Thesis Ref. No._____

ISOLATION AND IDENTIFICATION OF METHICILIN RESISTANT *S. AUREUS* FROM BOVINE MASTITIC MILK IN DAIRY FARMS OF BAHIR DAR AND ITS SURROUNDING NORTH WEST ETHIOPIA

MSc Thesis

Addis Ababa University, College of Veterinary Medicine and Agriculture, Department of Veterinary Microbiology, Immunology and Veterinary Public Health

BY

ALEMAYEHU BITEWA

June 2015

Bishoftu, Ethiopia

ISOLATION AND IDENTIFICATION OF METHICILIN RESISTANT *S. AUREUS* FROM BOVINE MASTITIC MILK IN DAIRY FARMS OF BAHIR DAR AND ITS SURROUNDING NORTH WEST ETHIOPIA

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

BY

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June 2015
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As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the Thesis prepared by **Alemayehu Bitewa** entitled: **ISOLATION AND IDENTIFICATION OF METHICILIN RESISTANT *S. AUREUS* FROM BOVINE MASTITIC MILK IN DAIRY FARMS OF BAHIR DAR AND ITS SURROUNDING NORTH WEST ETHIOPIA** and recommended that it be accepted as fulfilling the thesis requirement for the Degree of Master of Science in **Veterinary Microbiology.**

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STATEMENT OF AUTHOUR

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Date of Submission: June 16, 2015

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LIST OF ABBREVATIONS

AGR   Accessory gene regulator

CLSI   Clinical Laboratory Standard Institute

CMT   California Mastitis Test

CNS   Coagulase Negative Staphylococci

CSA   Central Stastical Agency

DNA   Deoxyribo Nucleic Acid

EFSA   European Food Safety Authority

MAR   Multiple Antibiotic Resistant

MRSA   Methicillin Resistant Staphylococcus aureus

NCCLS   National Committee for Clinical Laboratory Standard

NMC   National Mastitis Committee

OR   Odds Ratio

PBP   Penicillin Binding Protein

PCR   Polymerase Chain Reaction

SCC   Staphylococus Chromosome Cassette

SE   Staphylococcus Enterotoxin

SPSS   Stastical Package for Social Science

SXT   Sulphamethoxazole-Trimethoprim

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ABSTRACT

Staphylococcus aureus is a common causative agent of bovine mastitis in dairy herds. The emergence of Methicillin-Resistant *Staphylococcus aureus* (MRSA) in dairy farms is significant and costly public health concern. *S. aureus* bovine mastitis is a common reason for therapeutic and/or prophylactic use of antibiotics on dairy farms. A cross sectional study was conducted from November 2014 to April 2015 in and around Bahir Dar Town, to isolate and identify Methicillin Resistant *S.aureus*, their resistance pattern to different antimicrobials and to identify risk factors associated with mastitis. A total of 311 lactating local (Fogera) and Crossbreed cows were included during the study period, and total of 1244 quarters were examined to detect clinical and subclinical mastitis by physical examinations of udder, milk and by California Mastitis Screening Test. The overall prevalence of mastitis at cow level was 62.06 % with 3.54% and 58.52 % of clinical and subclinical mastitis prevalence respectively. From a total of 1244 quarters examined, 4.82% were blind teats, 1.93 % and 40.51 % of quarters were affected by clinical and subclinical mastitis respectively. In this study, the subclinical mastitis was higher than clinical mastitis. The occurrence of mastitis varied significantly ($p<0.05$) between cross breeds and local Fogera breeds by 78.64% and 29.52% respectively. The univariate logistic regression showed that among potential risk factors considered from the farm attributes, breed, age, farm floor type and previous treatment history had significant ($p < 0.05$) effect on the prevalence of mastitis. However, lactation stage, parity and milking hygiene were not a significant ($p > 0.05$) potential risk factors. From 193 mastitis infected lactating cows, 528 milk samples were cultured and twenty-nine *Staphylococcus aureus* were isolated. The isolated *S.aureus* was tested with eight different types of antibiotics to identify their resistance pattern. The present result showed a significant association of resistance, particularly to penicillin G 95.8%, streptomycin 73.1%, tetracycline 72.2%, amoxicillin 61.5% and vancomycin 52.4%. In this study, 75.7 % *S. aureus* isolates were resistant for Cefoxtin, which is an indication of existence high level of MRSA prevalence. There were also observed resistance for other multidrugs, mainly to penicillin G, streptomycin and tetracycline because of production of β-lactamase and inactivate potency of antibiotics. The present study revealed higher prevalence of mastitis and occurrence of multidrug

resistance *S. aureus* specifically which belongs to the MRSA which are dependent on multiple associated risk factors. *S. aureus* to various antibiotics indicated that existence of alarming level of resistance of frequently isolated mastitis bacteria to commonly used antimicrobial agents in the study area. Hence, regular resistance follow-up, using antimicrobials sensitivity tests helps to select effective antibiotics and to reduce the problems of drug resistance developments towards commonly used antimicrobials.

Key words: *Antimicrobial susceptibility, Dairy, Mastitis, MRSA, Multidrug resistant, S. aureus*

1. INTRODUCTION

Ethiopia has the largest cattle population in Africa with an estimated population of 49.3 million. Cows represent the biggest portion of cattle population of the country, around 42% of the total cattle heads are milking cows (CSA, 2008). However, milk production often does not satisfy the country's requirements due to a multitude of factors. Mastitis is among the various factors contributing to reduced milk production (Biffa *et al.*, 2005).

Bovine mastitis is an infectious inflammation or irritation of the mammary glands that interferes with the normal flow and quality of milk (Pyorala, 2003). Among mastitis causing pathogens, the *Staphylococcus aureus* bacterium is a major pathogen of intramammary infections in dairy cattle (Erskine, 2001). It is an important opportunistic pathogen both in humans and in dairy cattle. *Staphylococcus aureus* is one of the most prevalent major mastitis pathogens in Ethiopia (Getahun, 2006).

S. aureus is the most important pathogen among *Staphylococci* species related to subclinical intramammary infections in dairy cows leading to severe economic losses in industry worldwide (Godden *et al.*, 2002). Although a variety of antibiotics can be used against this organism, *S. aureus* mastitis has been found to respond poorly to antibiotic treatment (Barkema *et al.*, 2006). The increased resistance of *S. aureus* isolates to several antimicrobial agents has been reported (Gentilini *et al.*, 2000).The determination of antimicrobial susceptibility of clinical isolates is required not only for therapy but also for monitoring the spread of resistant strains throughout the populations. ß-lactam antibiotics are the most frequently used in intramammary infusion therapy. Bacterial resistance mechanisms to this class of antibiotics include production of ß-lactamase and low-affinity penicillin-binding protein 2a (PBP 2a) determined by the presence of the chromosomal gene *mec*A. The latter, designated for methicillin resistance, precludes therapy with any of the currently available ß-lactam antibiotics, and may predict resistance to several classes of antibiotics (Moon *et al.*,2007).

Some of these *S.aureus* bacteria have become resistant to several antibiotics, mainly penicillin's. At first, most of the resistance to penicillin was a result of penicillinases, produced by the bacteria. Therefore, methicillin, which is a β-lactam antibiotic and insensitive to penicillinases, was widely used to cure *S.aureus* cases in humans. However, soon after its introduction in the 1950s the first methicillin-resistant isolates were found in the UK (Jevons, 1961; Barber, 1961).

MRSA was initially reported as a nosocomial pathogen in human hospitals (hospital-associated MRSA) and was isolated from patients with compromised immune systems undergoing medical procedures. MRSA accounts for 30 to 40% of all hospital-acquired infections and for 40% to 70% of *S. aureus* infections in intensive care units (Gordon and Lowy, 2008).

The first report of MRSA in animals was in milk from Belgium cows with mastitis (Morgan, 2008). With more advanced studies, MRSA it was possible to determine strains strictly related to animals, such as those found in pigs, which were named LA-MRSA (livestock-associated Methicillin-Resistant *Staphylococcus aureus*) in 2010 (Vanderhaeghen *et al.*, 2010).

 Mechanism of MRSA resistance to methicillin, is caused by an altered penicillin-binding protein, which makes it resistant to all β-lactam antimicrobials. *S.aureus* can become methicillin -resistant by the introduction of an exogenous mobile staphylococcal chromosomal cassette (mec) (SCCmec) gene. *S. aureus* strains were once nearly uniformly insusceptible to semi-synthetic penicillinase-resistant β-lactams e.g. methicillin, and oxacillin, the most commonly used class of antibiotics for skin infection. These strains were termed 'Methicillin Resistant *Staphylococcus aureus,* or MRSA, a term that implied cross-resistance to all β-lactams including all penicillins, aminoglycosides, macrolides, lincosamides, and cephalosporins. Antibiotic-resistant *S. aureus* isolates pose a severe challenge to both veterinary and health professions and dairy cattle producers because they have a negative impact on therapy (Sears and McCarthy,2003 ; Brouillette and Malouin, 2005).

The usage of antibiotics correlates with the emergence and maintenance of antibiotic-resistant traits within pathogenic strains (Shitandi and Sternesjo, 2004). These traits are coded for by particular genes that may be carried on the bacterial chromosome, plasmids, and transposons or on gene cassettes that are incorporated into integrons (Rychlik, 2006), thus are easily transferred among isolates.

Multiple antibiotic resistant *S. aureus* strains were isolated from milk obtained from cattle samples in many parts of the world (Pesavento *et al.,* 2007). Staphylococci are normal inhabitants of the skin and mucous membranes of animals and humans. Pathogenic strains are usually coagulase-positive (Mahon *et al*., 1995) and have been found to cause disease in their hosts throughout worldwide (Matsunaga *et al.*, 1993).

In Ethiopia there are few studies in Hawassa by Daka *et al.* (2012) and in Adama by Abera *et al*. (2013). However Bahir Dar is high milk-shed area, no study has been reported regarding the MRSA in dairy mastitis.

Therefore, the present study considered with the following general and specific objectives:-

General Objectives:
- To determine the prevalence of bovine mastitis in the study area.
- To asses associated risk factors with mastitis infection

Specific Objectives:
- Isolation and identification of *Staphylococcus aureus* from mastitis cow's milk.
- To identify multi-drug resistance effects of *Staphylococcus auerus*
- To identify the occurrence of *MRSA strains* using antibiotic sensitivity test.

2. LITERATURE REVIEW

2.1. Bovine Mastitis

Ethiopia has the largest cattle population in Africa with an estimated population of 49.3 million. Cows represents the biggest portion of cattle population of the country, around 42% of the total cattle heads are milking cows (CSA, 2008).However, milk production often does not satisfy the country's requirements due to a multitude of factors. Mastitis is among the various factors contributing to reduced milk production (Biffa *et al.*, 2005).

Bovine mastitis is an infectious inflammation or irritation of the mammary glands that interferes with the normal flow and quality of milk (Pyorala**,** 2003). Pathogens those invade the mammary glands, develop and multiply, producing some toxic substances that results in inflammation, reduced milk production and altered milk quality, leading to a clinical condition known as mastitis (Rall *et al.*, 2013).

Mastitis can occur in any mammalian species, including humans, it is a major concern of the dairy industry, which relies on the consistent and efficient production of milk by lactating ruminants such as dairy cattle (Erskine, 2001).Bovine mastitis can be caused by a wide variety of opportunistic and pathogenic microorganisms including, but not limited to, *staphylococcus aureus,* various coagulase-negative staphylococcal species, *streptococc*i (*Streptococcus agalactiae*, *Streptococcus dysgalactiae),* Mycoplasma species, Coliforms, *Corynebacterium bovis* and various species of yeast (Quinn *et al.*, 1999; Radostitits *et al.*, 2000).

Intramamary infections can result in mastitis, which is either sub-clinical or clinical. Clinical mastitis is a form of disease, which can be directly observed, with symptoms including milk clotting, redness and hardness of the teat, and poor milk letdown. Subclinical mastitis is generally defined, as the absence of visible symptoms but characterized by measured *somatic* cell count of greater than 2.5×10^5 cells/ml (Schukken *et al.*, 2003) or the presence of a known pathogen in the secreted milk as detected by culture.

Bovine intramammary infections are single most costly disease on dairy farms. Depending on individual circumstances, each case of clinical mastitis costs approximately hundred dollar, accounting for treatment costs and lost production (Fetrow *et al.*, 2000).

While clinical cases often receives the most direct attention, there is an ample evidence which suggests that sub-clinical cases, as detected by elevated milk somatic cell count, can negatively affect production (Gill *et al.*,1990; Degraves and Fetrow,1993), but the estimated magnitude of this loss varies greatly between studies. The average loss appears to be approximately 0.5 kg/day for every two-fold increase in milk somatic cell count (Hortet and Seegers, 1998). Sub-clinical infection also acts as reservoirs of pathogen within the herd and may develop into clinical mastitis; there is limited information on the economic loss due to mastitis. However, the few data available indicate that the loss is significant (Bishi, 1998; Mungube, 2001).

2.2. Bovine Mastitis Caused by *Staphlococcus aureus*

2.2.1. General characteristics of Staphlococcus aureus

Staphylococci are gram-positive bacteria, with diameters of 0.5–1.5 μm and characterized by individual cocci, which divide in more than one plane to form grape-like clusters. There are 32 species and eight sub-species in the genus *Staphylococcus*, many of which preferentially colonise the human body (Kloos and Bannerman, 1994). *S.aureus* are described "coagulase positive, β-haemolytic, maltose and mannitol fermenting organisms, forming pigmented colonies" .This bacterium is a non-motile, non-spore forming, facultative anaerobes that grow by aerobic respiration or fermentation (KLoos and Bannerman, 1995).

Staphlococcus aureus strains can be distinguished by their production of catalase, haemolysins, coagulase and heat-stable nuclease. *Staphlococcus aureus* is also highly osmotolerant, with an ability to grow on media supplemented with 7.5% NaCl (Kloos and Bannerman, 1995; Quinn *et al.*, 1999). Staphylococci are commonly identified by their

ability to produce coagulase, and blood clot. This distinguishes from coagulase positive staphylococcus, *S.aureus*, *S.intermedus* and *S.hyicus* from the other species; such as *S.epidermides*, that are coagulase negative *Staphylococi* (Harries *et al*., 2002). Colonies of *Staphlococcus aureus* are typically round, shiny, golden-yellow surrounded by a zone of double-haemolysins on blood agar (Quinn *et al*., 1999).

2.2.2 Mastitis due to Staphylococcus aureus

Among the bacterial mastitis pathogens, *S. aureus* standout for its resistance to antibiotic treatment and its propensity to recur chronically. As contagious mastitis pathogen, the primary reservoir of *S. aureus* in a herd is infected cattle, which shed the pathogen into their milk. *S.aureus* is a usual cause, although it may be difficult to demonstrate the presence of the organism in the peracute cases especially when necrotic tissues are invaded by *E.coli* and *Clostridium* species (Smith, 2002).

A major longitudinal surveys (1991-1995) of New York and Pennsylvania herds detected *S. aureus* in 9.1% of all cows sampled (Wilson *et a*l., 1997). A similar longitudinal survey (1994-2001) of Wisconsin herds found 9.7% positive samples for *S. aureus* (Makovec and Ruegg, 2003). In Ontario, Canada, it was found that almost 20% of all cows experienced one or more episodes of clinical mastitis in each lactation, and 6.7% of these cases were associated with *S.aureus* (Sargeant *et al*., 1998).

