Thesis Ref, No._____

ISOLATION AND ANTIMICROBIAL SUSCEPTIBILITY OF *STAPHYLOCOCCUS AUREUS AND* OCCURRENCE OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS (*MRSA*)* IN MASTITIC DAIRY COWS IN THE SELALE/FITCHE AREA, NORTH SHOWA, ETHIOPIA

MSc Thesis

By

SHIMELS TESFAYE

Addis Ababa University, College of Veterinary Medicine and Agriculture, Department of Veterinary Microbiology, Immunology and Veterinary public health

JUNE, 2014

DEBRE ZEIT, ETHIOPIA

ISOLATION AND ANTIMICROBIAL SUSCEPTIBILITY OF *STAPHYLOCOCCUS AUREUS AND* OCCURRENCE OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS (*MRSA*)* IN MASTITIC DAIRY COWS IN THE SELALE/FITCHE AREA, NORTH SHOWA, ETHIOPIA

MSc Thesis

By

SHIMELS TESFAYE

A thesis submitted to the college of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfilment of the requirements of the degree of Masters of Science in Veterinary Microbiology

JUNE, 2014

DEBRE ZEIT, ETHIOPIA

ADDIS ABABA UNIVERSITY, COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE, DEPARTMENT OF VETERINARY MICROBIOLOGY, IMMUNOLOGY AND VETERINARY PUBLIC HEALTH

As members of the Examining Board of the final Msc open defense, we certify that we have read and evaluate the thesis prepared by: Shimels Tesfaye Megersa entitled a study on Isolation and antimicrobial susceptibility of *staphylococcus aureus and* occurrence of methicillin resistant *staphylococcus aureus (*mrsa*)* in mastitic dairy cows in the selale/fitche area, north showa, Ethiopia and recommend that it be accepted as fulfilling the thesis requirement for the degree of :Masters of Science in Veterinary Microbiology.

Prof. (Dr.) Mahendra Pal. Chairman	_____ signature	_____ Date
Dr.Workineh Shibeshi External examiner	_____ signature	_____ Date
Dr.Takele Abayineh Internal examiner	_____ signature	_____ Date
Dr.Bruk Tesfaye Major Advisor	_____ signature	_____ Date
Dr.Tesfaye Sisay Co- Advisor	_____ signature	_____ Date
Dr.Bedaso Mamo Department chairperson	_____ signature	_____ Date

TABLE OF CONTENTS PAGE

DEDICATION

This thesis manuscript is dedicated to all my family members especially to my beloved mother Senait Asefa W/gebreil.

STATEMENT OF AUTHOR

First, I declare that this thesis is my effortful work that all sources of materials used for this have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirement for an advanced (MSc) degree at Addis Ababa University, college of veterinary medicine and agriculture and it can then be deposited at the university, college of veterinary medicine and agriculture library for borrowing according to the rule of the library. On the other hand, I solely declare that this thesis is not submitted to any other body anywhere for the award of any academic degree, diploma, or certificate.

Brief quotations from this thesis are allowable without special permission provided if an accurate acknowledgement of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the dean of the college when in his or her judgment.

In all other instances, however permission must be obtained from the author.

Name= Shimels Tesfaye Sign=_____

Addis Ababa University, College Of Veterinary Medicine And Agriculture

Date of submission: June,__/2014

ACKNOWLEDGEMENTS

The work incorporated in this thesis was undertaken using the thematic research grant allocated by institute of biotechnology, Addis Ababa University. I am grateful to the university in particular and government of Ethiopia, in general, for providing me the research. Oromia Regional State, Merti woreda administration and Merti woreda livestock development and health office are also highly acknowledged for sponsoring me to attend this postgraduate programme.

I am highly indebted to my academic advisors, Dr.bruke Tesfaye and Dr. Tesfaye Sisay for their technical advice, provision of materials, and their help throughout the work, time devotion to review the manuscript and encouragement, for their invaluable comments and suggestion on the editorial and scientific aspect of this paper and Dr.bedaso Mamo head of veterinary microbiology, immunology and veterinary public health department at college veterinary medicine and agriculture for his all round helpful motivation during my study.

I wish to extend my profound gratitude to Salale dairy farm manager and workers for their full cooperation during my work within the farm and also Fitche, Debre-tsige and Muketuri dairy owners, milk collection centres workers and woredas' animal health clinic workers. Without the cooperation and enthusiasm shown by them, the study would not have been possible.

I am highly indebted to my wife Rahel Nigatu for her inspirational support and love throughout the time.

Finally yet importantly, I would like to express my deepest heartfelt thanks and appreciation to my family for their overall support and family hood interaction throughout my live and to my friends at Addis Ababa University for their full support and lovely time I have spent with.

LIST OF ABBREVIATIONS

µm	Micrometer
CA-MRSA	Community-associated methicillin-resistant *S. aureus*
CL	Confidence interval
CLSI	Clinical and laboratory standard institute
CMT	California mastitis test
CNS	Coagulase negative Staphylococci
CPS	Coagulase positive Staphylococci
CSA	Central statics agency
DD	Disc diffusion
DHI	Dairy herd improvement program
DNA	Deoxy ribonuclotide
Fc	Fragment crystallizable region of antibody
FDA	Food and drug control authority
H_2O_2	Hydrogen peroxide
HA-MRSA	Hospital-acquired methicillin-resistant *Staphylococcus aureus*
Kms	Kilometers
LA-MRSA	Livestock-associated methicillin-resistant *S. aureus*
m.a.s.i	Meters above sea level
MDR	Multi drug resistant
MLST	Multilocus sequence typing
Mm	Millimeters
MRCoNS	Methicillin resistant coagulase negative Staphylococci
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSA	Manitol salt agar
Nacl	Sodium chloride
NAHMS	National Animal Health Monitoring System
NCBI	National center for Biotechnology Information
NMC	National mastitis council
NT	Non-typable

LIST OF ABBREVIATIONS (Continued…..**)**

O_2	Oxygen molecule
^oC	Degree centigrade
OR	Odds ratio
ORSA	Oxacillin -resistant *Staphylococcus aureus*
PAB	Purple agar base
PBP2a	Penicillin-binding protein2a
PCR	Polymerase chain reaction
PH	Power of hydrogen
SCC	Somatic cell count
SCCmec	Exogenous mobile staphylococcal chromosomal cassette
SE	Staphylococcal enter toxin
SPSS	Statistical Package for the Social Sciences
UK	United Kingdom
USA	United States of America
USDA	United States of America Department of Agriculture
X^2	Chi-square

LIST OF TABLES

LIST OF FIGURES

LIST OF TABLES IN THE APPENDIX

LIST OF FIGURES IN THE APPENDIX

ABSTRACT

Bovine mastitis is of the most significant problems associated with high milk production loss in Ethiopia. Staphylococcus aureus is still a major cause of bovine mastitis. MRSA is any strain of Staphylococcus aureus that has developed resistance to beta-lactam antibiotics. It has been known by a significant public health problem infected animals with MRSA might serve as a potential source of human infection. This study was carried out with the aim that to determine the prevalence of Staphylococcus aurous mastitis from the total of clinical and sub-clinical mastitis and identify associated risk factors, isolates and identify Staphylococcus aurous and MRSA strains from mastitic milk samples and conduct in vitro antimicrobial susceptibility test on the isolates. Quarter milk samples from cows were examined to determine the prevalence of Staphylococcus aureus, MRSA and different antibiotic resistant pattern were determined in a cross-sectional study design. A total of 403 samples were collected and screened for the presence of S. aureus. The overall prevalence of mastitis at cow and quarter levels were 128 (83.1%) and 403 (65.42%) respectively. A total of 164 (40.69%) S. aureus isolates were obtained and out of these isolates 60 (36.6%) were found MRSA isolates during this study. The risk factors of mastitis like age group and pregnancy status had no effect on ($p>0.05$) Staphylococcus aureus isolation whereas, stage of lactation and previous mastitis history had significant effect on ($p<0.05$) isolates of S. aurous. A total of 61 isolate of S. aureus species were tested for antimicrobial sensitivity for 12 different types of antibiotics. The S. aureus isolates showed highest sensitivity towards Amoxicillin-clavulinic acid (80%), Chloroamphenicol (78.7%), Nitrofuration (73.8), Cefoxitin (67.2%), Sulphamethoxazole-trimethoprim (59%) and also uncommonly 70.1% of S. aureus isolates were found resistant to Vancomicin. The most frequent multidrug resistance pattern consisting of three drugs is exhibited for, gentamicin, ceftazidime and streptomycin with a resistance of 7 (9.46%) of the isolates. Sixty four (86.46% of the isolates) were resistant to different combinations of two or above tested antibiotics.

Keywords: *Mastitis, Staphylococcus aureus, Methicillin resistant S. aureus, antimicrobial susceptibility test, Multidrug resistance, dairy cow milk, Ethiopia*

1. INTRODUCTION

Staphylococcus aureus is a ubiquitous pathogen and a common cause of invasive and life threatening infections of animals and human beings. In human, it is the most common cause of community-associated cellulitis (Brook and Frazier, 1995; Diekema *et al*., 2001) endocarditis (Hoen *et al*., 2002), and is a common cause of bacteremia (Weinstein *et al*., 1997; Diekema *et al*., 2001; Javaloyas *et al*., 2002). In animals, it is commonly associated with mastitis leading to contamination of milk and dairy products (Oliver *et al*., 2005). *S. aureus* is present in a variety of locations in the dairy farms, in many occasions it was isolated from swabs taken from the cows head, skin swabs, legs and nasal mucosa (Zadoks *et al*., 2000). Furthermore *S. aureus* was found on the milkers' hands as well as on the nasal mucous membrane of the humans working at the dairy farms, in bedding and the drinkers (Benić *et al*., 2012). However an infected udder quarter remains the main reservoir of the bacteria, which transmitted mostly during the milking time. Recent researches show that many biotypes and genotypes exist on the dairy farms (Zadoks *et al.,* 2002*;* Smit*h et al.,* 2005*)*. Variability of the genotypes could be explanation for different success in controlling *S. aureus* mastitis in dairy farms.

Because of the widespread use of antibiotics, resistance profile of microorganisms is increasing among bacterial populations. Antimicrobial resistance is a main public health worry worldwide. Public hazards associated with the consumption of antibiotic contaminated milk could be allergic responses, changes in intestinal flora and development of antibiotic resistant pathogenic bacteria (Thirapatsakun, 1999). The expansion of resistance both in human and animal bacterial pathogens has been allied with the widespread remedial use of antimicrobials or with their administration as growth promoters in animals. Further transfer of antimicrobial resistant bacteria such as *S . aureus* to humans via the food chain has been reported (Angulo *et al*., 2004). Although there are a many reasons which compromise antibiotic treatment of *S. aureus* mastitis, resistance of bacteria toward antibiotics is one of the most important. Special attention is paid to the resistance against methicillin because it is coded by the gen *mecA* which is coding resistance against almost all beta-lactam antibiotics. It should be emphasised that beta-lactam antibiotics are widely used in mastitis treatment (Benić *et al*., 2012).

It is stated that, Methicillin-resistant *Staphylococcus aureus* (MRSA) was first emerged in hospitals during the 1970s in humans and has recently become a worldwide public health problem (USDA and NAHMS, 2011). But in livestock MRSA was first reported in cows in 1972, when Devriese and co-workers found 5.2% of Belgian dairy farms MRSA positive (Devriese *et al*., 1972; Juhasz-Kaszanyitzky *et al*., 2007). Thirty years later, MRSA became a sporadically reported finding from bovine mastitis (Gindonis *et al*., 2013). Currently, increasing evidences point to domestic animals including food animals as reservoirs and shedders of MRSA, and transmission between host species also may be possible (USDA and NAHMS, 2011). Over the past decade, a growing number of MRSA isolates have been reported in companion and food animals and in their human associates, including pet owners, farmers, and veterinary personnel (Lee, 2003).

MRSA strains have been observed to be multi-drug resistant, such as aminoglycosides, macrolides, lincosamides, streptogramins, tetracyclines, etc., which are often used in the treatment of mastitis (Wang *et al*., 2008; Kumar *et al*., 2010). The evolutionary processes enhance the pathogenic and antimicrobial-resistant properties of *S. aureus* strains. However, a limited diversity of *S. aureus* strains or clones cause most of the mastitic infections in each geographical region, as these isolates are better adapted to infect animals (Moon *et al*., 2007). Various molecular techniques have been explored and used to analyze the pathogenesis and distribution of pathogenic genes in strains of *S. aureus* (Fitzgerald *et al*., 2001; Løvseth *et al*., 2004). Identification of MRSA strains by the use of different microbiological and molecular tools may help for early prevention of pathogenic MRSA strains.

Although *S. aureus* is a common mastitis pathogen and among the leading causes of food borne bacterial infections, MRSA appears to be relatively rare in foods originating from animals, and there is little evidence to suggest that MRSA is common in milk (Normanno *et al*., 2007). From a number of epidemiological studies of Staphylococcal mastitis conducted only few of them were done on MRSA strains from milk samples in Ethiopia. Even those few studies were limited to specific areas like Addis Ababa, Bahir Dar, Asela and Sebeta (Mekuria

et al., 2013). Even though it is high milk production area, few studies has been reported from Selale area of the Oromia regional state.

Therefore, this study was carried out with the aim that:

- To determine the prevalence of *Staphylococcus aurous* mastitis from the total of clinical and sub-clinical mastitis and identify associated risk factors
- To isolate and identify *Staphylococcus aurous* and MRSA strains from mastitic milk samples obtained from Selale area, North Showa, Ethiopia
- To conduct *in vitro* anti*microbial susceptibili*ty test on the isolates of *Staphylococcus aurous*.

2. LITRATURE REVIEW

2.1. Bovine mastitis

Today in Ethiopia, there is a national drive to alleviate the existing food deficit by devising different agricultural strategies including improvements of the productivity of livestock sector. With this aim, dairy production improvement is the main concern but in this sector bovine mastitis still remains one of the most significant problems associated with high milk production loss in Ethiopia (Fekadu, 1995; Mekonnen *et al., 2005*). Even though the production loss due to mastitis different from study to study based on dairy management factors, in general it is evidenced that affected dairy cows may loss 15% of their production and the affected quarter shows a 30% reduction in productivity (Heeschen, 1997) which causes financial loss as a result of reduced milk yield, discarded milk following antibiotic therapy, veterinary expense and culling of mastitis cows (Radostitis *et al*., 2007).

As with most infectious disease, generally mastitis risk factors depend on three components; exposure to microbes, cow defence mechanism, environmental and management factors (Quinn *et al*., 2002). Besides improving herd health and dairy management, the controls of mastitis in dairy herds are accomplished in part with the aid of antibiotics (NMC, 2004). Public hazards associated with the consumption of antibiotic contaminated milk results in allergic responses, changes in intestinal flora and development of antibiotic resistant pathogenic bacteria (Thirapatsakun, 1999).

2.2. Mastitis due to Staphylococcus aurous

Although mastitis can be caused by 137 microorganisms, other microorganisms which may be responsible for mastitis include *Streptococcusagalactiae*, *Strep.uberis*, *Enterobacteraerogenes*, *Actinomycespyogenes*, *E. coli*, *Klebsiella* spp., certain fungi and yeasts (Gruet *et al.,* 2001), but in dairy animals, *Staphylococcus aureus* is still a major

cause of bovine mastitis and the disease is responsible for substantial economic losses in the dairy industry world-wide (Athar, 2007; Saei *et al*., 2009).

2.2.1. General characteristics of Staphylococcus aurous

The family *Staphylococcaceae* includes the genera *Staphylococcus* and *Micrococcus. Staphylococcus aureus* is the best characterized species among the staphylococci, a genus of Gram-positive, A-T rich cocci comprising over 50 species and sub-species according to the National Center for Biotechnology Information NCBI Taxonomy browser (NCBI, 2014). *S. aureus* grows in grape-like clusters. This bacterium is a non-motile, non-spore forming, facultative anaerobe (Quinn *et al*., 2002).

Staphylococci do not produce endo-spores but are highly resistant to drying, especially, when associated with organic matter such as blood, pus, and other tissue fluids. They are quite resistant to desiccation and high osmotic conditions. These properties facilitate their survival in the environment and growth in food products. Staphylococci organisms are usually readily killed at cooking, pasteurization temperatures and survives frozen storage. On the other hand, Staphylococcal enterotoxins (SEs) are extremely heat stable (Silva *et al*., 2000; Quinn *et al*., 2002).

Staphylococcus aureus strains can be distinguished by their production of catalase, haemolysins, coagulase and heat-stable nuclease; *S. aureus* is also highly osmo-tolerant, with an ability to grow on media supplemented with 7.5%NaCl (Kloos and Bannerman, 1995; Quinn *et al.,* 2002)**.** Colonies of *Staphylococcus aureus* are typically round, shiny, golden-yellow surrounded by a zone of double-haemolysis on blood agar (Quinn *et al.,* 2002).

The *Staphylococcus* genus is classified into two major groups based on their ability to produce coagulase, protein that affect fibrinogen of the blood-clotting cascade: Coagulase positive staphylococci (CPS) and coagulase-negative staphylococci (CNS), which do not produce coagulase. *S. aureus, S. intermedius and S. hicus* are the species that belong to CPS and

constitute the most significant human and animal pathogens (Turutoglu *et al.*, 2005; Morrison, 2008). The CNS is not highly virulent but is an important cause of infections in certain high-risk groups (Lourdes *et al*., 2004).

