reported

an overall prevalence of 28.8% in Bahir Dar and 29.5% around Wolaita Sodo, respectively. Since mastitis is a complex disease and the difference in results could be due to difference in management system of the farm, difference in drug usage and the geographical locations of the studies.

The prevalence of clinical and subclinical mastitis in the present study is 10% and 46% respectively. In the current study the rate of sub-clinical mastitis (46%) is higher than that of the clinical mastitis (10%) which is in agreement with the reports of Abera *et al.* (2013) (36.7% subclinical and 10 % clinical mastitis) in Adama town. The result of subclinical mastitis (46%) is higher than that of Abera *et al.* (2013) with a prevalence of 36.7%. 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 clinical symptoms and secretes apparently normal milk. Therefore farmers 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 occurrence of mastitis in front quarter and hind quarter was 11.7% and 22% respectively. Mastitis positive cow at quarter level with breed difference was insignificant. This shows that breed difference is not as risk factors for the occurrence of mastitis in hind quarters than front quarters rather than the teat is exposed to contamination. However milking hygienic practice at quarter level mastitis is significant in the hind quarter and has contribution for the occurrence of the infection.

Therefore hind quarter is more affected than the front quarter and the current result is comparable with the finding of 19% in quarters by Kerro and Tareke (2003). 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.

The association between mastitis and age of cows was significant. All of the isolated MRSA were from old age category. Old cows in this study were more susceptible to mastitis infection than young and adult cows. 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).

In this study breed is not significantly influenced on the occurrence of mastitis. In contrast to this study breed has significant influence on the occurrence of mastitis, Almaw *et al.* (2009) in Gondar town and its surroundings, Sori *et al.* (2005) in and around Sebeta. Mastitis occurrence among breeds might reflect the differences in management rather than a true genetic difference (Radostits *et al.*, 2007).

The occurrence of mastitis has significant association with parity of the animal. The increased occurrence of mastitis with parity in the current study is in agreement with the previous reports of 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 occurrence of mastitis during early lactation as compared to late lactation stages was insignificant. This study was in contrast to previous reports of Mulugeta and Wassie (2013); Biffa *et al.* (2005) and Tamirat (2007) showed that higher infection in cows is occurred at early stage of lactation followed by late and medium stages of lactation. 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 low dry cow therapy, 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.

The current study shows the occurrence of mastitis is insignificantly associated with previously treated animals. In contrast to Tacconelli *et al.* (2008), 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. The opposite finding of this research may be due to the absence of proper udder washing and teat dipping, increased presence of potential pathogens on the skin of the teat which can easily penetrate into the teat canal and multiply and antibiotic resistance ability of most pathogens.

In this study floor system had a significant influence on the occurrence of 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 (soil) floors when compared with concrete(cement) floor types. 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.

The prevalence 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 millers using a common udder cloth and milking of subclinical mastitic cows first, which could be vectors of spread especially for contagious mastitis (Radostitis *et al.*, 1994).

In the present study Microbiological examination of milk from lactating dairy cows shows the presence of *S. aureus*. *Staphylococcus aureus* isolated at a rate of 73.3% and 42% in clinical and

subclinical mastitis infections, respectively is in line with that of Bedada and Hiko (2011), Workineh *et al.* (2002) and kerro and Tareke (2003) who reported 39.1%, *S. aureus* isolates at Assela, 39.2% at Addis Ababa and 40.3% at Southern Ethiopia respectively. This is higher result when it is compared with the findings of Abebe *et al.* (2013) who reported 15.5% at Addis Ababa. 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 isolates of *S. aureus* in clinical mastitis are significantly higher than subclinical mastitis. This result 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, amoxicillin, tetracycline, and cefoxitin. This is in accordance with the findings of Abebe *et al.* (2013) who reported resistance of *S. aureus* to penicillin (94%), tetracycline (73.8%) around Addis Ababa. The current finding is in line with the finding of Abera *et al.* (2013) around Adama who recorded 94.4% to penicillin. The present study has demonstrated the existence of alarming level of resistance of *S. aureus* to commonly used antimicrobials (pencillin G and tetracycline including amoxicillin) in the study farms. The results were in accordance with reports from earlier studies in other countries (Jakee *et al.*, 2008; Edward *et al.*, 2002 and Gentilini *et al.*, 2002) suggesting a possible development of resistance from prolonged and indiscriminate usage of some antimicrobials.