A survey of exclusively heifer in Nowegian dairy herds found that 44.3% of clinical mastitis cases were caused by *S. aureus* (Waage *et al.,* 1999). The mastitis caused by *S. aureus* is characterized by significantly lower cure rates compared with infections caused by other microorganisms. This phenomenon is mainly a result of unusually frequent acquisition of antibiotic resistance mechanisms among these group of bacteria and their ability to form biofilm or slime (Cramton, 1999). Biofilm production is considered the major reason for recurrence and for the difficulty in eradicating infections of mammary glands (Melchior, 2006).

2.2.3. Transmission and ecology

Staphylococcus aureus has been isolated from not only the nares of dairy cattle, but also from the teat skin, udder skin, belly and genitals, as well as from many environmental sites including dairy personnel, feed, bedding and milking equipment (Roberson *et al.,* 1999).

There is considerable evidence from herd surveys that *S.aureus* tends to spread within a herd, and that certain strains tend to predominate. In Canadian survey, Sabour (2004) found that 58.6% of herd samples contained only a single *S.aureus* strain.

In a study, which employed, molecular identification methods to discriminate between strains isolated from different sites, Zaoks *et al.,* 2002 determined that *S.aureus* strains from bovine intra -mammary infection were most likely to be isolated from milk than any other sample site such as bovine skin or equipment.

Sommerhauser *et al.* (2003) were able to reduce *S.aureus* intra -mammary infection within herds by implementation of control methods, which limited intra-herd transmission of the pathogen. Use of antimicrobial sanitizer on teat ends before and other milking ,thorough sanitization of milking equipment milk transport lines, and segregation of cows with known intr-mammary infections are all considered to be good general practices in preventing the transmission of mastitis pathogens (Pankey, 1989,Fox and Gay,1993) .

2.3. Epidemiology of Staphylococcal Bovine Mastitis in Ethiopia

The majority of intramamary infection due to *S.aureus* is subclinical. The incidence of clinical mastitis due to *S.aureus* is dependent on its prevalence of infection in the herd. With an effective mastitis control program, the most common causes of clinical mastitis are the environmental pathogens (Busato *et al.*, 2000). However, in some herds with a low rolling SCC, incidence of clinical mastitis due to *S.aureus* ranges from 190-240 case/100cows/year, with about 47% of the clinical cases being *S.aureus* (Jones *et al.*, 1998; Radostitis *et al*., 2007).

A number of epidemiological studies of bovine mastitis were carried out in Ethiopia, and from the species of organisms that were isolated from the various studies, *S.aureus* contributed the major share (Husderra, 1997; Workineh *et al.,* 2002; Kerro and Tareke, 2003; Araya, 2004; Getahun, 2006).

The species of organisms that were isolated from both, clinical and subclinical mastitis, *Staphlococci* were the major pathogens, out of which S.aureus contribute the major share accounting for, 34%(16) and 49.43%(43) from clinical and sub-clinical mastitis respectively (Araya,2004). From two major Ethiopian dairy farms isolated 119 mastitis causing pathogens, (40.5%) *S.aureus* were predominate isolates (Workineh *et al*., 2002). Similarly, Kerro and Tereke (2003) reported a 39.24% rate of isolation of *S.aureus* in the dairy farms of Ethiopia.

2.4. Pathogenesis of *Staphylococcus aureus* Mastitis

S.aureus, to cause mastitis initially must gain access to the mammary gland through the teat canal and then has to avoid removal by the flushing of the fluids during the milking processes. Therefore, the ability to adhere to the epithelial cells and extracellular matrix proteins is instrumental to colonize the gland and develop the pathologic process. The adhesion mechanism of *S.aureus* is complex and includes multiple proteins able to specifically recognize components of the microbial surface that recognize adhesive matrix molecules (Patti *et al*.,1994), allowing bacterial anchorage in normal and inflamed tissues (Foster *et al*.,1998).

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

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

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

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

2.5. Diagnosis of *Staphylococcus aureus* Mastitis

Confirmatory diagnosis of *S.aureus* mastitis is based on bacterial isolation. A herd screen may be performed on bulk milk, but this lacks the quantitative precision to be anything other than a raw guide to the presence of *S.aureus* inta-mammaryinfection with the herd (Radostitis *et al*., 2000).To estimate more closely the significance of *S.aureus* on farm, culture of milk-selected cows is important. A California mastitis test aids identification of infected quarter(s) for culture in high (somatic cell count) in cows (Quinn *et al.,* 1999; Radostitis *et al*., 2000).

There are standard microbiological techniques and procedure for selection and identification of *S. aureus*. Strict aseptic procedures must be following while collecting milk samples in order to prevent contamination with many microorganisms present on the skin of cow's flanks, udder and teats, on the hands of the sampler and in the barn. A loop of milk is streaked on 5% sheep blood agar and plates are incubate aerobically at 37^O_C and examined after 24 hours of incubation for growth. Mannitol salt agar and Baird-parker medium are specifically selective for *Staphylococci* (Quinn *et al.*, 1999).

Colony characteristics, microscopic appearance, and catalase test and oxidative-fermentative (O-F) test are recommended for identifying *Staphylococci.* Confirmation of *S.aureus* is based on the ability to clot rabbit plasma (Coagulase test) and degree of maltose fermentation on purple agar base with 1% maltose (NMC, 1990; Quinn *et al.*, 1999).

2.6. Control and Prevention *Staphylococcus aureus* Mastitis

A successful *S.aureus* control program will eliminate existing infections, prevalent new intra-mammary infections, and have a system for on-going monitoring of the infection status of the herd (Owens *et al.*, 1997; Radiostititis *et al.*, 2000).

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

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

standard recommendation (schukken *et al.,* 2003).

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

2.7. Definition of MRSA

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

2.7.1. Strains of MRSA

Hospital-associated MRSA (HA-MRSA)

MRSA was first detected in a UK hospital in 1961, and was detected a few years later in U.S. hospitals and other healthcare facilities where the widespread use of antibiotics selected for bacteria carrying resistance genes (Gordon and Lowy, 2008). Until the 1990s, MRSA was almost exclusively an issue in hospitals and long-term care facilities, affecting surgical patients, other aged or ill residents, and some healthcare workers. Some MRSA infections occurred in non-hospitalized persons but these were traced to close contacts with persons who had been hospitalized. Central Disease Control classified MRSA infections as Hospital acquired). If they were detected in patients 48 hours after admission to a hospital or were detected in patients with a recent history of hospitalization, surgery, dialysis, or an indwelling catheter. Due to the high rate of

antibiotic usage in healthcare facilities, HA-MRSA are often resistant to many classes of antibiotics (tetracycline, sulfa-drugs, and gentamicine, etc.) in addition to the β-lactams (Lee, 2003).

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

Community associated MRSA (CA-MRSA)

MRSA infections were largely confined to immune compromised individuals or individuals with healthcare exposure. In 1997, death of four healthy children from MRSA pneumonia and sepsis heralded the arrival of a new type of MRSA. Soon thereafter, MRSA cases grow rapidly across continents; the majority of cases were confined to few clone lineages that were markedly different from HA-MRSA, shared a small sized Type IV SCC*mec* cassette, and encoded the genes for the Panton-Valentine Leukocidin (David and Daum ,2010):

Livestock Associated MRSA (LA-MRSA)

The scope of MRSA infection is not limited to human medicine only but also in Veterinary Medicine (Lee, 2003). Livestock associated refers mainly to the clonal spread of a certain MRSA strain (ST398) that colonizes from different food producing animal species and may cause infections in humans. Companion animals and horses may be colonized with a variety of strains due to their close contact with humans. Thus, these species may act as carriers of MRSA originating from humans (Morgan, 2008).

During the period 1970-2000, MRSA has been sporadically isolated from animals, in particular cows, small companion animals, and horses. With the exception of some equine isolates, the nature of these cases suggested a human origin and no epidemics have been reported (Leonard and Markey, 2008).

At the end of 20th century both the scientific community and policy makers were convinced that MRSA in human medicine had nothing to do with animal husbandry but was a problem only based on the antimicrobial use in human medicine. The situation has now changed, with an increased number of reports on LA-MRSA in livestock, especially swine and veal calves (Leonard and Markey, 2008). Livestock Associated Methicillin-Resistant *S. aureus* (LA-MRSA) belonging to the clonal complex 398 (LA-MRSA CC 398) is considered to be zoonotically important because of its capacity to colonize a wide range of hosts (Paterson *et al*., 2012).

2.7.2. MRSA in Food Producing Animals

Until the 1990s, Methicillin-Resistant *Staphylococcus aureus* (MRSA) was traditionally considered a pathogen causing nosocomial infections, being the so-called HA-MRSA (Hospital-Associated Methicillin-Resistant *Staphylococcus aureus*). However, over time, cases of MRSA-positive individuals were observed who never had contact with hospital services, and strains from these individuals were identified and named CA-MRSA (Community Associated Methicillin-Resistant *Staphylococcus aureus*) (Kluytmans, 2010).

In 2003 in the Netherlands, a new MRSA strain arose in patients that could not be typed through PFGE (pulsed field gel electrophoresis) with *Sma*I, with resistance to digestion by this enzyme (Bens *et al*., 2006), being called since then NT-MRSA (non type-able Methicillin-Resistant *Staphylococcus aureus*).

Investigations of this NT-MRSA intensified, and it was observed that these patients carrying this strain had previous contact with pigs and the geographic distribution of cases showed clusters near pig farms (Van Loo *et al*., 2007).

In 1972, the first reported MRSA infection was observed in a cow with mastitis in Belgium (Devriese *et al*., 1972). Since then, pigs (Voss *et a*l., 2005; Khanna *et al*., 2008), horses (Witte *et al*., 2007; Cuny *et al*., 2008), poultry (Persoons *et al*., 2009) and calves (Mooij *et al*., 2007) have been identified as new reservoirs for MRSA. With more advanced studies, it was possible to determine strains strictly related to animals, such as those found in pigs, which were named LA-MRSA (Livestock-Associated Methicillin-Resistant *Staphylococcus aureus*) in 2010 (Vanderhaeghen *et al*., 2010).

In recent years, there has been increased concern about antibiotic resistant strains of *S. aureus* and development of resistance has been attributed to the extensive therapeutic use of antimicrobials or to their administration as growth promoters in food animal production (Normanno *et al.*, 2007). Isolates of *S. aureus* are frequently resistant to methicillin and essentially all other β-lactam antibiotics (Lee, 2003).

In the 1990s, a major change in the epidemiology of MRSA has been observed, with the appearance of cases affecting people with no epidemiological connection to hospitals; strains that cause such infections are referred to as community-acquired or community associated MRSA (EFSA, 2009).The MRSA infections are more difficult to treat with standard antibiotics and thus more dangerous. MRSA strains were first isolated in 1961, the year in which methicillin was licenced (Jevons, 1961) and has since emerged as a serious concern in human medicine.

2.7.3. MRSA as a Zoonotic Disease

Staphylococcus aureus is usually commensal on human skin, but it is one of the most important pathogens, causing illnesses ranging from minor skin infections to life-threatening diseases such as pneumonia and bacteraemia (Deleo and Chambers, 2009). As well as humans, livestock animals are also infected with *S. aureus,* resulting in bovine mastitis and chicken arthritis, which threaten food safety and cause food poisoning (Vanderhaeghen *et al.,* 2010). Notably, the increasing incidence of antibiotic resistant *S. aureus* strains, such as Methicillin-Resistant *S. aureus* (MRSA) and Vancomycin-

Resistant *S. aureus* , has become a major concern, because *S. aureus* is one of the most common causes of nosocomial infections (Deleo and Chambers, 2009).

The strains originating from companion animals are originally human strains, and that the infection with this MRSA type is considered humanosis. On the other hand, the strains originating from livestock (Livestock Associated - LA) are often divergent from human strains and the infection with this type of LA-MRSA could be considered zoonosis, and in this case, MRSA would be an emergent zoonotic agent. Within this context, veterinarians, cattle farmers and pet owners are considered risk groups for acquiring MRSA (Morgan, 2008).

The first known case of MRSA transmission between cows and a person was reported by Juhász-Kaszanyitzky *et al.*(2007). Cows with mastitis have been the most likely to harbor MRSA, and they may be related to horizontal transfer via wet hands of colonized or infected dairy farm workers, and selection by the use of antibiotics to treat mastitis (Morgan, 2008). MRSA strains isolated from cows with subclinical mastitis were phenotypically and genotypically indistinguishable from the strain from the person who worked with these animals. These strains were determined as ST1, *spa* type t127, SCC*mec* IVa. These strains epidemiologically related, indicating transmission from cow to human or from human to cow and through the food chain (Kluytmans, 2010). MRSA strains have been isolated in many countries from cows' or small ruminants' milk and various dairy products (Juhasz-Kaszanyitzky *et al.*, 2007; Ateba *et al.*, 2010).

2.7.4. Transmission of MRSA

Transmission routes of MRSA are probably similar to those of other *S. aureus* strains, but there are likely to be differences in efficiency of host colonization following exposure (Kawada *et al.*, 2003). MRSA can be transmitted from person to person, as well as from animals to humans and *vice-versa*. Transmission usually occurs by direct contact, often via the hands, with colonized or infected people or animals (Ferreira *et al.*, 2011). Infection in the hospital, the hands and nostrils of colonized individuals are the major

sources of MRSA transmission. MRSA is released into the hospital environment through aerosol, skin cells or stools of infected patient (Klotz *et al.,* 2005).

Contaminated source of infection in the hospital include medical instrument, beddings, clothing, furniture's, toiletries and the atmosphere (Dancer, 2008, Gehanno *et al.,* 2009) found similar strain of MRSA in patients of a hospital and the room atmosphere. While Loeffler *et al.*(2005) was reported MRSA in environmental samples collected from small animal veterinary hospital and equine veterinary hospital. Although, hospital cleaning reduces MRSA contamination of the environment, in some cases it does not eliminate it. In human hospitals, colonized and infected patients are the main reservoirs of MRSA, which is typically spread from patient to patient via hands of staff (EFSA, 2009). Carrier animals serve as reservoirs of MRSA and they may transmit the pathogens to other animals or humans (Cuny *et al.,* 2010).

Some MRSA lineages tend to predominate in specific geographical regions and show host specificities; therefore, they tend to be associated with animals more than with humans and *vice-versa* (Sung *et al.*, 2008).

CC398 is the MRSA lineage most often associated with asymptomatic carriage in intensively reared food-producing animals, primarily in pigs, but also in cattle and perhaps in poultry (EFSA, 2009). Although this strain is mainly found to colonize animals without causing clinical diseases, in a few isolated cases, it caused clinical infections in animals. Colonization with livestock associated MRSA, especially CC398, has been reported frequently in people who work with such animals, i.e. farmers, veterinarians and their family members (Leonard and Markey, 2008; Cuny *et al.*, 2009).

2.7.5. Antibiotic Resistance Mechanism of Staphylococcus aureus

Methicillin is classified under narrow spectrum beta-lactamase resistance penicillins. It acts by interfering primarily with the synthesis of bacterial cell wall and produce effect by binding to penicillin binding proteins (PBPs). PBPs essentially involved in the maintenance of normal cell morphology and viability of bacteria. Drugs occupy the

active site of transpeptidase enzyme (PBP) and inactivate it. Inactivation of transpeptidation in cell wall synthesis leads to blockage of cell wall synthesis (Walther *et al.,* 2008).