2.2.2. Transmission of Staphylococcus aurous mastitis

The bacteria persist in mammary glands, teat canals and teat lesions of infected cows and are contagious (Petersson-Wolfe *et al*., 2010). Environmentally, *S. aureus* is present in a variety of locations on the dairy farms, and several studies suggested that the possible explanation for this 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). Most of the time infection is spread at milking time when *S.* aureus-contaminated milk from an infected gland comes in contact with an uninfected gland and the bacteria penetrate the teat canal (Petersson-Wolfe *et al*., 2010), through contaminated milking machines, clothes and hands of milker's or machine operators (Radostitis *et al*., 2007). Heifers infected during gestation that carry infections through calving represent an important reservoir from which *S. aureus* can spread to uninfected herd mates. There is considerable debate surrounding the route of *S. aureus* infection in heifers prior to first calving, but Mullarky *et al.*(2010) in there review stated that calves fed colostrums from an *S. aureus*-infected dam is a likely source of transmission.

2*.2.3. Pathogenesis of Staphylococcus aurous mastitis*

A large number of commonly accepted virulence factors are associated with *S. aureus* but it is yet to be elucidated which of these are important for infection of the bovine udder. Virulence factors may be divided in three functional categories: Factors that mediate adhesion of bacteria to host cells; those that produce tissue damage; and those that protect the bacteria against the host's immune system and antibiotics (Franco *et al*., 2008).

Staphylococcus aurous bacteria produce various enzymes and toxins that destroy cell membranes and can directly damage milk-producing tissue. Initially, the bacteria damage the tissues lining the teats and gland cisterns within the quarter, which eventually leads to formation of scar tissue. The bacteria then move up into the duct system and establish deep-seated pockets of infection in the milk secreting cells (alveoli). This is followed by the formation of abscesses that wall-off the bacteria to prevent spread but allow the bacteria to avoid detection by the immune system. The abscesses prevent antibiotics from reaching the bacteria and are the primary reason why the response to treatment is poor (Mullarky *et al.*, 2010; Petersson-Wolfe *et al*., 2010)**.**

The capability of this bacteria to resist phagocytosis, for example,'protein A' in the bacterial cell wall of some *S. aureus* strains binds to the Fc portion of antibody molecules making the bacteria unrecognisable to neutrophils ,even if phagocytosed, S. *aureus* may survive and even multiply inside phagocytes (Green and Bradley, 2004). The capsule has been also shown to promote *S. aureus* virulence in several animal infection models (Sordelli *et al*., 2000). Capsulated *S. aureus* strains are more resistant to phagocytises than non capsulated strains, and allow the bacteria to remain in the infected hosts.

2.2.3. Clinical futures of *Staphylococcus aurou*s on mastitis

The clinical futures of Staphylococcal mastitis can be expressed by wide spectrum of clinical signs, from mild cases without clinical signs to extreme cases with lethal exit. Clinical signs vary with the severity of the disease and generally include pain, heat and swelling of the affected quarter or half of the gland and abnormality of milk either as clots or flakes and wateriness of the liquid phase (Miffin, 2004). Per acute form of infection is often seen as a gangrenous mastitis with lethal exit. Acute and sub-acute cases resemble mastitis caused by other pathogens. These cases often lead to chronic forms of infections (*Green and Bradley,* 2004; Benić *et al*., 2012). Chronic and sub-acute cases are the most common forms and from the herd health point of view they are the most important, and are often associated with fibrosis, abscessation and blocked ducts within the gland (Green and Bradley, 2004).

2.*2.4.* Isolation and identification of *Staphylococcus aurous*

Diagnosis of *S. aureus* infections helps in developing preventive strategies and in determining where deficiencies in mastitis control procedures may occur. Diagnosis of *Staphylococcus aureus* mastitis is based on bacterial isolation and it is best achieved through the use of laboratories accredited for mastitis culture. The best method to detect *S. aureus* mastitis is to (1) identify all high somatic cell count(SCC) cows (more than 200,000 cells/ml), (2) perform the California Mastitis Test (CMT)(Quinn *et al.,* 2002),on those cows to determine which quarter or quarters are affected, and (3) culture the milk from those quarters (Arnold and Bewley, 2011). It is extremely important to identify infections early in order to prevent spread and to increase the chance of cure.

Final identification of staphylococci organisms and species assignment can be done based on Gram staining, catalase test, sugar fermentation and coagulase test (Quinn *et al*., 2002)

Gram's staining

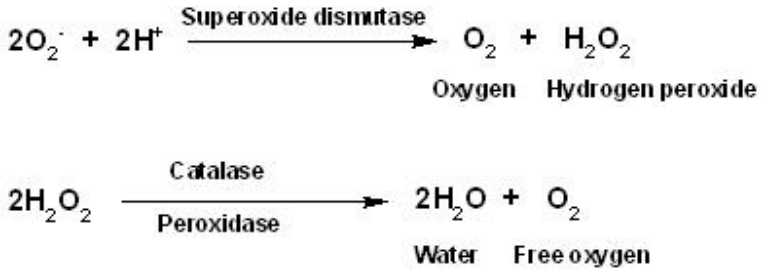
Gram staining is a differential staining technique that imparts different colors to different bacteria or bacterial structures. Usually it differentiates bacteria into two groups; gram positive and gram negative. Hence after the gram staining, the gram positive cells appear as purple and gram negative cells appear as pink (Quinn *et al*., 2002). The study of morphological features and staining characteristics help in the preliminary identification of the isolate. The Gram stained smears from typical colonies that showed Gram-positive cocci occurring in bunched, grapelike irregular clusters which will be taken as presumptive *Staphylococcus* species.

Catalase Test

The inability of strict anaerobes to synthesize catalase, peroxidase, or superoxide dismutase may explain why oxygen is poisonous to these microorganisms. In the absence of these enzymes, the toxic concentration of H_2O_2 cannot be degraded when these organisms are cultivated in the presence of oxygen. Organisms capable of producing catalase rapidly degrade hydrogen peroxide which is a tetramer containing four polypeptide chains, which are usually 500 amino acids long. It also contains four porphyrinheme groups (ie, iron groups) that will allow the enzyme to react with the hydrogen peroxide.

The enzyme catalase is present in most cytochrome containing aerobic and facultative anaerobic bacteria. Catalase has one of the highest turnover numbers of all enzymes such that one molecule of catalase can convert millions of molecules of hydrogen peroxide to water and oxygen in a second.

Catalase production and activity can be detected by adding the substrate H_2O_2 to an appropriately incubated (18- to 24-hour) tryptic soy agar slant culture. Organisms which produce the enzyme break down the hydrogen peroxide, and the resulting O_2 production produces bubbles in the reagent drop, indicating a positive test. Organisms lacking the cytochrome system also lack the catalase enzyme and are unable to break down hydrogen peroxide, into O_2 and water and are catalase negative.



Oxidase Test

Oxidase test is an important differential procedure that should be performed on all gram-negative bacteria for their rapid identification. The test depends on the ability of certain bacteria to produce indophenol blue from the oxidation of dimethyl-p-phenylenediamine and α-naphthol. This method uses N,N-dimethyl-p-phenylenediamine oxalate in which all Staphylococci were oxidase negative. In presence of the enzyme cytochrome oxidase (gram-negative bacteria) the N,N-dimethyl-p-phenylenediamine oxalate and α-naphthol react to indophenol blue. Oxidase test usually used as differentiation for Saphylococci (oxidase negative) from Micrococci (oxidase posative) (Baker, 1984).

Coagulase Test

Coagulases are enzymes that clot blood plasma by a mechanism that is similar to normal clotting. The coagulase test identifies whether an organism produces this exo-enzyme. This enzyme clots the plasma component of blood. The only significant disease-causing bacteria of humans that produce coagulase are *Staphylococcus aureus*. Thus this enzyme is a good indicator of the pathogenic potential of *S. aureus*. In the test, the sample is added to rabbit plasma and held at 37° C for a specified period of time. Formation of clot within 4 hours is indicated as a positive result and indicative of a virulent *Staphylococcus aureus* strain. The absence of coagulation after 24 hours of incubation is a negative result, indicative of an avirulent strain (Hebert *et al.,* 1988).

Mannitol salt agar (MSA)

Mannitol salt agar (MSA) is both a selective and differential medium used in the isolation of staphylococci. It contains 7.5% sodium chloride and thus selects for those bacteria which can

tolerate high salt concentrations. MSA also distinguishes bacteria based on the ability to ferment the sugar mannitol, the only carbohydrate in the medium.

Staphylococci can withstand the osmotic pressure created by 7.5% NaCl, while this concentration will inhibit the growth of most other gram-positive and gram-negative bacteria (Koch, 1942). Additionally, MSA contains mannitol and uses phenol red as a pH indicator (pK = 7.8) in the medium. At pH levels below 6.9, the medium is a yellow color. In the neutral pH ranges (6.9 to 8.4) the color is red; while above pH 8.4, the color of phenol red is pink (Gerhardt *et al*., 1981). When mannitol is fermented by a bacterium, acid is produced, which lowers the pH and results in the formation of a yellow area surrounding an isolated colony on MSA. A non-fermenting bacterium that withstands the high salt concentration would display a red to pink area due to peptone breakdown (Mahon and Manusekis**,** 1995).

Purple agar Base (1% maltose fermentation)

Purple Agar Base is used for studying carbohydrate fermentation reactions, particularly in the identification of gram-negative enteric bacteria and pathogenic Staphylococci groups on addition of the desired carbohydrate (Ewing, 1986; Forbes *et al*., 1998). These media are recommended by FDA (FDA Bacteriological Analytical Manual, 2005) for fermentation studies of sugars (1% maltose fermentation). Beef extract and peptone special supply the essential nutrients especially nitrogen sources to the growing organisms. Sodium chloride maintains the osmotic balance of the medium. Bromocresol purple is the pH indicator, which turns yellow at acidic pH. Gas production is evident by splitting of agar. The acid produced during the fermentation of carbohydrate causes bromocresol purple, the pH indicator to turn yellow. If the carbohydrate is not utilized or fermented, the color of the medium remains unchanged or becomes more alkaline (darker purple) due to decarboxylation of the amino acids present in the medium. It is recommended (McFadden, 1985) to add carbohydrate in 1% concentration to avoid possible reversion reactions except glucose (dextrose). Purple agar base (PAB) with the addition of 1 percent maltose was used to differentiate the pathogenic staphylococci, particularly the coagulase-positive isolates. The identification was based on the

fact that *S. aureus* rapidly ferment maltose and the acid metabolic products cause the pH indicator (bromocresol purple) to change the medium and colonies to yellow. *S. intermedius* gives a weak or delayed reaction and *S. hicus* did not ferment maltose but attacks the peptone in the medium producing an alkaline reaction (a deeper purple) around the colonies. The rapid fermentation (24hrs) was considered as *Staphylococcus aurous* isolates *(*Quinn *et al*., 2002).

2. 2.*5.treatment of Staphylococcus aurous mastitis*

Many approaches have been taken to the treatment of *S. aureus* mastitis including intra-mammary antimicrobial therapy, systemic antimicrobial therapy, and vaccination in conjunction with antimicrobial therapy (Middleton, 2013).

Staphylococcus aureus has the ability to resist conventional antibiotic therapy. Indeed, the poor response to therapy has resulted in a multitude of treatment protocols and is always the source of some discussion (Green and Bradley, 2004). One over-riding difficulty is that most licensed treatment regimens for clinical mastitis are probably too short to produce good results for *S. aureus* mastitis. The major treatment strategies are antibiotic treatment during lactation (parenteral antibiotic therapy in combination with intra-mammary treatments for *S. aureus* mastitis (Radostitis *et al*., 2007), particularly to improve penetration of inflamed or intracellular sites), antibiotic treatment during the dry period (bacteriological cure rates from antibiotic therapy during the dry period are usually in the region 40–80% (Green and Bradley, 2004),and therefore the dry period is the time of choice for treating *S. aureus* mastitis) and Culling a chronically infected cow with *S. aureus* mastitis, which achieves both a reduction in herd prevalence and also a reduction in the risk of subsequent spread of infection (Radostitis *et al*., 2007). Roy and Keefe (2012) in a systematic review of the literature on the treatment of *S. aureus* mastitis during lactation also concluded that extended intra-mammary therapy for 5-8 days was the best therapeutic option.

2.2.6. *Staphylococcus aurous prevention and control*

The foundation of infection control for staphylococcal infections is the same as for control of virtually all other relevant veterinary pathogens, a good general infection control programme (Weese, 2012). It is certainly possible to maintain a low herd prevalence with <2% cows infected (in one or more quarters) with *S. aureus*. Since in the majority of herds, the most important reservoirs of the organism are infected cows, most control procedures are based on reducing the probability of spread between cows. These can be achieved through carrying out treatment; culling, drying off infected quarters and ensuring new heifers/cows are uninfected (Green and Bradley, 2004). However, there are other potential sources of *S. aureus* associated with the environment and these may be of special significance in particular herds in which the condition is difficult to control with the traditional methods. Sources of *S. aureus* outside the mammary gland are also the reason why it is virtually impossible to eradicate *S. aureus* mastitis from a commercial dairy unit.

2.2 Over view of Methicillin-resistant staphylococcus aurous (MRSA)

Methicillin-resistant *Staphylococcus aurous* (MRSA) is a bacterium responsible for several difficult-to-treat infections in humans (Bagcigil *et al*., 2007). It is also called multidrug-resistant *Staphylococcus aurous* and Oxacillin-resistant *Staphylococcus aureus* (ORSA). MRSA is any strain of *Staphylococcus aureus* that has developed, through the process of natural selection, resistance to beta-lactam antibiotics, which include the penicillins (methicillin, dicloxacillin, nafcillin, oxacillin, etc.) and the cephalosporins (Deurenberg and Stobberingh, 2008).

Methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged globally as a significant public health/antimicrobial resistance problem both in human and veterinary medicine. It has been well known as a nosocomial agent, later also as a community-associated pathogen. In recent years, the so-called non-typable (NT)-MRSA (also called Livestock-associated MRSA, LA-MRSA) has become an additional focus of concern.

S. aureus can cause severe blood infections and necrotizing fasciitis in humans, wound infection and mastitis in cattle, horses, pigs and goats, exudative epidermitis in pigs, pyoderma in horses, dogs and cats, pyemic sheep (Quinn *et al*., 2002 ; Resch *et al*., 2008).

In human MRSA causes a wide range of infections such as skin infections, fatal septicaemia, pneumonia and food poisoning as well as life threatening postsurgical infections. MRSA is especially troublesome in hospitals, prisons and nursing homes, where patients with open wounds, invasive devices, and weakened immune systemsare at greater risk of infection than the general public (Vanderhaeghen *et al*., 2010).

2.2.1 History of MRSA

The history of MRSA is mainly situated in human medicine and started in 1961, when MRSA was first isolated in a UK hospital and soon after methicillin was introduced into human medicine to treat penicillin-resistant staphylococci (Fitzgerald *et al*., 2001). From then onwards, MRSA began to spread in hospitals all around the world.

Hospital-Acquired MRSA (HA-MRSA)

In the beginning of the present century, it was shown that the majority of the MRSA known international epidemic hospital strains, named Hospital-Acquired MRSA (HA-MRSA), belonged to only five CCs: CC5, CC8, CC22, CC30 and CC45 (42), and that they generally possessed one of the larger SCC*mec*-types I-III, partly explaining their resistance to most clinically used antimicrobial agents (Tenover *et al*., 2001)

Community-Associated or Community-Acquired MRSA (CA-MRSA)

While the problems with HA-MRSA were not at their full width yet, a second phase in the history of MRSA dawned halfway the 1990s, when MRSA infections involving strains

different from HA-MRSA were increasingly documented in non-hospitalized patients (Udo *et al.,* 1993; Herold *et al.,* 1998; Bukharie *et al*., 2001; Chambers, 2001). Such cases, called Community-Associated or Community-Acquired MRSA (CA-MRSA), have since been reported worldwide. It is hypothesized that the evolution of CA-MRSA is due to the acquisition of *mec* DNA by previously methicillin-susceptible strains that circulated in the community but in recent studies have shown that patients who have acquired CA-MRSA infections do not have typical MRSA risk factors, such as recent hospitalization, kidney dialysis, residence in a long-term health care facility, or intravenous drug use(Fey *et al*., 2003). Fey *et al*. (2003) also confirmed that the prevalence of CA-MRSA in the United States was unknown until recent time. Analysis of the genetic background of CA-MRSA strains also has shown a clear distinction from typical HA-MRSA, as they predominantly belong to ST1, ST8, ST30, ST59, ST80 and ST93 (Vandenesch *et al*., 2003). In addition, CA-MRSA mostly possess the smaller SCC*mec*-types IV and V (Okuma *et al.,* 2002; Tristan *et al*., 2006), which is assumed to be at least partly explanative for the generally more antimicrobial susceptible phenotype of CA-MRSA.

Livestock-associated MRSA (LA-MRSA)

Looking into the epidemiological aspects of animal MRSA, food production animals (cattle, pigs and poultry, further referred to as livestock) are of particular concern.

Although LA-MRSA is the most important MRSA clone residing in livestock, it is not the only MRSA type that has been reported in livestock. MRSA was first reported in cows in 1972, when Devriese and co-workers found 5.2% of Belgian dairy farms MRSA positive (Devriese *et al*., 1972; Juhasz-Kaszanyitzky *et al*., 2007). Thirty years later, MRSA became a sporadically reported finding from bovine mastitis (Gindonis *et al*., 2013).Since then, the evolution of antibiotic resistance in *S. aureus* strains is a serious cause of concern in dairy animals (Wang *et al.*, 2008).

