Thesis ref. no……...

ISOLATION AND PHENOTYPIC CHARACTERIZATION OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) FROM SELECTED DAIRY CATTLE MASTITIS INFECTION IN AND AROUND BATU TOWN, ETHIOPIA

MSc. THESIS

BY

ABINET KETEMA

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

JUNE, 2015

BISHOFTU, ETHIOPIA

ISOLATION AND PHENOTYPIC CHARACTERIZATION OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) FROM SELECTED DAIRY CATTLE MASTITIS INFECTION IN AND AROUND BATU TOWN, ETHIOPIA

Msc. thesis

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

Abinet ketema

June, 2015

Bishoftu, Ethiopia

Addis Ababa University

College of veterinary medicine and agriculture department of microbiology, immunology and veterinary public health

As members of the Examining Board of the final MSc. open defense, we certify that we have read and evaluated the Thesis prepared by Abinet Ketema Alemu Entitled "**Isolation and phenotypic characterization of Methicillin-resistant *staphylococcus aureus* (MRSA) from selected dairy cattle mastitis infection in and around Batu town, Ethiopia"** and recommend that it be accepted as fulfilling the thesis requirements for the degree of Masters of Science in Veterinary Microbiology.

Dr. Gezahegn Mamo (PhD)	_____	_____
Chairman	Signature	Date
Dr. Workineh shibeshi (PhD)	_____	_____
External Examiner	Signature	Date
Dr. Akelilu Feleke	_____	_____
Internal Examiner	Signature	Date
1. Dr. Addisu Demeke	_____	_____
Major Advisor	Signature	Date
2. Dr. Biruk Tesfaye	_____	_____
Co-Advisor	Signature	Date
3. Dr. Bedaso Mamo	_____	_____
Department chairperson	Signature	Date

TABLE OF CONTENTS PAGES

Table of content (continued)

Table of content (continued)

STATEMENT OF THE AUTHOR

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

Brief quotations from this thesis are allowable without special permission provided if an accurate acknowledgement of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the dean of the collage when in his or her judgment. In all other instance, however permission must be obtained from the author.

Name: Abinet ketema sign: ----------

College of veterinary medicine and agriculture, Bishoftu.

Date of submission…….

ACKNOWLEDGEMENT

First, I would like to express my deepest gratitude to my academic advisor Dr. Addisu Demeke for his guidance, patience, and helpfulness throughout my studies. In addition, I extend my thanks to Dr. Biruk Tesfaye for his consultation and encouragement.

Special thanks to Dr. Tesfaye Sisay for provision of all the necessary materials required for the research work.

Am also great full to Dr. Birhanu, for his support and permission to take samples from diary cattle's managed at Adami Tullu agricultural research center. I extend my thanks to Dr. Fikadu who convince dairy cattle owners and arrange a time managing schedule to take samples from Bulbula Woreda.

I owe my thanks to Ato Kassay and Miss Tesfanesh for their advice on technical laboratory expertise. It is also the pleasure to extend the gratitude to Addis Ababa University microbiology Laboratory, College of Veterinary Medicine for its laboratory material support during the study period.

Lastly, I thank all the dairy farmers who allowed me to get samples from their cows and also gave the data that has enabled me produce this report.

LIST OF ABBREVIATIONS

agr	Accessory gene regulator
CA-MRSA	Community associated-Methicillin-resistant *Staphylococcus aureus*
CFU	Colony forming unit
CMT	California mastitis test
CRF	Coagulase reacting factor
DNA	Deoxyribose nuclic acid
EPS	Exopoly-saccharides
FnBPs	Fibronectin-binding protein
HA-MRSA	Hospital acquired-Methicillin-resistant *Staphylococcus aureus*
ICUs	Intensive care units
IMI	Intra-mammary infections
LA-MRSA	Livestock associated-Methicillin-resistant *Staphylococcus aureus*
MAR	Multiple antibiotic resistant
MCRA	Modified Congo Red Agar
MDR	Multi Drug Resistance
MHC	Major histocompatibility complex
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSCRAMMs	Microbial surface components recognizing adhesive matrix Molecules
MSSA	Methicillin-susceptible *Staphylococcus aureus*
PBP	Penicillin-binding protein
PMN	Polymophonuclear neutrophils

PNAG	Poly-N-acetyl β 1, 6 glucosamine
PVL	Panton-Valentine leukocidin
S. aureus	*Staphylococcus aureus*
Sags	*Staphylococcal* super antigens
SPSS	Statistical package for the social science
SCC	*Staphylococcal* cassette chromosome
TSST	Toxic shock syndrome toxin

LIST OF TABLES PAGES

LIST OF FIGURES PAGES

LIST OF APPENDIXES PAGES

ABSTRACT

Among the animal diseases that require antibiotic treatment in dairy herds, mastitis is the commonest one. Mastitis pathogens like Staphylococcus aureus have received recent attention due to antimicrobial resistance potential. A cross-sectional study was carried out from, November 2014 to may 2015 to determine the prevalence and phenotypic characteristics of Methicillin Resistant Staphylococcus aureus (MRSA) isolated from mastitis lactating dairy cattle in and around Batu, Ethiopia. A total of 216 lactating cows were examined clinically as well as by California mastitis test (CMT) to detect clinical and sub clinical mastitis. Of the total dairy cattle examined, 92 (42.59%) were found to be positively reactive to CMT. From the total count, the clinical and subclinical mastitis forms were 6.48% (14/216) and 36.11% (78/216) respectively. Among the total 864 quarter examined, 12(1.39%) quarters were found blind and overall prevalence of mastitis 172(19.9%) at quarter level was recorded. From the total infected quarter, the front and hind quarter share 68(7.87%) and 104(12.03%) respectively. The collected milk samples (California Mastitis Test Screened) were cultured on sheep blood agar and confirmed by other biochemical tests commonly used for Staphylococcus aureus identification. For all except breed and floor type, the multivariable logistic regression analysis for intrinsic and extrinsic risk factors showed significant value for the prevalence of mastitis in the study area ($P<0.05$). Productions of biofilm on the isolates were evaluated on Modified Congo Red Agar (MCRA) and only red colonies detected, indicating a bacterium without biofilm production. Pure S. aureus isolates were subjected to antimicrobial susceptibility test and 12(32.43%) MRSA have been identified from a total of 37 S. aureus using cefoxitin through disk diffusion method. The isolates were found highly sensitive to Kanamycin 35 (94.6%), however they were least sensitive to Amoxicilin 9(24.3%). In the present study the Multiple Antibiotic Resistance Index analyses revealed that an existence of isolates originated from an environment where several antibiotics are used MAR Index > 0.2. Generally, the study showed that mastitis is an important problem and a potential growing of MRSA will be serious threat for dairy industry in the study area. Therefore, there is a need for building specific measures to tackle the problem and antimicrobial sensitivity testing should be implemented more often.

***Key word**: Biofilm, Cefoxitin, dairy cattle, Mastitis, MRSA, risk factors, Staphylococcus aureus*

1. INTRODUCTION

Ethiopia is a country with a human population estimated to 85 million within annual population growth rate, 3.5% (CSA, 2010). Livestock represent a major national resource and form an integral part of the agricultural production system. Milk production is one of the county resources and it is a biologically efficient system that converts feed and roughages to milk (Yohannes, 2003).

However, despite large number of dairy cows, milk production does not satisfy the nations' demand for milk. Different constraints have been listed as causes of low milk production in Ethiopia. Among these, mastitis is the major one. Mastitis (inflammation of mammary gland) is among important health problems in dairy cattle. It has been considered one of the most important threats affecting the dairy industry throughout the country (Mungube *et al*., 2004). In addition, there is a danger that the bacterial contamination of milk from affected cattle may render it unsuitable for human consumption by causing food poisoning or in rare cases provide a mechanism of spread of disease to humans (Bitew *et al*., 2010).

Evidence to date shows that affected dairy cows may loss 15% of their production and the affected quarter a 30% reduction in productivity. *Staphylococcus aureus* is one of the important causative agents of mastitis all over the world (Cabral *et al*., 2004); causing both sub-clinical and clinical form of mastitis in cattle (Pradeep *et al*., 2003).The chief reservoir of this bacterium is an infected udder. The organism is well adapted to survive in the udder and usually establishes mild sub clinical infection of long duration by shedding into milk from infected quarters (Tsegaye, 1988).

The bacterium produces a variety of exoproteins that contribute to its ability to colonize mammary gland (Salasia *et al*., 2004), different membrane-damaging toxins, four hemolysins (alpha-, beta-, gamma-, and delta-hemolysin) and leucocidin. Beta and alpha hemolysins are the most important in pathogenesis of the intra-mamarian infections (Park *et al*., 2004). Staphylococcal protein A is a membrane-bound exoprotein characterized and well known for its ability to bind to the Fc region of immunoglobulins of most mammalian (Alonso & Dagget, 2000).

The ability to form biofilm is an important factor in several *Staphylococcus aureus* infections, since bacteria living in biofilms are resistant to antimicrobials and also to immune system clearance (Fux *et al*., 2005).

Transmission occurs mainly at milking time through contaminated milking machines, clothes and hands of milkers or machine operators (Radostitis *et al*., 1994). Clinical signs vary with the severity of the disease and generally include pain, heat and swelling of the affected quarter or half of the gland and abnormality of milk either as clots or flakes and wateriness of the liquid phase (Miffin, 2004).

The remarkable ability of *S. aureus* to acquire drug resistance has lead to the emergence of Methicillin-resistant *S. aureus* (MRSA). This resistance is caused by an alternative penicillin-binding protein, called PBP2a. PBP2a is encoded by the *mec*A gene located in the mobile genetic element called *Staphylococcal* cassette chromosome (SCC*mec*) (Vanderhaeghen *et al*., 2010). Until the 1990s, methicillin-resistant *Staphylococcus aureus* (MRSA) was traditionally considered a pathogen causing nosocomial infections, being the so-called HA-MRSA (healthcare-associated methicillin-resistant *Staphylococcus aureus*) (Bens *et al.,* 2006).

Methicillin-resistant *S. aureus* are divided into three groups according to their epidemiological and genetic characteristics: hospital acquired (HA-MRSA), community-associated (CA-MRSA), and livestock-associated (LA-MRSA). The groups differ in sensitivity to antibiotics, in the location and size of the chromosomal cassette (SCC*mec*), and in the presence of Panton-Valentine leukocidin gene (PVL) (Huber *et al*., 2010).