Mutation on *mecA* gene, which results in modification to PBP - 2a, results in drugs not binding to target site and organism, becomes resistant to β-lactams and other antibiotics with the same target site. MRSA uses efflux phenomenon resulting in continuous pumping of antimicrobial drugs out of bacterial cell. Alteration in outer-membrane proteins, which limit the access of drugs to cell (Stevens, 2004).

PBP2a - mediated resistance in MRSA suggested to take various forms and may arise from overproduction of PBP, acquisition of a foreign PBP with low affinity, recombination of susceptible PBP with more resistant varieties, point mutations within PBPs that consequently lower their affinity for β-lactams (Deurenberg *et al.*, 2007).

Development of resistance to Methicillin and other beta-lactams by *Staphylococcus aureus* was first *mecA* reported in 1961, which marked the appearance of Methicillin-Resistant *S. aureus* (MRSA). Methicillin resistance requires the presence of *mecA* gene. This gene is chromosomally located on a mobile genetic element called the Staphylococcal Cassette Chromosome (SCC). Further *mecA* gene encodes for penicillin-binding protein 2a (PBP-2a) and responsible for synthesis of penicillin-binding protein 2a (PBP2a; also called PBP2a) a 78-kDa protein. Expression of PBP-2a is controlled by *mecR1* and *mecI* regulator genes located upstream of gene. A mutation in the *mec* regulators leads to expression of *mecA* gene (PBP-2a). A PBP2a substitute for the other PBPs and, because of its low affinity for all β-lactam antibiotics, enables staphylococci to survive exposure to high concentrations of these agents. Thus, resistance to methicillin confers resistance to all β-lactam agents, including cephalosporin (Deurenberg *et al.*, 2007).

The confirmation of the presence of the *mecA* gene, has until recently been the 'golden standard' for detection of MRSA worldwide. However, a novel *mecA* homologue (with approximately 70% similarity to the *mecA* gene) that also confers methicillin resistance

was identified in *S. aureus* isolates from dairy cattle and humans. Additional genes, which are also found in susceptible isolates, can affect the methicillin resistance phenotype in *S. aureus*, resulting in heterogeneity of resistance and making detection of resistance difficult (Montanari *et al*., 1990; De lencastre and Tomasz ,1994).

Some strains of *S. aureus* possess an alternative resistance mechanism, attributable to the hyper-production of the *S. aureus* β-lactamase enzyme, which inactivates the antibiotic agents by hydrolysing the β-lactam ring of penicillin and cephalosporin compounds (Brown *et al*., 2005). Vancomycin was the only antibiotic available for treating MRSA infections. However, vancomycin-resistant MRSA strains, including some community acquired-MRSA strains, have increasingly been reported, thereby causing public health concern (CLSI, 2006; Tenover and Goering, 2009).

2.7.6. Identification of MRSA isolates

S. aureus (including MRSA strains) are cluster forming, facultative aerobic, Gram-positive cocci with intrinsic ability to ferment carbohydrates, producing white to deep yellow pigmentation on solid culture media. They also ferment mannitol turning mannitol salt agar yellow. The organisms produce deoxyribonuclease (DNase) and catalase enzymes and coagulase proteins used for their identification (Bannerman, 2004).

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

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

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

A range of factors, including the growth medium, the NaCl concentration and temperature (Reller et al., 2009), influences the results of the disk diffusion test. Commercial minimum inhibition concentration tests and automated antimicrobial susceptibility testing systems are widely used for MRSA detection. A commercial agglutination test based on the detection of PBP2a is also available for screening of methicillin resistance and Polymerase Chain Reaction (PCR) (Kluytmans et al., 2002) achieves definitive identification of MRSA upon detection of the mecA gene.

2.7.7. Control and Prevention measure of MRSA

The indiscriminate exposure of humans and animals to antibiotics created problem through acquisition and dissemination of MRSA, which limit the choice of treatment. Most antibiotics used for treatment of MRSA infection has been reported to developed resistance (Ayliffe, 1997).

In order to manage the risk of antibiotic resistance in humans and animals, decolonization of carriers and monitoring of resistant strains through susceptibility test will surely help. The use of antibiotic to treat infection should depend on the result of antimicrobial susceptibility testing, although most strains appear ineffective during treatment even when sensitive in routine susceptibility test. Antibiotics such as trimethoprim-sulphamethoxazole, clindamycin and doxycycline are reported to be effective in the treatment of CA-MRSA infection (Burke and Warren, 2014).

New drugs such as oritavancin, telavancinomadacycline, tedizolid and dalbavancin have a promising impact on the treatment of MRSA. Other existing agents such as fosfomycin and fusidic acid are under investigation for potential used in the treatment of MRSA infection (Burke and Warren, 2014).

As the MRSA epidemic becomes life threatening and beyond antibiotic therapy, development of vaccine to combat the disease became important (Cimolai, 2006). The first attempt to develop *S. aureus* vaccine was by the use of Streptococcus pneumonia and hemophilus influenza vaccine model (Hu *et al.,* 2013). Continuous attempts were made by different institutions like University of Chicago and the Absynth biologics, which uses clotting factors to produce abscess and membrane protein, respectively (Cheng *et al.*, 2010). However, trial on mice did not produce the desired result of abscess development and antibody production (Hu *et al.,* 2013).

Recently, Russell (2012) suggested the role of polyvalent pneumococcal vaccine to develop vaccine for staphylococcal infections. Therefore based on published data, researches are still been conducted on MRSA vaccine development but no established vaccine is available. In the absence of preventive measures such as vaccination, basic hygiene control options that will reduce MRSA colonization or infection in humans and animals are necessary. Basic hygiene, good husbandry and biosecurity measures on farms, abattoirs and food processing units have a tendency to reduce the spread of MRSA in animal population. Individuals with frequent animal contact should be educated on the risk of MRSA transmission in animals or their environment. In hospitals, hygienic measures particularly hand washing before and after contact with contaminated surfaces and the avoidance of close contact with discharges from nose, mouth and wounds of infected human and animals will surely reduce the chances of transmission (Cimolai,2006).

Decolonization of MRSA positive carriers' either through the use of antibiotic therapy (chlorhexidene or murocidin) or culling of affected animals or product will reduce the spread in the environment. Isolation of all suspect cases of MRSA infection, no entry to waiting room and hospitalize in isolation area prevents transmission from animal to

animal and hand hygiene and related measures like correct hand washing, use of alcohol-based hand sanitizers, cover wounds and skin lesions, use gloves, masks, eye protection, disposable aprons for contact with wounds, body fluids or other contaminated materials, Strict asepsis during surgery and Screening staff for MRSA colonization are highly significant for MRSA transmission from animal to human and vice versa (Morgan, 2008).

3. MATERIALS AND METHODS

3.1. Description of the Study Area

The study was conducted in and around Bahir Dar Town. Bahir Dar is the capital city of the Amhara region is located about 565 km North West of Addis Ababa at an elevation of the maximum 2276 to minimum 1660 meter above sea level with estimated to 170.052 square kilometers, is located between 11º27´1.82 to 11º39´ 7.56 N and 37º16´ 36.75 E to 37º31´48.06 E (Figure 1). It has summer rainfall, the highest rainfall is between June to September and a winter dry season (December to March) with mean annual rainfall at 1200-1600mm, and mean temperature 10-20^O_C (Bureau of Agriculture, 2006).

The livestock population of Amhara Region is estimated to be 13,766,923 Cattle, 8,825,061 Sheep, 5,102,580 Goat, 4,17,324 Horse, 2,398,190 Donkey,134,168 Mule, 60,576 Camel and 4,610,770 Poultry respectively (ANRSLRDPA, 2013/14).
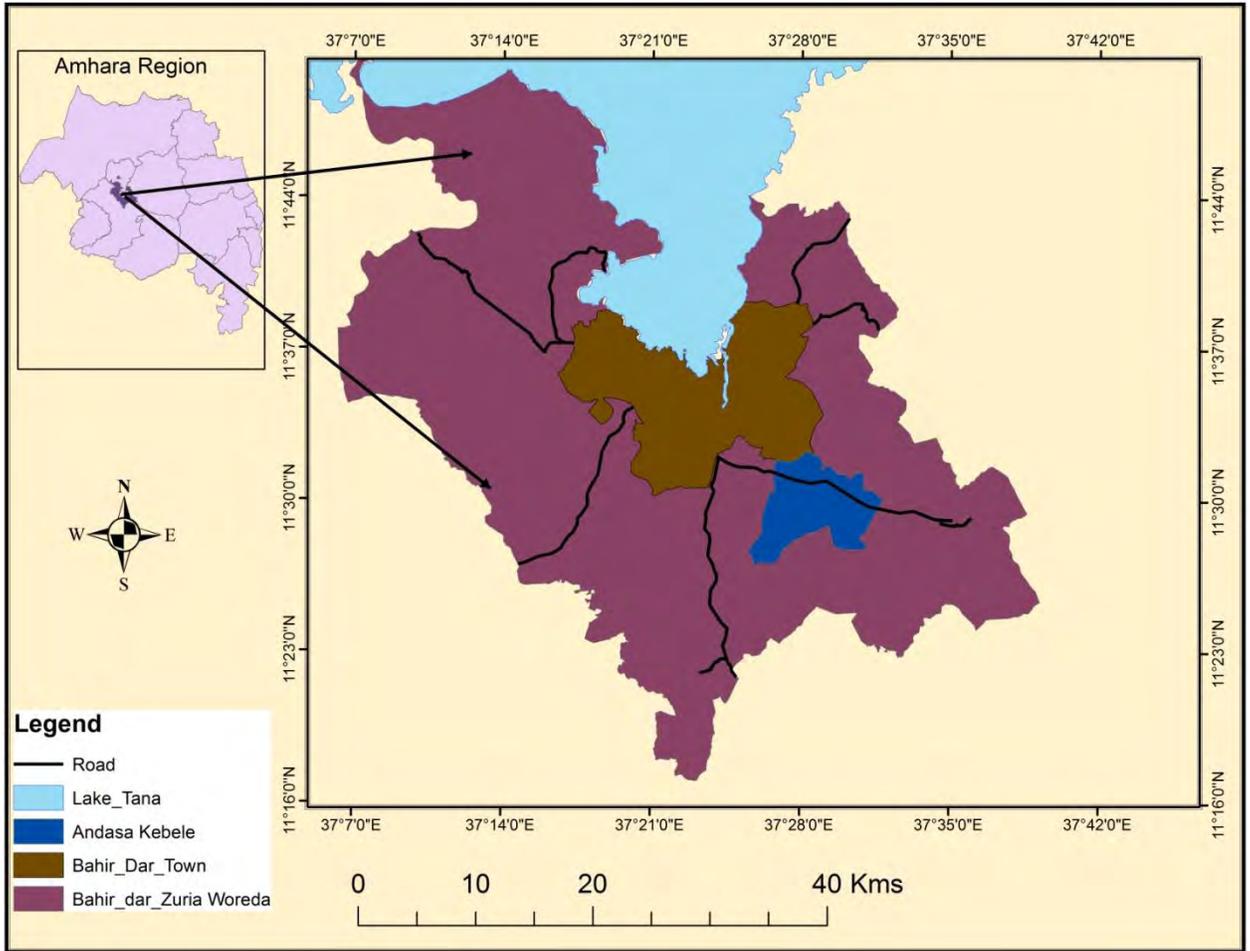
Figure 1: Map of the Study Area

3.2. Study Design

A cross sectional type of study was conducted from November 2014 to May 2015 to isolate and identify Methicillin resistant *Staphylococcus aureus.*

3.3. Study Population

Two breeds of dairy cattle, cross breed (Holstein-Friesian X   Fogera zebu and Local Fogera) were included in this study.

3.4. Sample Size

3.4.1 Sample size determination and sampling strategy

The sample size was calculated according the formula given by Thrusfield (2005). It was calculated by taking 28.2% estimated prevalence from previous study report by Bitew *et al.* (2010) and 95% confidence levels and 5% precision level. Simple random sampling method was considered to select the individual dairy cow.

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

Where N= required sample size,

Pexp = expected prevalence

d^2 = desire absolute precision

The sampling frame from the study site indicates most of the farms were smallholder dairy farms having an average of 8 lactating cows each and from Andassa Livestock Research Center. Therefore, all the lactating cows from 39 small-scale dairy farms and Andassa Livestock Research Center were considered for study that consists of 311 lactating cows. Of which 105 indigenous Fogera and 206 Holstein-Zebu crosses.

3.5. Study Methodology

3.5.1. Questionnaire survey

Data on each sampled cow was collected using a properly designed questioner format for determining the associated risk factors leading to MRSA. This includes age, parity, and stage of lactation, breed, previous history of mastitis treatment, barn floor type, milking hygiene, milking practice and other relevant information related to other managemental practices related to mastitis were gathered. Udder and milk abnormality (injuries,

swelling, milk clots and abnormal secretions, etc) were also recorded (Annex 1). Drug usage practice in the study area was also collected to evaluate its contribution to the emergence of antimicrobial resistance strains from Bahir Dar veterinary clinic.

Clinical Inspection of the Udder

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

California Mastitis Test (CMT)

The California mastitis test was conducted to diagnose the presence of subclinical mastitis and it was carried out according to standard procedures. A squirt of milk from each quarter of the udder was placed in each of four shallow cups in the CMT paddle and an equal amount of the reagent was added. A gentle circular motion was applied in a horizontal plane. Positive samples showed gel formation within a few seconds. The result was scored based on the gel formation and categorized as negative if there was no gel formation, or positive if there was gel formation ranging from +1 to +3 (Appendix II). If at least one quarter was positive by the CMT then the cow was considered as positive (Quinn *et al.*, 1994).

3.5.2. Milk sampling

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

Milk samples were collected from each of clinically and sub clinically mastitis functional quarters of the selected cows for bacterial isolation. About 10 ml of milk was aseptically collected from each mastitis positive quarter using sterile universal bottle. Then, samples were transported in an icebox to Bahir Dar Regional Veterinary laboratory for microbiological examination. If immediate inoculation is not convenient, samples were kept at 4°C until cultured for isolation.

3.5.3. Laboratory examination

I. Culturing and biochemical tests

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

II. Antimicrobial susceptibility testing

 Determining of the type antibiotic for invitro sensitivity test, retrospective data were compiled on the type of antibiotics used to treat mastitis and other infectious diseases in the region of the study area. In addition to, the selection of the types of antimicrobial agents was made based on clinical considerations including frequent use of the drug in the study area and availability.

The *Staphylococcus aureus* isolates were tested for anti-microbial susceptibility by disc diffusion method (Quinn *et al.,* 1999). It is stated in the absence of methicilin the best alternative is to use Cefoxitin for MRSA identification (NCC LS, 2011).
The following antibiotics were used for testing: Cefoxtin (30μg), vancomycin (30μg), penicillin G (10μg), tetracycline (30μg), streptomycin (10μg), chloramphenicol (30μg),

sulphamethoxazole trimethoprim (30μg) and amoxacilin (30μg) Oxoid Company (Hampshire, England). Colonies isolated from pure culture were transferred into a test tube of 5 ml peptone and suspension was made and incubated at 37°c for 8 hours. The turbidity of the suspension was adjusted comparing with that of 0.5 McFarland standards. Muller-Hinton Agar plate was prepared and a sterile cotton swab was dipped into the suspension and swabbed on the surfaces of Muller-Hinton Agar plate. Then, the antibiotic discs were placed on the agar plate using sterile forceps and pressed gently to ensure the complete contact with the agar surface. The plates were read after 24 hours of incubation at 35 ^{O}C under aerobic condition. The isolates were classified in accordance with the guideline of the National Committee for Clinical Laboratory Standards (CLSI, 2006) as susceptible, intermediate or resistance for each antibiotic tested according to the manufacturer's instructions by measuring the zone of inhibition around the antibiotic disc. Intermediate results were considered as resistant (Huber *et al*., 2011). Multiple antibiotic resistant (MAR) phenotypes were recorded for isolates showing resistance to three and more antibiotics (Rota *et al*., 1996).