2.2.2 MRSA as zoonosis

MRSA remains a significant public health problem, even though, in several countries, with the paradigm of the Scandinavian and The Netherlands, successful infection control measures have been applied. The spread of MRSA among companion and food-chain animals has not been completely. The details of animal MRSA carriage are not completely known, and different rates have been published, reflecting transient carriage, a diversity of applied protocols, and the variation in MRSA epidemiology between countries.

Several reports suggest that animals may serve as reservoirs for MRSA infection of humans (Loeffler and Lloyd, 2010). The ways by which animal MRSA can be transmitted to humans are direct contact with the animals, environmental contamination, and eating or handling contaminated meat. As when MRSA spreads between humans, direct contact is an important way by which animal MRSA can be transmitted. As a result, those who have direct contact with farm animals carrying LA-MRSA have the highest risk of acquiring farm animal MRSA (Mekuria *et al*., 2013). A previous study showed that 50% of humans living on Dutch pig farms were carriers and some developed serious infections (Kluytmans *et al*., 1995). MRSA can also be transmitted between cattle and farmers, and between chickens and farmers. Veterinarians are also at risk if the animals that they are handling are infected with MRSA (Garcia *et al*., 2012). With regard to humans in contact with farm animals, Voss *et al*. (2005) reported in 2005 that Dutch pig farmers were at a 760-fold risk of being colonized with LA-MRSA as compared with the general Dutch population. In an international study, Wulf *et al*. (2008) found MRSA in 12.5% of veterinarians originating from all over the world. In Switzerland, the MRSA prevalence in veterinarians was 3% in 2009 (Huber *et al.,* 2009). These studies strongly suggest that people working with livestock are at potential risk of becoming MRSA carriers, and hence have an increased risk of suffering infections caused by MRSA. Recently, the results of Price *et al*. (2012) strongly suggested that LA-MRSA CC398 originated in humans as MRSA. The lineage appears to have undergone rapid radiation in conjunction with the jump from humans to livestock, where it subsequently acquired tetracycline and methicillin resistance. The first outbreak of LA-MRSA ST398 was reported in

a Dutch hospital; none of the patients had had contact with pigs or veal calves (Wulf *et al*., 2008).

2.2.3 *Epidemiology of MRSA in dairy animals*

The predominant Staphylococcal species colonizing or infecting animals vary according to animal species. While *S. aureus* and CNS are commonly isolated from milk, Methicillin-resistant staphylococci have been infrequently isolated from cases of clinical and subclinical bovine mastitis (Huijsdens *et al*., 2006; Monecke *et al*., 2007; Wang *et al*., 2008). Furthermore, the origin of MRSA intra-mammary infections in dairy cattle has been difficult to define. Devriese and Hommez (Devriese and Hommez, 1975) reported that MRSA infections in dairy cattle were most likely of human origin. In the review of Cohn and Middleton (2010), it is stated that the study by a Hungarian had reported that MRSA isolates from dairy cattle with subclinical mastitis and a farm worker were phenotypically and genotypically indistinguishable suggesting cross-species transmission, but still not definitively identifying the origin of infection (Juhasz-Kaszanyitzky *et al.,*2007). Recently, methicillin-resistant *S. epidermidis* was isolated from a cow with mastitis but it was unclear whether the isolate was of bovine or human origin (Walther and Perreten, 2007). Although MRSA can be isolated from cattle, infection is relatively rare. The emergence of MRSA ST398 in swine and the association of human colonization with ST398 among persons associated with veal calf operations suggest that, at least in some types of cattle rearing facilities, cattle may serve as a reservoir for MRSA colonization or infection.

2.2.4 Transmission of MRSA in dairy animals

Many domestic animal species can become colonized or infected with MRSA and might serve as a potential source of human infection. *Staphylococcus aureus* has been isolated not only from the nares and skins of dairy personnels and veterinarians in charge and mastitic milk, but also from the nares of dairy cattle, teat skin, udder skin, belly and genitals, as well as from many environmental sites including feed, bedding and milking equipment (Roberson *et al.,* 1998; Roberson, 1999).

It was first thought that the transmission of MRSA was solely from human to animal, with MRSA colonization and infection typically occurring with contact between the hands of the human and anterior nares (nostrils) of the animal. There is now increasing evidence that MRSA can be transmitted in both directions, from animal to human (zoonotic) and human to animal (reverse zoonotic) (Lee, 2003). Once exposed to MRSA, animals can become colonized, and may serve as reservoirs to transmit the infection to other animals and also to their human handlers (Simoons-Smit *et al*., 2000). It has been shown that even apparently healthy animals may be MRSA reservoirs, and therefore may pose a risk to their handlers (Manian, 2003). This has been documented in the general community, and is becoming increasingly documented in health-care settings and in animal environments (eg, veterinary clinics and hospitals, farms, and slaughterhouses). Data have indicated that owners and veterinary personnel who come into contact with MRSA-colonized or MRSA-infected animals may become colonized by MRSA (O'Mahony *et al*., 2005). There is then a risk that subsequent contact with susceptible animals or human beings will transfer MRSA infection. Transmission of MRSA is by direct contact or nosocomial infection. It can spread among humans and animals and between species either by direct physical contact or indirectly through clothing, towels, equipment, food, air, or surfaces contaminated by infected or colonized persons or animals (Weese *et al*., 2006).Generally, the routes of infection are: person-person, air-born transmission, animal contact, contaminated equipment and surfaces and contaminated food. An important and previously unrecognized means of community-associated MRSA colonization and transmission is during sexual contact (Calfee, 2011).

2.2.5 Microbiological identification of MRSA isolates from milk

In the diagnosis of MRSA, when infection is suspected, appropriate samples should be collected for culture and susceptibility testing. These samples which are diagnosed could be Milk and milk products and refrigerated samples survive well during routine transport. In vitro susceptibility tests on cultured samples now use either oxacillin or cefoxitin in place of methicillin (CLSI, 2012).

In human medicine, several detection methods based on phenotypic expression of the mecA gene have been evaluated for diagnosing MRSA including antimicrobial susceptibility testing, PBP2a latex agglutination test kit, and cefoxitin disk diffusion test (Diab *et al*., 2008).

In the study of Diab *et al*. (2008) stated that, Both PBP2a latex agglutination and Cefoxitin disk diffusion assays exhibited sensitivity of 100% for detection of both MRSA and MRCoNS (methicillin resistant coagulase negative Staphylococci) and specificities of 100% and 75% (PBP2a assay) and 90% and 100% (cefoxitin DD) for identification of methicillin-sensitive isolates, respectively.

2.*2.6.* MRSA *prevention and control in dairy animals*

Most aspects of methicillin resistant staphylococcal control are also basic infection control practices that are applicable to a wide range of pathogens, and development of a good general infection control programme (rather than focusing solely on MR staphylococci) is probably the most important factor for reducing MR staphylococcal transmission (Weese, 2012). Numerous reports on MRSA control in humans have been published and many of the principles may also be applied to control in animals. However, caution is necessary for extrapolating these human guidelines to animals, as disease epidemiology can differ significantly (Leonard and Markey, 2008).

It has been observed that exposure to antimicrobials is a risk factor for the acquisition and dissemination of MRSA in humans and also most probably in animals. In this respect, strategies for prevention and management of MRSA in animals should be, as much as possible, related to the use of antimicrobials. If the antimicrobial treatment is necessary in individual cases for the sake of animal welfare, the risk of the emergence of wider resistance in MRSA strains colonizing animals needs to be managed, especially considering zoonotic aspects. Options to manage this risk include the non-use of antimicrobials except as a last resort strategy, decolonization in humans, and isolation of animals during treatment, and monitoring the effects of treatment in strain resistance through selective culture and susceptibility tests (Catry *et al*., 2010).

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 (Muto *et al*., 2003). Special attention should be paid to dust in stables; Animal owners should be informed about the risks and necessary precautions.

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, and in some cases the infected quarter must be prematurely dried-off. If the antimicrobial treatment is chosen, it is necessary to evaluate its risk-benefit compared with other alternatives. 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 (Jayarao and Cassel, 1999).

2.3. Multi-drug resistance mechanism *Staphylococcus aurous*

Resistance to chemical antibiotics in *S. aureus* was documented only a few short years after the introduction of penicillin into general use in 1944 (Livermore, 2000).

MRSA produces a specific penicillin-binding protein20 (or PBP2a) that possesses reduced affinities for binding to β-lactam antibiotics resulting in β-lactam antibiotic resistance (Berger-Bachiand Rohrer, *2002)*. PBP20 is encoded by the meca gene carried by a large mobile genetic element (Kwon *et al*., 2005), staphylococcal cassette chromosome mec (sccmec), which is integrated at the 30 ends of orfx on the chromosomes of MRSA strains. The Sccmec element contains the mec gene complex composed of the meca gene and its regulators, and the

ccr gene complex that encodes site-specific recombinases, ccra and ccrb, which are responsible for the mobility of sccmec (Kwon *et al*., 2005).

Study of early isolates of MRSA showed that a key genetic component responsible for resistance, *meca,* is not native to the *S. aureus* genome. The staphylococcal chromosome cassette *mec* (SCC*mec***)** has been characterized as a novel, mobile resistance element that differs from both transposons and bacteriophages (Berger-Bachiand Rohrer, *2002)*. MRSA typically spreads through clones; however, it is known that the *mec* gene has been transmitted between *S. aureus* strains and, possibly, between other staphylococcal species (Berger-Bachiand Rohrer, *2002*; Chu *et al*., 2012*)*.

However, not all *meca* clones are resistant to methicillin, and overall resistance levels in a population of MRSA depend on efficient production of PBP 2a, which is modulated by a variety of chromosomal factors. This explains why MRSA resistance levels range from phenotypically susceptible to highly resistant (Berger-Bachiand Rohrer, *2002).*

According to Kwon *et al*.(2005) although MRSA strains isolated from humans have been well characterized and examined by analyses of their sccmec complex, antimicrobial resistance patterns and multilocus sequence typing (MLST), but there have been few studies about MRSA isolated from animals or livestock products and their sccmec complex characteristics.

3. MATERIALS AND METHODS

3.1. Description of the study area

The study was carried out in the Selale area which includes Girar-Jarso, Debre- Libanos and Wuchale Woredas where the home towns are Fitche, Debre-Tsige and Muketuri towns, respectively. The study was done in and around the three towns.

Fitche/ Girar-Jarso woreda

Fitche is the administrative centre of the Semien Shewa Zone of Oromiya Region and in Girar-Jarsoworeda.The woreda lies along the highway to Amhara National Regional State in the Northwestern direction at a distance of 112 km from Addis Ababa. The GirarJarsoworeda has an altitude of 1300 to 3419 m.a.s.l and astronomically the woreda occupies 9035'-10000'N latitude and 38039'-38039'E longitude.According to Fitche Station meteorological data the average rainfall amount of the woreda is about 1200mm, and maximum and minimum rainfall is about 1115mm and 1651mm, respectively. Temperature of the woreda ranges from a minimum of 11.5^0C to a maximum of 35^0C.Agro-ecologically, the woreda is categorized into three: Dega, Woina-Dega and Kolla constituting 52%, 41% and 7% of the total area of the woreda, respectively.

The total livestock population is estimated as 119650 cattle, 33250 sheep, 17295 goat, 2290 horses, 380 mules, 14650 donkey, 72270 chicken and 1915 bee hives. The main source of income is obtained from sale of livestock and livestock products and livestock by-products (such as kubet), crops, and firewood and eucalyptus (Girar-Jarso woreda Livestock Production, Marketing and Health Office, 2013).

Debre-Tsige/DebreLibanose

Debre-Tsige is a town of Debre-Libanos Woreda located 89 km from Addis Ababa.

Debre-Libanos District is located 9^o 48N and 38^o 44E.The altitude of the District is between 1500-2700m a.s.l, its minimum and maximum temperature vary from 19^oC to 23^oC.It gets

bimodal rain fall that ranges from 800-1200mm and the predominant soil type are black soil (56%) and red soil (38%).Two agro ecologies are found in the area and mixed agricultural activities are performed. According to Debre-Libanos woreda Livestock office, there are about 80,796 head of cattle, 84,507 goats, 23,723 sheep, 10,899 equines, 1,894 camels and 75,305 poultryin thedistricts. All of these livestock species are reared mainly by smallholder farmers under intensive, semi intensive and extensive production system (Debre-Libanose Woreda Livestock Production, Marketing and Health Office, 2013**).**

Mukaturi/Wuchale

Mukaturi is a town of Wuchale Woreda located 78 km north-west of Addis Ababa.Agro-ecologically, the woreda is categorized into three: Dega, Woina-Dega and Kolla constituting 87%, 11% and2% of the total area of the woreda, respectively. Its altitude ranges from 1000-3000 m.a.s.i. The annual mean rain fall reaches 1028mm. The mean maximum and maximum temperature are 11.23^{0}C and 20.86^{0}C, respectively. The climate of the area is favorable for crop and livestock production like the neighboring woredas.

Figure 1: Map of Selale area (rough sketch). Map source: Encarta 2014

3.2 Study population and animals

The study population was selected dairy cows in Muketury dairy farm and medium and small holder dairy farms in and around Fitche, Debre-tsige and Muketuri towns which consisted of 154 milking cows selected from these areas.

3.3 Study design

3.3.1 Study type

A cross-sectional type of study design was used to isolate *Staphylococcus aureus* at cow and quarter level, occurrence of Methicillin resistance*Staphylococcus aureus* (MRSA) and also to evaluate their antibiotic susceptibility patterns from samples of mastitic cow's milk obtained from Muketury dairy farm and Fitche, Debre-tsege and Muketury towns small and medium scale dairy farms from October 2013-April 2014.

3.3.2 Sampling method and determination of sampling size

The large scale dairy farm (Muketury dairy farm) was selected purposively and but for medium and small holder dairy farms a sampling frame containing the list of current members of Fitche, Debre-tsige and Muketuri towns milk and milk products marketing cooperatives were acquired from the cooperatives offices from each towns at the beginning of the study. Based on this the farms under study from the sampling frame was selected by using a stratified random sampling procedure and stratification was done in similar with previous reports(Lemma *et al*., 2001; Tesfaye, 2008) as small holder (<5 heads of dairy cattle), medium size (6-50) heads of dairy cattle and large scale (> 50 heads of dairy cattle). A total of 58 dairy farms (only one large scale, 12 medium and 45 small scale dairy farms were selected randomly and included in the study. The study animals in Muketury dairy farm was selected by systematic random sampling method whereas for medium and small holder dairy farms.

Moreover, on site, questionnaires were prepared to assess basic information on each farm, which includes the number of cows, breed type, age, stage of lactation, lactation status,

pregnancy status and the previous mastitis history used were requested and filled before collecting samples.

To calculate the sample size the following parameters were used: 95% level of confidence (CL), 5% desired level of precision and withthe expected prevalence of mastitis 89.5% and 63.1% at cow and quarter level, respectively, by Argaw and Tolosa (2007) atsmall holderdairy farmsin Selale, North Shewa Zone in milk sample and the sample sizes were determined using the formula given in Thrusfield (2005).

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

Where: n = required sample size, Pexp = expected prevalence, d = desired absolute precision

Using the above formula, accordingly, the calculated values for sample size were equal to144 dairy cows and 358 quarters. However, not to lose the chance of sampling 10 dairy cows were added to the sample size which equals to 154 dairy cows and also all teat quarters of dairy cows (N=616)included under this study were involved in this study.

3.4 data collection method

3.4.1 Screening methods

Clinical examination

Clinical cases were recorded at the time of milk sampling purposively. Theses Clinical mastitis cases were diagnosed on the basis of manifestation of visible signs like inflammation of udder, warm and swollen with painful upon palpation and/or gross changes in milk was well considered otherwise chronic mastitis when misshaped, atrophied, hard and fibrotic quarters were examined (Radostitis *et al*., 2000; Quinn *et al*., 2004).

Screening using California Mastitis Test (CMT)

Subclinical mastitis was diagnosed based on CMT results and the nature of coagulation and viscosity of the mixture show the presence and severity of the infection, (milk and CMT reagent) following the procedures in Quinn *et al*. (2002). Before sample collection for bacteriological examination, milk samples were examined for visible abnormalities and were screened by the CMT. From each quarter of the udder, a squirt of milk sample (2mm) was placed in each of the cups on the CMT paddle and an equal amount of 3% CMT reagent was added to each cup and mixed well. Reactions were graded as 0 and trace for negative, +1, +2 and +3 for positive (Quinn *et al.,* 2002).

3.4.2 Bacteriological examination of milk samples

i. Collection, transportation and handling

Milk samples were collected from each of clinically and sub clinically mastitic non-blind quarters of the selected cows according to the National Mastitis Council Guideline (2004). After milking out and discarding the first two drops, about 2ml of milk was tested on CMT paddle from each quarter and about 25ml of milk was aseptically collected from each mastitis positive quarter using sterile universal bottle. Then samples were transported to microbiology laboratory, College of Veterinary medicine and Agriculture, the Addis Ababa University in an ice box for microbiological examination. Since immediate inoculation was not convenient, samples were kept at 4°C until cultured for isolation.