Resistance to Penicillin G is a great concern; since this antibiotic represents the main antibiotic groups recommended for Staphylococcal mastitic infection. The 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). The resistance of *S. aureus* to penicillin and cefoxitin may be attributed to the production of beta lactamase enzyme that inactivates penicillin and closely related antibiotics. Around 50% of mastitis causing *S. aureus* strains produces beta-lactamase (Green and Bradely,

2004). 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 G, cefoxitin, amoxaclin and tetracycline was evident. High percentage of *S. aureus* was resistant to penicillin G, amoxacilin, tetracycline, cefoxitin, and to some extent streptomycin. In the current study area cefoxitin resistant isolates was isolated from milk and is in line with the findings of Derese *et al.* (2012). All cefoxitin resistant *S. aureus* were also resistant to penicillin G (100%). Out of the 25 cefoxitin resistant *S. aureus* isolates, 100% were also resistant to amoxicillin. This is an indicator of MRSA (Daka *et al.*, 2012). In this study, gentamycin is the drug of choice for treating MRSA followed by erythromycin.

## 6. CONCLUSION AND RECOMMENDATIONS

The overall prevalence of mastitis in the study area is 56% (10% clinical and 46% subclinical) in cows and 33.7% at quarter level and different risk factors are found to be associated with mastitis. Among these different risk factors, age of the animal, milking hygiene, parity and floor type were critical for the occurrence of mastitis. Mastitis caused by *S. aureus* is one of the major problems of dairy cows in milk production in the study area. It was found that the majority of the tested isolates were resistant to the various antimicrobial agents especially penicillin G, ceftiofur, tetracycline, amoxicillin and to some extent streptomycin. It was also observed that most proportions of the isolates were susceptible to gentamycin followed by erythromycin. In this study all ceftiofur resistant *S. aureus* were also resistant to penicillin G. The possible explanations for the high record of most 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 concluding remarks the following recommendation are forwarded

- Mechanisms to control the risk factors associated to the disease should be implemented;
- There should be regular antimicrobial sensitivity testing to select effective antibiotics;
- Veterinarians should reduce repeated use of drugs to minimize drug resistance development;
- Awareness should be created among veterinarians, dairy farm owners and dairy workers on the effect of MRSA;

- *Staphylococcus aureus* mastitis control strategy should be implemented and promoted in the study area;
- Use of gentamycin is better to treat mastitic infected cows in the study area;
- Application of molecular techniques is mandatory to identify drug resistant gene of MRSA.

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## 8. APPENDICES

### Appendix A: Questionnaire format

#### 1. General information

Dairy Farm name: \_\_\_\_\_ Owners Name: \_\_\_\_\_  
 Address \_\_\_\_\_ Date of sample Collection \_\_\_\_\_

#### 2. History of cow

Breed \_\_\_\_\_ Body condition: Good \_\_\_\_\_ medium \_\_\_\_\_ 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: 1-5 \_\_\_\_\_ above 5 \_\_\_\_\_

Gross milk quality: watery \_\_\_\_\_ blood tinged \_\_\_\_\_ clots/flakes \_\_\_\_\_ normal \_\_\_\_\_

Sample collected from: HR \_\_\_ HL \_\_\_ FR \_\_\_ FL \_\_\_ CMT score: HR \_\_\_ HL \_\_\_ FR \_\_\_ FL \_\_\_

### 3. 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 \_\_\_\_\_

### 4. Housing

Floor: concrete \_\_\_\_\_ soil \_\_\_\_\_ sloppy \_\_\_\_\_ leveled \_\_\_\_\_

Roof: metal sheet \_\_\_\_\_ grass \_\_\_\_\_ Wall: concrete \_\_\_\_\_ mud \_\_\_\_\_ other \_\_\_\_\_