3.6. Data Management and Statistical Analysis

Processing of data was done by computer software. Data was coded and entered to MS Excel spreadsheet and checked for accuracy. After validation, it was transferred and processed using computer software SPSS version 20 for analysis. Pearson's chi-square tests were used when appropriate to analyze the proportions of categorical data. Odd ratio and 95% CI were computed, the 95% confidence level was used, and results were considered significant at (P < 0.05).

4. RESULTS

4.1. Prevalence

A cross sectional study was carried out from October 2014 to April 2015 on lactating dairy cows of smallholder dairy farms in and around Bahir Dar. A total of 311 (105 Indigenous zebu and 206 cross breeds) dairy cows were included for the investigation of MRSA strains from mastitis cases of dairy cattle. Out of total lactating cows examined, 193 (62.06%) were found to be affected with clinical and subclinical mastitis infection. From the cases, 162(78.64%) were Crossbred while 31 (29.52%) were local Fogera breed (Table1). The prevalence of mastitis shows a significant difference (P< 0.000) between crossbreed and local Fogera breeds (Table 1).

Table 1: Prevalence of mastitis at breed level in cross breed and local zebu of lactating cows

Breed	No. of animal examined	No. of positive (%)	X^2	(p-value)
Crossbred	206	162(78.64%)		
Local Fogera	105	31(29.52%)	71.26	0.000
Total	311	193(62.06%)		

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The prevalence of mastitis between crossbreed and local Fogera breeds has high significant (P< 0.000).

From a total population of lactating cows examined the overall prevalence of mastitis at cow level was 193(62.06%), with the prevalence of 182 (58.52%) and 11 (3.54%) subclinical and clinical mastitis, respectively (Table 2). X^2 value between clinical and sub-clinical is 219.67 and (P<0.05) highly significant variation. The subclinical mastitis is significant (p=0.000) between breeds.

Table 2: Prevalence of Subclinical and clinical mastitis at breed level

Form of mastitis	Breed	No. of animals examined	No. of positive (%)	X^2	(p-value)
Subclinical	Cross breeds	206	152(73.79)		
	Local Fogera	105	30(28.57)	58.58	0.00
	Total	**311**	**182(58.52)**		
Clinical	Cross breed	206	10(4.85)	3.10	0.078
	Local Fogera	105	1(0.95)		
	Total	**311**	**11(3.54)**		

X^2 value between clinical and sub-clinical is 219.67 and (P<0.05) highly significant variation. The subclinical mastitis is significant (p=0.000) between breeds of cow but clinical mastitis insignificant P=0.078 (P>0.05) between breeds.
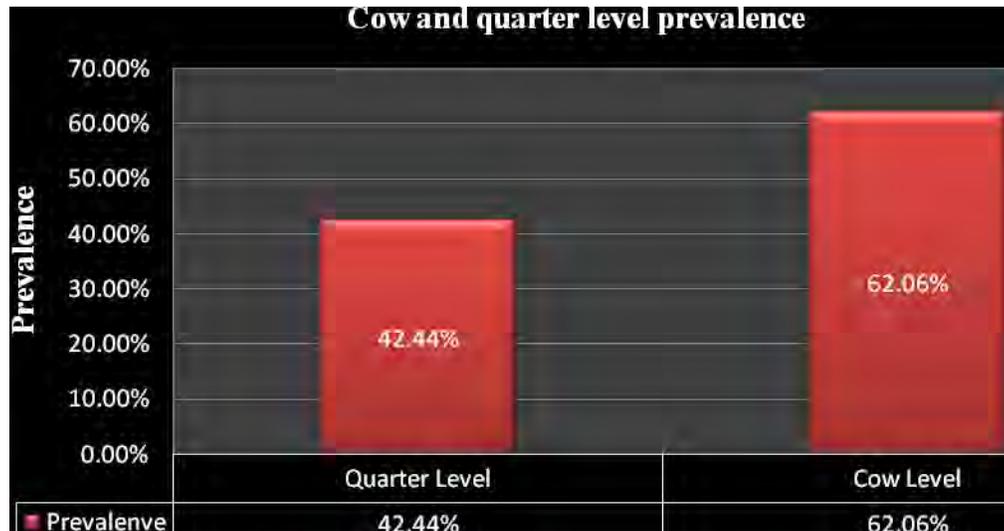
Figure 2: Cow and Quarter level prevalence
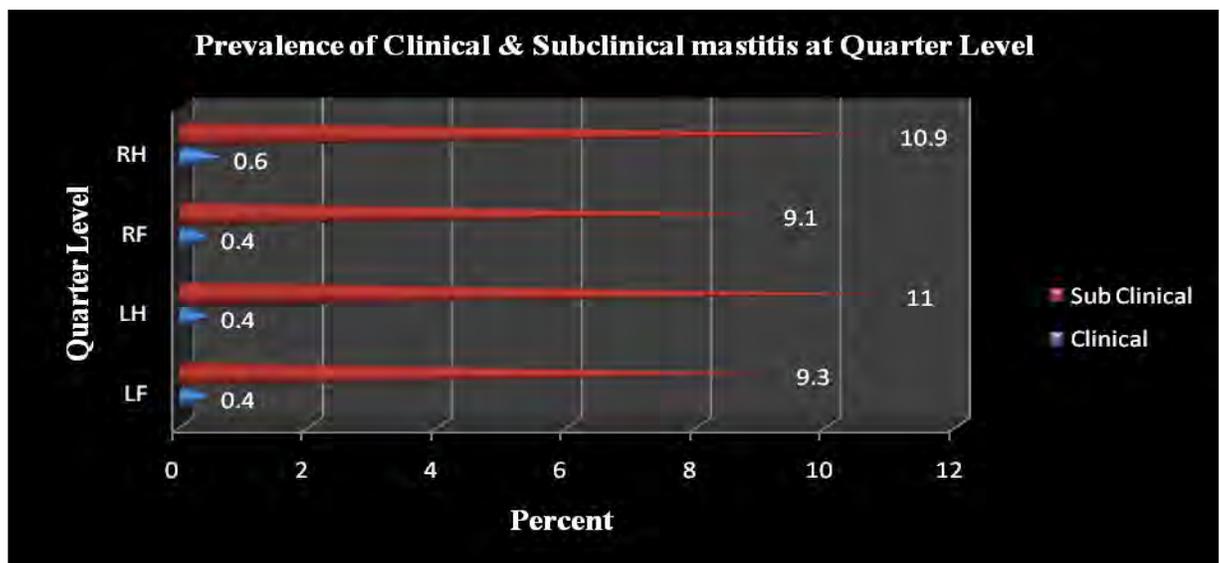
LF = left front, LH= Left hind, RF =Right front, RH = right hind

Figure 3: Prevalence of clinical and sub-clinical mastitis at quarter level

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Out of the 1244 quarter examined, 60 (4.82%) quarters were found blind, and the quarter and cow level prevalence were 42.44% (528) and 193(62.06%) respectively. From the total quarter level prevalence, 32.3% was caused by sub clinical mastitis (Figure 2 &3).

4.2. Risk Factors Associated with Mastitis Prevalence

The questionnaire survey and observation data result shows breed, age, and previous mastitis treatment history and floor type are among the potential risk factors, which are associated with mastitis disease in dairy farms (Table 3).

Table 3: Result of Univariate logistic regression of attribute risk factors with mastitis

Factor	Categories	Total no. examined	No (%) positives	OR	p-valve	CI
Age(years)	Young(≤ 3)	106	25(23.58%)			
				3.27	0.000	2.358-4.54
	Adult(4-7)	123	93(75.06%)			
	Old(> 7)	82	75(91.46%)			
Breed	Cross	206	162(78.64%)			
				0.089	0.000	0.053-0.152
	Zebu	105	31(29.52%)			
Parity	1-2	146	65(44.52%)			
				1.205	0.214	0.897-1.620
	3-4	80	53(66.25%)			
	≥ 5	85	75(88.23%)			
Lactation period	Early	101	43(42.57%)			
				1.029	0.831	0.791-1.339
	Mid	119	90(75.63%)			
	Late	91	60(65.93%)			
Previous mastitis treatment history	Yes	122	88(72.13%)			
				0.390	0.0001	0.241-0.630
	No	189	105(55.56%)			

Floor system	Concrete	250	180(72.0%)			
	Mud	61	13(21.31%)	0.743	0.000	0.386-0.643
Milking hygiene	Good	134	72(53.73%)			
	Poor	177	111(62.71%)	1.371	0.1641	0.879-2.138

Based on the above univariate logistic analysis the p-values of age group, breed, previous mastitis treatment history related, and floor system (P<0.05) which showed significant correlation with occurrence mastitis and the rest are non-significant (p>0.05).

4.3. Occurrence of *S. aureus* from Mastitic Lactating Cows

From 193 mastitis, infected lactating cows (clinical 11 cows subclinical 182 cows of 528 milk samples) were cultured and twenty-nine *Staphylococcus aureus* were isolated. The isolates of *Staphylococcus aureus* in subclinical and clinical mastitis was 29 (15.02%). *S. aureus* was isolated at a rate of 28 (14.50%) and 1(0.51%) from subclinical and clinical mastitis infections, respectively. Statistically no significant association was observed on the occurrence of Staphylococcal mastitis (Table 4).

Table 4: Number and percentage of *S.aureus* isolated from clinical case and CMT positive subclinical cows (528)quarters

Form of mastitis	No of examined	No of *S.aureus* isolated	Prevalence
Clinical	11(26)	1	0.51%
Subclinical	182(502)	28	14.50%
Total	193(528)	29	15.02%

$X^{2=}$, 0.32   OR=, 0.55   df=, 1   p-value =0.57

4.4. Antibiotic Susceptibility Test

All the isolated Twenty-nine *S.aureus* was tested for susceptibility to selected antibiotics. Antimicrobials used in this study were, Amoxicillin, Sulphamethoxazol trimethoprim, tetracycline, chloramphenicol, Cefoxitin, Vancomycin ,Penicillin G, and Streptomycin. The present study has demonstrated the existence of the levels of resistance of *S. aureus* to commonly used antimicrobial agents in the study area. 75.7% of the *S.aureus* was found to be resistance to Cefoxtin, which shows the prevalence of MRSA.

The resistance profile of penicillin G, streptomycin, tetracycline, amoxicillin and vancom -ycin were 95.8%, 73.1%, 72.2%, 61.5%and 52.4%, respectively (Fig.4).
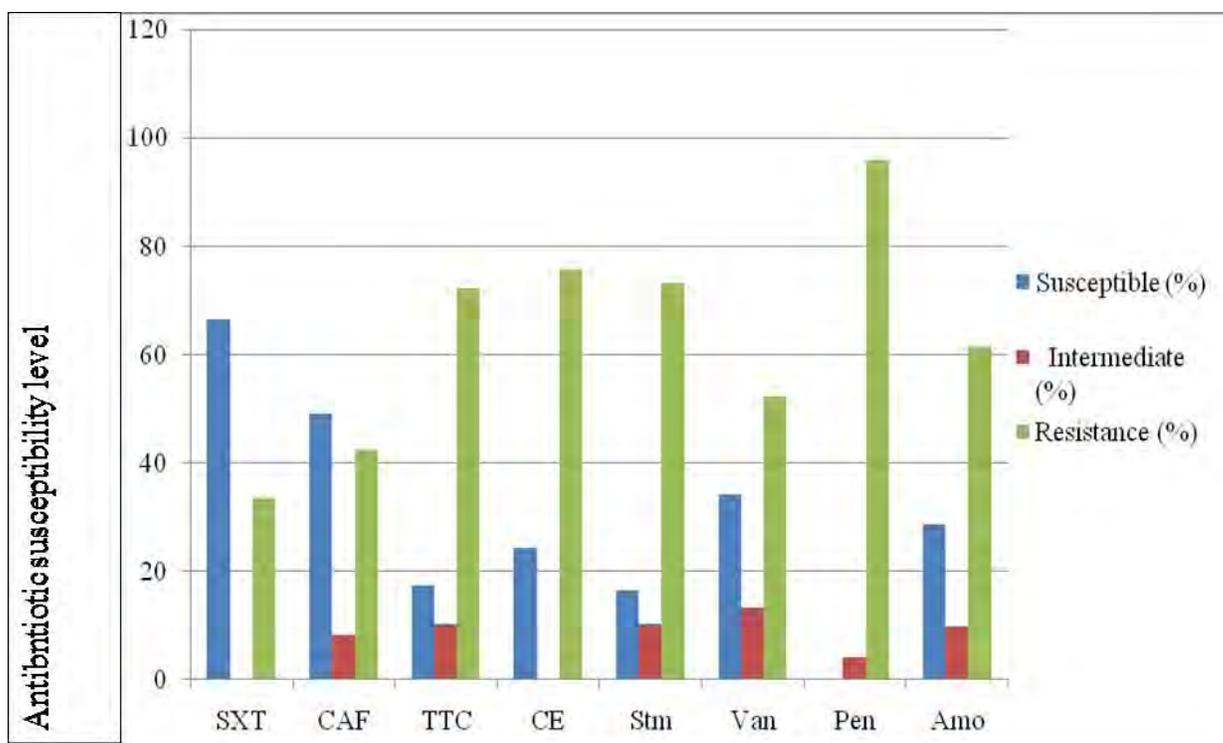
Figure 4: Antibiotic-resistance profiles of *Staphylococcus aureus is* isolated from mastitis milk

Key- SXT= Sulphamethoxazole Trimethoprim Caf= Chloramphenicol TTC= Tetracycline CE= Cefoxitin Stm= Streptomycin Van= Vancomycin Pen= Penicillin G, Amo= Amoxicillin

4.5. Association of MRSA with Age of Cows and Previous Treatment

Occurrence of *S.aureus* relation with age of cows were 6 (20.7%), 18 (62.06%) and 5(17.24%) in young, in adult and old age group respectively. Older cows more often harbor multidrug resistant than younger cows. Adults and old age category dairy cows were found to be positive for multidrug resistant *S. aureus* of which. 72.1% and 100% respectively (Table 5). MRSA was also found to be associated with previous treatment history of the animal. It shows that 72.4% of the isolate had previous history of treatment (Table 6). All of the isolated MRSA were from adult and old age category.