3.4.3 Culture procedure

Isolation and identification of *Staphylococcus aureus* and Methicillin Resistance *S. aurous (*MRSA) was conducted in the microbiology laboratory at Addis Ababa University College of veterinary medicine and agriculture. On arrival in the laboratory, aliquots (centrifuged milk sample) of 0.01 ml of milk were streaked on blood agar (Oxoid, UK) containing 7% sheep blood for isolation of Staphylococci. The incubation was done aerobically at 37 °C for 24-48

hrs. The presence of more than 3 colonies of a similar morph-type was accepted as positive bacteriological finding (Ebrahimi *et al*., 2010). Identification of the bacteria on primary culture was made on the basis of colony morphology, haemolytic characteristics, Gram stain reaction including shape and arrangements of the bacteria, catalase test and oxidase test. In addition, growth characteristics on Mannitol salt agarand purple agar and tube coagulase test were conducted for specifically identifies *staphylococcus* species.

3.4.4 Isolation and identification of Staphylococcus aurous species

Final identification of staphylococci organisms and *Staphylococcus aurous* species assignment were done based on Gram staining and biochemical tests such as catalase test, oxidase test, Mannitol sugar fermentation, Coagulase test and 1% maltose fermentation.

Gram's staining

All suspected cultures of *Staphylococcus* species were subjected to Gram's stain and observed under a light microscope for Gram's reaction, size, and shape and cell arrangements. The Grams stained smears from typical colonies that showed Gram-positive cocci occurring in bunched, grapelike irregular clusters were taken as presumptive *Staphylococcus* species.

Catalase test

Pure culture of the isolates to be tested for catalase were picked up by bacteriological loop from the agar plate and mixed with a drop of 3% hydrogen peroxide on a clean slide. When the organism was positive, bubbles of oxygen was liberated within a few seconds. Those positive cocci were considered as Staphylococci (Quinn *et al*., 2002).

Oxidase test

A piece of filter paper was moistened in a petridish with 1 percent aqueous solution of tetramethyl –p-phenylenediamianedihydrochloride. The test colony was streaked firmly across the filter paper with a glass rod. The disappearance of dark purple color along the streak on the filter paper was considered as Staphylococcus (Quinn *et al*., 2002).

Mannitol salt Agar (Mannitol fermentation)

The colonies that were confirmed by gram's staining reaction, haemolysis on the blood agar, colony characterization ,catalase positive and oxidase negative were selected and streaked on Mannitol salt agar plate and incubated at 37^0C and examined after 24-48 h for growth. The presence of growth and change of PH in the medium (red to yellow) was regarded as presumptive identification of *Staphylococcus aurous* or coagulase positive *Staphylococcus aurous*.

Phenol red pH indicator detected the acidic metabolic product of mannitol. Fermentation of mannitol by *S. aureus* causes yellow discolouration of the medium with in 24 hrs of incubation (Quinn *et al*., 2002) (Appendix-5).

Coagulase test

The coagulase tests used were both slide coaglase and tube coagulase tests. The presumptively identified *Staphylococcus aurous* from mannitol salt Agar were sub-cultured to nutrient agar plate and after 24 hours culture colonies of Staphylococcus *aurous* was picked by bacteriological loop and placed on clean slide with a small drop of distilled water and emulsified. The test suspension was treated with a drop of rabbit plasma and mixed well with a needle for 5-10 seconds. Those forming Clumping of cocci were taken as positive (Quinn *et al*., 2002).

For those slide coagulase negative isolates, the tube coagulase test was performed in sterile tubes by adding 0.5 ml of selected isolates of *Staphylococcus* grown on Brain Heart Infusion

broth (BHI) at 37^0C for 24 hours to 0.5 ml of rabbit plasma (Quinn *et al*., 2002). The reaction was considered positive, if any degree of clotting from a loose clot to a solid clot that is immovable when the tube is inverted (tilted) was visible within the tube and no degree of clotting would be taken as negative.

Purple agar Base (1% maltose fermentation)

Purple agar base (PAB) with the addition of 1 percent maltose was used to differentiate the pathogenic staphylococci, particularly the coagulase-positive isolates. The suspected culture was inoculated on PAB media plate (Defico) with 1% of maltose and incubated at 370C for 24-48 hours. The identification was based on the fact that *S. aureus* rapidly ferment maltose with in 24 hrs and the acid metabolic products cause the pH indicator (bromocresol purple) to change the medium and colonies to yellow. The rapid fermentation (24hrs) was considered as *Staphylococcus aurous* isolates *(*Quinn *et al*., 2002).

3.4.5 Phonotypical isolation of MRSA strains

MRSA was identified phenotypically by using Cefoxitin disk diffusion method (CLSI, 2012). There are several methods for detection MRSA including routine methods such as disk diffusion, MIC determination (broth dilution-test) oxacilin screening agar, and recently developed methods like disk diffusion using Cefoxitin instead of Oxacillin ,latex agglutination and CHROMagar MRSA (Brown *et al*., 2005). According to Karami *et al. (*2011) in his findings, all phenotypic methods had high sensitivity and specificity for detection of MRSA. However, Cefoxitin disk diffusion method in comparison to other methods had higher specificity. In the study of Diab *et al*. (2008) stated that Cefoxitin disk diffusion assays exhibited sensitivity of 100% and specificity of 90% for identification of methicillin-sensitive isolates.

The disk diffusion test was performed for all *S. aureus* isolates (N=164). For susceptibility testing, direct colony suspension of the isolates was adjusted to a turbidity equivalent to a 0.5 McFarland standard and resistance to Cefoxitin disk was determined for isolated strains on

Mueller-Hinton agar following the Clinical and Laboratory Standards Institute guideline (CLSI, 2012).

Finally, the diameters of the zone of inhibition around the disks were measured to the nearest millimeter using caliper, and the isolates were classified as susceptible, intermediate and resistant according to the interpretative standards of Clinical and Laboratory Standards Institute (CLSI, 2012)(Appendix 8).

3.4.6 Antibiotic susceptibility testing

Antimicrobial susceptibility testing was performed for *S. aureus* isolates (N=61) according to the criteria of the Clinical and Laboratory Standards Institute (CLSI, 2012). For susceptibility testing, direct colony suspension of the isolates was adjusted to a turbidity equivalent to a 0.5 McFarland standard Susceptibility to antimicrobial agents were determined for isolated strains by the disk diffusion method on Mueller-Hinton agar following the Clinical and Laboratory Standards Institute guideline (CLSI, 2012).

For susceptibility test antimicrobials from each subclass (CLSI, 2012) and antimicrobials which are commonly used for treatment of bovine mastitis were selected. Thus a total of twelve antimicrobials were used in this study. The antibiotic discs used were, Cefoxitin (Fax/30 µg), Amoxicillin-clavulinic acid (AMC/30 µg), Ceftazidime (CAZ/30 µg), Vancomicin (VA/30µg), Gentamicin (CN/10 µg), Streptomycin (S/10µg), Ciprofloxacin (CIP/5 µg), sulphamethoxazole-trimethoprim (SXT/25 µg),Chloroamphenicol (C/30 µg), Nitrofuration (F300 µg) and Clindamycin (DA/10 µg) (Oxoid, UK) and Bacitracin (B/10 µg) (Himedia, India).

Finally, the diameters of the zone of inhibition around the disks were measured to the nearest millimeter using caliper, and the isolates were classified as susceptible, intermediate and resistant according to the interpretative standards of Clinical and Laboratory Standards Institute (Quinn *et al*., 2002; CLSI, 2012)(Annex-). Moreover, isolates showing resistance to two or more antimicrobial subclass were considered as multidrug resistant (Intrakamhaeng and Komutarin, 2012).

3.4.7 Data Collection and Statistical analysis

Microsoft Excel was used for data management, computation of descriptive statistics and drawing graphs. Computation of descriptive statistics was conducted using SPSS version 20.0 software. Descriptive statistics such as percentages, proportions and frequency distributions were applied to compute some of the data. Prevalence of *Staphylococcus* and *Methicillin resistant Staphylococcus aurous*in milk samples were computed.

Independent variables such as type of farm, breed type, age, stage of lactation, pregnancy status, type of mastitis, history of previous mastitis status and disinfectant used were used to be interpreted against dependent variable of *S. aureus* isolation.

The Pearsons chi-square ($\chi 2$) test at a significance level of 5% and 95% CI was used to determine the degree of association between risk factors and the occurrence *S .aureus and* significant variation between the prevalence of *S. aureus and MRSA*. Furthermore, univariate logistic regression was used to see the association of the potential risk factors with occurrence *S. aureus* and MRSA for those risk factors significant for Pearson's chi-square ($\chi 2$) test. The degree of association between risk factors and the occurrence *S .aureus* were analyzed using odds ratio (OR). In all analysis, associations were considered to be significant when $P<0.05$.

4. RESULTS

4.1 Prevalence of mastitis

In this study a total of 616 quarters were examined from 154 cows, most of Holstein Fresian (N=146, 94.80%) and only few of them (N=8, 5.20%) cross breeds. Under these clinical examination and CMT screening were used to determine the prevalence of mastitis.

4.1.1 Prevalence of Mastitis at cow level

The overall prevalence of mastitis at cow level as determined by CMT and clinical examination was 128 (83.1%) from a total population of 154 cows; the prevalence of subclinical and clinical mastitis were 89 (57.8%) and 29 (25.3%), respectively (Fig. 2).

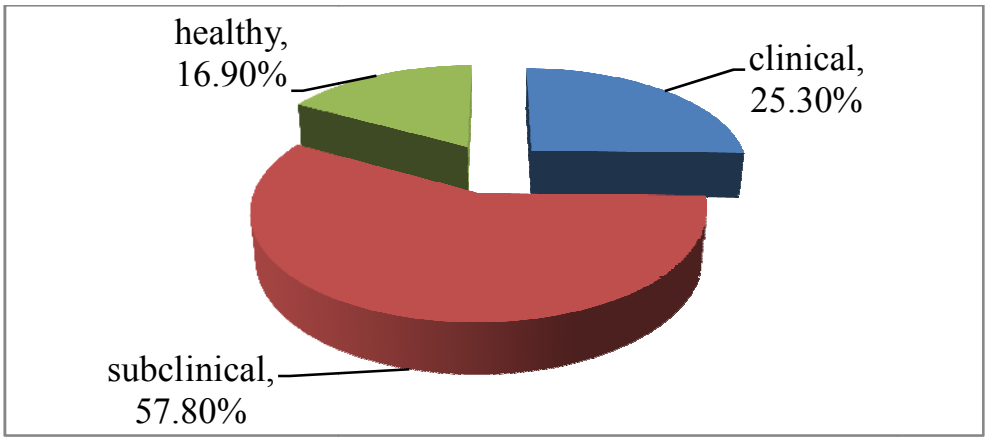


Fig. 2 Prevalence of different types of mastitis (N=154*)*

4.1.2 Prevalence of mastitis at quarter level

Out of 616 quarters examined using CMT and clinical examination methods, a total quarter of 403 (65.42%) were found affected by mastitis, 45 (7.31%) and 358 (58.12%) clinical and subclinical respectively. The quarter level prevalence of subclinical mastitis was 74 (12.01%),

100 (16.23%), 95 (15.42%) and 89(14.45%) from left front, lest hind, right front and right hind quarters, respectively (Table2.).

Table 1 Prevalence of clinical and sub-clinical mastitis at quarter level using CMT and clinical examination

Form of mastitis	**Quarter level**				**Total**
	LF	LH	RF	RH	
Clinical	8(1.30%)	12(1.95%)	15(2.44%)	10(1.62%)	45 (7.31%)
Sub-clinical	74(12.01%)	100(16.23%)	95(15.42%)	89(14.45%)	358 (58.12%)
Total	82(13.31%)	112 (18.18%)	110(17.86%)	99(16.07%)	403(65.42%)

N=616

4.1.3 Prevalence of Staphylococcus aureus caused sub-clinical and clinical mastitis at cow level

The overall cow level *S.aureus ca*used mastitis was 66 (51.56%) and the contribution of *Staphylococcus aureus* to subclinical and clinical mastitis were 36(28.1%) and 30(23.4%) respectively.

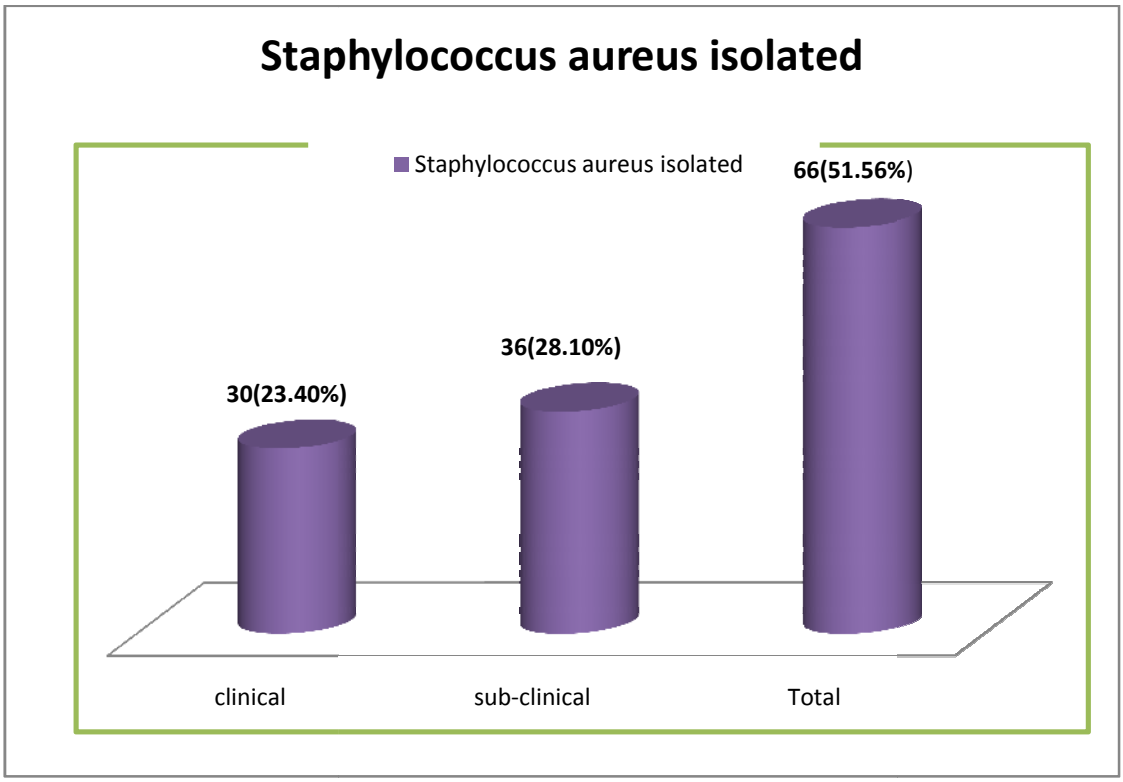


Fig 3 *Prevalence of Staphylococcus aureus caused sub-clinical and clinical mastitis at cow level*

4.1.3 Prevalence of Staphylococcus aureus caused sub-clinical and clinical mastitis at quarter level

The overall prevalence of *Staphylococcus aureus*caused mastitis at quarter level was 164 (40.69%) and the prevalence of *Staphylococcus aureus* sub-clinical and clinical mastitis at quarter level was 32.00% and 8.68% respectively (Table 2).

Table 2 *Prevalence of Staphylococcus aureus caused sub-clinical and clinical mastitis at quarter*

Type of mastitis	***S. aureus isolated* at quarter level**				**Total**
	LF	**LH**	**RF**	**RH**	
clinical	6(1.49%)	8(1.97%)	13(3.23%)	8(1.97%)	35(8.68%)
sub-clinical	20(4.96%)	42(10.42%)	40(9.93%)	27(6.70%)	129(32.00%)
Total	26(6.45%)	50 (12.4%)	53(13.15%)	35(8.68%)	164(40.69%)

N=403

4.1.4 Prevalence of MRSA at quarter level

MRSA was identified using Cefoxitin disk diffusion method (CLSI, 2012). The overall prevalence of Methicillin resistance *S. aureus* was 60 (36.6%). From the four quarters the right hind (12.8%) shows more prevalent and followed by left hind (10.4%), right front (8.5%) and Left front (4.9%), respectively (Table 3).

Table 3 Prevalence *of MRSA at quarter level*

Prevalence of MRSA	**Quarter level**				**Total**
	LF	**LH**	**RF**	**RH**	
Count	8	17	14	21	60
% of Total	4.9%	10.4%	8.5%	12.8%	36.6%

N=164

4.1.5 Differences between mastitis prevalence and MRSA prevalence

The difference between mastitis prevalence and prevalence of MRSA at quarter levels was statistically strongly significant ($p<0.05$).

Table 4 *Differences between mastitis prevalence and MRSA prevalence* using X^2 correlation

Variables Categories		**N**	**Prevalence of MRSA *S. aurous***	**X^2**	**p-value**	**OR**	**CIof OR**
Prevalence of mastitis at quarter level	Positive	403	60(14.89%)	85.20	0.000	41.34	**12.664-134.96**
	Negative	213	104 (48.83%)				
	Total	616					

4.1.6 Risk factors associated with isolation *of Staphylococcus aureus* using X^2 correlation

The questionnaire result on the risk factors of mastitis like age group and pregnancy status reveled that they had no effect on ($p>0.05$) *Staphylococcus aureus* isolation. But stage of lactation and previous mastitis history had significant effect on ($p<0.05$) isolates *of S. aurous*.