MRSA was reported in veterinary setting after detection of drug resistant isolates from cattle in Belgium in 1970s (Devriese *et al*., 1972) and it has emerged as an organism of zoonotic importance when scientists proclaimed the transmission of animal MRSA strains to humans (Lee, 2003). Juhasz-Kaszanyitzky *et al*. (2007) first reported the evidence of direct MRSA transmission between cattle and humans. *Staphylococcus* present in milk may serve as a reservoir for human infections, thus allowing these microorganisms to persist and spread in the community.

Mastitis as a disease, has received little attention in Ethiopia, especially the sub clinical form which is mainly caused by *S. aureus* (Mekonnen *et al*., 2005; Hundera *et al*., 2005) and efforts have only been concentrated on the treatment of clinical cases (Girma, 2001). The disease is insufficiently investigated and information relating to its magnitude, distribution and risk factors is scant. Such information is important to envisage when designing appropriate strategies that would help to reduce its prevalence and effects (Mekebib *et al*., 2009; Megersa *et al*., 2010).

Mastitis is obviously an important factor that limits dairy production. The disease should be studied as it causes financial loss as a result of reduced milk yield, discarded milk following antibiotic therapy, veterinary expense and culling mastitis cows (Hillerton, 1987).

Antimicrobial susceptibility testing is one of the strategies which could reduce the prevalence of multi drug resistant mastitis causative microbes. In some part of our country drug sensitivity test have been performed on *S. aureus* isolated from mastitis dairy cattle. Mekuria *et al*. (2013) in Addis Ababa, Abera *et al*. (2010) in Adama town, Sori *et al*. (2011) Jimma Town, South West Ethiopia and Daka *et al*. (2012) in Hawassa area, South Ethiopia have made recent publications mainly focusing on drug susceptibility profile of *S. aureus* isolates.

Therefore, the objectives of this study were:

- To identify farm-level risk factors associated with bovine mastitis in and around Batu town.
- To isolate *S. aureus* from dairy cattle mastitis infection, investigate their antimicrobial resistance profile and potential for biofilm production.
- To identify MRSA from isolated *S. aureus* and determine factors leading to MRSA infection in the study area.

2. LITERATURE REVIEW

2.1. *Staphylococcus aureus*

Staphylococcus aureus is one of the most significant pathogens causing disease in animals and human. The term *S. aureus* means "Golden Cluster Seed" and it is also called as "golden staph". This microorganism was first isolated in 1884 by Scottish surgeon (Sir Alexander Ogston) from surgical abscesses (Ogston, 1884).

Staphylococcus aureus is aerobic and facultative anaerobic, oxidase-negative, catalase-positive, non-motile, fermentative, and non-spore forming bacteria. On solid culture medium, it forms glistening, smooth, raised, and circular colonies. The single colony can reach up to the size of about 4-6 mm in diameter when grown on non-selective media. Variation is found in the colony color and may range from orange to grayish or grayish-white in color (Carter *et al*., 1994).

2.2. *Staphylococcus aureus* and its significance in dairy cattle Mastitis

Staphylococcus aureus is one of the significant causes of udder infection in dairy animals (Sargeant *et al*., 1998). Intra-mammary infections (IMI) with this pathogen may lead to clinical and sub-clinical mastitis and is usually associated with the increase in somatic cell number. *S. aureus* is a problem in a variety of locations and under different management styles. Contagious mastitis pathogens, in particular *S. aureus* are reportedly a problem in hand milked dairy herds (Oliver *et al*., 1975).

2.2.1. Staphylococcus aureus intramammary infection

An infection like IMI can be considered a condition where adverse colonization by a microbe has occurred in the host animal. Inflammation, the host's response to the microbe, is seen as swelling, redness, pain, heat and interference in the normal function of the affected organ. The general term for inflammation of the mammary gland is mastitis, which can occur with or without infection. Development of *S. aureus* infection in the bovine mammary gland can be divided into the following stages: entry and attachment of

S. aureus bacteria in the mammary gland, interaction between the bovine immune system, evasion of immune defense, survival and tissue invasion. Entry of *S. aureus* into the teat canal can lead to IMI, but this depends on certain conditions as for any infection: the initial number of bacteria, access of the microbe to the target tissue, virulence of the strain and immunity of the host (Projan and Novick, 1997).

2.2.2. Entry and attachment of Staphylococcus aureus into the mammary gland

Entry of a sufficient number of *S. aureus* bacteria via the teat canal into the mammary gland is required for the development of natural IMI. The infective dose of *S. aureus* in bovine IMI is not known precisely, but quantities as small as 10 cfu (colony forming unit) have caused infection in an experimental model where bacteria were infused directly into the teat duct. However, infection did not develop in all cows in experimental infection models, which suggests that *S. aureus* does not always multiply sufficiently in the mammary gland, but other predisposing factors need to be present (Schukken *et al*., 1999).

Attachment of *S. aureus* to host cells or extracellular matrix molecules is a critical step for colonization and intra-mammary infection. Adherence to these substances is thought to occur by non-specific physicochemical mechanisms and by specific bacterial host-cell binding. The bacteria must then resist flushing of milk until adherence to the epithelial cells lining, the ductules and alveoli of the mammary gland (Frost *et al*., 1977).

S. aureus adheres to bovine mammary epithelial cells better than most other bacterial species and the presence of milk enhances adherence (Mamo and Froman, 1994). Attachment is best to fully mature cells, such as keratinized epithelial cells, compared with the cells of the deeper epidermis. Adherence to teat canal cells depends on the origin of the cell type (Sutra and Poutrel, 1994). *S. aureus* adheres particularly well to the cells of the upper part of the mammary gland. The bacterium is also able to adhere to fat globules, which allows dissemination to the upper part of the gland by floating (Sandholm *et al*., 1989).

Teat traumas increase the risk of colonization of the teats by *S. aureus*. Epithelial damage reveals the underlying sub epithelial components, e.g. fibrinogen and collagen, which

allows Staphylococcal surface proteins to mediate adherence to the host cell matrix (Patti *et al.,* 1994).

Exotoxins secreted by *S. aureus* can also be involved in the epithelial damage. Even minor traumas, such as those from milking, can facilitate entry of *S. aureus* into the teat. Callusing of the teat canal prevents tight closing and extremely callused teats were shown to be more susceptible to *S. aureus* mastitis (Zadoks *et al.,* 2001).

2.2.3. Staphylococcus aureus and host immune system

The second line of defense comprises the innate immune system that includes defense cells (neutrophils, macrophages, natural killer cells, and dendritic cells) and humoral factors (lactoferrin, lysozyme, lactoperoxidase, protein A, and complement) present in the teat duct and in the milk (Rainard and Riollet, 2003). Bacterial colonization leads to influx of somatic cells, primarily polymophonuclear neutrophils (PMN), as response to cytokine production triggered by the bacteria. Phagocytosis and killing by leukocytes play the main roles in host defense against *S. aureus* infection. Numerous exoproteins, like Sags secreted by *S. aureus*, can interfere with phagocytosis (Verhoef, 1997).

Phagocytosing cells express ingested staphylococci or their products in association with major histocompatibility complex II (MHC II) molecules on their surface in order to present them to T cells. Accumulation of neutrophils and capsule formation often surrounds gland areas infected by *S. aureus* and the bacteria remain sheltered within furuncles or abscesses. *S. aureus* may avoid intracellular death and survive within the host cells (Mullarky *et al.,* 2001), where it is protected from the host defense mechanisms and effects of antimicrobials. *S. aureus* is not regarded as an intracellular microorganism but replication within bovine mammary epithelial cells has been demonstrated (Almeida *et al*., 1996).

In the intracellular environment, *S. aureus* can fall into nutritional stress but still survive. These so-called small colony variants grow slowly and have typical colony morphology, due to altered metabolism, but they are infective and are highly resistant to antimicrobials (Brouillette *et al*., 2004).

2.3. Virulence factors and biofilm production in *Staphylococcus aureus*

In the broadest sense, any factor produced out of the bacterial cytoplasm allowing survival within or on a host organism in a non-symbiotic manner can be regarded as a virulence factor (Projan and Novick, 1997). *S. aureus* can produce numerous putative virulence factors that allow the microbe to adhere to eukaryotic membranes, resist phagocytosis, lyses eukaryotic cells and trigger the production of a cascade of host immune-modulating molecules. Among bovine *S. aureus* isolated from mastitis, production of enterotoxins or genes encoding these toxins have been the most studied (Salasia *et al*., 2004; Fueyo *et al*., 2005).

Many suggest that virulence of *S. aureus* vary among strains (Smith *et al*., 1998; Raimundo *et al*., 1999) but the possible role of specific virulence factors in this phenomenon is poorly understood. Virulence factors of *S. aureus* are not essential for bacterial cell division and growth, and many of these are needed only in certain circumstances. Continuous secretion of the virulence factors would be uneconomic for the microorganism. Therefore, expression of *S. aureus* surface proteins and exoenzymes is a coordinated process, which depends on the phase of bacterial growth and environmental stimuli (Horsburgh, 2008).

S. aureus can produce several adhesins or microbial surface components recognizing adhesive matrix Molecules (MSCRAMMs), which have been shown to mediate attachment to various cell surface proteins such as collagens, elastin, fibrinogen, fibronectin, bone sialoprotein, laminin and thrombospondin (Foster and Hook, 1998). Ligations to these proteins enable *S. aureus* to colonize tissues and initiate infection (Brouillette *et al.,* 2003).

Fibronectin-binding protein (FnBPs) has been demonstrated to be involved in cell invasion and to mediate adhesion to platelets via fibronectin and fibrinogen (Heilmann *et al*., 2004). *S. aureus* is capable of invading eukaryotic cells and phagocytosing cells, and antibiotics are unable to reach the intracellular bacteria. In this invasion *S. aureus* uses its surface-expressed FnBPs and α1β5-integrin on the host cell surface (Sinha *et al*., 1999).

Alpha-haemolysin (α-toxin), frequently produced by bovine *S. aureus* IMI isolates (Akineden *et al*., 2001), is toxic to bovine mammary cells, causes erythrolysis, disturbs the host cell ion balance, is dermonecrotic and neurotoxic (Dinges *et al.,* 2000).

Beta-haemolysin (β-toxin), probably produced by most of the bovine strains (Aarestrup *et al.,* 1999), functions as sphingomyelinase of the erythrocytes. Susceptibility of erythrocytes to β-haemolysin varies depending of the sphingomyelin content of the target cell. Based on *in vitro* studies, β-haemolysin has been thought to increase the damaging effects of α-haemolysin and enhance the attachment of *S. aureus* on bovine mammary epithelial cells (Cifrian *et al*., 1996).