Table 5: **Drug resistance pattern of *S. aureus* and age of cows**

Age of Cows	*S. aureus* isolated	Resistance pattern					
		One drug	Two drug	Multidrug	P-value	Df	X^2
Young	6	4(66.66%)	2(33.33%)	0(0%)			
Adult	18	2(11.11%)	3(16.67%)	13(72.1%)	0.003	4	14.99
Old	5	0(0%)	0(0%)	5(100%)			
Total	29	6	5	18			

X^2 = 14.99   Df = 4   P-value = 0.003 statistically significant (P<0.05).Multidrug resistance prevalence were =18*100/29 = 62%

Table 6: Association of Cefoxitin resistance pattern with previous treatment

Cefoxtin resistance	Previous mastitis treatment		
	Yes	No	total
Positive	21	4	25
Negative	2	2	4
Total	23	6	29

X^2 = 2.43, df = 1, P-value = 0.119, OR= 5.25, statistically insignificant (P>0.05)

4.6 Retrospective data

Retrospective data were compiled on the type of antibiotics used to treat mainly mastitis diseases in the study area. List of antibiotics used to treat clinical mastitis cases from July 2011- April 2015 were gathered from casebook records in Bahir Dar Veterinary Clinic (Table 7).

Table 7: Mastitis cases and type of drugs used for therapy in Bahir Dar Veterinary Clinic (2011 – 2015).

No.	Commercial name	Common name	No. of mastitic case treated	%
1	Pen- Strep	Procaine penicillin BP 200mg and Dihydrostreptomycin BP 250mg	109	53.96
2	Procaine Penicillin	Procaine penicillin 4 million IU	19	9.41
3	Benzanthine Penicillin	Benzantine penicillin	7	3.5
4	Intramamary Infusion/Lactaclox/	Ampicillin(as sodium salt) 75mg Cloxacillin(as sodium salt)200mg	38	18.81
5	Oxytetracycline 20%	Oxytetracycline hydrochloride 200mg	21	10.39
6	Oxytetracycline 10%	Oxytetracycline hydrochloride 100mg	8	3.96
		Total	**202**	**100.00**

Retrospective data were compiled on the type of antibiotics used to treatment of mastitis lactating cows in study area, with the aim of additional criteria to select antibiotic discs for invitro susceptibility test. The data indicated that the Penicillin and streptomycin combination, oxytetracycline and intamammary infusion of ampicillin were the only commonly used antibiotics to treat mastitis cases.

5. DISCUSSION

A cross sectional study was conducted on bovine mastitis in Bahir Dar and its surroundings to determine the occurrence and major risk factors associated with bovine MRSA in mastic infection. A total of 311 cows, 105 lactating local zebu and 206 Holstein x Local Zebu crossbreds from smallholder farms were investigated.

The overall prevalence of mastitis at cow level in this study, was in agreement with previous finding of other researchers, Tolla (1996) 61.1% in South Wello and Geresu (1989) 63% in Addis Ababa. However, the finding was lower than the previous finding of Abaineh (1997) who reported 65% in Fiche, Abera *et al*.(2013) who reported 66.6% in Adama, 68.1% by Zerihun (1996) in Addis Ababa, (Mekibib *et al*.,2010) 71.0% in Holeta, and 85.6% by Nesru (1986) in Dire-Dawa. On the other hand, this result is higher than previous reports of Bitew *et al*. (2010) of (28.2%) in Bahir Dar Ethiopia. This variability in prevalence of mastitis between different reports could be attributed to differences in farms management practice or to differences in study methods agro-climatic condition.

The present study showed that breed was significantly ($p < 0.05$) associated with the occurrence of mastitis. This is in line with Bitew *et al*.(2010) who reported in Bahir Dar, between Cross and Fogera breed, Lakew *et al* .(2009) in cross and local Arsi breed and Biffa *et al*.(2005) found significant difference between local Zebu, Holstein-Frisian and Jersey breeds in Ethiopia. Increased milk yield from genetic selection may be accompanied in genetic susceptibility to mastitis (Schutz, 1994). Therefore, the lower prevalence in local Fogera breeds in this study could be associated with difference in genetically controlled physical barrier like streak canal sphincter muscles, keratin in the teat canal or shape of teat end where pointed teat ends are prone to lesion (Seykora and Mcdaniel, 1985). In addition to physical barriers, the difference in occurrence of mastitis in these breeds could arise from differences in cellular immunity (Erskine, 2001).

The prevalence of clinical mastitis at cow level is in line with Almaw (2004), Bitew *et al.*(2010) who reported 3.9%, and 4.8% respectively in Bahir Dar and 3.9% by Abera *et al.*, 2013) in Adama, Ethiopia. However this finding was lower than reports made by Kerro and Tareke (2003) in southern and Hundera *et al.*(2005) in central Ethiopia with a rate of 10% and 16.11% respectively.

The prevalence of subclinical mastitis at cow level in both breeds was in agreement with Zerihun (1996) 55.1% in Addis Ababa dairy farms, where as prevalence of subclinical mastitis in cross breed was significantly associated with the occurrence of mastitis and this result was closely related with Machang and Muyungi (1998) who reported 67% in Tanzania. However, Kivaria *et al.* (2004) reported higher prevalence 90.3% in lactating cows in smallholder dairy farms in Tanzania.

The occurrence of subclinical mastitis in local breed at cow level was comparable with Temesgen (1999) 25% in Mekele, Ethiopia. The current study as well as in other similar studies, overwhelming cases of mastitis were subclinical as compared to clinical mastitis in both breeds (Kassa *et al.*, 1999; Hussein, 1999; Workineh *et al.,* 2002; Kerro and Tareke, 2003). In Ethiopia, the subclinical form of mastitis received little attention and efforts have been concentrated on the treatment of clinical cases (Hussein, 1999) while the high economic loss could come from subclinical mastitis.

The quarter level mastitis recorded in the present study was in line with the report of Abera *et al.*(2013) and Mekibib *et al.* (2010) who reported 42% and 44.8% in Adama and Holeta town respectively. The current finding was also comparable with the report of Nesru *et al.* (1999) 37% in Addis Ababa; but higher than Biffa *et al.*(2005) and Almaw (2004) who reported 28.2% and 17.9% respectively and Kerro and Tareke,(2003) who found 19 % in different area of Ethiopia. This variation might be due to the complex effect of mastitis in the management system of the farm, breeds of cattle and geographical location of the study area.

The higher mastitis infection was observed in hindquarters than front quarters. This is in agreement with previous report of Kerro and Tareke, 2003. This might be due to

hindquarters are highly prone to contamination with dirty. In addition to this, large amount of milk is produced from hindquarters and as a result, the pressure on teat canal forces the canals to be opened widely which allows entrance of microbes. In the present study, the proportion of blind quarters to be (4.82%), which is close to the finding of Mungube (2001) and Almaw (2004) in Ethiopia who reported 3.75% in Addis Ababa and 3.8% in Bahir Dar, respectively. This could be an indication of serious mastitis problem in dairy farms and absence of culling that should have served to remove a source of mammary pathogens/or infections.

The current study showed significantly higher mastitis infection as the ages of cows increases. This finding was in agreement with the report of Kerro and Tareke, (2003) who found that, the risk of clinical and subclinical mastitis infection increases with the advancing age of the cow. This might be due to the increased opportunity of infection with the time and the prolonged duration of infection, especially in herd without mastitis control program (Radostitis *et al*., 2007).

The higher the parity number, the higher prevalence was observed in the studies of Tesfaye, (1995) and Biffa *et al*.(2005). According to Erskine (2001) report, primiparious cows have more effective defense mechanism than multiparious cows. But the present study showed a lower prevalence of mastitis at 3-4 births followed by in cows that gave 1-2, births and higher in cows that gave more than 5 births, however the occurrence of mastitis and parity revealed insignificant variation. This variation might be due to the influence of breed and management of the farm.

The highest occurrence of mastitis was observed in cows at mid stage of lactation. This result was in agreement with the work of Nesru ,(1999), and Kerro and Tareke (2003) who reported higher prevalence of mastitis in cows at mid and late stage of lactation. However, Mungube et *al*. (2004) and Biffa *et al*. (2005) reported higher prevalence of mastitis in early stage of lactation. The variations in the stages of lactation among different studies might be related to age variation, parity and breed of the sampled animals as indicated by Getahun *et al*. (2008).

The occurrence of mastitis in house with concrete floors significantly higher as compared to muddying floors. This finding was differ with the report of Fekadu *et al.* (2005) and Abera *et al.* (2013) in South Ethiopia and Adama town. This variation could be attributed to the management, lower number of samples examined in muddy housed animals in the study area, and the existence of *S. aureus* in a wide extremes of temperature, moisture in the environment and readily colonize teat orifice, damaging roughed epithelium (NMC, 1990).The main sources of infection are udder of infected cows transferred via milker's hand, towels and environment (Radostitis *et al.*,1994).

Isolation of *S.aureus* from clinical and CMT positive milk samples were done in this study. The microbiological examination of milk from lactating cows for *S. aureus* revealed similar result with the report of Abebe *et al.*(2013) 15.5% at Addis Ababa, Almaw(2004) 16.67% in Bahir Dar, and closely agreed with the report of Bitew *et al.*(2010) 20.3% in the same area, but higher than Bishi (1998) and Hussien (1999) who reported 9% and 10.69% respectively in Addis Ababa dairy farms. This finding was lower as compared to the result of Workineh *et al.* (2002) and Kerro and Tareke (2003) who reported 39.2% and 40.3% at Addis Ababa, and Southern Ethiopia respectively. The possible explanation for the variation might be S. *aurues* is a contagious pathogen transmitted from one cow to another by contact with animals during unhygienic milking procedures (Rowe, 1999).

The isolation of *S. aureus* in subclinical mastitis showed higher prevalence than clinical mastitis. This could be due to the adaptation of S. *aureus* to survive in the udder and establish chronic and subclinical mastitis infection by shedding into milk, which serves as source of infection for other healthy cows during the milking process. Subclinical mastitis infections are generally not treated during the lactating period because of low cure rates and economic costs associated with treatment and withdrawal period for milk (Radostatis *et al.* (1994). Statistically, insignificant difference has been observed in the status of *S. aureus* between clinical and subclinical mastitis as a cause of mastitis, this could be because of very few number of clinical case samples and isolates of the *S. aureus* during the study period.

Furthermore, in the present study *S. aureus* showed resistance, to penicillin G, cefoxtin, streptomycin, tetracycline, amoxicillin and vancomycine. This is in line with the finding of Abebe *et al.*(2013) who reported resistant of penicillin G 96.7% and tetracycline 73.8% around Addis Ababa, and Abera *et al* .(2010) 94.4% resistance to penicillin G in Adama; in addition to this study has demonstrated the existence of alarming level of resistance of *S. aureus* to commonly used antimicrobials (penicillin G,streptomycin and tetracycline) in dairy farms. This results were in accordance with reports from other countries (Edward *et al.*, 2002 and Jakee *et al.*, 2008) and this could be due to prolonged and indiscriminate usage of some antimicrobials. Penicillin G was showed very great resistance to *S. aureus*. Since this antibiotic represents the main antibiotics group recommended for Staphylococcal mastitis treatment and regular use of antibiotics for the treatment of cows, may result in the spread of resistant because penicillin and tetracycline are the only most commonly used antimicrobials for the treatment of other infections as well as mastitis in veterinary practice in Ethiopia.

According to Green and Breedely (2004) 50% of mastitis causing *S.aureus* strains produces beta lactamase and develop resistant to various antimicrobials such as:- penicillin and cefoxtin may be attributed to the production of ß-lactamase, an enzyme that inactivate penicillin and closely related antibiotics. Antibiotic resistant genes carried on plasmid and transposons which can pass from one staphylococcal species to another and the most common strategy used by *S. aureus* to circumvent the action of the penicillins is by the production of enzyme ß-lactamase, which hydrolyses the beta-lactam ring, rendering the entire compound inactive (Kernodle , 2000).

Staphylococcal bovine mastitis appearance of multidrug resistance against to certain antibiotics groups in a specific region might be due to their frequent and long-term use of antibiotics (Sabour *et al.*, 2004; Moon *et al.*, 2007).

In the current study the numbers of Methicillin (Cefoxtin) resistance isolates were obtained from mastitis milk in the study area which was lower than that of Daka *et al.* (2012) who found 60.3% but it was comparable with finding of Joshi *et al.*(2014) who reported prevalence of MRSA (Cefoxtin,11.25%) in Nepal which is well known that

Cefoxtin is not used for veterinary practice in the study area. However, the cross transmission between human and animals (Juhasz-Kasanyitzky et al., 2007) could explain Methicillin resistance.

S. aureus has a tendency to rapidly acquire antibiotic resistance to different classes of antibiotics. The present study showed similar result with the finding of Abera *et al*.(2013) , who reported lower résistance of chloramphenicol and sulphamethoxazole trimethoprim in Adama town. The reason why chloramphenicol and sulphamethoxazole trimethoprim were less resistance could be infrequent use in the study area in veterinary clinics, and perhaps in human medicine. Similar suggestion was given by Jaims *et al*. (2002) that the development of antimicrobial resistance is nearly always as result of repeatedly use of therapeutics or indiscriminate use.

MRSA strains have developed multi-drug resistance worldwide with broad diversity in prevalence rate in different regions (Normanno *et al.*, 2007). *S.aureu*s revealed high percentage of resistant to multi-drugs as age increases in the present study. Even though, an antibiotic therapy is an important tool in the scheme of mastitis control, extensive use of antibiotics can lead to development of resistance among different bacterial strain (Radostits *et a*l., 2000). The association between multi-drug resistance and age of cows of MRSA strains were significantly associated. In the current study, multidrug resistance status was increases as age of cows increased .The possible suggestion for this may be as age of cows advance of having chance of treatment with antibiotics.

Vancomycin is one the drug choice for treatment and control of MRSA, but in the present study recorded resistance. This is in agreement with the finding of DAka *et al*.(2012) 57% isolated *S.aureus* resistant to vancomycin in Hawassa town. However, this result is very high as compared to Tariku *et a*l.(2011) who reported   *S.aureus* resistance to vancomycine was 3% in dairy farm in Jimma Town, South Ethiopia. Vancomycin was the only antibiotic available for treating MRSA infections. However, vancomycin-resistant MRSA strains, including some community acquired-MRSA strains, have increasingly been reported, thereby causing public health concern (CLSI, 2006; Tenover and Goering, 2009).

The retrospective data collected from veterinary clinic in the study area from 2011 - 2015 of five years period, indicated that about 202 mastitis cases brought to the clinic for the treatment of mastitis in the present study area. Out of the total cases 53.96% cases were treated with penicillin-streptomycin combination, 18.81% of intramammary infusion of ampicillin, 10.39% oxytetracyline hydrochloride 200mg and 9.41% Procaine penicillin 4 million IU respectively, which were frequently used drug in clinics. This showed that the commonly used antimicrobials in veterinary clinic in the area closely in agreement with drug resistance pattern which found in this study. Frequently usage of antibiotics correlates with the emergence and maintenance of antibiotic-resistant traits within pathogenic strains of bacteria (Shitandi and Sternesjo, 2004). The possible explanation for high record for multidrug resistant *S.aureus* in dairy farms may be due to the uncontrolled use of antimicrobial and lack of drugs sensitivity test in the dairy farms.