Table 5 Association between risk factors *with Staphylococcus aureus* caused mastitis at cow level using chi-square test

Variables Categories		**N**	**Prevalence of *S. aurous***	**X^2**	**df**	**p-value**
Age group	<3yrs(young)	13	6(46.2%)	0.872	3	**0.832**
	3-6 yrs(youg-adult)	68	31(45.6%)			
	6-10 yrs(adult)	58	24(41.4%)			
	>10 yrs(old)	15	5(33.3%)			
Stage of lactation	Early	16	8(50.0%)	8.790	3	**0.032**
	Mid	59	26(44.1%)			
	Late	54	16(29.6%)			
	Dry	25	16(64.0%)			
Previous mastitis history	Not-infected	109	40(36.7%)	5.780	1	**0.016**
	Infected	45	26(57.8%)			
Pregnancy status	Non-pregnant	132	53(40.2%)	2.762	1	**0.097**
	Pregnant	22	13(59.1)%			

4.1.7 Association between risk factors with Staphylococcus aureus isolates caused mastitis at cow level using a univariate logistic regression test.

Those risk factors that had significant effect ($p<0.05$) on the isolates of *Staphylococcus aureus* using X^2 correlation were titled in univariate logistic regression analysis. The result showed that the effect of stage of lactation at early and dry period and previous mastitis exposure had significant effect ($p<0.05$) for the occurrence of Staphylococcal mastitis whereas lactation stages at mid and late periods had no significant ($P<0.05$) effect on the occurrence of *S. aureus*.

Table 6 Association between risk factors *with Staphylococcus aureus isolates*

Variables	**Categories**	**N**	**Prevalence (%)**	**P=value**	**OR**	**CI of OR**
Stage of lactation	Early	16	8(50.0%)	0.038		
	Mid	59	26(44.1%)	0.377	1.778	0.496-6.366
	Late	54	16(29.6%)	0.098	2.256	0.860-5.922
	Dry	25	16(64.0%)	0.005	4.222	1.547-11.524
Previous mastitis history	**Not-infected**	**109**	**40(36.7%)**	**0.017**	**2.361**	**1.163-4.793**
	Infected	**45**	**26(57.8%)**			

The questionnaire results on the risk factors of mastitis like age group, pregnancy status, stage of lactation and previous mastitis history reveled that they had no effect on ($p>0.05$) methicillin(Cefoxitin) resistant *Staphylococcus aureus* isolates.

Table 7 Association between risk factors *with methicillin resistant Staphylococcus aureus (MRSA) isolates.*

Age group	<3yrs(young)	13	1(7.7%)	1.041	3	0.791
	3-6 yrs(youg-adult)	68	4(5.9%)			
	6-10 yrs(adult)	58	4(6.9%)			
	>10 yrs(old)	15	2(13.3%)			
Stage of lactation	Early	16	1(6.2%)	3.558	3	0.313
	Mid	59	3(5.1%)			
	Late	54	3(5.6%)			
	Dry	25	4(16.0%)			
Previous mastitis history	Not-infected	109	7(6.4%)	0.292	1	0.589
	Infected	45	4(8.9%)			
Pregnancy status	Non-pregnant	132	92(6.8%)	0.147	1	0.702
	Pregnant	22	2(9.1%)			

4.2 In-vitro *antimicrobial* susceptibility results

A total of 61 isolates of *S. aureus* species which were isolated from clinical and subclinical mastitis cases were tested for antimicrobial sensitivity for 12 different types of antibiotics.

In the present study, *S. aureus* isolates were found variably resistant to the antibiotics tested. The *S. aureus* isolates showed highest sensitivity towardsAmoxicillin-clavulinic acid (80%), Chloroamphenicol (78.7%), Nitrofuration (73.8), Cefoxitin (67.2%), Sulphamethoxazole-trimethoprim (59%). In the other groups, the pattern clearly indicated that *S. aureus* isolates were highly resistant to Clindamycin (73.8%), Bacitracin (72.1%) and Vancomicin (70.5%**).**

Also intermediate sensitivity of *S. aureus* isolates was highest towards streptomycin (19.7%), Clindamycin (8.2%) and followed by Gentamycin (4.9%) and ciprofloxacin (3.3%) (Fig 2).

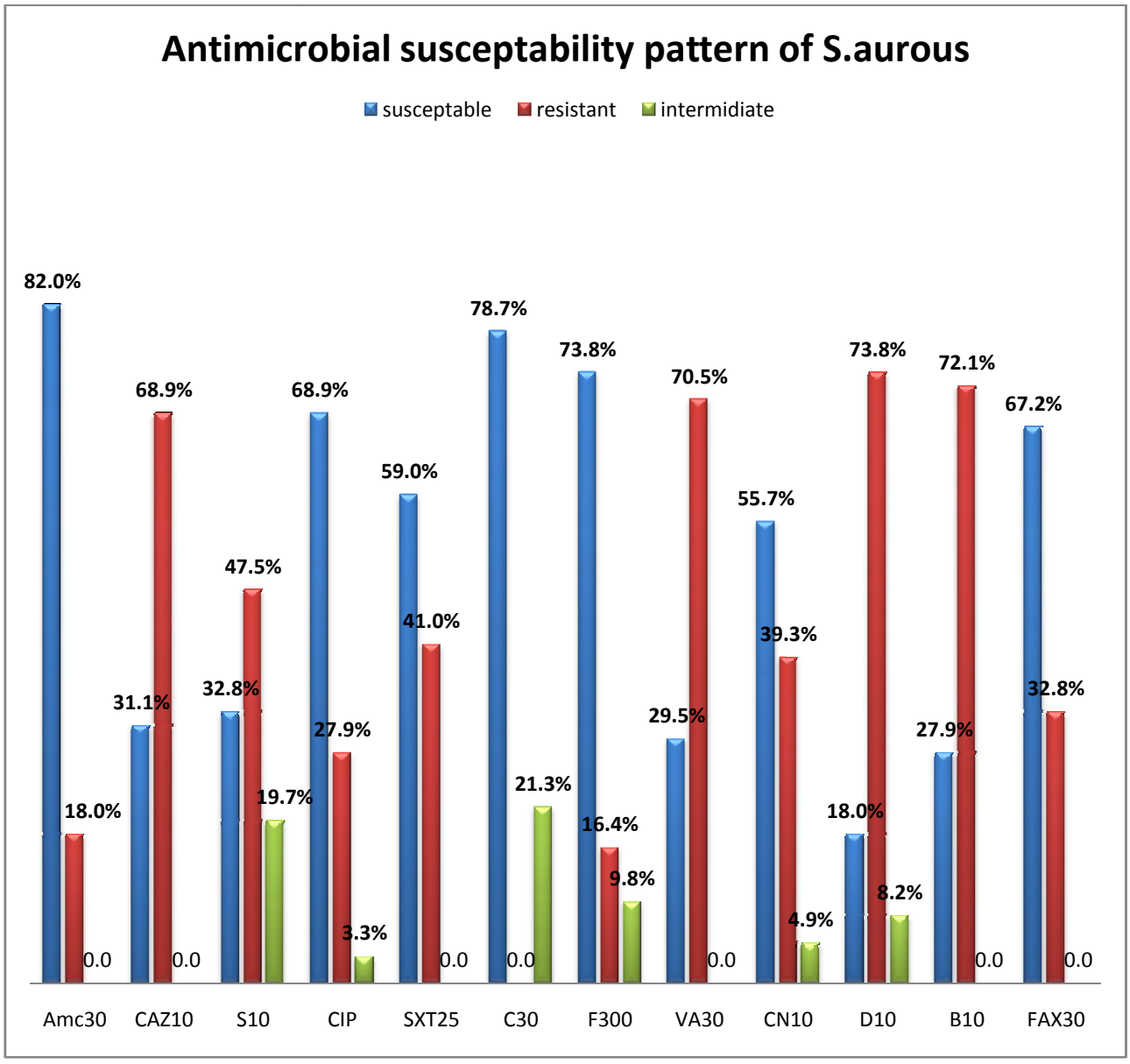


Fig.4. Susceptibility pattern of S. aureus isolated isolates susceptibility pattern of S. aureus isolated ted from cow milks (N=61)

Key:-Cefoxitin (Fax30), Amoxicillin-clavulinic acid (AMC30), Ceftazidime (CAZ30),Clindamycin (DA10),Vancomicin (VA30),Gentamicin (CN10), Streptomycin (S10),Ciprofloxacin(CIP5),Sulphamethoxazole-trimethoprim (SXT25),Bacitracin (B10),Nitrofuration (F300) and Chloroamphenicol (C30).

The most frequent multidrug resistance pattern consisting of three drugs is exhibited for, gentamicin, ceftazidime and streptomycin with a resistance of 7 (9.46%) of the isolates. Sixty four (86.46% of the isolates) were resistant to different combinations of two or above tested antibiotics (Table 7).

Table 8 Antibiograms of the total *S. aureus* isolates for multi-drug resistance

Antibiotic	Resistant strains	
	Number	%
DA	1	1.35%
CAZ	3	4.05%
CN	3	4.05%
S	3	4.05%
AMC, S	1	1.35%
VA, B	1	1.35%
DA, SXT	3	4.05%
F, CAZ	2	2.70%
AMC, B, S	1	1.35%
VA, B, CAZ	2	2.70%
CN, CAZ, S	7	9.46%
DA ,CAZ, FAX	2	2.70%
VA, DA, B	3	4.05%
VA, DA ,AMC	1	1.35%
AMC ,B, S, VA	2	2.70%
FAX ,SXT, S, DA	1	1.35%
VA ,DA ,B, CIP	3	4.05%
VA, DA, B, CAZ	2	2.70%
S, CN ,CAZ, S	3	4.05%
VA ,CN, DA, S	1	1.35%
VA ,CN, B, S, SXT	2	2.70%
DA, B, CAZ, CIP, SXT	1	1.35%
VA, DA ,B, CIP, CAZ	1	1.35%
VA, CN, DA, B, CAZ	3	4.05%
VA, CN, DA, B, F	1	1.35%
VA, DA, B,CAZ ,F, FAX	2	2.70%
VA, CN, DA, B, AMC, S	1	1.35%
VA, CN, DA, B, SXT, S	3	4.05%
VA, CN, DA, B, SXT, FAX	1	1.35%
VA, DA, B, CAZ, CIP, SXT, FAX	5	6.76%
S, FAX, SXT, S,CAZ ,B, DA	3	4.05%
VA, DA, B, CAZ, CIP, SXT, FAX	1	1.35%
VA, DA ,B, AMC, CAZ, CIP, SXT, FAX	3	4.05%
VA ,DA, B, AMC ,CAZ, CIP, SXT, F	2	2.70%
Total	74	100%

5. DISCUSSISION

In the present study, the overall prevalence of mastitis at cow and quarter levels in large, medium and small holderdairy farms of the Selale/Fitche area is in line with the finding of Argaw and Tolosa (2007) conducted in the same area 89.5% and 63.1% at cow and quarter level, respectively atsmall holderdairy farmsin Selale. Similarly, it was closely comparable with the findings of Muluneh (2013), Duguma *et al*. (2013) andMekibib*et al. (*2010), who reported 72.16% and 42.23% at Alage dairy farm 81.1% and 80.88% at Holleta agricultural research centre, and also71.0% and 44.9% in dairy farms of Holeta town, respectively. In addition, the lower occurrence of mastitis in local breeds in addition to genetic factors could also be one indication for higher occurrence of mastitis prevalence in areas where exotic breeds and their hybrids well adapted and in our study area exotic breeds (Holstein Fresianbreeds) are well adapted. The report from Biffa *et al*. (2005) confirmed that Holstein Fresian pure breeds were affected at a higher rate both by clinical (26.3%) and subclinical (30.1%) mastitis than local breads.

5.1 Prevalence of clinical and subclinical mastitis at cow and quarter level

Clinical mastitis is only the 'tip of the iceberg'. Subclinical mastitis is by far the more costly disease in the majority of herds, and is often defined as the presence of a microorganism in combination with an elevated somatic cell count (SCC) of the milk. In this study sub clinical mastitis is more prevalent than clinical mastitis at cow and quarter level and the same per individual quarter levels. This also provides further support of other studies in different region of the country which have concluded that sub-clinical mastitis is more prevalent than clinical mastitis Biffa *et al*. (2005), Mekibib *et al. (*2010) and Duguma *et al*. (2013) who reported 23.0% and11.9%, 48.6% and 22.4% and 73.3% and 7.8% sub clinical and clinical mastitis, respectively. This is likely to be partly influenced by virulence of the circulating bacterial strains and the levels of immunity of the cows to these pathogens. In addition most smallholder farmers are not well informed or don’t know about sub-clinical mastitis and they were surprised during our field work when they saw CMT positive milk reaction while it appeared to them normal milk before the test was conducted.

The prevalence of mastitis at quarter level both clinical and subclinical agrees with the findings of Duguma *et al*. (2013) who found 5.59% and 75.3% clinical and subclinical mastitis in Holleta agricultural research centre. However, it is lower for subclinical (32%) and comparable for clinical (10%) reported by Abera*et al*. (2013) in Asella government dairy farm.

5.2 Staphylococcal isolates in bovine mastitis

In this study, the isolation ***S. aureus*** from clinical and CMT positive milk samples from each quarter were done. The findings of this study are in line with the findings which were done around Sebeta (44.03%) by Sori *et al*. (2005), in Holleta agricultural research centre (43.3%) by Duguma*et al*. (2013), in Hawassa area (48.75%) by Daka*et al*. (2012), in and around Holeta town (47.1%) by Mekibib*et al. (*2010) and in DebreZiet area (39.5%) by Addis *et al.* (2011). It was also closely comparable with findings of Lakew*et al*. (2009), Ndegwa*et al.* (2000) and Bedada and Hiko (2011) who reported 41.1% and 43.3%, 39.1% in dairy cows, respectively.However, the present findings are lower than that of Hussein*et al*. (1997), Bishi (1998) and Mekuria*et al*. (2013) who reported 10%, 9% and 16.2% prevalence in Addis Ababa, respectively.The high prevalence of this organism may be associated with its frequent colonization of teats, its ability to exist intracellular and localize within micro abscesses in the udder and hence resistant to antibiotic treatment (MacDonald, 1997). The bacteria usually establish chronic, subclinical infections and are shed in the milk, which serves as a source of infection for other healthy cows during the milking process. Transmission among cows increase whenever there is lack of effective udder washing and drying, post- milking teat dip and drying, inter-cow hand-washing and disinfection, washing clothes and milking machine cups (Radostitis *et al*., 2007). Therefore, the *S. aureus* occurrence at a considerable high percentage indicates the alarming situation for dairy farms.

5.3 MRSA isolates in bovine mastitis

Methicillin-resistant *Staphylococcus aureus* (MRSA) are the strains of *S. aureus* that are resistant to all the β lactam antibiotics. MRSA has received a lot of attention in recent years as a zoonotic organism when studies suggested the possibility of animals serving as reservoirs for human MRSA infection (Joshi *et al*., 2014). In this study larger numbers of Methicillin (Cefoxitin) resistant isolates were obtained from the mastitic milk in this study area which is lower than that of Daka *et al*. (2012) who found 60.3% but it was higher than the findings of Joshi *et al*. (2014) who reported lower prevalence of MRSA (Cefoxitin, 11.25%) in Nepal which is well known that Cefoxitin is not used for veterinary practice in the study area. However, Methicillin resistance could be explained by the cross transmission between human and animals (Juhász-Kaszanyitzky *et al*., 2007).

In the study of Diab *et al*. (2008) stated thatCefoxitin disk diffusion assays exhibited sensitivity of 100% andspecificity of 90% for identification of methicillin-sensitive isolates.Detection of mecA gene and PBP2' may be a further requirement to justify this exceptionally high level of cefoxitin resistant isolates which is not included in the present study.

In this study, it was also observed that MRSA isolates were also resistant to beta-lactam antibiotics such as clindamycin (73.8%), Bacitracin (72.1%), Ceftazidime (68.9%) and vancomicin (70.1%).This result is also in line with Daka *et al*. (2012) who found MRSA strain resistant to oxacillin, penicillin, ampicilin and 26.5% of the oxacillin resistant *S. aureus* were found resistance to vancomicin.

In this study the difference between mastitis prevalence and prevalence of MRSA at quarter levels was statistically significant ($p<0.05$). The strong difference between mastitis and MRSA may be due to that occurrence of MRSA is not always related to the intramammary infection. Only a few mammary infections of the dairy cow by MRSA strain have been reported and no information is available on the interaction between the bacteria and the mammary gland (pilla *et al*., 2012). In addition the occurrence of mastitis can be related to more than 137 micro-

organisms: includes bacteria, certain fungi and yeasts (Gruet *et al.,* 2001). But methicillin-resistant staphylococci have been infrequently isolated from cases of clinical and subclinical bovine mastitis (Huijsdens *et al*., 2006; Monecke *et al*., 2007; Wang *et al*., 2008). Furthermore, the origin of MRSA intra-mammary infections in dairy cattle has been difficult to define.

5.4 Effects of risk factors on the occurrence of *S. aureus* and MRSA isolates.

Many reports on treatment trials of *S. aureus* mastitis do not contain detailed descriptions of hostfactors of the treated animals, or of the strains causing the infections that are treated (Barkema *et al*., 2006). Risk factors associated with isolation of *Staphylococcus aureus* using X^2 correlationlike age group and pregnancy status revealed that they had no effect on *Staphylococcus aureus*isolation. This observation agrees with the findings of Mekuria*et al*. (2013),Grace *et al*. (2009) and Denis *et al.* (2008). Whereas, the stage of lactation and previous mastitis history had significant effect on isolation *of S. aureus* which do not agree with the report of Mekuria *et al*. (2013).