Delta-haemolysin has surfactant or channel-forming properties and it intensifies haemolytic effects of β-haemolysin (Dinges *et al.,* 2000). Haemolysin γ is toxic to monocytes and macrophages and lyses red blood cells and phagocytes (Projan and Novick, 1997).

The family of leukocidins comprises toxins that damage membranes of phagocytosing host defense cells by inducing Ca2+ influx and subsequent pore formation. Leukocidins are composed of two subunits of related proteins, class S (slow eluting) and F (fast-eluted) proteins, the toxic effect depending on the synergistic action of both of these proteins (Kamio *et al.,* 1993). Leukocidins are more leukotoxic than γ-haemolysin (Prevost *et al*., 1995).

The family of staphylococcal super antigens (Sags) includes enterotoxins and enterotoxin-like proteins (SEA-SEE, SEG-SER, and SEU), toxic shock syndrome toxin (TSST), and exfoliatins ETA and ETB. SAgs bind to the outer surface of the MHC class II proteins and outside the site in T-cell receptors, to which antigens normally bind. The normal antigen presentation mechanism is by passed and consequently as much as 30% of the T-cells are activated instead of the normally of 0.1% activated T-cells (Fleischer and Schrezenmeier, 1988). This results in massive and uncontrolled release of cytokines, and in capillary leak, hypovolemic shock and multi-organ failure as seen in the toxic shock syndrome. SAgs can stimulate macrophages and monocytes to produce several inflammatory mediators, including tumour necrosis factor-α, nitric oxide, interleukin-6 and IL-10. Furthermore, in the presence of interferon-γ, the macrophages become cytolytic (McCormick *et al*., 2001).

Protein A, a cell wall-associated protein, interacts with several host factors including immunoglobulins G, A, E, tumour necrosis factors, and platelets attenuating opsonisation and phagocytosis (Gomez *et al*., 2004). Binding of IgM associated with B cells by protein A induces apoptosis, a mechanism leading to death of host cells such as polymorphonuclear cell (PMN) and macrophages (Goodyear and Silverman, 2004).

Most *S. aureus* strains produce a capsule and slime that can enhance adhesion to endothelial cells, inhibit phagocytosis due to their aggregate size, and hide the bacteria from antimicrobials and antibodies when subsequent opsonisation and phagocytosis fail (Lee *et al*., 1994; Verhoef, 1997). Biofilm is a structural complex of bacteria in which they are enclosed and composed of self-made polymeric matrix. These make connections to inert and free living surfaces which enhance the secure growth in the environment (Prakash *et al*., 2003).

Exopoly- saccharides (EPS) provide physical/ chemical barrier and prevent the adsorption of antibodies or different antibiotics. EPS also bind the antibiotics that are attempting to reach across the biofilm because these are negatively charged and act as ion-exchange resin, bacteria embedded in biofilm results in decreased growth rate of the bacteria and smaller size of the cells which make the cells less pervious to antibiotics, as all antimicrobial drugs are more operative in destroying the fast growing cells (Thien and O' toole, 2001).

Certain mastitis pathogens shows varied prevalence of infection with age or period of lactation, these may be due to the effect of variation in milk composition or any other factor during the lactation period, which makes the cattle more prone to certain bacterial infection. Milk composition varies with in a lactation period, between lactation and may also vary seasonally due to change in feed (payorala and taponen, 2009).

Poly-N-acetyl β 1, 6 glucosamine (PNAG), a surface polysaccharide is a key component of the staphylococcus biofilm matrix which is synthesized by proteins encoded by the intercellular adhesion ica operon and it can elicit a protective immune response (Maira-Litran *et al*., 2004). Another biofilm consti- tuent among mastitis isolates is the Bap protein but it is not so much common (Cucarella *et al.,* 2001). Quorum- sensing is the principal controlling mechanism of the biofilm formation. Bacteria that use quorum

sensing constitutively produce and secrete certain signaling molecules (called *autoinducers* or *pheromones*). These bacteria also have a receptor that can specifically detect the signaling molecule (inducer). When the inducer binds the receptor, it activates transcription of certain genes, including those for inducer synthesis. There is a low likelihood of a bacterium detecting its own secreted inducer. Thus, in order for gene transcription to be activated, the cell must encounter signaling molecules secreted by other cells in its environment. The *S. aureus* quorum sensing system is encoded by the accessory gene regulator (*agr*) locus and the responsible molecule that it produces and senses is called an auto inducing peptide (Hoeswill, 2008).

2.4. Reservoirs and transmission

Dairy cows, among other domestic artiodactyls, are considered to be temporary hosts of *S. aureus*, in which the microbe is frequently present as a contaminant that can multiply and persist for short periods. *S. aureus* has been isolated from practically all external surfaces of healthy cows (Matos *et al*., 1991 and Roberson *et al*., 1994), udder skin being the preferred site (Jorgensen *et al*., 2005). Long-term colonization by *S. aureus* on teat skin and several other body sites, primarily mucosal external orifices, have been observed in heifers (Roberson *et al.,* 1994) suggesting persistent colonization. It's likely that a calf has its first contact with *S. aureus* already at birth via the genital area of the mother (Kloos, 1997).

Infected milk is generally considered to be the primary source of the microbe, and milking liners are the main vector of transmission, since they have been frequently shown to be contaminated with similar *S. aureus* strains to those in infected milk (Zadoks *et al.,* 2002).

Traumatized sites such as abrasions on teats, legs, bends and navel, typically infected by *S. aureus*, are regarded as secondary sources of *S. aureus* causing IMI. *S. aureus* is regarded as a clonal organism as the populations consist of groups of genetically related strains with a common ancestor. *S. aureus* diversifies more often by point mutation than by horizontal gene transfer (Feil *et al*., 2003). Most studies have demonstrated that a given herd usually harbours a limited number of *S. aureus* strains, often with one or a few strains predominating (Mork *et al.*, 2005; Rabello *et al*., 2007).

2.5. Over view of Methicillin Resistant *Staphylococcus aureus* (MRSA)

2.5.1. Historical background of MRSA

Soon after the introduction of penicillin, around 1945, the majority of the *S. aureus* population had become resistant to penicillin through the production of beta-lactamase, an enzyme that hydrolyzes penicillin. In the late 1950s, the beta-lactamase resistant methicillin was introduced in human medicine. Methicillin resistance is caused by the acquisition of the *mec*A gene. This gene encodes an alternative penicillin-binding protein, called PBP2A, which has a low affinity for beta-lactam antibiotics (Vanderhaeghen *et al*., 2010).

2.5.2. Types of MRSA

Different types of MRSA may be distinguished based on epidemiological groups. This can be a simplistic approach since in some cases strains of MRSA have spread between the groups (Morgan, 2008).

I. Hospital Associated MRSA (HA-MRSA)

HA-MRSA infections occur most commonly in immune compromised individuals in hospitals and health care centers. MRSA are regarded as HA-MRSA when infections caused by them are likely to be acquired in health care settings when they emerge at least 48 hours after admission in patients having particular risk factors such as prolonged hospital stay, care in intensive care units (ICUs), prolonged antibiotic treatment, surgical interventions, and/or close contact with MRSA-positive individuals (Salgado *et al*., 2003).

II. Community Associated MRSA (CA-MRSA)

CA-MRSA strains were first reported in the late 1990s; these cases were defined by a lack of exposure to the health care setting. CA-MRSA infections occur in otherwise healthy people without a recent history of hospitalization or clinical presentation, and are usually associated with skin and soft tissue infection. Risk factors for CA-MRSA include crowding, frequent contact, compromised skin, contaminated surfaces and shared items,

and poor hygiene. To date the commonest human associated MRSA is USA300 (Naimi *et al*., 2003).

III. Livestock Associated MRSA (LA-MRSA)

LA-MRSA refers mainly to the clonal spread of a certain MRSA strain (ST398) that colonizes different food animal species (including horses) 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 (a so called "humanosis") (Morgan, 2008).

Livestock associated MRSA belong to the clonal complex 398 (LA- MRSA CC 398) is considered to be zoonotically important because of its capacity to colonize a wide range of hosts (Paterson *et al*., 2012). Bovine and human MRSA strain distinguishable by phenotyping and genotyping methods have been found providing evidence for MRSA transmission between human and cattle (Hata *et al*., 2010).

2.6. Mechanisms of antibiotic resistance in MRSA

MRSA strains are resistant to all cephalosporins, cephems and other β-lactams, such as ampicillin-sulbactam, amoxicillin-clavulanic acid, ticarcillin-clavulanic acid, piperacillin-tazobactam and the carbapenems. This group of organisms is also frequently resistant to most of the commonly used antimicrobial agents, including the aminoglycosides, macrolides, chloramphenicol, tetracycline and fluoroquinolones (Lee, 2003).

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

proteins are inactivated. PBP2a is encoded by the *mecA* gene, which is located in the staphylococcal cassette chromosome (SCC*mec*) (Katayama *et al*., 2000).

The exact mode of transfer of *mecA* gene still remains debatable. Initially, various MRSA strains were thought to have a single common ancestor due to different pigmentation than those of MSSA strains. However, it has been shown that the horizontal transfer of Staphylococcus cassette chromosomes element (SSCmec) is rare apart from the fact that horizontal transfer has a major part in the evolution of *S. aureus* (Kuroda *et al*., 2001). Any antimicrobial agent ineffective against MRSA increases the chances of acquiring MRSA, but certain groups of antibiotics including quinolones and cephalosporins have been identified as potential suspects (Muller *et al*., 2003 and Hori *et al*., 2002).

2.7. Identification and typing of MRSA

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

Disk diffusion test is the common method for detection of MRSA. This test is performed by applying the bacterial inoculums on to the surface of Muller Hinton agar plates. Commercially prepared, fixed –concentration, antibiotic –impregnated paper disk are placed on the inoculated ager surface. After appropriate incubation, the zones of growth inhibition around the antibiotics disks are recorded and resistance is evaluated according to the clinical laboratory standards institute (CLSI, 2009).

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

Definitive identification of MRSA is achieved up on detection of the *mecA* gene by polymerase chain reaction (Lee *et al*., 2004). In order to harmonize monitoring of MRSA in animals and foods in EU, EFSA proposed that the MRSA definition should be made by the examination for the presence of *mecA* or the recently described *mecC* using multiplex polymerase chain reaction or, in isolated negative for these genes, by phenotypical tests for resistance to cefoxitin (FSA, 2012).

Pulse-field gel electrophoresis of DNA fragments restricted with the *SmaI* enzyme is considered to be the gold standard for typing MRSA isolates (Moussa *et al*., 2011). However it is important to follow uniform standard protocols to achieve types of *Scc mec* internationally comparable results. There is no consensus regarding to the best methods for typing MRSA strains. Application of any typing method required carful assessment of its suitability and an individual approach depending upon the purpose of the study (Mehndiratta and Bhalla, 2012).