6. CONCULUSION AND RECOMMENDATIONS

Mastitis was found to be one of the major constraints to dairy production in extensive dairy farms. Management factors are highly responsible for the successful mastitis control in dairy farms. This study revealed that host risk factors significantly influence the prevalence of mastitis. Different risk factors are associated with bovine mastitis in the study area, among these; breed type, floor type and age of the animal were prominent. The Crossbred cows were affected more than the local Fogera. The prevalence of *S. aureus* mastitis at cow and quarter level indicates that mastitis one of the major problems of dairy cows in milk production in the study area. It was found the major of the tested isolates of *S. aureus* were resistant to the various antimicrobial agents particularly penicillin G, cefoxtin, tetracycline, streptomycin and amoxicillin. As the retrospective data collected from veterinary clinic utilization antimicrobial for the treatment of mastitis cases as well as for other infection in the study area predominately, depend on Penicillin and Streptomycin combination and ampicillin intramamary infusion, which is related with a great resistant level of *S.aureus* in the present study. In the present observation *S.aureus* isolates showed multidrug resistance primarily to penicillin G, cefoxtin, tetracycline and streptomycin because of resistance to beta lactams and frequently use. The findings suggest that multidrug resistance *S,aureus* are present in higher concentrations in the dairy farms of the area which indicated that MRSA spread with the farm.

Generally, the present study revealed higher prevalence of mastitis and occurrence of multidrug resistance *S. aureus* specifically which belongs to the MRSA which are dependent on multiple associated risk factors. *S. aureus* to various antibiotics indicated that existence of alarming level of resistance of frequently isolated mastitis bacteria to commonly used antimicrobial agents in the study area. Therefore, it is very important to implement general mastitis control method with a systematic application in vitro antibiotic susceptibility test prior to the use of antibiotics in both treatment and prevention of intra mammary infections.

Based on the above conclusion the following points are forwarded:-

- Different epidemiological factors that interplay in mastitis occurrence should be studied routinely which ultimately downgrading the prevalence of the disease.
- Hygiene during milking procedure is mandatory which reduce the transmission of the disease.
- There should be regular antimicrobial sensitivity test to select effective and alteration of antibiotics to reduce the problems of drug resistance development towards commonly used antibiotics
- Awareness creation of dairy farms owners, dairy workers and veterinarians on the effect of MRSA should made.
- Risk factor controlling mechanisms should be implemented.
- Veterinarian and medical researchers in collaboration should make further investigation on public health significance of MRSA associated with bovine mastitis at regional and national level.
- Medical practitioners should be encouraged to choose antibiotic based on susceptibility test and to wear protective equipment during surgery and handling of patients to reduce contamination and spread of the disease.
- Furthermore, impacts and dynamics of generic of antibiotics should be investigated using molecular method.

7. REFERENCES

Abaineh, D. (1997): Treatment trials of sub clinical mastitis with a polygonaceae herb. Proceeding of the 11th Conference of Ethiopia Veterinary Association. Addis Ababa. Ethiopia. Pp. 66-75.

Abebe, M., 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. *Afr. J. Microb. Res.,* **7**: 3501-3510.

Abera, M., Demie B., Aragaw K., Regassa F. and Regassa, A. (2010): Isolation and identification of *Staphylococcus aureus* from mastitic milk and their drug resistance patterns in Adama town, Ethiopia. *J.Vet. Med. Anim. Hlth.,* **2**: 29-34.

Abera, M., Demie, B., Araga, K., Regassa, F. and Regassa, A. (2013): Isolation and identification of *Staphylococcus aureus* from bovine mastitic milk and their drug resistance patterns in Adama town, Ethiopia. *Afr. J. Dai. Far. and Mil. Prod.,* **1**:019-023.

Almaw, G. (2004): Cross sectional study of bovine mastitis in and around Bahir Dar and antibiotic resistance patterns on major pathogens. MSc thesis, Addis Ababa Ababa University, Faculty of Veterinary Medicine, Ethiopia.

Anderson, J.C. (1976): Mechanism of staphylococcus virulence in relation to bovine Mastitis. *Br. Vet .J.* **132**:229-321.

ANRSLRDPA (2013/14).Amhara National Regional State of Livestock resource development promotion Agency.

Araya, M. (2004): The effect of herbal preparations of *Staphylococcus aureus* and *streptococcus agalactiae* isolated from clinical bovine mastitis. Msc.Thesis, Addis Ababa University, Faculty of Veterinary Medicine, Debre-Zeit, Ethiopia.

Ateba, C.N., Mbewe, M., Moneoang, M.S., Bezuidenhout, C.C. (2010): Antibiotic-resistant Staphylococcus aureus isolated from milkin the Mafikeng Area, North West province, South Africa. *S .Afr.J Sci.*, **106**:1-6.

Ayliffe, G. (1997): The progressive intercontinental spread of Methicillin-resistant *Staphylococcus aureus.Clin.Infec.Dis.*,**24**:74-79.

Bannerman, T. L. (2004): Staphylococcus, Micrococcus and other catalase-positive cocci that grows aerobically In: Manual of Clinical Microbiology P. R. Murray, E. J. Baron, J. H. Jorgensen, `M. A. pfaller and R. H. Yolken eds. 8th edition, ASM Press Washington DC.

Barber, M. (1961): Methicillin-resistant Staphylococci. *J. Cl. Path.*, **14**: 385-393.

Barkema, H.W., Schukken, Y.H. and Zadoks, R.N. (2006): Invited review: The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. *J. Dai. Sci.*, **89**: 1877–1895.

Bens, C.C.P.M., Voss, A. and Klaassen, C.H.W. (2006): Presence of a novel DNA methylation enzyme in methicillin-resistant *Staphylococcus aureus* isolates associated with pig farming leads to un interpretable results in standard pulsed-field gel electrophoresis analysis. *J. Cl. Micr.*, **44:** 1875-1876.

Biffa, D., Debela, E. and Beyene, F. (2005): Prevalence and risk factors of mastitis in lactating dairy cows in southern Ethiopia. *Int. J. Appl. Res. Vet. Med.*, **3**:189-198. *J. Biosci.,* **36** :175–188.

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 University, Berlin, Pp 1-50.

Bitew, M., Tafere, A. and Tolosa, T. (2010): Study on Bovine Mastitis in Dairy Farms of Bahir Dar and its Environs. *J. Anim. Vet. Adv.*, **9**:2912-2917.

Brouillette, E. and Malouin, F. (2005): The pathogenesis and control of *Staphylococcus aureus* induced mastitis, Study models in the mouse, *Micro. Infct.,***7:**560-568.

Brown, J., Edwards, I. Hawkey, M., Morrison, D. Ridgway, L., Towner, J. and Wren, D. (2005): Guidelines for the laboratory diagnosis and susceptibility testing of methicillin resistant *Staphylococcus aureu*s (MRSA). *J. Antim. Chemor*., **56**:1000–101.

Bureau of Agriculture (2006): Livestock resource development and animal health Development Annual Report, Bahir Dar, Ethiopia. 855p.

Burke, S.T. and Warren, E.R. (2014): New pharmacological treatments for methicillin-resistant *Staphylococcus aureus* infections. *Expert opinion on pharmacotherapy*, **15**: 483-491.

Busato, A., Tracheal, P., Schallibaum, M. and Blum, J.W. (2000) : Udder health and risk factors for subclinical mastitis in organic dairy farms in Switzerland. *Prev. vet. Med.*, **28**:205-220.

Carter G.R.(1984): Diagnostic Procedures in Veterinary Bacteriology and Mycology. 4th ed. U. S. A. Charles Thomas Publishers: Springfield, Ill.

Central Statistical Agency (CSA) (2008): Federal democratic republic of Ethiopia, central statistical agency. Agricultural sample survey, Vol. 2, report on livestock and livestock characteristics.

Chambers, H.F. and Deleo, F.R. (2009): Waves of resistance *Staphylococcus aureus* in the antibiotic era. *Nat. Rev . Microbiol,* **7:** 629–641.

Cheng, A.G., McAdow, M., Kim, H.K., Bae, T., Missiakas, D.M., Schneewin (2010): Contribution of coagulases towards *Staphylococcus aureus* disease and protective immunity. *PLoS. Pthog*., **6**:1001-036.

Cimolai, N. (2006): Community-acquired MRSA infection: An emerging trend. *BCMJ.*, **48**: 116-120.

Clinical and Laboratory Standards Institute (CLSI) (2006): Investigation and control of vancomycin intermediate and resistant *Staphylococcus aureus*. A guide book for health departments and infection control personnel, Wayne, PA.

Clinical and Laboratory Standards Institute (CLSI) (2009): Performance standards for antimicrobial susceptibility testing: Nineteenth informational supplement, M100–S19, CLSI, Wayne, PA.

Cramton, S. E., Gerke, C., Schnell, N. F., Nichols, W.W. and Gotz, F. (1999): The intercellular adhesin locus is present in *Staphylococcus aureus* and is required for biofilm formation. *Inf. Imm.,* **67**: 5427-5433.

Cuny, C., Friedrich, A., Kozytska, S., Layer, F. and N¨ubel, U. (2010): Emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) in different animal species. *Int. J. Med. Microbiol.,* **300** :109–117.

Cuny, C., Kuemmerle, J., Stanek, C., Willey, B., Strommenger, B. and Witte, W. (2006): Emergence of MRSA infections in horses in a veterinary hospital. Strain characterisation and comparison with MRSA from humans. *Euro. Surv*., **11**(1).

Cuny, C., Nathaus, R., Layer, F., Strommenger, B., Altmann, D.and Witte, W. (2009): Nasal colonization of humans with methicillin-resistant *Staphylococcus aureus* (MRSA) CC398 with and without exposure to pigs. *PLoS ONE,* **4**:6800.

Daka, D., Solomon, G. and Dawit, Y. (2012): Antibiotic-resistance Staphylococcus aureus isolated from cow's milk in the Hawassa area, South Ethiopia. *An. Cli. Microb. and Antimicr.*, **11**:26

Dancer SJ (2008): Importance of the environment in meticillin-resistant *Staphylococcus aureus* Acquisition: the case for hospital cleaning. *Lnct. Inf. Dis*.,**8** : 101-113.

David MZ and Daum RS. (2010): Community-associated methicillin-resistant *Staphylococcus aureus*: epidemiology and clinical consequences of an emerging epidemic. *Clin. Microbiol Rev.*, **23**:616–687.

De Lencastre H, Tomasz, A.(1994): Reassessment of the number of auxiliary genes essential for expression of high-level Methicillin resistance in *Staphylococcus aureus*. *Antimicrob. Agents Chemthr.*, **38**: 2590–2598.

DeGraves,J .and Fetrow, J.(1993):Economics of mastitis and mastitis control. *Vet. Clin. North. Am. Food Anim. prct.*, **9**:421-434.

Deleo, F.R., and Chambers, H.F. (2009): Reemergence of antibiotic-resistant *Staphylococcus aureus* in the genomics era. *J. Clin. Invs.,* **119:** 2464–2474.

Deurenberg, R. H., Vink, C., Kalenic, S., Friedrich, A. W., Bruggeman, C. A. and Stobberingh, E. E. (2007): The molecular evolution of methicillin-resistant *Staphylococcus aureus. Clin. Microbi. Infe.,* **13**: 222–235.

Devriese, L.A., Van Damme, L.R. and Fameree, L. (1972): Methicillin(cloxacillin)-resistant *Staphylococcus aureus* strains isolated from bovine mastitis cases. *Zbl. Vet. Med. B.,* **19**:598-605.

Dingwell, R.T., Leslie, K.E. and Duffield, T.F. (2003): Efficacy of intramammary tilmicosin and factors for cure of *Staphylococcus aureus* infection in the dry period. *J. Dai. Sci.*, **86**:159-168.

Edward, M., Anna, K., Michal, K., Henryka, L. and Krystyna, K. (2002): Antimicrobial susceptibility of staphylococci isolated from mastitic cows. *Bul. Vet. Inst.*, Pulawy. Pp 289-294.

Erskine, R. J. (2001): Mastitis control in dairy herds. In: Radostits, O. M. (ed.): Herd Health: Food and Animal Production. 3rd ed. Philadelphia: W. B. Saunders Company. Pp 397 – 435.

European Food Safety Authority (EFSA) (2009): Assessment of the Public Health significance of methicillin resistant *Staphylococcus aureus* (MRSA) in animals and foods. Scientific Opinion of the Panel on Biological Hazards. *EFSA. J.,* **993**:1-73.

Fekadu, B., Demelash, B. and Etana, D. (2005): Prevalence and risk factors of mastitis in lactating dairy cows in Southern Ethiopia. *Int. J. Appl. Res. Vet. Med.,* **3**:189-198.

Ferreira, P., Anderson, L., Correa, T., Lyman, R., Ruffin, F., Reller, B. and Fowler, V. (2011): Transmission of MRSA between companion animals and infected human patients presenting to outpatient medical care facilities. *PLoS One*, **6**:269-278.

Fetrow, J., Stewart, S., Eicker, S., Farnsworth, R. and Bey, R. (2000): Mastitis: an economic consideration. In: Proceedings of the 2000 annual meeting of the National Mastitis Council. National Mastitis Council, Inc., Madison, WI. Pp3-47.

Foster, T.J. and Hook, M. (1998): Surface protein adhesins of *Staphylococcus aureus*. *Trnd. Micrbi.,* **6:** 484–488.

Fox, L.K. and Gay, J.M. (1993): Contagious mastitis. *Vet. Clin. Nor.Am .Anim .prc.*, **9**: 475-487.

Gehanno, J., Louvel, A., Nouvellon, M., Caillard, J.F. and Pestel-Caron, M. (2009): Aerial dispersal of meticillin-resistant *Staphylococcus aureus* in hospital rooms by infected or colonized patients. *J. Hsp. Inf.,* **71**: 256-262.

Gentilini, E., Denameiel, G., Liorente, P., Godaly, S., Rebuelto, M. and DeGregorio, O.(2000): Antimicrobial susceptibility to *S.aureus* isolated from bovine mastitis in Argentina. *J. Dia. Sc*i.,**83**:1244-7.

Geressu, B. (1989): Major bacteria causing bovine mastitis and their sensitivity to common antibiotics. *Ethiopia J. Agr. Sci.* **11** (2).

Getahun, K. (2006): Bovine mastitis and antibiotic resistance patterns of major pathogens in small Holder dairy farms in the centeral highlands of Ethiopia. Msc. Thesis, Addis Ababa University, Fucalty of Veterinary Medicine, Debr-Zeit, Ethiopia.

Getahun, K., Kelay, B., Bekana, M. and Lobago, F. (2008): Bovine mastitis and antibiotic resistance patterns in Selalle smallholder dairy farms, central Ethiopia. *Trop. Ani. Hlth. and Prod.,* **40**: 261–268.

Gill, R., Howard, W.H., Leslie, K.E. and Lissmore, K. (1990): Economics of mastitis control. *Dai. Sci.*, **61**:27-43.

Godden, S.M., Jansen, J.T., Leslie, K.E., Smart, N.L and Kelton, D.F.(2002): The effect of sampling time and sample handling on the detection of *Staphylococus aureus* in milk from quarters within subclinical mastitis. *Cana. Vet. J.*, **43** :38-42.

Gordon, J. and Lowy, D. (2008): Pathogenesis of methicillin resistant *Staphylococcus aureus* infection. *Clin. Infec. Dis.*, **46**:350–359.

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

Harris, G., Foster, S. and Richards, G. (2002): An introduction to *Staphylococus aureus* and techniques for identifying and quantifying *Staphylococcus aureus* adhesions. *Rev.*, **4**:39-60.

Hata, E., Katsuda, K., Kobayashi, H. Uchida, I., Tanaka, K. and Eguchi, M. (2010): Genetic variation among *Staphylococcus aureus* strains from bovine milk and their relevance to methicillin resistant isolates from humans. *J. Cli. Micro.*, **48**:2130-2139.