Those risk factors that had significant effect ($p<0.05$) on the isolation of *Staphylococcus aureus* using X^2 correlation were titled in univariate logistic regression analysis. The result showed that the effect of stage of lactation at early and dry period and previous mastitis exposure had significant effect ($p<0.05$) for the occurrence of Staphylococcal mastitis. Whereas, lactation stages at mid and late periods had no significant ($P<0.05$) effect on the occurrence of *S. aureus*. In the previously infected animals, the Staphylococcal isolates which were responsible to the previous infection were not eliminated by the effect of various antibiotics which was related to the development of drug resistance by Staphylococci organisms. But mainly, mastitis is a complex disease involving interactions of several factors, mainly of management, environment, and factors relating to animal and causative organisms.

The questionnaire results on the risk factors of mastitis like age group, pregnancy status, stage of lactation and previous mastitis history revealed that they had no effect on methicillin (Cefoxitin) resistant *Staphylococcus aureus* isolation.

5.5 Antimicrobial susceptibility pattern

The presence of antibiotic-resistant *S. aureus* has been reported to negatively affect the treatment of its associated infections in humans and animals. Therefore, it was believed that an investigation of the antibiotic-resistance profiles of these isolates may serve as a tool for proper drug administration and effective drug choices and also the health risks that humans may encounter when infected by antibiotic-resistant strains.

The causes of antibiotic resistance is explained by several authors and reported numerous mechanisms of conferring resistance such as: antibiotic-resistant genes, mutation, clonal evolution and plasmid transfer, target site alteration of ribosome, metabolic pathway alteration, efflux pumps and enzymatic cleavage of antibiotics (Jayaraman, 2009; Malhotra-Kumar *et al*., 2010). Recently, Durai *et al. (*2010) has discussed the causes of these resistance as a results of a protein (penicillin-binding protein 2a [PBP2a]).

The observations made in the present study unequivocally proved that *S. aureus* showed resistance to all antimicrobials tested except for chloroamphenicol (C30). This shows that the existence of alarming level of resistance of *S. aureus* to almost all commonly used antimicrobials in dairy farms and human medicine. In the present study, only three drugs have shown less resistant, 0 to 25% of the total isolates tested. These drugs were chloroamphenicol (0%), nitrofuration (16.4%**)** andAmoxicillin-clavulinic acid (18%). The reason why these antimicrobials were less resistant might be that they are not used in the study area in veterinary services, and even not frequently used perhaps in human medicine. Similar suggestion was given by Mekuria*etal*. (2013) that the development of antimicrobial resistance is nearly always as a result of repeated therapeutic and/or indiscriminate use of them. However, most of the isolates were resistant to Streptomycin, other β-lactams and Vancomicin (Macrocyclic Peptide**)**. This is due to the fact that not all of the above mentioned drugs are used for veterinary cases but it might indicate transfer of resistant strain among environment, livestock and human (Mekuria*et al*., 2013). The present study showed that the resistance of *S. aureus* to amoxicillin-clavulinic acid (18%), Sulphamethoxazole-trimethoprim (41%), Chloroamphenicol (0%), Gentamycin (39.3%) and Clindamycin (73.8%) observed in milk

samples. It disagrees with the observation made by Mekuria*etal*. (2013) in the case of amoxicillin-clavulinic acid (35.7%), Chloroamphenicol (23.8%), Sulphamethoxazole-trimethoprim (21%), Gentamycin (19%), Clindamycin (16.7%) observed in milk samples taken from dairy cows around Addis Ababa and higher than the findings reported by Daka*et al*.(2012) in Hawassa area. The probable explanation could be that *S. aureus* strains have the capacity to change their resistance behavior to the exposed antimicrobials.

Furthermore, the resistance profile of *S. aureus* to Clindamycin (73.8%), Bacitracin (72.1%), Vancomicin (70.1%) and Ceftazidime (68.9%) in dairy cows was found to be high. This in line with Daka*et al*. (2012) who reported that the β-lactam drugs to which a large proportion of the isolates were resistant. This is due to the fact that resistance of *S. aureus* to these drugs may be attributed to the production of β-lactamase, an enzyme that inactivates pencillin and closely related antimicrobial. It is believed that about 50% of mastitis causing *S. aureus* produces β-lactamase (Green and Bradely, 2004). Uncommonly 70.1% of *S. aureus*isolates were found resistant to Vancomicin but in a study conducted in South Africa, 100% of the isolates from two commercial farms were susceptible to Vancomicin (Larsen *et al*., 2000). Resistance to Clindamycin (73.8%), Bacitracin (72.1%), and Ceftazidime (68.9%) is thus used as a marker to assess the susceptibility of *S. aureus* isolates against other beta-lactam antibiotics.

There are several different combinations of the tested antibiotics which give very huge numbers of multidrug resistance patterns by the organism. The drug combinations resisted by the isolates contain one up to nine of the antibiotics tested (Table 7).

The most frequent multidrug resistance pattern consisting of three drugs is exhibited for gentamicin, ceftazidime and streptomycin with a resistance of 7 (9.46%) of the isolates. It was found that almost above 86.46% of the isolates were fond resistant to different combinations of two and above tested antibiotics. This result almost comparable the finding of Wubishet *et al*. (2012) who found that (55.5% of the isolates) were resistant to different combinations of two of the tested antibiotics and almost 100% of isolates were resistant to two or more than two combinations. This multi drug resistance occurred might be due to administration of

multiple antibiotics for prophylaxis or infection, discriminate use of antibiotics in the farms and another possibility is that cattle are being treated with antibiotics for other conditions, thereby selecting for resistant populations of *S. aureus.* Such multi drug resistance may apparently be occurred which may ultimately replace the drug sensitive microorganisms from antibiotic saturated environment

6. CONCLUSIONS AND RECOMMENDATIONS

Still the prevalence of mastitis was high both at cow and quarter level in the study area like previous reports from different areas in Ethiopia. The isolation of 40.69% *S. aureus* from the infected milk sample shows that *S. aureus* remains the major role player on the high prevalence of mastitis especially for subclinical mastitis. Phonotypical isolation MRSA isolates also shows that high prevalence of MRSA in mastitic milk at the study area which may present a potential public health risk and MRSA may spread among animals and between animals and humans as previously reported by other authors. Resistance of MRSA to multiple antibiotics is a more realistic risk to animal and public health if found to cause invasive diseases other than MRSA.

The observations made in the present study unequivocally proved that *S. aurous* showed resistance to all antimicrobials tested except for chloroamphenicol (C30). This shows that the existence of alarming level of resistance of *S. aurous* to almost all commonly used antimicrobials in dairy farms and human medicine.

A large proportion of the *S. aureus* isolates obtained were resistant to two or more antibiotic combinations. This indicates that the presence of higher prevalence of multidrug *S. aureus* in dairy cows may result on great risk for consumers and individuals who have contact with animals.

Based on above conclusions the following recommendations are forwarded:

- Extensions packages that increase farmers' awareness on subclinical mastitis would be helpful in mastitis control and improve farmers' income.

- Therefore, careful monitoring of the resistance status of *S. aureus* in dairy environments is needed, as *Staphylococcus aureus* transmission is dynamic and involves humans, animals, and likely the farm production environment.

- The high level of MAR *S. aureus* result in this study and the implications there for warrant for further investigation.

- The occurrence of multidrug resistance *S. aureus* should be under consideration during selection of antimicrobials for treatment of mastitis.

7. REFERENCE

Abera B., Lemma D. and Iticha I. (2013): Study of bovine mastitis in asella government dairy farm of Oromia Regional state, South Eastern Ethiopia.Int. J. Curr.Res. Aca. Rev.,**1** (2):134-145.

Addis M., pal M. and Kyule M.N. (2011): Isolation and Identification of *Staphylococcus* Species from raw bovine milk in Debre Zeit, Ethiopia**. 4: (**2), 45-49**. (**Med well publisher)**.**

American Food and Drug Administration (FDA) (2005): Bacteriological Analytical Manual, 18th Ed. AOAC, Washington, DC.

Angulo F., Nargund V.N., Chiller T.C. (2004): Evidence of an association between use of antimicrobial agents in food animals and antimicrobial resistance among bacteria isolated from humans and human health consequences of such resistance. *J. Vet. Med*. **51**: 374-379.

Argaw K. and Tolosa T. (2007): Prevalence of sub clinical mastitis in small holder dairy farms in Selale, North Shewa 144 Zone, Central Ethiopia. *Inter. J. Vet. Med*. ISSN: , **5**:1937-8165

Arnold M. and Bewley J. (2011): *Staphylococcus aureus* Mastitis. Cooperative extension service university of Kentucky, college of agriculture, lexington, ky 40546, UK.

Athar M., (2007): Preparation and evaluation of inactivated polyvalent vaccines for control of mastitis in dairy buffaloes. Ph.D. Thesis, Department of Clinical Medicine and Surgery, University of Agriculture Faisalabad, Pakistan.

Bagcigil F.A., Moodley A., Baptiste K.E., Jensen V.F., Guardabassi L. (2007): Occurrence, species distribution, antimicrobial resistance and clonality of methicillin- and erythromycin-resistant staphylococci in the nasal cavity of domestic animals. *Vet. Microbiol*., **121**:307–315.

Baker J. S. (1984): Comparison of various methods for differentiation of *staphylococci* and *micrococci*. *J. Clin. Microbiol*., 1**9**: 875–879.

Bedada B.A and Hiko A. (2011): Mastitis and antimicrobial susceptibility test at Asella, Oromia Regional state, Ethiopia. *J. Microbiol. Antimicrob*. **3**: 228-232.

Benić M., Habrun B. and Kompes G. (2012): Clinical and epidemiological aspects of cow mastitis caused by *Staphylococcus aureus* and its methicillin-resistant strains. Medical Sciences Croatian Veterinary Institute, Zagreb,Croatia ,**37** :113-122.

Berger-Bachi B. and Rohrer S. *(2002):* Factors influencing methicillin resistance in staphylococci. *Arch. Microbiol.,* **178***:*165-71.

Biffa D., Etana D. and Fekadu B. (2005): Prevalence and Risk Factors of Mastitis in Lactating Dairy Cows in Southern Ethiopia. *Intern. .J. Appl. Res. Vet. Med.,* Hawassa College of Agriculture, Debub University, Hawassa, Ethiopia, **3**: (3), 189-198.

Bishi A. (1998): Cross-sectional and longitudinal prospective study of bovine mastitis in peri -urban and urban dairy production systems in the Addis Ababa region, Ethiopia. MSc Thesis, Faculty of Veterinary Medicine, Addis Ababa University School of Graduate studies and Freje Uiversity, Berlin, Pp 1-50.

Brook I. and Frazier E.H (1995): Clinical features and aerobic and anaerobic microbiological characteristics of cellulites. *Arch. Surg.*, **130**:786-792.(PubMed Abstract Publisher Full Text).

Brown D.F., Edwards D.I., Hawkey P.M., Morrison D., Ridgway G.L., Towner K J, *et al (*2005): Guidelines for the laboratory diagnosis and susceptibility testing of methicillin-resistant *Staphylococcus aureus* (MRSA). J. Antimicrob. Chemother.,**56** (6):1000-18.

Bukharie H.A., Abdelhadi M.S., Saeed I.A., Saeed I.A., Rubaish A.M. and Larbi E.B. (2001): Emergence of methicillin-resistant *Staphylococcus aureus* as a community pathogen. *Diagn. Microbiol. Infect. Dis.,* **40**: 1-4.[PubMed].

Calfee D.P. (2011): "The epidemiology, treatment and prevention of transmission of methicillin-resistant *Staphylococcus aureus*". *J. Infus. Nurs*., **34** (6): 359–64.

Catry B., Van Duijkeren E., Pomba M.C., Greko C., Moreno M.A., Pyörälä S., Ružauskas M., Sanders P., Threlfall, E.J.,Ungemach F., Törneke K., Muñoz-Madero C. and Torren-Edo J. (2010): Reflection paper on MRSA in food-producing and companion animals: epidemiology and control options for human and animal health. *Epidemio.l Infect*., **138:**626–644.

Chambers H.F (2001): The changing epidemiology of *Staphylococcus aureus*? *Emerg.*

Chu C., Changyou Y., Yanhaui L. and Yaochi S. (2012): Genetically divergent methicillin-resistant *Staphylococcus aureus* and sec-dependent mastitis of dairy goats in Taiwan. *Bio.Med. Cent. Vet. Rese.*, **8**:39.

Clinical and Laboratory Standards Institute (CLSI) (2012): Performance Standards for Antimicrobial susceptibility testing; Twenty-second informational supplement: CLSI document M100-S22. Clinical and Laboratory Standards Institute, Wayne, PA, USA, **32** (3):70-90.

Cohn L.A. and Middleton J.R. (2010): A veterinary perspective on Methicillin-resistant Staphylococci: State-of-the –art-Review. *J. Vet. Emerg. Crit. Care*, **20(**1): 31–45. DOI: 10.1111/j.1476-4431.2009.00497.x.

Daka D, Solomon G/ S, Yihdego D. (2012): Antibiotic-resistance *Staphylococcus aureus* isolated from cow's milk in the Hawassa area, South Ethiopia. *Ann. Clin. Microbiol. Antimicrob*., **11**:26-37.

Denis O., Suetens C., Hallin M., Ramboer I., Catry B., Gordts B., Butaye P. and Struelens M.J. (2008): *Staphylococcus aureus* ST398 in swine and pig farmers in Belgium. 18th *Eur. Congress of Clin. Microbiol. and Infect. Disea. (ECCMIC)*. Barcelona, Spain, 55-73.

Deurenberg R.H. and Stobberingh E.E. (2008): The evolution of *Staphylococcus aureus*. *Infec. Genet Evol.,* **8:** 747-763.

Devriese L.A., Van- Damme L.R. and Fameree. L. (1972): Methicillin (cloxacillin)-resistant Staphylococcus aureus strains isolated from bovine mastitis cases. In: Gindonis *et a*l. (eds): Occurrence and characterization of Methicillin resistant staphylococci from bovine mastitis milk samples in Finland. *Zentralbl Vet*. *Armed B*., **19**:598–605.

Diab M., El-Damarawy M. and Shemis M. (2008): Rapid identification of methicillin-resistant staphylococci bacteremia among intensive care unit patients. Medscape J. Med. 28; 10(5):126.

Diekema D.J., Pfaller M.A., Schmitz F.J, Smayevsky J., Bell J., Jones R.N.and Beach M. (2001): Survey of infections due to Staphylococcus species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the sentry Antimicrobial

Surveillance Program, 1997–1999. *Clin. Infect. Dis.*, **32**(2)**:**S114-S132. PubMed Abstract | Publisher Full Text

Duguma A., Tadele T.and Aster Y. (2013): Prevalence of clinical and sub-clinical mastitis on cross bred dairy cows at Holleta Agricultural Research Center, Central Ethiopia. *Glob. J. Vet. Med. Res. 027*, **1** (1): 026-030.

Durai R., Ng P. C. H. and Hopue H. (2010): Methicillin-resistant *Staphylococcus aureus*: an update. *Aorn J.,* **91**(5): 599-609.

Ebrahimi A., Naser S., Somayeh S. and Pezhman M. (2010): Characteristics of Staphylococci isolated from mastitic goat milk in Iranian dairy herds. In: Deinhofer, M. and Pernthaner, A. (eds): *Staphylococcus spp*.as mastitis-related pathogens in goat milk. *Vet. World,* **3** (5): 205-208 from department of Pathobiology, School of Veterinary Science, Shahrekord University, Shahrekord, Iran.

Ewing W. H., (1986): Edwards and Ewings identification of Enterobacteriaceae , 4th ed. Elsevier Science Publishing Co, Inc., New York, N.Y.

Fekadu K., (1995): Survey on the prevalence of bovine mastitis and the predominant causative agents in chaffa valley. Proceedings of the 9th conference of Ethiopian veterinary Association: Addis Ababa, Ethiopia. Pp.101- 111.

Fey P. D., Saïd-Salim B., Rupp M. E., Hinrichs S. H., Boxrud D. J. Davis C. C. Kreiswirth B. N. and Schlievert P. M. (*2003):* Comparative Molecular Analysis of Community- or Hospital-Acquired Methicillin-Resistant *Staphylococcus aureus***.** *J. Clin. Microbiol. ,***41** (8): 3806-3815.

Fitzgerald J.R., Sturdevant D.E., Mackie S.M., Gill S.R. and Musser J.M. (2001): Evolutionary genomics of *Staphylococcus aureus*: insights into the origin of

methicillin-resistant strains and the toxic shock syndrome epidemic. *Proc. Natl. Acad. Sci*., USA, **98**: 8821–8826.

Forbes B. A., Sahm A. S. and Weissfeld D. F. (1998): Bailey and Scotts Diagnostic Microbiology, 10th Ed. Mosby, Inc., St. Louis, Mo.

Franco J.C., González L.V., Gómez S.C., Carrillo J.M. and Ramírez J.J.C. (2008): Virulence factors analysis of *Staphylococcus aureus* isolated from bovine mastitis in México. *eGnosis*, ISSN, **6** (7): 1665-5745. www.e-gnosis.udg.mx/vol6/art7.

Garcia-Graell C., Antoine J., Larsen J., Catry B., Skov R. and Denis O. (2012): Livestock veterinarians at high risk of acquiring methicillin-resistant *Staphylococcus aureus* ST398. *Epidemiol. Infect.*, **140:** 383–389.