2.8. Zoonotic nature of MRSA

Several studies have been done to determine the degree to which MRSA plays a role in zoonosis or humanosis. It has been observed that usually 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 orginating 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).

Bovine and human MRSA strains indistinguishable by phenotyping and genotyping methods have been found providing evidence for MRSA transmission between human and cattle (Hata *et al*., 2010; Juhasz-Kaszanyitzky, 2007). MRSA infected cattle acts as a reservoir and later transmit the infections to other animals and humans. Transmission of animal MRSA to veterinary personnel has been found and it is more common for large animal personnel than small animal personnel (Wulf *et al*., 2008; Hanselman *et al*., 2006). Animal to human transmission occurs through direct contact, environmental contamination

and through handling of infected animal's product (Nunang and Young, 2007) whereas human to animal transmission is still unclear (Weese, 2010).

2.9. Prevention and control of MRSA infection

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

Good hygiene is an important general preventive and control measure, both in homes and human and animal healthcare environments, because environmental contamination with MRSA acts as a reservoir for infection. Known MRSA-positive animals should be nursed apart from other animals, with strict washing of the hands, gloves and gowns if in close contact. Recording the history of contact with human or animal MRSA, as well as an early culture of a wound non-responsive to first-line therapy allows for earlier recognition of MRSA. Animal owners should be informed about the risks and necessary precautions (Morgan, 2008).

The majority of serious MRSA infections are treated with two or more antibiotics that, in combination, often still are effective against MRSA (for example, vancomycin, linezolid, rifampin, sulfamethoxazole-trimethoprim and others). Minor skin infections, however, may respond well to topical mupirocin (Bactroban). The earlier the appropriate diagnosis and therapy is instituted for MRSA, the better the prognosis. Recent data showing vancomycin resistance, increasing numbers of MRSA isolates with higher vancomycin minumum inhibitory concentrations, and an apparent increase in vancomycin clinical failures have brought vancomycin's utility into question (CDC, 2007).

3. MATERIALS AND METHODS

3.1. Study area

The study was conducted in and around Batu town. Is a town and separate woreda in central Ethiopia and located on the road connecting Addis Ababa to Nairobi in the East Shewa Zone of the Oromia Region of Ethiopia. Batu has a latitude and longitude of 7°56′N 38°43′E/ 7.933°N 38.717°E with an elevation of 1643 meters above sea level. Adjacent to Lake Ziway (Lake Dambal), the economy of the town is based on fishing and horticulture. The livestock population in the area is estimated to be 21,400 cattle, 9,600 sheep, 850 goats, 158 horses, 266 donkey and 45,620 chickens. Batu is also home to a prison and a caustic soda factory. The 2007 national census reported a total population for Batu of 43,660, of whom 22,956 were men and 20,704 were women. The majority of the inhabitants said they practiced Ethiopian Orthodox Christianity, with 51.04% of the population reporting they observed this belief, while 24.69% of the population were Muslim, 0.42% practiced traditional beliefs, and 22.07% of the population were Protestant. The 1994 national census reported this town had a total population of 20,056 of whom 10,323 were males and 9,733 were females (CSA, 2007).

3.2. Study design

A cross-sectional study was conducted in which 216 lactating dairy cattle were examined for the presence of clinical and sub clinical mastitis to isolate *S. aureus*, investigate their biofilm production potential and perform in-vitro antimicrobial susceptibility test for identification of MRSA from November 2014 to May 2015 in selected dairy farms located in and around Batu town.

3.3. Study population

Study animals included were three breed of lactating dairy cattle (Holstein Friesian, Jersey and Borena breed cows) found in and around Batu town.

3.4. Sample size

A total of 216 dairy cattle were considered from nineteen small holder and large scale dairy farms having an average of 2 to 8 and 23 to 55 lactating cows respectively. Purposive sampling technique was applied on all available dairy cattle in the study area. Batu, Adami tulu and Bulbula were the targeting site for the sample collection.

3.5. Study methodology

3.5.1. Questionnaire survey

Data regarding the different potential risk factors (age, parity, lactation stage, housing conditions, previous history of mastitis, milking hygiene, tick infestation presence of teat lesion, floor type, antibiotic use and milk abnormalities like clotting and abnormal secretion) were collected for 216 lactating cows from farm records when available and by interviewing a translated Amharic version questionnaire to the farm owner when not.

3.5.2. Sampling method and transporting

Milk samples were collected according to the National Mastitis Council Guideline (1990). After a quarter had been washed with tap water and dried (in cases when there was a considerable amount of dirt to be removed) the teat end was swabbed with cotton soaked in 70% ethyl alcohol. Lactating cows were tested for the presence of clinical and sub clinical mastitis, Udder attachment, any physical abnormalities such as swelling of the udder, presence of lesions, anatomical malformations and tick infestation were recorded. The milk was examined for its color, odor, consistency and other abnormalities prior to milking.

The California mastitis test was carried out as described by Quinn *et al.* (2004). After discarding the first three milking streams a squirt of milk, about two ml from each quarter was placed in each of four shallow cups in the CMT paddle. An equal amount of the commercial CMT reagent was added to each cup. A gentle circular motion was applied to the mixtures in a horizontal plane for 15 seconds. Based on the thickness of the gel formed by CMT reagent-milk mixture, test results were scored as 0 (negative/trace), +1 (weak positive), +2 (distinct positive), and +3 (strong positive). Positive CMT cows were defined as having at least one CMT positive quarter.

Approximately 10 ml of milk was then collected aseptically from clinical and subclinical (CMT positive) mastitis cattle into sterile universal bottles. The collected samples were transported using icebox to microbiology laboratory of College of Veterinary Medicine, Addis Ababa University. Milk samples from mastitis cows were immediately cultured or stored at 4°C for a maximum of 24 hour until it was cultured on blood agar media.

3.6. Bacteriological procedures

3.6.1. Culturing and biochemical test

Milk samples were bacteriologically examined according to the procedures employed by Quinn *et al*. (1999). In refrigerated milk samples, bacteria may be concentrated in the cream layer and held with in clumps of fat globules (NMC, 1990). Hence dispersion of fat and bacteria was accomplished by warming the samples at 25 ^{0} C for15 minutes and shake before plating on the blood agar base. A loopful of milk sample collected from each infected quarter was inoculated separately on blood agar base enriched with 5-7% defibrinated sheep blood. The inoculated plates were then incubated aerobically at 37 ^{0}C for 24 to 48 hours. Identification of the bacteria on primary culture was made on the basis of colony morphology, pigmentation, hemolytic characteristics, Gram stain reaction including shape and arrangements of the bacteria. Then the bacterium positive for the desired characteristics were inoculated on nutrient agar and tested for catalase, O-F tests, tube coagulase test, growth characteristics on Mannitol salt agar and purple agar base.

3.6.2. Biofilm production test

Phenotypic characterization of biofilm production was performed as proposed by Freeman *et al*. (1989). A pure one isolate of *S. aureus* colony from 18-20 hour growth on nutrient agar were streaked on Modified Congo Red Agar (MCRA) plates and incubated aerobically for 24-48 hours. Then it was left at room temperature for more than 4 days, to observe if the bacterium colonies form a delayed black pigmentation. Positive result shows when the Congo red dye interacts with certain polysaccharides; forming black colonies on MCRA, where as non biofilm producers form red colonies.

3.7. Anti-microbial susceptibility test

All identified *S. aureus* isolate were subjected to antimicrobial susceptibility test using the disk diffusion method on Mueller-Hinton agar according to protocol from Clinical Laboratory Standards Institute CLSI (2007). Two or three colonies from the agar plates were inoculated in peptone water and incubated for 4-6 hours at 37°C. Turbidity standard

of the inoculums was maintained to 0.5 McFarland units by addition of normal saline if turbidity was higher. These inoculums were plated evenly on Muller Hilton Agar plates. Then the antibiotic disk was transferred aseptically on to the surface of the inoculated medium. The plates were incubated at 35°C for 24 hours. For identification of MRSA, cefoxitin (30 µg) disk was used. *S. aureus* isolates exhibiting resistance to cefoxitin (having zone of inhibition ≤ 21 mm) were identified as MRSA as suggested by CLSI (2007). Along with cefoxitin, other commercially available antibiotic discs were also used to assess the antibiotic sensitivity pattern of *S. aureus*. Other discs used were: Streptomycin (10µg), Compound sulphonamides (300 µg), oxytetracycline (30 µg), ceftraiaxone (30 µg), kanamycin (30 µg), Amoxacilin (30 µg) and Nalidixic acid (30 µg).The inhibition zone was reported as the diameter of the zone surrounding the individual disk in which bacterial growth was absent. Based on this, the isolates were defined as resistant, intermediate and susceptible according to the guide lines of the NCCLS (1997).

3.7.1. Identification of MDR (Multi Drug Resistance)

Multi Drug Resistance is defined as resistance to all the tested antibiotics in at least three of the antibiotics. The Multi Drug Resistance (MDR) character of the isolates was identified by observing the resistance pattern of the isolates to the antibiotics (Rota *et al*., 1996).

3.7.1. MAR (Multiple Antibiotic Resistance) Index

The MAR Index of an isolate is defined as a/b, where a represents the number of antibiotics to which the isolate was resistant and b represents the number of antibiotics to which the isolate was subjected. The MAR indexes of the isolates were calculated and noted and if bacteria having MAR Index > 0.2 indicates originate from an environment where several antibiotics are used (Jayaraman *et al*., 2012).

3.8. Data management and statistical analysis

Data from the questionnaires was coded and entered into a Microsoft ® 2007. From the data, risk factors relevant to the emergence of multiple drug resistant *S. aureus* was chosen and entered into a Microsoft Excel along with the laboratory data for MRSA. The data was then exported to SPSS windows version 20.0 (SPSS) for appropriate statistical analysis.

Chi square ($\chi 2$) were used to measure the association between the different risk factors and occurrence of MRSA in dairy cattle. Odds ratio and 95% CI computed and the 95% confidence level was used. Results were reported as statistically significant if p-value is less than 5 % ($P< 0.05$).

4. RESULTS

4.1. Prevalence

Of the total 216 lactating cows examined during the study period 92 (42.59%) had mastitis, of which 6.48% (14/216) and 36.1% (78/216) showed clinical and subclinical mastitis, respectively (Figure 1).