Manure removal: daily \_\_\_\_\_ weekly \_\_\_\_\_ monthly \_\_\_\_\_ other (specify) \_\_\_\_\_

General hygiene: Good \_\_\_\_\_ bad \_\_\_\_\_

### 5. Mastitis situation

Can you differentiate healthy udder from diseased udder? Yes \_\_\_\_\_ no \_\_\_\_\_

Is there previous mastitis problem in the farm? Yes \_\_\_\_\_ no \_\_\_\_\_

Do you treat mastitis case as they occur? yes \_\_\_\_\_ no \_\_\_\_\_

What Person treating mastitis? Vet professional \_\_\_\_\_ myself \_\_\_\_\_

Is there any alternative measure? \_\_\_\_\_ what are they? \_\_\_\_\_

## 6. Drug usage

Do you name drugs used for mastitis treatment \_\_\_\_\_

Is there problem of cure after therapy? yes \_\_\_\_\_ no \_\_\_\_\_

Do you have knowledge about dry cow therapy? Yes \_\_\_\_\_ no \_\_\_\_\_

Do you practice dry cow therapy? yes \_\_\_\_\_ no \_\_\_\_\_

Appendix II. Format used for recording data in the field

Appendix table 1. Farm visit data collection format

| Clinical Mastitis |    |    |    | Sub-Clinical Mastitis |    |    |    |
|-------------------|----|----|----|-----------------------|----|----|----|
| RF                | RR | LF | LR | RF                    | RR | LF | LR |
|                   |    |    |    |                       |    |    |    |
|                   |    |    |    |                       |    |    |    |

RF= Right Front, RR= Right Rare, LF= Left Front, LR= Left Rare

Appendix table 2. CMT results and Interpretation

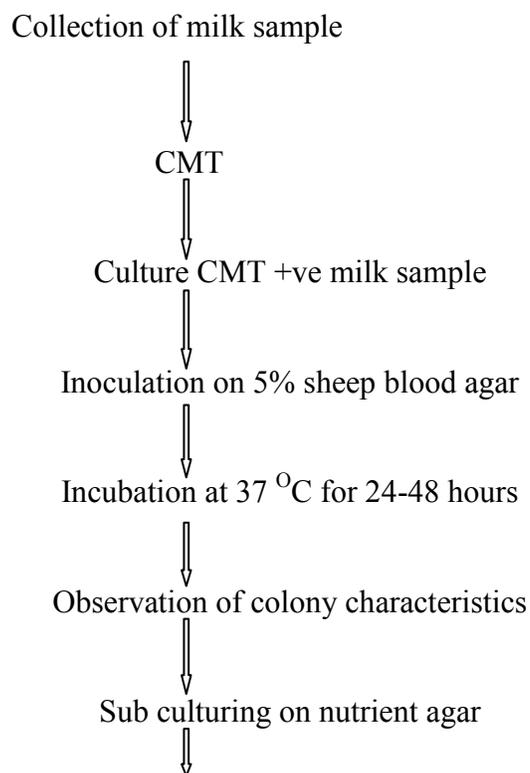
| Score | Interpretation | Visible reaction      |
|-------|----------------|-----------------------|
| 0     | Negative       | Milk fluid and normal |

|    |                   |  |
|----|-------------------|--|
| 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.<br>cohesive with a convex surface |

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

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



Incubation at 37 °C for 24 hours



Primary identification

- ✓ Gram staining
- ✓ Catalase test
- ✓ Coagulase test

Secondary identification

- ✓ Growth on manitol salt agar
- ✓ 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 violet 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.

### **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% H<sub>2</sub>O<sub>2</sub> is added. An effervescence of oxygen gas, within a few seconds, indicates a positive reaction.