Gerhardt P., Murray R. G. E., Wood W. A. and Krieg N. R**.** (1981): Methods for general and molecular bacteriology. ASM Press, Washington, D.C.

Gindonis V., Taponen S., Myllyniemi A.L., Pyörälä S., Suvi N., Saara S., Laura L. and Merja R. (2013): Occurrence and characterization of Methicillin resistant staphylococci from bovine mastitis milk samples in Finland. *Acta .Veterinaria Scandinavica*, **55**:61.

Grace M.L., Susan S.H., Sheryl L.R., Virginia L.H., Stephen I., Pelton K.K., William P.H., Marc L., Alexander J.M. and Jonathan A.F. (2009): Epidemiology and risk factors for *Staphylococcus aureus* colonization in children in the post-PCV7 era. *BMC Infect. Dis***.** **9**: 110.

Green M. and Bradley A. (2004): *Staphylococcus aureus* mastitis in cattle. Clinical Forum, *UK. VET. J*., **9** (4):1-9.

Gruet P., Maincent P., Berthelot X. and Kaltsatos V. (2001): Bovine mastitis and intramammary drug delivery: review and perspectives. *Adv. Drug Del. Rev*., **50**: 245-259.

Herold B.C., Immergluck L.C., Maranan M.C., Lauderdale D.S., Gaskin R.E., Boyle-Vavra S., Leitch C.D.and Daum RS. (1998): Community-acquired methicillin-resistant *Staphylococcus aureus* inchildren with no identified predisposing risk. *JAMA: the Journal of the American MedicalAssociation* , 279: 539-598

Hoen B., Alla F., Selton-Suty C., Alla F., Selton-Suty C., Béguinot I., Bouvet A., Briançon S., Casalta J.P., Danchin N., Delahaye F., Etienne J., Le Moing V., Leport C., Mainardi J.L., Ruimy R. and Vandenesch F.(2002): Changing profile of infective endocarditis: results of a 1-year survey in France. *J.A.M.A*., **288:75**-81.(PubMed Abstract).

Huber H., Koller S., Giezendanner N., Stephan R. and Zweifel C. (2010): Prevalence and characteristics of methicillin-resistant *Staphylococcus aureus* in humans in contact with farm animals, in livestock and in food of animal origin, Switzerland, 2009. *Eur. Surveill 2010,* **15**: pii19542. *Infect. Dis.,* **7**: 178-182.

Huijsdens X.W., van-Dijke B.J., Spalburg E., van Santen-Verheuvel M. G.**,** Heck M. E.**,** Pluister G. N.**,** Voss A.**,** Wannet W. J.B. and de Neeling A. J. (2006): Community acquired MRSA and pig-farming. Ann. Clin. Microbiol. Antimicrob., **5**:26.

Hussien N., Yehualashet T. and Tilahuan G. (1997): Prevalence of mastitis in different local and exotic breeds of milking cows. *Ethiope J. Agric. Sci*. **16**: 53-60.

Intrakamhaeng M. And Komutarin T. (2012): Antibiotics resistance and RAPD-PCR typing of multidrug resistant MRSA isolated from bovine mastitis cases in Thailand. *Scie. Asia*, **38**: 30–35.

Javaloyas M., Garcia-Somoza D. and Gudiol F. (2002): Epidemiology and prognosis of bacteremia: a 10-years study in a community hospital**.** *Scand. J. Infect. Dis.,* **34:**436-441. (PubMed Abstract | Publisher Full Text).

Jayarao B.M. and Cassel E.K. (1999): Mastitis prevention and milk hygiene practices adopted by dairy practitioners. *Large Anim. Pract*., **20:**6–14.

Joshi L.R., Tiwari A., Devkota S.P., Khatiwada S., Paudyal, S. and Pande K R. (2014): Prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in dairy farms of Pokhara, Nepal. *Inter. J. Vet. Sci*., **3**(2): 87-90. www.ijvets.com.

Juhasz-Kaszanyitzky E., Janosi S., Somogyi P., Dan A., Van derGraaf-van Bloois L., Van Duijkeren E.and Wagenarr J.A. (2007): MRSA transmission between cows and humans. *Emerg. Inf. Dis.,* **13**:630-632.(PMC abstract).

Karami S., Rahbar M. and Yousefi J.V. (2011): Evaluation of Five Phenotypic Methods for Detection of Methicillin Resistant *Staphylococcus aureus* (MRSA**).** *Iranian J. Pathol*., **6** (1), 27 – 31.

Kloos W.E., Bannerman T.L., Murray P.R., Baron E.J., Pfaller M.A., Tenover F.C. and Yolken, R.F. (1995): *Staphylococcus* and *Micrococcus*. *In*: Manual of Clinical.

Kluytmans J., van- Leeuwen W., Goessens W., Hollis R., Messer S., Herwaldt L., Bruining H., Heck M., Rost J., van Leeuwen N., *et al.* (1995): Food-initiated outbreak of methicillin-resistant *Staphylococcus aureus* analyzed by phenol and genotyping. J. *Clin. Microbiol*., **33:** 1121–1128.

Koch F. E**.** (1942): Electivnährboden für Staphylokokken. Zentr. Bakt. Parasitenk. I Orig. **149:**122–124.

Kwon N. H., Kun T. P., Jin S. M., Woo K. J. and So H. K. (2005): Staphylococcal cassette chromosome mec (SCCmec) characterization and molecular analysis for methicillin-resistant Staphylococcus aureus and novel SCCmec subtype IVg isolated from bovine milk in Korea. *J. Antimicrob. Chemother.,* **56**:624–632.

Lakew M**.**, Tolesa T**.**, Tigrie W**.** (2009): Prevalence and Major bacterial causes of bovine mastitis in Assela, South Eastern, Ethiopia. *Trop. Anim. Health Prod*.**, 41**: 1525-1530.

Larsen H.D., Sloth K.H., Elsberg C., Enevoldsen C., Pedersen L.H., Eriksen N.H., Aarestrup F.M.and Jensen N.E.(2000): The dynamics of Staphylococcus aureus intramammary infections in nine Danish dairy herds. *Vet. Microbiol*., **71**:89-101. (PubMed Abstract).

Lee H.L. (2003): Methicillin (Oxacillin)-resistant *Staphylococcus aureus* strains isolated from major food animals and their potential transmission to humans. *Appl. Environm. Microbiol.***, 69:**6489-6494.

Leonard F. C. and Markey B.K. (2008): Methicillin-resistant *Staphylococcus aureus* in animals: *A rev.Vet.J,.* **175**: 27–36.

Livermore D.M. (2000): Antibiotic resistance in staphylococci. *J. Antimicrob. Agents.,***16:** 53-10.

Loeffler A. and Lloyd, D.H. (2010): Companion animals: a reservoir for methicillin resistant*Staphylococcus aureus* in the community? *Epidemiology and Infection*.,**138**: 595-605.

Lourdes M., Cunha R., Sinzato Y. and Silveira L. (2004): The medical significance and identification of coagulase negative staphylococci. *J. Clin. Microbiol.,* **99:** 855-860.

Løvseth A., Loncarevic S. and Berdal K.G. (2004): Modified multiplex PCR method for detection of pyrogenic exotoxin genes in staphylococcal isolates. In: Chu C., Changyou Y., Yanhaui L. and Yaochi S. (eds): Genetically divergent methicillin-resistant *Staphylococcus aureus* and sec-dependent mastitis of dairy goats in Taiwan. *J. Clin. Microbiol*., **42**:3869-3872.

MacDonald U.S. (1997): Streptococcal and Staphylococcal mastitis. *J. Am. Vet. med. Assoc.* 170:1157.

MacFaddin J.F. (1985*):* Media for isolation-cultivation-identification-maintenance of medical bacteria, 1st ed. Williams & Wilkins Co., Baltimore, MD., **1**: 263, 535, 540, 564, 620.

Mahon C. R. and Manusekis **G. (**1995): Textbook of diagnostic microbiology. W.B. Saunders Company, Philadelphia, P 1062.

Malhotra-Kumar R.,Yadav B. R. And Singh R. S. (2010): Genetic determinants of antibiotic resistance in *Staphylococcus aureus* isolates from milk of mastitic crossbred cattle. *Curr. Microbiol*., **60**: 379-386.

Manian F.A. (2003): Asymptomatic nasal carriage of mupirocin-resistant methicillin-resistant *Staphylococcus aureus* in a pet dog associated with MRSA infection in household contacts. *Clin. Infect. Dis.,* **36**: 26-28.

Mekibib B., Furgassa M., Abuna F., Megersa B. and Regassa A. (2010): Bovine mastitis: prevalence, risk factors and major pathogens in dairy farms of Holeta town, Central Ethiopia. *Vet. World*, **9**(3): 397 - 403.

Mekonnen H., Workineh S., Bayleyegne M., Moges A. and Tadele K. (2005): Antimicrobial susceptibility profile of mastitis isolates from cows in three major Ethiopian dairies. *Rev. Med. Vet.,* **176**: 391-394.

Mekuria A.S., Daniel A., Yimtubezinash W. and Genene T. (2013): Identification and antimicrobial susceptibility of *Staphylococcus aureus* isolated from milk samples of dairy cows and nasal swabs of farm workers in selected dairy farms around Addis Ababa, Ethiopia. *African J. micriobiol. Res.,* **7**(27):3501-3510.

Middleton J. R. (2013): *Staphylococcus aureus* mastitis: have we learned anything in the last 50 years? NMC Regional Meeting Proceedings, University of Missouri, Columbia, Missouri, USA.

Monecke S., Kuhnert P., Hotzel H., Slickers P. and Ehricht R. (2007): Microarray based study on virulence-associated genes and resistance determinants of *Staphylococcus aureus* isolates from cattle. *Vet. Microbiol*., **125** (1–2):128–140.

Moon J.S., Lee A.R., Kang H.M., Lee E.S., Kim M.N., Paik Y.H., Joo Y.S. and Koo H.C. (2007): Phenotypic and genetic antibiogram of methicillin-resistant staphylococci isolated from bovine mastitis in Korea. *J. Dairy Scie*. **90**:1176–1185.

Morrison R. (2008): The coagulase test in the identification of pathogenic staphylococci. *J. Appl. Bacteriol.,* **25**: 432-435.

Mullarky I. *K.,* Wolfe P.C. S. and Jones G. M. (2010*): Staphylococcus aureus* Mastitis: Cause, Detection, and Control. *Paper revi.,* Virginia cooperative Extension, Virginia Polytechnic Institute and State University, Pp 404-229**.**

Muluneh Y. (2013): Subclinical mastitis due to *S. aureus* at Alagae dairy farms: prevalence, quarter milk yield loss and antimicrobial susceptibility: *MSc thesis*. College of veterinary medicine and Agriculture, Bishoftu, Ethiopia.

Muto C.A., Jernigan J.A., Ostrowsky B.E., Richet H.M., Jarvis W.R., Boyce J.M. and Farr B.M. (2003): "SHEA guideline for preventing nosocomial transmission of multidrug-resistant strains of *Staphylococcus aureus* and enterococcus". *Infect. Control Hosp. Epidemiol*., **24** (5): 362–86.

National Centre for Biotechnology Information (NCBI): Taxonomy browser (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax .cgi?id=1279). Accessed 21, January 2014.

National Mastitis Council (NMC) (2004): Procedures for Collecting Milk Samples. In:Microbiological Procedures for the Diagnosis of Bovine Udder Infection and Determination of Milk Quality ,*NMC publication*.

Ndegwa E., Mulei C.M.and Munyna S.J. (2000): Prevalence of Sub-clinical mastitis in dairy goats in Kenya. *J. S. Afr. Vet. Assoc.* **71**: 25-27.

Normanno G., Corrente M., La Salandra G *et al.* (2007): Methicillinresistant Staphylococcus aureus (MRSA) in foods of animal origin produced in Italy. *Int. J. Food Microbiol.* **117**(2):219-222.

O'Mahony R., Abbott Y., Leonard F.C., Markey B.K., Quinn P.J., Pollock P.J., Fanning S. and Rossney A.S. (2005): Methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from animals and veterinary personnels in Ireland. *Vet. Microboiol*., **109:** 285–296.

Okuma K., Iwakawa K., Turnidge J. D., Grubb, W. B., Bell, J. M., O'Brien F. G., Coombs, G. W.*et al.* (2002): Dissemination of new methicillin-resistant *Staphylococcus aureus* clones in the community. *J. Cli. Microbiol.,* **40**: 4289-4294.

Oliver S.P., Jayarao B.M., Almeida R.A. (2005): Food borne pathogens in milk and the dairy farm environment: Food Safety and Public Health Implications. *Food borne Pathog. Dis.,* **2**:115-129.

Petersson-Wolfe C. S., Mullarky I. K. And *Jones* G. M. (2010): *Staphylococcus aureus* Mastitis: Cause, Detection, and Control. Virginia cooperative extension, College of Agriculture and Life Sciences, Virginia Polytechnic Institute and State University, Pp 404-229.

Pilla R., Castiglioni V., Gelain M. E., Scanziani E., Lorenzi V., Anjum M. and Piccinini R.(2012) : Long-term study of MRSA ST1, t127 mastitis in a dairy cow. J. *British Vet. Asso.,* doi:10.1136/vr.100510.

Price L.B., Stegger M., Hasman H., Aziz M., Larsen J., Andersen P. S., Pearson T., Waters A. E., Foster J. T., Schupp J. and Gillece J. *et al*. (2012): *Staphylococcus aureus* CC398: host adaptation and emergence of methicillin resistance in livestock. *M. Bio*; **3**: pii: e00305–pii: e00311.

Quinn P. J., Markey B. K., Carter M. E., Donnelly W. J. C., Leonard F. C. and Maghir, D. (2002): Veterinary Microbiology and Microbial Disease. Blackwell Science Ltd., London, Pp 191-208.

Radostits, O.M., C.C. Gay, D.C. Blood and Hinchlif, K.W. (2007): Mastitis. In: Veterinary medicine, 9th ed. Harcourt Ltd, London, Pp 174-758.

Resch M., Nagel V. and Hertel C. (2008): Antibiotic resistance of coagulase-negative staphylococci associated with food and used in starter cultures. *Int. J. Food Microbiol*., **127**:99-104.

Roberson J.R., Fox L.K., Hancock D.D., Gay, J.M. and Besser, T.E. (1998): Sources of intramammary infections from *Staphylococcus aureus* in dairy heifers at first parturition. *J. Dairy Sci.,* **81**: 687-93.

Roberson R. (1999): The epidemiology of *Staphylococcus aureus* on dairy farms. *In*: Proceedings of the 1999 Annual Meeting of the National Mastitis Council. National Mastitis Council, Inc., Madison, W.I., P 38.

Rowe J. (1999): Milk quality and Mastitis. In: Small ruminant for mixed practitioner. Western Vet. Confe., Lasvagas, Pp 152-156.

Roy J.P. and Keefe G. (2012): Systematic Review: What is the best antibiotic treatment for *Staphylococcus aureus* intramammary infection of lactating cows in North America? *Vet. Clin.North Am. Food Anim. Pract*., **28**:39-50.

Saei H.D., Ahmadi M., Marian K. and Batavani R.A. (2009): Molecular typing of Staphylococcus aureus from bovine mastitis based on polymorphism of the coagulase gene in the north west of Iran. *Vet. Microbiol*., **137**: 202-206.

Silva W., Destro M., Landgraf M. and Franco B. (2000): Biochemical characteristics of typical and atypical *Staphylococcus aureus* in mastitic milk and environmental samples of Brazilian dairy farms. *Brazilian J. Microbiolo.,* **31**: 103-106.

Simoons-Smit A.M., Savelkoul P.H., Stoof J., Starink T.M.and Vandenbroucke-Grauls C.M. (2000): Transmission of *Staphylococcus aureus* between humans and domestic animals in a household. *Eur. J. Clin. Microbiol. Infect. Dis*., **19:**150–225.

Smith E.M., Green L.E., Medley G.F., Bird H.E., and Fox L.K., Schukken Y.H. et al (2005): Multilocus sequence typing of intercontinental bovine *Staphylococcus aureus* isolates. *J. Clin. Microbiol*., **43**: 4737-4743.

Sordelli D., Buzzola M., Gomez M., Steele-Moore L., Berg D., Gentilini E., Catalano M., Reitz A., Tollersrud T., Denamiel G., Jeric P.and Lee J. C. (2000). Capsule expression by bovine isolates of *Staphylococcus aureus* from Argentina: genetic and epidemiological analyses. *J. Clin. Microbiol*., **38**(2):846-850.

Sori H., Ademe Z.and Sintayehu A. (2005): Dairy Cattle Mastitis In and Around Sebeta, Ethiopia. *Intern. J. Appl. Res. Vet. Med .,* **3** (4): 332-338.

Tenover F.C,Arbeit R. D.,Goering R. V.,Mickelsen P. A., Murray B. E., Persing, D. H. Swaminathan B. (1995): Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. Guest commentary. *J. Clin. Microbiol.,* **33**: 2233-2239.

Tenover F.C., Biddle J.W. and Lancaster M.V. (2001): Increasing resistance to Vancomicin and other glycopeptides in *Staphylococcus aureus*. *Emerg. Infect. Dis.,* **7**: 327-332.

Thirapatsakun T. (1999): Mastitis management. *In*: small holder dairying in the tropics. *In*: Hunt and Chantalakhana (Eds). ILRI, Kenya, Nairobi, 299-339.

Thrusfield M. (2005): Veterinary Epidemiology. 3rd edition. Blackwell Science Ltd., UK., Pp 233-250.