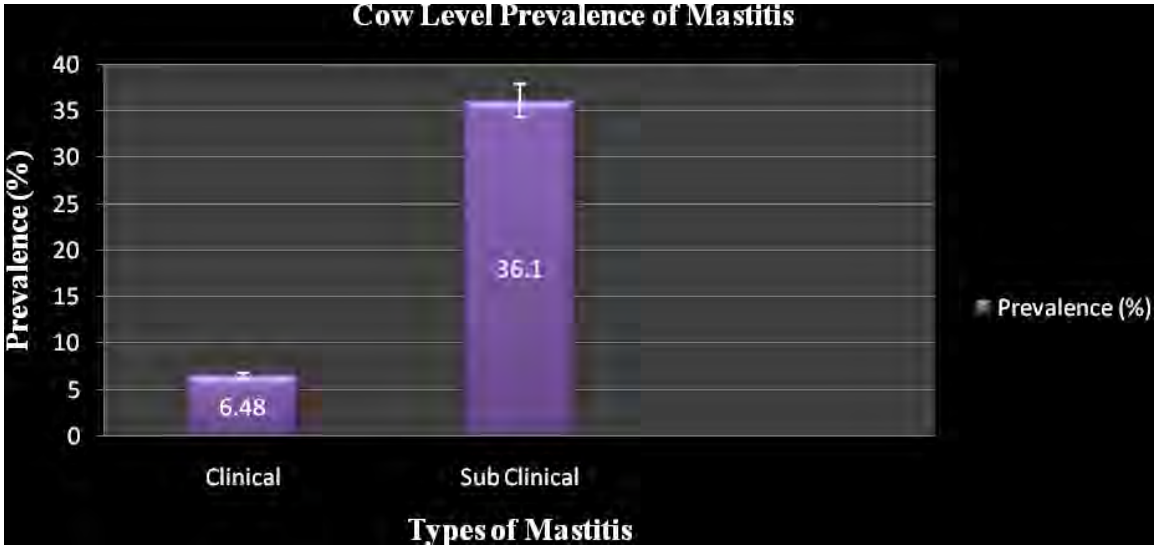


Figure 1: Prevalence of clinical and subclinical mastitis at cow level.

A total 864 quarters were investigated and among these, 852 (98.61%) quarters were found to be patent while the remaining 12 (1.39%) were blind. At the quarter level, 172 (19.9%) of them were positively reactive for CMT, however 681 (78.81%) were not. The proportion of right front (RF), right hind (RH), left front (LF) and left hind (LH) quarters that reacted positively for CMT was 35 (4.05%), 53 (6.13%), 33 (3.82%) and 51 (5.90%) respectively (Table 1).

Table 1: Prevalence of mastitis at quarter level

Quarter	Number of examined	Blind Quarter	Negative Quarter	Positive Quarter	x^2	p-value
RH	216	4(0.46%)	159(18.40%)	53(6.13%)	62.128	0.000
LH	216	6(0.69%)	159(18.40%)	51(5.90%)	51.638	0.000
RF	216	1(0.12%)	180(20.83%)	35(4.05%)	29.174	0.000
LF	216	1(0.12%)	183(21.18%)	33(3.82%)	32.528	0.000
Total	**864**	**12(1.39%)**	**681(78.81%)**	**172(19.9%)**		

RH-right hind, LH-left hind, RF-right front, LF- left front

Form the entire observed dairy cattle during the study period, a total of 25 (2.9%) and 147 (17%) infected quarters were identified from clinical and sub clinical mastitis case respectively (Figure 2).

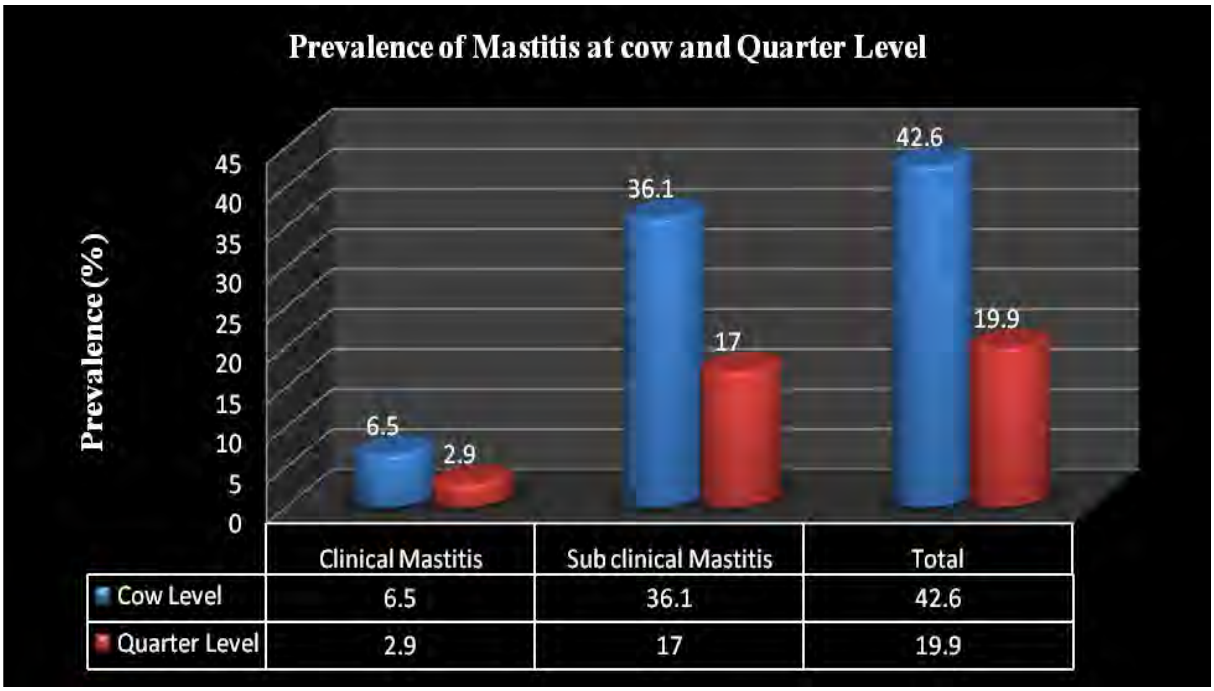


Figure 2: Prevalence of mastitis at cow and quarter level.

4.2. Risk factors associated with dairy cattle mastitis

4.2.1. Intrinsic risk factors

Prevalence of mastitis related to the specific risk factors were determined as the proportion of affected cows out of the total examined. As indicated in (table 2) age, parity and lactation stage were found to be having significant difference on the prevalence of bovine mastitis (P< 0.05).

Table 2: Multivariable logistic regression analysis for selected intrinsic risk factors with the occurrence of mastitis.

Risk-factors	No. examined	No. positive (%)	OR	P-value	CI
Breed type					
HF	109	58(53.21%)	1.475	0.699	0.480-4.533
Borena	68	22(32.35%)	1.174		0.327-4.216
Jersey	39	12(30.77%)	Ref.		Ref.
Age group (Years)					
Young(<4)	92	21(22.82%)	0.224	0.019	0.035-1.447
Adult(4-7)	56	30(53.57%)	0.249		0.063-0.988
Old(>7)	68	41(60.29%)	Ref.		Ref.
Parity number					
Few(1-2)	94	21(22.34%)	0.613	0.043	0.016-1.163
Moderate(3-4)	56	30(53.57%)	1.214		0.028-2.430
Many(>4)	66	41(62.12%)	Ref.		Ref.
Lactation-stage (months)					
Early(1-3)	58	9(15.52%)	0.876	0.042	0.189-4.060
Mid(4-6)	88	48(54.55%)	2.547		0.826-7.856
Late(>6)	70	35(50%)	Ref.		Ref.

HF: Holstein Friesian, OR**:** odd ratio, CI**:** confidence interval

4.2.2. Extrinsic risk factors

Management factors like milk hygiene, floor type and antibiotic used were evaluated as risk factors that influence the prevalence of bovine mastitis. All except flood type were found having significance on the prevalence of bovine mastitis in the study area (P< 0.05) (Table 3).

Table 3: Multivariable logistic regression analysis for selected extrinsic factors with the occurrence of mastitis.

Risk-factors	No. examined	No. positive (%)	OR	P-value	CI
milking Hygiene	69	14(20.29%)	0.620	0.024	0.142-2.702
Good	147	78(53.06%)	Ref.		Ref.
Poor					
Floor type					
Concrete	128	41(32.03%)	0.966	0.149	0.308-3.037
Muddy soil	88	51(57.95%)	Ref.		Ref.
Antibiotic use					
Yes	56	15(26.79%)	0.335	0.005	0.106-1.056
No	160	77(48.13%)	Ref.		Ref.

OR**:** odd ratio, CI**:** confidence interval

4.3. Number and percentage of *S. aureus* isolated from mastitis dairy cows

A total of 172 quarters of milk samples from 92 CMT positive dairy cattle were collected and cultured. Positive growths of *S. aureus* isolated were 37. Thus, overall prevalence of *S. aureus* from the whole dairy cattle was 17.13%, from these 9 (4.17%) and 28 (12.96%) were from clinical and sub clinical mastitis respectively (Table 4).

Table 4: *Staphylococcus aureus* isolates by the mastitis type (n= 216).

	Mastitis type		
Isolated bacteria	Clinical (n=14)	Subclinical (n=78)	Total No. of isolate
Staphylococcus aureus	9 (4.17%)	28 (12.96%)	37 (17.13%)

$X^2 = 66.91$ df = 1 p-value= 0.000

4.4. Biofilm production test

A total of 37 pure isolated *S.aureus* were subjected for biofilm production on a Modified Congo Red Agar. However none of the isolate shows a black colony with dry crystalline consistency (0%), rather they were found forming a red colony which indicates a bacterium without biofilm production (Table 5).

Table 5: *Staphylococcus aureus* isolates and Biofilm production status

	Biofilm production on MCRA	
Number of isolated bacteria	Positive	Negative
37	0 (0%)	37 (100%)

MCRA: Modified Congo Red Agar

4.5. Invitro antibiotic sensitivity test

The antimicrobial susceptibility tests carried out in this study indicated the existence of susceptibility and resistance of *S. aureus* to some of the antimicrobial 12 (32.43 %) of *S. aurues* was found to be resistance to cefoxitin, indicating the existence of MRSA (Table 6).

Table 6: Summary of antimicrobial susceptibility test result for *S. aureus* isolates (n=37).