Appendix figure 2. Slide Catalase test

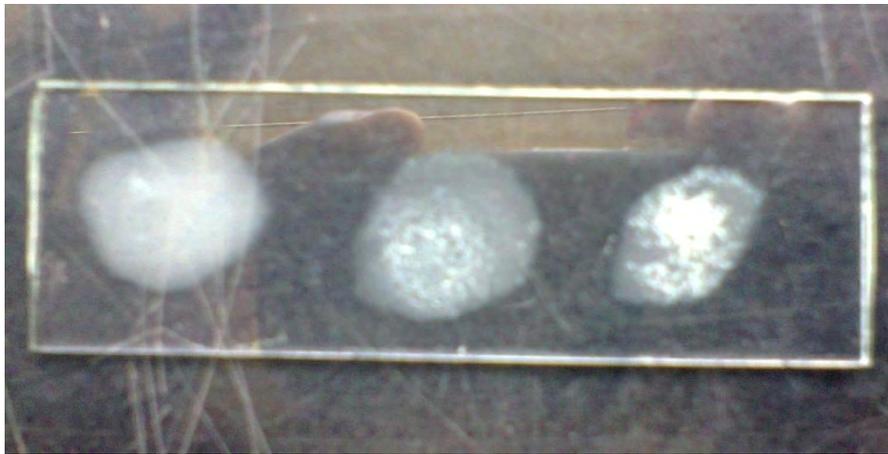
### **The Slide coagulase test**

Principle:

This method measures bound coagulase. The bound coagulase is also known as clumping factor. It cross-links with  $\alpha$  and  $\beta$  chain of fibrinogen in plasma to form fibrin clots those deposits on the cell wall. As a result, individual coccus sticks to each other and clumping is observed.

Procedure:

1. Divide the slide into two sections with grease pencil. One should be labeled as “test” and the other as “control
2. Place a small drop of distilled water on each area
3. Emulsify one or two colonies of Staphylococcus on blood agar plate on each drop to make a smooth suspension
4. The test suspension is treated with a drop of citrated plasma and mixed well with a needle
5. Do not put anything in the other drop that serves as control. The control suspension serves to rule out false positivity due to auto agglutination
6. Clumping of cocci within 5-10 seconds is taken as positive.
7. Some strains of S.aureus may not produce bound coagulase, and such strains must be identified by tube coagulase test.



Appendix figure 3. Slide coagulase test

### **The Tube Coagulase Test**

Procedure:

1. Three test tubes are taken and labeled “test”, “negative control” and “positive control”.
2. Each tube is filled with 1 ml of 1 in 10 diluted rabbit plasma.

3. To the tube labeled test, 0.2 ml of overnight broth culture of test
4. Bacteria are added.
5. To the tube labeled positive control, 0.2 ml of overnight broth culture of known *S.aureus* is added
6. To the tube labeled negative control, 0.2ml of sterile broth is added.
7. All the tubes are incubated at 37oC and observe the suspensions at half hourly intervals for a period of four hours.
8. Positive result is indicated by gelling of the plasma, which remains in place even after inverting the tube.
9. If the test remains negative until four hours at 37oC, the tube is kept at room temperature for overnight incubation.