Tristan A., Bes M., Meugnier H., Lina G., Bozdogan B., Courvalin P *et al.* (2007): Global distribution of Panton-Valentine Leukocidin-positive methicillin resistant *Staphylococcus aureus*. *Emerg. Infect. Dis.,*; 13: 594-600.(http://www.cdc.gov/eid/content/13/4/594.htm).

Turutoglu H., Tasci F. and Ercelik S. (2005): Detection of *Staphylococcus aureus* in milk by tube coagulase test. *J. Appl. Microbiol.*, **49**: 419-422.

U.S. Department of Agriculture's (USDA) and National Animal Health Monitoring System (NAHMS)) (2011): Methicillin-resistant *Staphylococcus aureus* in Bulk tank Milk on U.S. Dairy, Operations, 2007. *Tech. brief*, United States Department of Agriculture Animal and Plant Health Inspection Service (APHIS), USA.

Udo E.E., Pearman J.W. and Grubb W.B. (1993): Genetic analysis of community isolates of Methicillin resistant *Staphylococcus aureus* in Western Australia. *J. Hospital Infect.*, 25: 97-108.

Vandenesch F., Naimi T., Enright M.C., Lina G., Nimmo G.R., Heffernan H. *et al* (2003): Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes: worldwide emergence. *Emerg. Infect. Dis.,* **9**: 978-984.

Vanderhaeghen W., Hermans K., Haesebrouck F. and Butaye P. (2010): Methicillin-resistant *Staphylococcus aureus* (MRSA) in food production animals. *Epidemiol. Infect*.,**138**: 606–625.

Voss A., Loeffen F., Bakker J., Klaassen C.and Wulf M. (2005): Methicillin-resistant *Staphylococcus aureus* in pig farming. *Emerg. Infect. Dis*., **11**: 1965–1966.

Walther C, Perreten V. (2007): Methicillin-resistant *Staphylococcus epidermidis* in organic milk production. J. Dairy Sci., **90**(12):5351.

Wang Y., Wu C.M., Lu L.M., Ren G.W.N., Cao X.Y. and Shen J. Z. (2008): Macrolide-lincosamide resistant phenotypes and genotypes of Staphylococcus aureus isolated from bovine clinical mastitis. *Vet. Microbiol*., **130**: 118–125.

Weese J. S. (2012): Staphylococcal control in the veterinary hospital. *Vet. Dermatol*., Department of Pathobiology, University of Guelph, Guelph, Ontario, Canada N1G 2W1**, 23**:292–e58.

Weese J.S., Dick H., Willey B.M., McGeer A., Kreiswirth B.N. and Innis B. (2006): Suspected transmission of methicillin-resistant *Staphylococcus aureus* between domestic pets and humans in veterinary clinics and in the household. *Vet. Microbiol*., **115**: 148–55

Weinstein M.P., Towns M.L., Quartey S.M., Mirrett S., Reimer L.G., Parmigiani G., Reller LB. *(1997)*: The clinical significance of positive blood cultures in the 1990s: a prospective comprehensive evaluation of the microbiology, epidemiology, and outcome of bacteremia and fungemia in adults. *Clin. Infect. Dis.,* **24:**584-602. (PubMed Abstract).

Wubishet B.L., Sabe Z.S., Alemu H.M. and Z/Mariam F.D. (2012): Antibiotic Susceptibility Pattern of *Staphylococcus aureus* Strains from Patients in Ethiopia. *Int. J. Pharm. Sci. Res*., **3**(12): 4889-4894.

Wulf M.W.H., Markestein A., van- der Linden F.T., Voss A., Klaassen C.and Verduin C.M.(2008): First outbreak of methicillin-resistant Staphylococcus aureus ST398 in a Dutch hospital, June 2007. Eur. Surveill. 2008, **13**: pii: 8051.

Zadoks R.W., Van Leeuwen H., Barkema O., Sampimon H., VerbrughY., Schukken H. and Van Belkum A. (2000): Application of pulsed-field gel electrophoresis and binary typing as tools in veterinary clinical microbiology and molecular epidemiologic analysis of bovine and human *Staphylococcus aureus* isolates. *J. Clin. Microbiol*. **38**: 1931-1939.

8. APPENDIXES

Appendix 1: Record sheet for Questionnaire

Serial number	Animal I. d	Farm type	Breed type	Age group	Stage of lactation	Pregnancy status	Previous mastitis history	Disinfection used	Mastitis type

Appendix 2: Interpretation of CMT findings

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

Source: Quinn et al. (2002)

Appendix 3: Record sheet for laboratory isolation and identification of *Staphylococcus aurous*

Serial number	**Sample code**	**Haemolysis on blood agar**	**Gram's result**	**Catalase test**	**Oxidase result**	**growth on MSA**	**Mannitol fermentation(MSA)**	**Coagulase result**	**Maltose fermentation**

Appendix 4: Differential tests used for identification of *Staphylococcus* species

Serial number	*Staphylococcus* species	Haemolysis	Pigment production	Coagulase test	Fermentation of sugar (MSA) and (PAB)	
1	*S. aureus*	+	+	+	+	+
2	*S. intermidius*	+	–	+	±	±
3	*S. hicus*	–	–	+	–	–
4	CNS	–	–	–	–	–

+ = 90% or more strains are positive, + = 90% or more strains are weakly positive, - =90% or more strains are negative.

Source: Quinn *et al. (2002*)

Appendix 5: Culture characteristics of the Staphylococcus organisms on mannitol salt agar (MSA) and purple agar base (PAB).

The photos below show culture characteristics of Staphylococcal organisms on mannitol agar (left) and PAB (right).

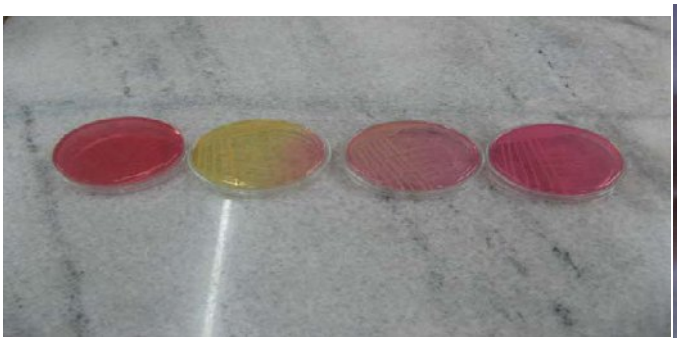


Photo 1=un-inoculated (control). Photo 4=maltose fermentation (yellow color)

Photo 2= there is growth and fermentation (pink-yellow) and non-fermented purple color

Photo 3= growth only, no fermentation

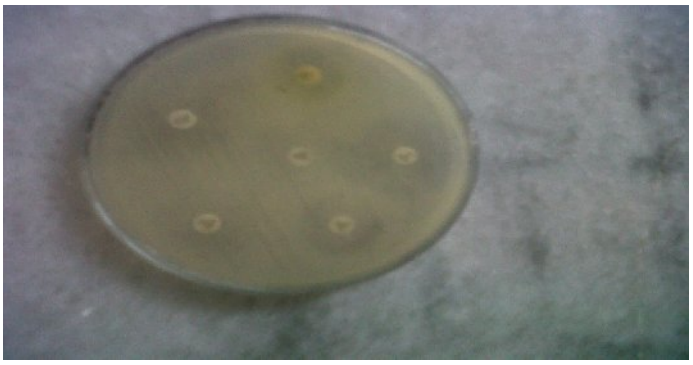


Photo 6= drug sensitivity pattern on Muller hinton agar

- Central disc (cefoxitine) resistant (zero inhibition)
- The right and upper parts show susceptibility patter

Appendix 6: Composition and preparation of media used for the study

Nutrient agar (HiMedia, India)

Nutrient Agar is used for the cultivation of less fastidious microorganisms, can be enriched with blood or other biological fluids

Typical formula (composition)

Ingredients	Gms / Litre
Peptic digest of animal tissue	5.00
Sodium chloride	5.000
Beef extract	1.500
Yeast extract	1.500
Agar	15.00
Final pH (at 25°C)	7.4±0.2

Instruction for use:-

Suspend 28 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Dispense as desired and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well before pouring.

Blood agar (HiMedia, India)

Sheep Blood Agar Base with added sheep blood gives improved haemolytic reactions of organisms.

Typical formula (composition)

Ingredients	Gms / Litre
Casein enzymic hydrolysate	14.000
Peptic digest of animal tissue	4.500
Yeast extract	4.500
Sodium chloride	5.000
Agar	12.500
Final pH (at 25°C)	7.3±0.2

Instruction for use:-

Suspend 40.5 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C and aseptically add 7% sterile sheep blood. Mix well and pour into sterile Petri plates.

Mannitol salt agar (Oxoid, England)

Typical formula (composition)

Ingredients	Gms / Litre
Lab-Lemco'powder	1.0
Peptone	10.0
Mannitol	10.0
Sodium chloride	75.0
Phenol Red	0.025
Agar	15.0
Final PH	7.5 + 0.2 at 250C

Instructions for use:-
Suspend111g in 1 litre distilled water and bring to the boil to dissolve completely. Sterilize by autoclaving at 1210C for15 minutes. Mix well before pouring. Into sterile petri -dishes.

Purple agar base (Difco, France)

Typical formula (composition)

Ingredients	Gms / Litre
Proteose pepton	10.0
Beef extract	1.0
Sodium chlorid	5.0
Bromcresol Purple	0.02
Agar	15.0
Final PH	6.8 + 0.2 at 250C

Instructions for use:

Suspend 31g of the powder in 1 litre of purified water. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Autoclave at 121^0C for 15 minutes. When preparing 0.5-1% carbohydrate fermentation, agar dissolves 5-10g of the desired carbohydrate in the basal medium prior to sterilization by autoclaving

Brain heart infusion broth (Oxoid, England)

A highly nutritious infusion medium recommended for the cultivation of streptococci, Neisseria and other fastidious organisms.

Typical formula (composition)

Ingredients	**gm/litre**
	12.5
Brain infusion solids	
Beef heart infusion solids	5.0
Proteose peptone	10.0
Glucose	2.0
Sodium chloride	5.0
Disodium phosphate	2.5
pH 7.4 ± 0.2 @ 25°C	

Instructions for use:-

Dissolve 37g in 1 litre of distilled water. Mix well and distribute into final containers. Sterilize by autoclaving at 121°C for 15 minutes.

Mueller-hinton agar (Oxoid, England)

An antimicrobial susceptibility testing medium which may be used in internationally recognized standard procedures.

Typical formula (composition)

Ingredients	gm/litre
Beef, dehydrated infusion from	300.0
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0
pH 7.3 ± 0.1 @ 25°C	

Instructions for use:-

Add 38g to 1 litre of distilled water. Bring to the boil to dissolve the medium completely. Sterilise by autoclaving at 121°C for 15 minutes.

Appendix 7: Flow chart followed for *Staphylococcus aurous* isolation (protocol)

Milk samples collection (CMT and Clinical positive (25ml))

▼

0.01ml of milk (loopful of milk) streaked on blood agar plate with 7% sheep blood

▼

Haemolysis, colony characterization observation

▼

Gram's staining (banch of grapes with cocci)

▼

Biochemical tests for confirmation

▼

Catalase test-3% H2O2 (positive)

▼

Oxidase(negative)

▼

Inoculate colonies on MSA and incubate at 37^0C for 24-48 hours, growth and change in the PH of the medium is confirmative for *Staphylococcus* classified as highly fermentative (*S. aureus*), weakly fermentative (*S. intermidius*) and non fermentative (*S. hicus* and CNS)

▼

Coagulase test to identify the most pathogenic CPS (*S. aureus, S. intermidius* and *S. hicus*) from CNS

▼

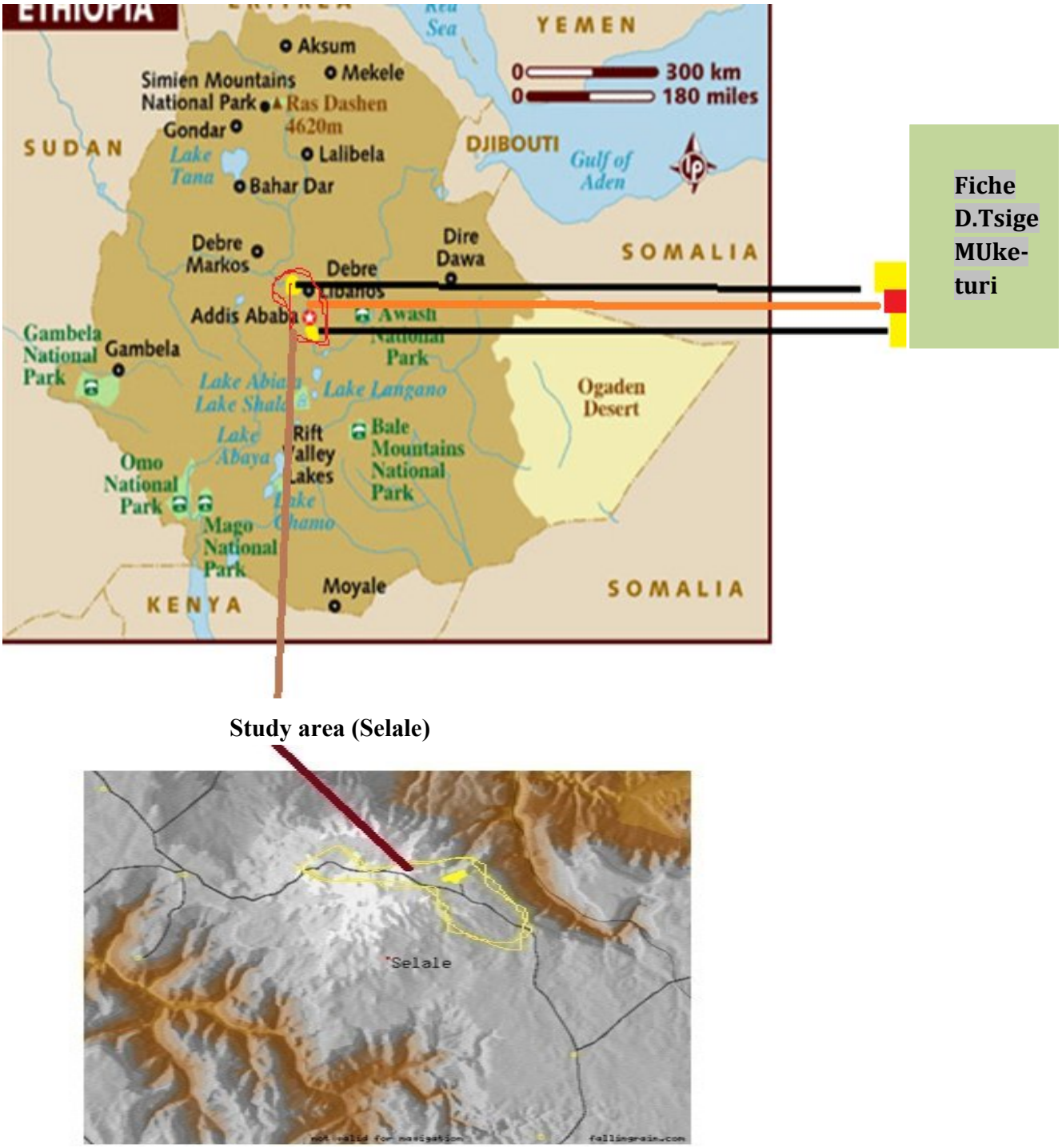
Inoculate CPS isolates on PAB media plate with 1% of maltose and incubate at 37^0C for 24-48 hours to differentiate the pathogenic staphylococci, particularly the coagulase-positive isolate. The identification was based on the fact that *S. aureus* rapidly ferment maltose to change the medium and colonies to yellow. *S. intermedius* gives a weak or delayed reaction and *S. hicus* did not ferment maltose.

Appendix 8: Antimicrobial sensitivity interpretation chart

Antimicrobial agent (symbol)	**Disc content**	**Zone diameter ,Nearest whole mm**			**Equivalent MIC break points(µg/ml)**		
		S	I	R	S	I	**R**
Cefoxitin (Fax)	30 µg	≥22	_	≤21	≤4	_	**≥8**
Amoxicillin-clavulinic acid (AMC)	20/10 µg	≥20	_	≤19	≤4/2	_	**≥8/4**
Ceftazidime (CAZ)	30 µg	≥18	15-17	≤14	≤8	16	**≥32**
Clindamycin (DA)	10µg	≥21	15-20	≤14	≤0.5	1-2	**≥4**
Vancomicin (VA)	30 µg	≥12	10_11	≤9	≤2	4-8	**≥16**
Gentamycin (CN)	10 µg	≥15	13-14	≤12	≤4	8	**≥16**
Streptomycin (S)	10 µg	≥15	12-14	≤11	-	-	**-**
Ciprofloxacin (CIP)	5 µg	≥21	16-20	≤15	≤1	2	**≥4**
Sulphamethoxazole-trimethoprim (SXT)	23.75/1.2	≥16	11-15	≤10	≤38/2	-	**≥76/4**
Bacitracin (B)	10 µg	≥13	9-12	8	-	-	**-**
Nitrofuration (F)	300 µg	≥17	15-16	≤14	≤32	16	**≥128**
Chloroamphenicol (C)	30 µg	≥18	13-17	≤12	≤8	16	**≥32**

Source: - Quinn *et al*. (2002) and CLSI, (2012)

Appendix 9: Map of the study area



Fiche D.Tsige MUke-turi