Antibiotics used	**S** **n (%)**	**I** **n (%)**	**R** **n (%)**
Streptomycin	30(81.1)	3(8.1)	4(10.8)
Compound sulphonamides	19(51.35)	_	18(48.65)
Oxytetracycline	20(54.1)	_	17(45.9)
Ceftraiaxone	32(86.5)	2(5.4)	3(8.1)
Cefoxitin	25(67.57)	_	12(32.43)
Kanamycin	35(94.6)	2(5.4)	_
Amoxicilin	15(40.5)	_	22(59.5)
Nalidixic acid	12(32.43)	9(24.33)	16(43.24)

S: Susceptible, I: Intermediate, R: Resistant, **- :** Negative

4.5.1. Association of Cefoxitin resistance with previous treatment

From the total of 92 CMT positive dairy cattle recorded, 15 of them were found to be treated with antibiotics. The antimicrobial susceptibility test result shows 12 (32.43%) isolate were found to be resistant to Cefoxitin. Majority of cefoxitin resistant 11 (91.66%) isolate were from mastitis cattle that had been taken antibiotic treatment (Table 7).

Table 7: Cefoxitin resistance with treatment history

	Previous mastitis treatment	
	Yes	No
	15	22
Cefoxitin resistance		
Positive	11	1

$X^2 = 15.51$ df= 1 P- value = 0.000

4.5.2. Antimicrobial profile and association of MRSA with age of cows

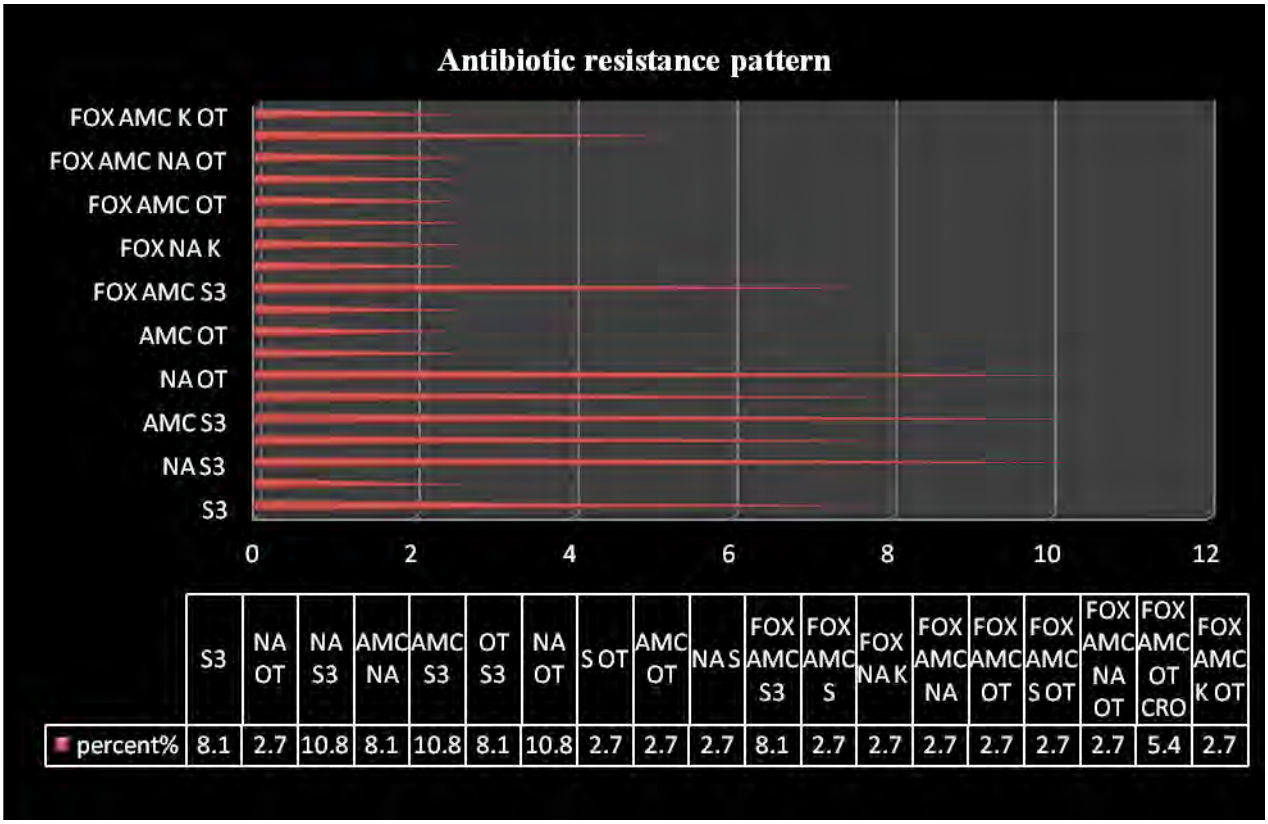
Occurrence of *S. aureus* relation with age were 9 (24.32%), 11 (29.73%) and 17 (45.95%) in Young, adult and old age groups respectively. Older cows more often harbor multidrug resistant *S. aureus* than younger cows. Most of adults and old age category group are found to be positive for multidrug resistant *S. aureus.* only11.11% of young were infected by unidrug resistant isolates while the remaining younger caws 88.89 % found carrying two drugs resistant *S. aureus* isolates (Table 8).

Table 8: Drug resistance pattern of *S. aureus* and age of cows

Age of Cows	***S. aureus* isolated**	**Resistance pattern**				
		One drug	**Two drug**	**Multi-drug**	**P-value**	**X^2**
Young	9	1(11.11%)	8(88.89%)	0(0%)		
Adult	11	2(18.18%)	7(63.64%)	2(18.18%)	0.004	11.41
Old	17	0(0%)	7(41.18%)	10(58.82%)		
Total	**37**	**3**	**22**	**12**		

4.5.3. Percentage and frequency of antimicrobial resistance pattern of S. aureus

From the total pure isolated *S. aureus* 3 (8.11%), 22 (59.45%), 7 (18.92%) and 5 (13.51%) of the isolates were resistant for 1-4 drugs, respectively (Figure 3).



AMC: Amoxicillin, NA: Naldic Acid, S3: Compound sulphonamide, FOX: Cefoxitin
CRO: Ceftriaxone, S: Streptomycin, OT: Oxytetracycline, K: Kanamycin

Figure 3: Percentage and antimicrobial resistance pattern of *S. aureus* isolate.

Out of the total isolate subjected to eight different class antibiotics about 32.4% was developed multi drug resistance, of which 18.9 % and 13.5% were multi resistant for three and four antibiotics respectively (Figure 4).

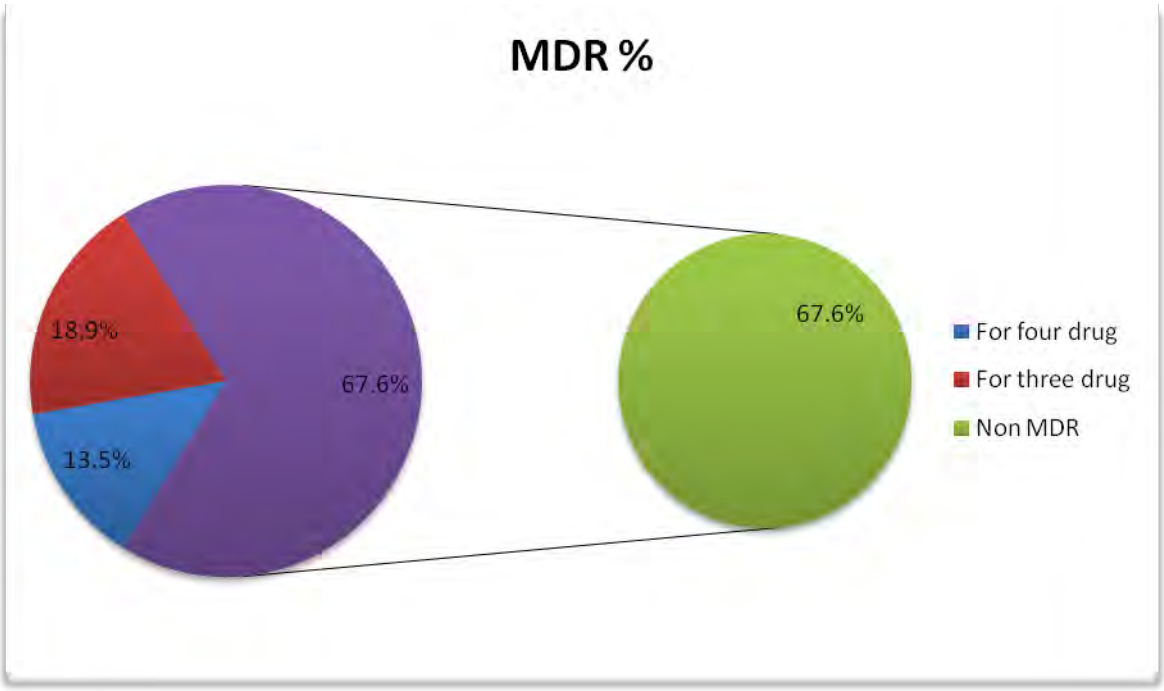


Figure 4: Proportions of Multi drugs resistance *S. aureus* tested for eight antibiotics

4.5.4. Multi antibiotic resistance index analysis

The MAR Index analysis (Figure 5) reveals that the isolate had a very high MAR index value (>0.2).