Appendix figure 4. Tube coagulase test

**Appendix table 3. Differentiation of Staphylococcus and micrococcus species.**

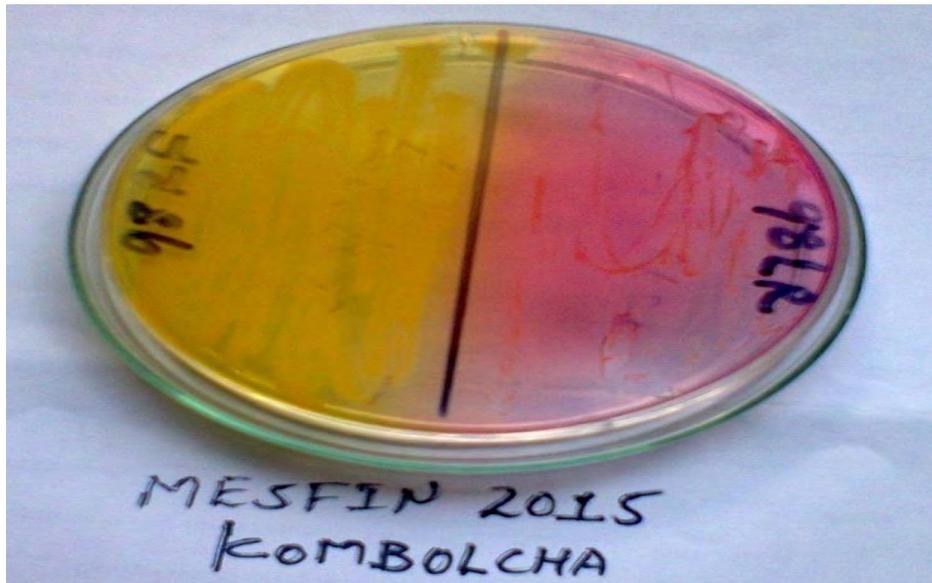
| test      | <i>S.aureus</i> | CNS | micrococcus |
|-----------|-----------------|-----|-------------|
| Catalase  | +               | +   | +           |
| Coagulase | +               | -   | -           |
| Hemolysis | +               | -   | -           |

|             |   |   |   |
|-------------|---|---|---|
| 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, and coagulase test were streaked on mannitol salt agar plate and incubated at 37 °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 5. Growth on mannitol salt agar (stph positive left and other stph spp right)

### Appendix V. Antibiotic sensitivity test

#### ➤ Preparation of inoculums

Inoculation of distinct colony in to 5ml nutrient b incubated at 35-37<sup>0</sup>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<sub>2</sub> 2H<sub>2</sub>O to 99.5ml of 1% (0.36N) H<sub>2</sub>SO<sub>4</sub>.

➤ 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. 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<sup>0</sup>c but not 37<sup>0</sup>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.

| Antimicrobial agents | disc potency | resistance | intermediate | susceptible |
|----------------------|--------------|------------|--------------|-------------|
| Streptomycin         | 10 $\mu$     | $\leq 11$  | 12-14        | $\geq 15$   |
| Tetracycline         | 30 $\mu$     | $\leq 14$  | 15-18        | $\geq 19$   |
| Penicillin G         | 10 U         | $\leq 20$  | 21-28        | $\geq 29$   |
| Ampicilin            | 10 $\mu$     | $<28$      | -            | $\geq 29$   |
| Cefoxitin            | 30 $\mu$     | $\geq 22$  | -            | $\leq 21$   |
| Gentamycin           | 10 $\mu$     | $\leq 12$  | 13-14        | $\geq 15$   |
| Erythromycin         | 15 $\mu$     | $\leq 13$  | 14-22        | $\geq 23$   |

Source (NCCL, 2011)



Appendix figure 6. Antibiotic sensitivity test

## Appendix VI. Medias used for bacteriological examination

### 1. Nutrient agar (Oxoid, England)

Composition (g/l): Lab-Lemco powder 1.0; Yeast extract 2.0 ; Peptone 5.0; Sodium chloride 5.0; Agar 15 ; pH:  $7.4 \pm 0.2$

Directions: Suspend 28 g in 1 liter of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at  $121^{\circ}\text{C}$  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:  $7.5 \pm 0$ .

Directions: Suspend 111g in 1 liter of distilled water and bring to the boil to dissolve completely.

Sterilize by autoclaving at  $121^{\circ}\text{C}$  for 15 minutes

3. Gram's reagent

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

4. . Muller Hinton Agar (Oxoid, England )

Composition (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  $121^{\circ}\text{c}$  for 15 minutes. Cool tubed medium in slanted position for

5. Blood agar

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. It is Autoclave at  $121^{\circ}\text{c}$  for 15 minute. Cool the base to  $45 - 50^{\circ}\text{c}$  and add 5 % sterile defiberinated sheep blood.