Hirsh, D.C. Maclachar, N.J. and Walker, R.L.(2004): Veterinary microbiology. 2nd ed. Blackwell science Pp 117-150.

Hortet, P. and Seegers, H. (1998): Calculated milk production losses associated with elevated somatic cell counts in dairy cows: Review and critical discussion. *Vet. Res.*, **29**:497-510.

Hu, C.J., Fang, R.H., Luk, B.T., Zhang, L. (2013): Nano particle-detained toxins for safe and effective vaccination *Nat. Nano. Technol.*, **8**: 933-938.

Huber, H., Giezendanner, N., Stephan, R. and Zweifel, C. (2011): Genotypes, antibiotic resistance profiles and microarray-based characterization of methicillin-resistant *Staphylococcus aureus* strains isolated from livestock and veterinarians in Switzerland. *Zoo. Pub. Hlth.,* **58**:343–49.

Hundera, S., Ademe, Z. and Sintayehu, A.(2005): Dairy cattle mastitis in and around Sebeta, Ethiopia. Intern. *J. Appl. Vet. Med.*, **3**:1525-1530.

Hussien, N.(1999): Cross sectional and longitudinal study of bovine mastitis in urban and periurban dairy system in the Addis Ababa region, Ethiopia. thesis, Faculty of Veterinary Medicine, Addis Ababa University School of Graduate Studies and Freie Universitat, Berlin, Philadelphia: sounders Pp153-158.

Jaims, E., Montros, L. and Renata, C. (2002): Epidemiology of drug resistance; the case of *Staphylococcus aureus* and Coagulase negative Staphylococci infections. *Salud Publica Mex.,* **44**:108-112.

Jakee, J., Ata, S.,Nagwa, M., Bakry, Sahar, A.,Zouelfakar, Elgabry, E. and Gad El-Said, W. (2008): Characteristics of *Staphylococcus aureus* strains isolated from human and animal sources. *Am-Euras. J. Agr. Envir. Sci.*, **4**:221-229.

Janson, J.(2006): Investigation of biological control strategies for the control of bovine mastitis caused by *Staphylococcus aureus* .PhD Thesis. The Faculty of graduate studies of University of Guelph Ottawa Canada.

Jevons, M.P. (1961): 'Celbenin'-resistant Staphylococci. *British Medical Journal*, **1**:124-125.

Jones, F., Kellum, E., Porter, S., Bell, M. and Schaffner, W. (2002): An outbreak of community acquired food borne illness caused by methicillin-resistant *Staphylococcus aureus*. *Emerg. Infe. Dis.,* **8**:82–84.

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

Juhász-Kaszanyitzky, E., Jánosi, S., Somogyi, P., Dán, A., Bloois, G., van Duijkeren, E. and Wagenaar, A. (2007): MRSA transmission between cows and humans. *Emerg. Inf. Dis.*, **13**:630-632.

Kassa, T., Wirtu, G. and Tegegne, A. (1999): Survey of mastitis in dairy herds in the Ethiopian central highlands. *Ethio. J. Sci.,* **22**:291-301.

Kawada, M., Okuzumi, K., Hitomi, S. and Sugishita, C. (2003): Transmission of *Staphylococcus aureus* between healthy, lactating mothers and their infants by breastfeeding. *J. Hum. Lact.*, **19**:411-417.

Kernodle, D.S.(2000): Mechanisms of resistance to β-lactam antibiotics. In: Gram-positive pathogens. Fischetti, V.A., Novick, R.P., Ferretti, J.J., Portnoy, D.A. and Rood, J.I. (Eds). American Society for Microbiology. Washington, DC, USA. Pp. 609-620.

Kerro, O. and Tareke, F.(2003): Bovine mastitis in selected areas of Southern Ethiopia. *Trop. Anim. Health and Prod.*, **35**:197-205.

Khanna, T., Friendship, R., Dewey, C. and Weese, J. S. (2008): Methicillin resistant *Staphylococcus aureus* colonization in pigs and pig farmers. *Vet. Micro.,* **28**:298-303.

Kivaria, F.M., Noordhuizen, J.P. and Kapaga, A.M., (2004): Risk indicators associated with subclinical mastitis in small holder dairy cows in Tanzania. *J. Tro. Ani. Hlth and Pro*., **36**:581-592.

Kloos, W.E. and Bannerman, T.L. (1994): Update on clinical significance of coagulase-negative staphylococci. *Clin . Microb. Rev.,* **7**: 117-140.

Kloos,W.E.andBannerman, T.L. (1995): *Staphylococcus* and *Micrococcus.* In: Murray, P. Baron, E.J., Pfaller, M.A., Tenover, F.C. and Yolken, R.F. l (Eds.) Manual of Clinical Microbiology. AM Press, Washington, DC, Pp. 289-298.

Klotz, M., Zimmermann, S., Opper, S., Heeg, K. and Mutters, R. (2005): Possible risk for recolonization with methicillin-resistant *Staphylococcus aureus* (MRSA) by faecal transmission. *Int. J. Hyg. Environ. Hlth..*, **208**: 401-405.

Kluytmans, A. (2010): Methicillin-resistant *Staphylococcus aureus* in food products: cause for concern or case for complacency. *Clin. Micr., Inf.,* **16**:11–15.

Kluytmans, J., Van Griethuysen, A., Willemse, P. and Van Keulen, P. (2002): Performance of CHRO Magar selective medium and oxacillin resistance screening agar base for identifying *Staphylococcus aureus* and detecting methicillin resistance. *J. Clin. Micro*., **40**:2480-2482.

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. Antimicro. Chemoth..,* **56**:624–632.

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

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

Leonard, C. and Markey, K. (2008): Meticillin-resistant *Staphylococcus aureus* in animals. A review: *Vet. J.*, **175:**27-36.

Loeffler, Anette and Boag, (2005): Prevalence of methicillin-resistant *Staphylococcus aureus* among staff and pets in a small animal referral hospital in the UK. *J. Antimicro. Chemotherapy*, **56** : 692-697.

Machang, U.R. and Muyungi, L.S.(1998): The occurrence of streptococci mastitis in dairy farm Morogoro area,Tanzania. *Bulletein of Animal health and production in Africa*,**36**:190-193.

Mahon, C.R. and Larsen, H.S. (1995): *Staphylococcus aureus*. In Textbook of diagnostic microbiology. Edited by Mahon C.R., Manuseseilis G Jr. New York, WB. Saunders Company, Pp. 325-330.

Makovec,J.A. andRuegg, P. (2003): Result of milk samples submitted for microbiological examination in Wisconsin from 1994 to 2001. *J.Dai. sci.*, **86**:3466-3472.

Matsunaga, T., Kamata, S., Kakiichi, N. and Uchida, K. (1993): Characteristics of *Staphylococcus aureus* isolated from per acute, acute and chronic bovine mastitis. *J. Vet. Med. Sci.,***55:**297-300.

McCarthy, N.L., Sullivan, P.S., Gaynes, R. and Rimland, D.(2010): Health care-associated and community-associated methicillin-resistant *Staphylococcus aureus* infections: a comparison of definitions. *Am. J. Infe. Contr.*, **38**:600–606.

Mehndiratta, L. and Bhalla, P. (2012): Typing of methicillin resistant Staphylococcus aureus: A technical review. *Indian J. Med. Microbiol., ***30**:16-23.

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. Wor.*, **9**: 397 - 403.

Melchior, M.B., Vaarkamp, H. and Fink-Gremmels, J. (2006): Biofilms A role in recurrent mastitis infections, *Vet. J*., **171**: 398–407.

Montanari, M.P., Tonin, E., Biavasco, F. and Varaldo, P.E. (1990): Further characterization of borderline methicillin-resistant *Staphylococcus aureus* and analysis of penicillin-binding proteins. *Antimicrob Agents Chemothe*r, **34**:911–913.

Mooij, T.A., Jenkins, J. and Thijssen, S. (2007): MRSA in calves. *Infec. ieziekten Bull,* **18**:234-236.

Moon, J.S., Lee, A.R., Kang, H.M., Lee, E.S., Kim, M.N., Paik, Y.H., Park, 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. Dai. Sci*.,**90**:1176-1185.

Morgan, M. (2008): Methicillin-resistant *Staphylococcus aureus* and animals: zoonosis or humanosis. *J. Antimicr. Chemoth,., **62**:1181-1187.

Mungube, E. O. (2001): Management and economics of dairy cow mastitis in the urban and periurban areas of Addis Ababa milk shed. MSc. Thesis, Addis Ababa University, Faculty of Veterinary Medicine, Debre-Zeit, Ethiopia.

Mungube,E.O.,Tenhagen,B.A.,Kassa,F.,Regassa,F.,Kyule,MN.,Greiner,M.andBaumann, M.R.O.(2004): Risk factors for dairy cows mastitis in the central highlands of Ethiopia. *Trop. Anim. Hlth and Prod*., **36**: 463-472.

National Committee for Clinical Laboratory Standards (*2011*)*: Performance standards for antimicrobial susceptibility testing, 7th informational supplement. Approved standard M100-S21.*

Nesru, H. (1986): A survey of bovine mastitis around Sebeta. DVM . Thesis, Faculty of Veterinary Medicine, Addis Ababa University, Ethiopia.

Nesru, H. (1999): A Cross-sectional and Longitudinal Study of Bovine Mastitis in Urban and Peri Urban Dairy System in the Addis Ababa Region. Free University of Berlin and Addis Ababa University, Ethiopia, MSc Thesis.

NMC (1990): National Mastitis Council (NMC) Microbiological procedures for the diagnosis of bovine udder infection. 3rd ed., Madison, Wisconsin: National Mastitis Council Inc., Pp 7-8.

Normanno, G., Corrente, M., Salandra, G., Dambrosio, A., Quaglia, C., Parisi, A., Santagada, G., Firinu, A., Crisetti, E. and Celano, G. (2007): Methicillin resistant *Staphylococcus aureus* (MRSA) in foods animals and their potential transmission to humans. *Appl. Environ. Microbl.,* **69**:6489-6494.

Owens, W.E., Ray, E. H., Watts, J. L. and Yancey, R. T. (1997): Comparison of success and results of antibiotic therapy during lactation and results of antimicrobial susceptibility tests for bovine mastitis, *J. Dai. Sci.,***80**: 313-317.

Pankey, J.W. (1989): Premilking udder hygiene. *J.Dai.Sci.*,**72**:1308-1312.

Paterson, K., Larsen, J., Harrison, M., Larsen, R., Morgan, J., Peacock, J., Parkhill, J., Zadoks, N. and Holmes, A. (2012): First detection of livestock-associated meticillin-resistant *Staphylococcus aureus* CC398 in bulk tank milk in the United Kingdom. *Euro. Surv.,* **17**:20337.

Patti, J.M., Allen, B.L., McGavin, M.J. and Hook, M. (1994): Microbial Surface components recognizing adhesive matrix molecule (MSCRAMM)-mediated adherence of microorganisms to host tissues. *Annu. Rev. Micro.*, **48**:585–617.

Perry, B.D., Cartert, F.W., Hill, M.E. and Milne, A.C. (1987): Mastitis and milk production in cattle in a communal land of Zimbabwe. *Brit.vet.J.* **143:44**-50.

Persoons, D., Van Hoorebeke, S., Hermans, K., Butaye, P., de Kruif, A., Haesebrouck, F. and Dewulf, J. (2009): Methicillin-resistant *Staphylococcus aureus* in poultry. *Emerg. Infect Dis*., **15**:452-453.

Pesavento, G., Ducci, B., Comodo, N. and L. Nostro, A. (2007): Antimicrobial resistance profile of *Staphylococcus aureus* isolated from raw meat: A research for methicillin resistant *Staphylococcus aureus* (MRSA**)**. *Food Cont.*, **18:**196-200.

Philpot, W.W. (1979): Control of mastitis by hygiene and therapy. *J. Dai .Sci*., **62**:168-176.

Pyorala**,** S**. (**2003): Indicators of inflammation in the diagnosis of mastitis. *Vet. Res*., **34:**565-78.

Quinn, J., Carter, E., Markey, B. and Carter, R. (1999): Mastitis. In: Clinical Veterinary Microbiology, Mosby International Limited, London, Pp 327-344.

Quinn, J.,Carter, E., Markey, B. and Carter, R. (2000): Clinical Veterinary Microbiology. Mosby: London, UK., Pp 21-66.

Quinn, J., Carter, E., Markey, B. and Carter, R.(1994): Clinical Veterinary Microbiology, Wilfe Publishing, London, Pp 95-101.

Quinn, P.J., Carter, M.E., Donnelly, W.J.C. and Leonard, F.C.(2002): Veterinary microbiology and microbial disease. Blackwell science, London Pp.465-472.

Radostitis, M., Blood, D. and Gay, C. (1994): Veterinary Medicine: A text book of the diseases of cattle, sheep, pigs, goats and horses. 8th ed., Bailliere Tindall: London. **8**:563-613.

Radostits, M., Gay, C., Blood, D. and Hinchillif, K. (2000): Mastitis In: Veterinary Medicine, 9th ed., Harcourt Limited, London, Pp 603-700.

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

Rall,V.L.M., Miranda, E.S., Castilho, I.G. and Camargo, C.H. (2013): Diversity of *Staphylococcus sp*ecies and prevalence of enterotoxin genes isolated from milk of healthy cows and cows with subclinical mastitis. *J. Dai. Sci*., **97**: 829-837.

Reller,B.,Weinstein, M., Jorgensen,H. and Ferraro, J. (2009): Antimicrobial susceptibility testing: a review of general principles and contemporary practices. *Clin. Infe. Dis*., **49**:1749-1755.

Roberson (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, WI. 38p.

Rota, C., Yanguela, J., Blanco, D., Carraminana, J., Arino, A. and Herrera, A. (1996): High prevalence of multiple resistances to antibiotics in 144 Listeria isolates from Spanish dairy and meat products. *J. Fo. Prot*., **59**: 938–943.

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

Russel, C.D. (2012): Staphylococcus Vaccination and MRSA. *Lancet Infect. Dis*., **12**: 586.

Rychlik, I., Gregorova, D. and Hradecka, H. (2006): Distribution and function of plasmids in Salmonella enteric. *Vet. Microb.,* **112**: 1-10.

Sabour, P.M. (2004): Molecular typing and distribution of *Staphylococcus aureus* isolates in Eastern Canada dairy herds. *J. Clin. micr*., **42**:3449-3455.

Sargeant, J. M., Scott, M. M., Leslie, K. E., Ireland and Bashiri, M. J. A. (1998): Clinical mastitis in dairy cattle in Ontario: frequency of occurrence and bacteriological isolates. *Can. Vet. J.,* **39**: 33-38.

Schukken,Y.H.,Wilson,D.J.,Welcome,F.,Garrison-Tikofsky,L.andGonzalez,R.N.(2003): Monitoring udder health and milk quality using somatic cell counts.*Vet.Res*.,**34**:579-596.

Schultz ,M.M.,(1994): Genetic evaluation of somatic cell count score for United States Dairy cattle .*J. Dai. Sci.,* Pp 2113-2129.

Sears, P.M, and McCarthy, K.K. (2003): Management and treatment of staphylococcal mastitis. *Vet. Cl. North Am. Food An. Pra.,* **19:**171-185.

Seykora, A.j.and McDaniel, B.T.,(1985): Udder and Teat Morphology related to mastitis. *J.Dai.Sci*.,**68**: 2087-2093.