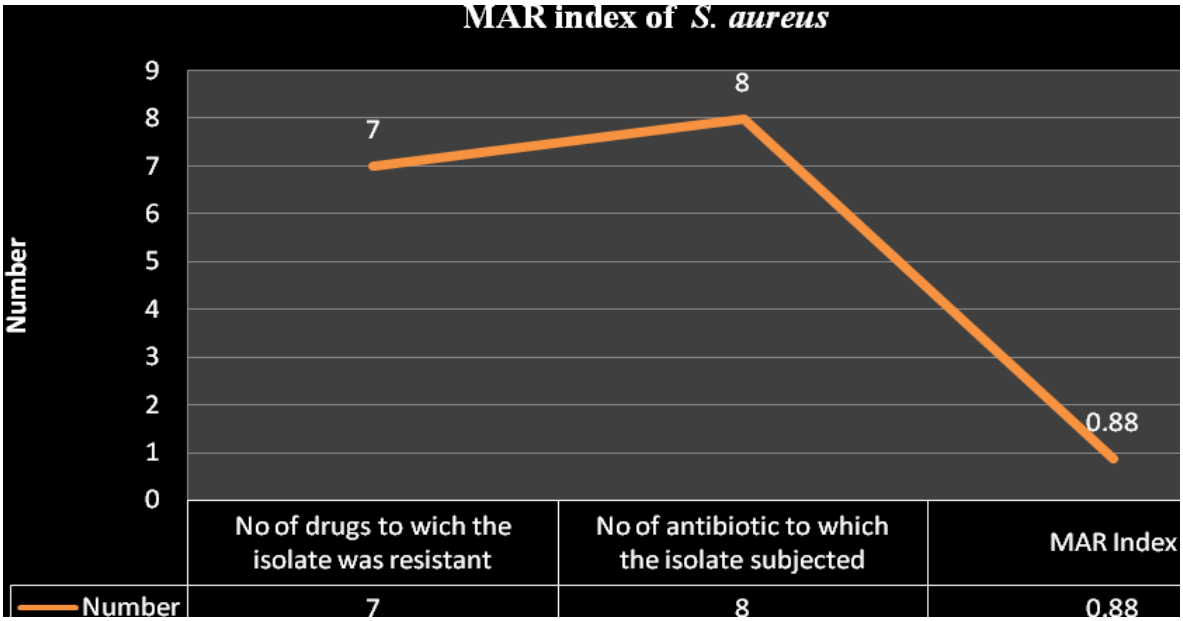


Figure 5: Multi antibiotic resistance index analysis of *S.aureus*

5. DISCUSSION

The study was designed to determine the prevalence, biofilm production and antimicrobial resistant profile of MRSA isolated from milk sample of mastitis dairy cattle. The overall 42.59 % mastitis prevalence reported in this study is comparable with earlier reports of 40% in cows by Kerro and Tareke (2003) in Southern Ethiopia. It was also relatively similar to the findings of Abdelrahim *et al.* (1990), who found a prevalence of 45.8% in Sudan. However, it was lower than previous reports (56.5%) by Duro *et el*. (2011) in Batu, (53.0%) by Takele (1987) in Arsi, 52.8% by Hundera *et al*. (2005) around Sebeta, 61.11% by Tolla (1996) in South Wollo, 63% by Biru (1989) and 68.1% by Zerihun (1996) in Addis Ababa. As mastitis is a complex disease involving interactions of various factors such as managemental and husbandry, environmental conditions, animal risk factors, and causative agents, its prevalence will vary (Radostitis *et al.,* 2007).

The prevalence of subclinical mastitis in this study was 36.1% which is closely comparable with 38.2% reported by Workineh *et al*. (2002). Clinical mastitis prevalence was 6.48% which is closely similar to report by Bish (1998) and Duro *et al*. (2011) who reported the prevalence of 5.3% in Addis Ababa and Batu respectively. However, the clinical and sub clinical mastitis was far lower than reported (65%) by Abaineh (1997) in Fiche and (25.1%) by Workineh *et al*. (2002) in Addis Ababa for sub clinical and clinical case respectively. This variation in prevalence between subclinical and clinical mastitis may be due to the fact that, the defense mechanism of the udder reduces the severity of the disease (Eriskine, 2001). In most developing countries including Ethiopia the sub- clinical mastitis received little attention and effort have been concentrated only on treatment of clinical cases (Hussein *et al*.,1997).

The mastitis prevalence of 19.9% in quarters reported in this study is in agreement with some earlier reports of 19% in quarters by Kerro and Tareke (2003). The quarter infection rate was much lower than the 39% quarter infection rate reported by Abdelrahim *et al*. (1990). The study shows that hind quarter are more exposed to mastitis infection comparing to the front quarter and these is due to the fact that the hind quarter are highly predisposed for contamination with dirt. In addition to this, large amount of milk is produced from hind quarters and as a result the pressure on the teat canal forces the canals

to be opened widely which allows entrance of microbes. The observation of blind quarters in this study might be an indication of a serious mastitis problem on the farms and of the absence of culling that should have saved to remove a source of mammary pathogens for the caws.

With regard to the bacteriological analysis of milk sample, the work revealed that from the entire CMT positive milk samples the presence of *S. aureus* were 17.13%. This finding was in agreement with seedy *et al*. (2010) who reported (17.2 %) in Egypt. The finding is also relatively comparable to Abebe *et al*. (2013) who reported 15% in Addis Ababa. However, it was greatly reduced than Ahamed and Mohammed (2007) who reported 52.5% in Egypt and G/Michael *et al*. (2013) 54.4% in Areka, Showing relatively higher prevalence of *S. aureus* in their study area. This variation may be due to season, manage-mental conditions at the farm area and difference in sample handling in the laboratory.

In the present study, the prevalence of *S. aureus* in sub clinical mastitis was significantly higher than clinical mastitis. *S. aureus* is adapted to survive in the udder and usually establishes chronic sub clinical infection of long duration from which it is shaded through milk serving as sources of infection for other healthy cows and transmitted during the milking process. Transmission among cows increase whenever there is lack of effective udder washing and drying, washing clothes, post-milking teat dip and drying, hand-washing and disinfection (Radostitis *et al*., 1994).

The results of analysis on selected risk factors showed that age was one of the predisposing factors. The occurrence of more cases of mastitis in older animals observed in the present study concurs with previous reports of Biffa *et al*. (2005) and Sargeant *et al*. (1998). This might be due to the increased opportunity of infection with time, the prolonged duration of infection and the physiological defense mechanism of the udder reduced with advancing age to overcome bacterial pathogens, so that pathogenic organisms get access to the glandular tissue and cause inflammation of mammary glands (Harmon, 1994; Radostits, 2003).

The prevalence of mastitis was not significantly influenced by breed and floor type ($P > 0.05$). It was however, highest in Holstein Friesian from breed type and in cattle managed at muddy floor.

The increased prevalence of mastitis with parity reported in the current study is in harmony with the previous reports by Biffa *et al*. (2005), Tamirat, (2007), Mekibib *et al*. (2010) and Haftu *et al*. (2012). This might be due to the increased opportunity of infection with time and the prolonged duration of infection, especially in a herd without mastitis control program (Radostits *et al*., 2007).

The chance of finding mastitis was higher in middle and late lactation stage comparing to early lactation stage. The increased prevalence of mastitis with advancing lactation stages agrees with previous investigations by Radostits *et al*. (2007) and Zerihun, (1996). Regime could be possibly among the major factors contributing to high prevalence at middle stage. During a dry period, due to low bactericidal and bacteriostatic qualities of milk, the pathogens can easily penetrate into the teat canal and multiply (Aylate *et al*., 2013).

In the present study, Cows at farms with poor milking hygiene standard were severely affected than those with good milking hygiene practices. Similar results were reported by Lakew *et al*. (2009) and Sori *et al*. (2005). This might be due to absence of udder washing, milking of cows with common milkers' and using of common udder cloths, which could be vectors of spread especially for contagious mastitis (Radostits *et al*., 2004).

The investigation also revealed higher Prevalence of mastitis in dairy cattle that hadn't been treated with antibiotics comparing to members who had. The possible explanation for this may be in some part of the study area the antibiotic treatment for mastitis infection is still in good governance by veterinary practitioners.

In the present study, the ability of all isolated *S. aureus* for biofilm production was nil. These might be due to high production of dipotassium hydrogen phosphate (K_2HPO_4) in milk of the dairy cattle which act as a buffering agent in milk and protect bacteria from adverse environmental conditions like P^H. However, the situation prevents the bacterium to start the initial step of biofilm production (Black *et al*., 2007).

The isolated *S. aureus* were typed for antibiotic resistance to obtain vital information that could help evolving a strategy for prevention and treatment of dairy cattle mastitis case in the study area. The *in vitro* disc sensitivity test showed that Kanamycin is relatively the most effective drug followed by Ceftraiaxone and Streptomycin. Closely Similar results

have been published by Belayneh *et al*. (2013) and Abera *et al*. (2010) in Adama showing (90%) and (86.1%) susceptibility for Kanamycin and Ceftraiaxone respectively. 91.59% Susceptibility for Streptomycin was also reported by Joshi *et al*. (2014) in Nepal. The reason why these antimicrobials were less resistant might be that they are not frequently used in the study area in veterinary services. Similar suggestion was given by Jaims *et al*. (2002) that the development of antimicrobial resistance is nearly always as a result of repeated therapeutic or indiscriminate use of them.

The poor inhibitory effect of oxytetracycline and Amoxicilin against *S. aurues* strain observed in this study were in accordance with the earlier studies reported by Girma *et al*. (2012) and Belayneh *et al.* (2013) who have reported (48%) and (62%) in west Harerghe and Adama respectively.

Prevalence of Cefoxitin resistance in the study area was found to be 32.43 %. This finding is closely comparable with Joshi *et al*. (2014) who have reported 37.82% Cefoxitin resistance *S. aurues* isolate in Nepal. All cefoxitin resistance *S. aureus* isolate was also found to be resistant to amoxicillin. Since MRSA strain have known to be resistance to β-lactams, the result revealed is an indicator for the existence of MRSA (Daka *et al*., 2012).The link of similar resistant pattern between this two antibiotics might be due to high use of beta-lactam antibiotics to treat the mastitis cases usually administered by charlatan practitioners without performing antibiotic sensitivity test.

In the presence study majority of cefoxitin resistance *S. aureus* was isolated from mastitis dairy cattle which had previous antibiotic treatment. The possible fair judgment for this could be that inappropriate implementation of antibiotics to treat mastitis case in some part of the study area leading to occurrence of an isolate which had a potential of drug resistance.

An increased occurrence of MRSA in association with age groups was found to be significant ($P< 0.05$). All identified MRSA isolate were from adult and older categories. These may be due to the fact that prolonged time of survival under low managemetal condition for dairy cattle leads to a possible chance of exposure to mastitis infection, so possibility of repeated antibiotic treatment will be relatively higher in advancing ages.

S. aureus strains have developed multidrug resistance worldwide with broad diversity in prevalence rate in different regions (Normanno *et al*., 2007). In the present observation (figure 3), 12 (32.43%) *S. aureus* isolate showed multidrug resistance primarily to Amoxicillin, Oxytetracycline, Naldic Acid and Compound sulphonamide. Similar finding by Mekuria *et al*. (2013) reported MRSA isolate with resistant to more than two of non-β-lactam antimicrobials.

The antimicrobial susceptibility test result (table 6) revealed that the isolated bacterium that were sujected to eight different abtibiotics found only non resistant to Kanamycin. Bacteria having MAR Index > 0.2 (figure 5) originate from an environment where several antibiotics are used (Tambekar *et al*., 2006).

6. CONCLUSION AND RECOMMENDATION

In the present study the prevalence of mastitis was one of a major health problem of dairy cows in the study area. The disease has also been reported in different parts of Ethiopia with varying prevalence. Data from questionnaire survey showed that majority of dairy cattle that have been observed in the study area were under poor management condition and most of the dairy cattle owner or assigned farm manager also found to be Unrelated and with low Experience to animal production.