Shitandi, A. and Sterneso, A. (2004): Prevalence of multidrug resistant *Staphylococcus aureus* in milk from large and small-scale producers in Kenya**,** *J. Dai. Sci*., **(87):**4145-4149.

Smith, P.B. (2002): Large Animal Internal Medicine 3rd ed. Mosb., Pp 1019-1032.

Stevens, D.L. (2004): Optimizing outcomes in methicillin_resistant *Staphylococcus aureus* infections: focus on nosocomial pneumonia and SSTI, highlights from a satellite symposium at the 11th annual international congress on infectious disease (ICID), Cancun, Mexico, Pp 1 - 8.

Strommenger, J., Kioppert, B., Wolter, W., Zschock, M., Sobiraj, A. and Failing, K. (2003): The epidemiology of *Staphylococcus aureus* infection from sub-clinical mastitis in dairy cows during a control programme.*Vet.Micr*.,**96**: 91-102.

Sung, M., Lloyd, H. and Lindsay, A. (2008): *Staphylococcus aureus* host specificity: comparative genomics of human versus animal isolates by multi-strain microarray. *Microb.,* **154**:1949- 1959.

Tariku, S.,Jemal,H., Molalegne,B.(2011):Prevalence andsusceptibility assay of *Staphylococcus aureus* isolated from bovine mastitis in dairy farms in Jimma town South West Ethiopia. *J. Anim. Vet. Adv.,***10**:745-749.

Temesgen, W. (1999): A Study on bovine mastitis in and around Mekelle. Faculty of Veterinary Medicine, Addis Ababa University, Debre Zeit, DVM, Thesis.

Tenover, C. and Goering, V. (2009): Methicillin-resistant *Staphylococcus aureus* strain USA300: origin and epidemiology. *J. Antimicrob. Chemo.Ther.,* **64**:441-446.

Tesfaye,H.D.(1995): Prevalence of bovine mastitis in Hoistein-Boran crosses and Indigenous zebu cows and invitro drug sensivitity test in and around Kombolcha. DVM.Thesis, Faculty of veterinary Medicine,AAU., Ethiopia.

Thrusflied,M.,(2005): Veterinary Epidemiology.3rd ed.Singapore: Black Well Science.Pp 182-198.

Tolla,T.(1996):Bovine mastitis inIndigenous zebu and Borena Holistein crosses in Southern,Wollo.Addis Ababa University,Facluty of Veterinary Medicine,Debre-Zeit, Ethiopia.

Van Loo,I., Huisdens, Tiemersma, E., de Neeling, A.,van de, Sande-Bruinsma, N., Bearijlan,D.,VossVandenesch,F.,Naimi,T.,Enright,M.C.,Lina,G.,Nimmo,G.R.,H effernan,H.,*et al*.(2003):Community-acquiredmethicillin-resistant *Staphylococcus aureus* carryingPanton-Valentine leukocidin genes: worldwide emergence. *Emerg.. Infec. Dis.,* **9**: 978–984.

Vanderhaeghen,W.,Cerpentier,TAdriaensen,C.,ViccaJ.,Hermans,K.andButaye,P.(2010): Methicillin-resistant *Staphylococcus aureus*(MRSA) ST398. associated with clinical and subclinical mastitis in Belgian cow. *Vet. Micr.*, **144:** 166 –171.

Voss,A.,Loeffen,F.,Bakker,J.,Klaassen,C.,Wulf,M.(2005):Methicillin-resistant *Staphylococcus aureus* in pig farming. *Emerg. Inf. Di*s.,**11**:1965–1996.

Waage,S.,Mork, T.,Roros, A., Aasl., D.,Hunshamar,A. and Odegaard, S.A.(1999) : Bacteria associated with clinical mastitis in dairy heifers. *J.Dai.Sd*., **82**: 712-719.

Walther B, Wieler L.H, Friedrich A.W, HanssenAM, Kohn, B., Brunnberg, L., Lubke-Becker, A.(2008): Methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from small and exotic animals at a university hospital during routine microbiological examinations. *Vt. Micrb*., **127**(1-2):171-178.

Wiegand, I., Hilpert,K. and Hancock, E.(2008): Agar and broth dilution methods to determine the minimal inhibitory concentration(MIC) of antimicrobial substances. *Nat. Protc*., **3**:163-175.

Wilson, D. J, Gonzalez, R. N., and Das, H. H. (1997): Bovine mastitis pathogens in New York and Pennnsylvania: prevalence and effects on somatic cell count and milk Production. *J. Dai. Sci*., **80**:2592-2598.

Witte, W.,Strommenger,B.,Stanek,C,Cuny,C.(2007): Methicillin resistant *Staphylococcus aureu*s ST398 in humans and animals, Central Europe. *Emer. Inf. Dis.,* 13:255-25.

Workeneh,S.,Bayleygne, M.,Mekonnen, H. and Potgieter, L. (2002): Prevalence and etiology of mastitis in cows from two major Ethiopian dairies. *Trop. Anim. Hlth.. Prod*., **34**:19-25.

Zadoks¸R.N., Allore, H.G. Hagcnnaars,T.J., Barkema, H.W.and Schukken, Y.H.(2002): A mathematical model of *Staphylococcus aureus c*ontrol in dairy herds. *J.Epi. Ifec*.,**129**:397-416.

Zerihun,T., (1996): A study on bovine subclinical mastitis at Stela dairy farm. Addis University, Faculty of Veterinary Medicine, Debre-Zeit, Ethiopia. DVM, Thesis.

Zingser,J.Day.YLopez, V., Grant,g., Bryan, I., Kearney,M. and huge- Jones,M.E.(1991): National survey of clinical and sub-clinical mastitis in Jamaican dairy herds. *Trop.Ani. Hlth. and pro*.,23:2-10.

8. LIST OF TABLES IN THE APPENDIXS

Appendix I. Questionnaire format

Owners Name: _____ Region_____Zone---------Woreda-------Date of sample Collection_____

1. Cow History:

- Breed_____ age___ calving date____   parity_____   previous history of mastitis___
- Tick infestation: present _____absent_____
- Teat lesion: present_____absent_____
- Gross milk quality: watery____blood tinged____clots/flakes___ normal___
- Sample collected from: RH___RF____LF___LH_____
- CMT score: RH___RF____LF___LH____

2. Milking practice

- Do you wash udder before milking? yes____ no____
- Do you dry after washing? yes____ no____
- Do you use the same cloth for both teats? yes____ no____
- Do you practice milking mastitis cows last? yes____ no____

3. Housing

- Floor type: concrete_____stone_____soil_____slopy_____leveled_____
- Roof: metal sheet_____ grass_____
- Wall: concrete_____mud_____others_____
- Manure removal: daily_____weekly____monthly____other (specify)_____

4. Drug usage

- Mention any drug you know used for treatment of any disease

- Name those used for mastitis treatment _____
- Is there problem of cure after therapy? _____
- Can you treat your animal by yourself _____ or take to the clinic _____

Appendix II Table 1. Farm visit data collection format for mastitis type

	RF		LF		RH		LH	
	Cli.	SCM	Cli.	SCM	Cli.	SCM	Cli.	SCM
Mastitis type								

Cli= clinical, SCM= subclinical

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

Appendix III Table 2. CMT results and Interpretation

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

cohesive with a convex surface

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

Gram stain (carter, 1984)

Procedure:

- Make a thin smear or film.
- Allow the film to dry in air.
- Fix the film by passing through the Bunsen flame several times.
- Flood the slide with crystal violent for 30 to 60 seconds.
- Pour of the stain and wash the remaining stain with iodine solution.
- Wash off the iodine and shake the excess water from the slide.
- Decolorize with acetone alcohol.
- Counter stain with safranin for 30 to 60 seconds and wash with water.

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

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

Procedure: a loopful of the bacterial growth is taken from the top of the colonies avoiding the blood agar medium. The bacterial cells are placed on a clean microscope slide and a drop of 3% H_2O_2 is added. An effervescence of oxygen gas, within a few seconds, indicates a positive reaction.

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

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

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

Appendix IV Table 3. Differentiation of mastitis causing Staphylococcus species and Micrococcus species.

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

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

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

The colonies that were confirmed by staining reaction, catalase test, OF test and coagulase test were streaked on mannitol salt agar plate, incubated at 37 OC, and examined after 24-48 h for growth. The presence of growth and change of pH in the media (red to yellow color) regarded as presumptive identification for *S.aureus* (Quinn *et al.,*2000).
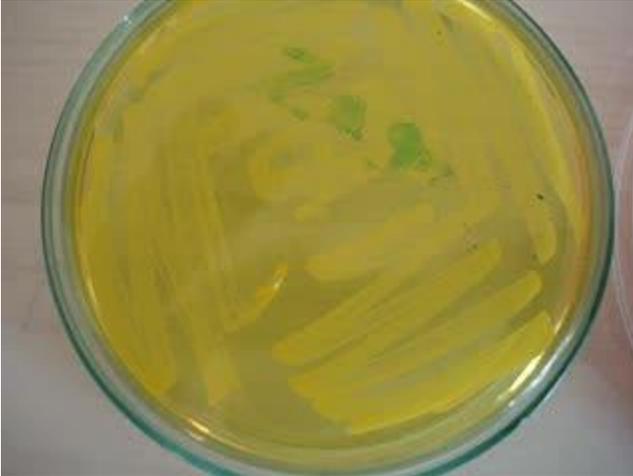
Appendix V Figure 1. *S.aureus*   Growth on Mannitol salt agar

Purple Base Agar Test

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

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

Procedure

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

Appendix VI Figure 2. Purple Base Agar test

A positives result on purple base agar. Right(*S. aureus*), Left (negative control).

Appendix VII Antibiotic sensitivity test

➢   Preparation of inoculums

Inoculation of distinct colony in to 5ml nutrient broth incubated at 35-37^{0}c for about 8 hours. Then the turbidity is compared with 0.5MacFarland standard. This standard is prepared by adding 0.5ml of 1 % (11.75g/liter) Bacl$_2$ 2H$_2$0 to 99.5ml of 1% (0.36N) H2SO4.

➢   Inoculation to Muller- Hinton Agar

Muller-Hinton Agar cooled to 50 ^{o}c and poured into a sterile petri dish on level surface to a depth of 4mm. this is equivalent to 60ml in 15cm plate and 25 ml in 10cm plate for slow growing bacteria 5 % defiberinated whole blood could be added. Then a sterile cotton swab on a wooden applicator stick is used to transfer the diluted bacterial suspension to a plate; excess fluid must be squeezed out by rotating the swab against the sides of the tube. The

plate is seeded uniformly by rubbing the swab against the entire agar surface in three different planes roughly 60 degrees to each others.

➢ Disc application

Within 15 minutes (time used to dry the inoculums) after the plates are inoculated, antibiotic impregnated discs are applied to the surface of the inoculated plates by hand using a sterile forceps. All discs gently pressed down on to the agar with forceps to ensure complete contact with the agar surface. The disc should no closer than 1.5 cm to the edge of the plate and they should rest 24 mm apart from each other. The large Petridishes accommodate 6 discs in outer ring and three in the center, where as no more than 5 should be placed in small plates (10cm plates). Incubate the plates inverted aerobically for 24 hours at 35^0c but not 37^0c.

➢ Interpretation

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

Appendix VIII Table 4. Zone of inhibition interpretation chart for Antimicrobials

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

Source (NCCL, 2011).   SXT = sulphamethoxazole trimethoprim

Appendix IX Figure 3. Antibiotic sensitivity test

Appendix X. Medias used for bacteriological examination

1. Nutrient agar (Oxoid, England)

   Composition (g/l): Lab-Lemco powder1.0; Yeast extract 2.0; Peptone 5.0;   Sodium

   chloride 5.0; Agar15 ; pH: 7.4 ± 0.2

   Directions: Suspend 28 g in 1 liter of distilled water. Bring to the boil to dissolve

   completely. Sterilize by autoclaving at 121^{0}C for 15 minutes.

   Mannitol salt agar (Oxoid, England)

   Composition (g/l): Lab-Lemco' powder 1.0 Peptone 10.0 Mannitol 10.0 Sodium

   chloride 75.0 Phenol red 0.025 Agar 15.0 pH: 7.5 ± 0.

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

   Completely. Sterilize by autoclaving at 121°C for 15 minutes.

2. Gram's reagent

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

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

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

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

4. Edwards medium, modified (Oxoid, England)

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

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

5. . Muller Hinton Agar (Oxoid, England)

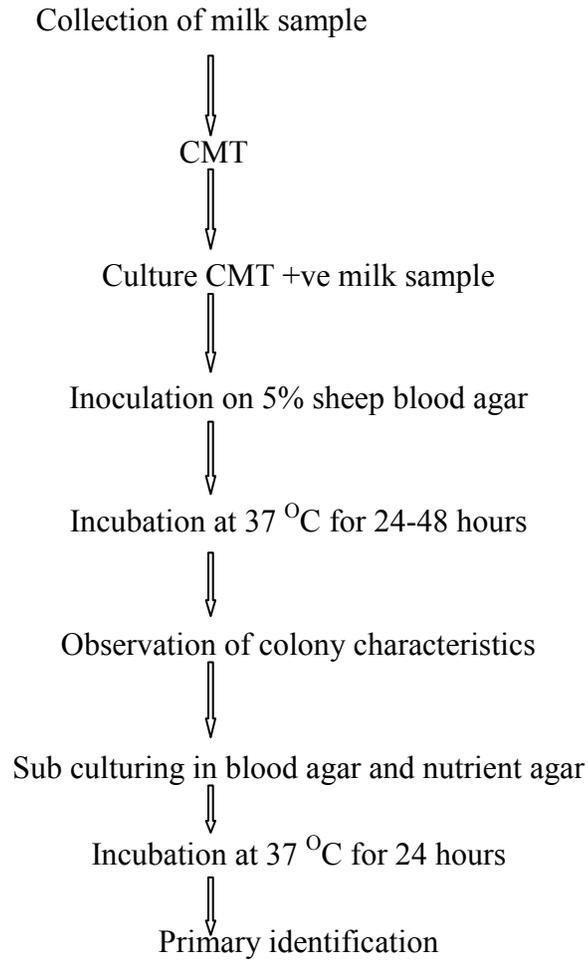Composition (g/l): beef extracts 2; acid hydrolysate of casein 17.5; starch1.5; agar 17. Direction: suspend 38 g of the powder in 1 liter of distilled water. Mix thoroughly, heat with frequent agitation and boil for 1 minute to completely dissolve the powder Autoclave at 121°c for 15 minutes. Cool tubes medium in slanted position for 121Oc for 15 minutes.

6. Blood agar (Oxoid, England)

Composition (g/l) hear muscle, infusion from (solids) 2.0; pancreatic digest of casein13.0; Yeast extract 5.0;sodium chloride 5.0;agar 15.0 .Direction: suspend 40 g of powder in 1 liter of distilled water. Mix thoroughly, heat with frequent agitation, and boil for 1 minute to completely dissolve the powder. Autoclave at 121^0c for 15 minutes. Cool the base to 45 - 50^0 c and add 5 % sterile defiberinated sheep blood.

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

Collection of milk sample

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CMT

↓

Culture CMT +ve milk sample

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Inoculation on 5% sheep blood agar

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

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Observation of colony characteristics

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Sub culturing in blood agar and nutrient agar

↓

Incubation at 37 ^{O}C for 24 hours

↓

Primary identification

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

Secondary bio-chemical tests

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

Drug sensitivity