According to the investigation Kanamycin, Ceftraiaxone and Streptomycin were showed relatively a good efficacy, there for could be the drug of choice for the study area. Since some of the trial discs specially oxytetracycline, Compound sulphonamides, and Amoxicilin shows low potency, so special attention should be given to those antibiotics because there could be a danger of drug failure to control mastitis in the study area.

Based on study and above conclusions, the following recommendations are forwarded:

- There is need to create farmers' awareness to improve efforts in using preventing measures including hygienic milking practice and briefing the effect of MRSA. Proper extension packages to dairy farm owners and strategic mastitis control programs should be implemented for controlling the prevalence of dairy cattle mastitis infection.
- Regular screening for the detection of subclinical mastitis and proper treatment of the clinical cases as well as appropriate treatment of cows during dry and lactation period should be practiced.
- Careful monitoring of the resistance status of *S. aureus* in dairy environment is needed, as *S. aureus* transmission is dynamic and involves animals and likely the farm production environment.
- Care has to be taken in selection of drugs before instituting treatment and application of an *in vitro* antimicrobial sensitivity testing should be implemented more often.
- Furthermore, impacts and dynamics of genetic antibiotic determinants should also be investigated using molecular methods.

7. REFERENCE

Abaineh D. (1997): Treatment trials of sub-clinical mastitis with a polygonaceae herb. Proceedings of the 11th Conference of Ethiopian Veterinary Association: Addis Ababa, Ethiopia, p_p 67–75.

Abera M., Demie B., Aragaw K., Regassa F. and A. Regassa. (2010): Isolation and identification of *Staphylococcus aureus* from bovine mastitic milk and their drug resistance patterns in Adama town, Ethiopia. *J. of Veterinary Medicine and Animal Health* Vol., **2**(3), pp 29-34.

Abdelrahim A. I., Shommein A. M., Suliman H. B. and Shaddard S. A. (1990): Prevalence of mastitis in imported Freisian cows in Sudan. *Rev. Elev. Med. Veterinary. Pays. Trop*., **42**: 512-514.

Akineden O., Annemuller C., Hassan A. A., Lämmler C., Wolter W. and schöck M. Z. (2001): Toxin genes and other characteristics of *Staphylococcus aureus* isolates from milk of cows with mastitis. *Clin. Diagn. Lab. Immunol*., **8**:959-964.

Almeida R. A., Matthews K. R., Cifrian E., Guidry A. J. and Oliver S. P. (1996): *Staphylococcus aureus* invasion of bovine mammary epithelial cells. *J. Dairy Sci*., **79**: 1021- 1026.

Alonso D.O. and Daggett V. (2000): Staphylococcal Protein A: Unfolding pathways, unfolded states, and differences between the B and E domains. *Proc. Natl Acad. Sci. USA*, **97**:133-138.

Ateba C., Mbewe M., Moneoang M. S., Bezuidenhout C. C. (2010): Antibiotic-resistant *Staphylococcus aureus* isolated from milk in the Mafikeng Area, North West province, South Africa. *S Afr. J. Sci*., **106**:12-13.

Aylate A., Fikiru H., Alemante M. and Aster Y. (2013): Prevalence of sub clinical mastitis in lactating caws in selected commercial dairy farms of Holeta district. *J. of Vet. Med. and Animal Health* vol., **5**(3), pp 67-72.

Belayneh R., Belihu K. and Wubet A.(2013): Dairy cows mastitis survey in Adama Town, Ethiopia. *J. of Vet. Med. and Animal Health. Vol.,* **5**(10): 281-287.

Biffa D., Debela E. and Beyene F. (2005): Prevalence and Risk factors of mastitis in lactating dry cows in southern Ethiopia. *Inter. J. Appl. Res. Vet. Med.,* **3**(3): 189-198.

Biffa D., Etana D. and Fekadu B. (2005): Factors Associated with udder infections in lactating cows in Southern Ethiopia. *Inter. J. Appl. Res. Vet. Med*., **3**: 189-198.

Biru G. (1989): Major Bacterial causing Bovine mastitis and their sensitively to common Antibiotics. *Eth J. Agri. Sc*., **11**: 47-54.

Bish A.B. (1998): Cross section and longitudinal prospective study of bovine clinical and sub clinical mastitis in peri-urban Dairy production system in Addis Ababa region, M. V. Sc. Faculty of Veterinary medicine, Addis Ababa University, Debre-Zeit, Ethiopia.

Bitew M., Tafere A. and Tolosa T. (2010): Study on Bovine mastitis in dairy farms of Bahirdar and its environs. J. Animal and Veterinary Advances, **9**(23): 2912-2917.

Black E., Huppertz P., Fitzgerald T. and Kelly G.F. (2007): Baroprotection of vegetative bacteria by milk constituent:a study of Listeria innocua . *International diary J.,* **17**: 04-110.

Boles B.R. and Horswill AR. (2011): Staphylococcal biofilm disassembly. *Trends in microbiology***, 19**(9): 449-455.

Brouillette E., Martinez A., Boyll B. J., Allen N. E. and Malouin F. (2004): Persistence of a *Staphylococcus aureus* small-colony variant under antibiotic pressure in vivo. FEMS *Immunol. Med. Microbiol*., **41**:35-41.

Brouillette E., Talbot B. G. and Malouin F. (2003): The fibronectin-binding proteins of *Staphylococcus aureus* may promote mammary gland colonization in a lactating mouse model of mastitis. *Infect. Immunol*., **71**: 2292-2295.

Cabral K.G., Lammler C., Zschock M., Langoni H., De Sa M EP., Victoria C. and Da Silva A. V. (2004): Pheno and genotyping of *Staphylococcus aureus*, isolated from bovine milk samples from Sao Paulo State, Brazil. Can. *J. Microbiol*., **50**: 901-909.

Carter G. R., Chengappa M.M. and Roberts A.W. (1994): Staphylococcus. In: Essentials of Veterinary Microbiology. 5th Ed, Williams Wilkins, Philadelphia, USA, p_p 115-120.

Catry B., Van Duijkeren E., Pomba MC., Greko C., Moreno MA., Pyorala S., Ruzauskas M., Sanders P., Threlfall EJ., Ungemach F., Törneke K., Munoz-Madero C. and Torren-Edo J. (2010): Scientific Advisory Group on Antimicrobials (SAGAM). Reflection paper on MRSA in food-producing and companion animals: epidemiology and control options for human and animal health. Epidemiol Infect, **138**: 626-644.

Centers for Disease Control and Prevention (CDC). (2007): Invasive methicillin-resistant *Staphylococcus aureus* infections among dialysis patients--United States. MMWR *Morb Mortal Wkly Rep*., **3**(56):197..

Cifrian E., Guidry A. J., Bramley A. J., Norcross N. L., Bastilda-Corcuera F. D., and Marquardt W. W. (1996): Effect of staphylococcal beta-toxin on the cytotoxicity, proliferation and adherence of *Staphylococcus aureus* to bovine mammary epithelial cells. *Vet. Microbiol*., **48**:187-198.

Clinical and laboratory standards institute (CLSI). (2009): performance standards for antimicrobial susceptibility testing: nineteenth informational supplement, M100-S1g, CLSI, wayne, PA.

CLSI. (2007): Performance standards for antimicrobial susceptibility testing; Seventh Information Supplement , 27, Clinical and Laboratory Standard Institute, Wayne, PA.

CSA. (2007): federal democratic republic of Ethiopia summary and statistical report: population and housing census, population size by sex and age.

CSA. (Central statistical agency) (2010): Live stock and live stock characteristics, Agricultural sample survey. Stat. Bull, **2**(468):107.

Cucarella C., Solano C., Valle J., Amorena B. and Lasa I. (2001): Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. *J Bacteriol*., **183**: 2888-2896.

Daka D., Solomon G. and Dawit Y. (2012): Antibiotic resistance staphylococcus aureus isolated from caw`s milk in the Hawassa area, south Ethiopia. *An. cli. microbiol. and Antimicrob*.,**11**:26

De silva ciombra M., Silva-carvalho M. C., Wisplinghoff H., Hall G. O., Tallent S., Wallace S., Edmond M. B., Figueredo A. M. and Wenzel R. P. (2003): Clonal spread of Methicillin- Resistant *Staphylococcus aureus* in a large geographic area of the United States. *J. Hosp. Infect*, **53**:103-110.

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

Dinges M. M., Orwin P. M., and Schlievert P. M. (2000): Exotoxins of *Staphylococcus aureus*. *Clin. Microbiol*. Rev., **13**:16-34.

Duro B. and Habtamu T. (2011): Study on prevalence of mastitis and its associated risk factors in lactating dairy cows in batu and its environs, Ethiopia. Global veterinarian, **7**(6): 632-637.

Eriskine R. J. (2001): Intramuscular administration of ceftiofursodiu versus intrammamary infusion of penicillin/ novobiocin for treatment of *Streptococcus agalactiae* mastitis in dairy cows. *J. Am. Vet. Med. Assoc*., **208**: 258-260.

European Food Safety Authority (EFSA). (2012): scientific report of EFSA: technical specifications on the harmonized monitoring and reporting of antimicrobial resistance in Methicillin resistant *Staphylococcus aureus* in food producing animals and food. *EFSA. J*., **10**: 2897.

Feil E. J., Cooper J. E., Grundmann H., Robinson D. A., Enright M. C., Berendt T., S. Peacock J., Smith J. M., Murphy M., Spratt B. G., Moore C. E. and Day N. P. (2003): How clonal is *Staphylococcus aureus*? *J. Bacteriol*., **185**: 3307-3316.

Fekadu B., Debela E. and Biffa D. (2005) : Prevalence and Risk Factors of Mastitis in Lactating Dairy Cows in Southern Ethiopia, p_p 189-198.

Fleischer B. and Schrezenmeier H. (1988): T cell stimulation by staphylococcal enterotoxins. Clonally variable response and requirement for major histocompatibility complex class II molecules on accessory or target cells.

Foster T. J. and Hook M. (1998): Surface protein adhesins of *Staphylococcus aureus*. Trends Microbiol, **6**:484-8.

Freeman D. J., Falkiner FR. and Keane CT. (1989): New method for detecting slime production by coagulase negative *staphylococci*. *J. Clin. Pathol*., **42**: 872-874.

Frost A. J., Wanasinghe D. D. and Woolcock J. B. (1977): Some factors affecting selective adherence of microorgansims in the bovine mammary gland. *Infect. Immunol*., **15**:245-253.

Fueyo J. M., Mendoza M. C., Rodicio M. R., Muniz J., Alvarez M. A. and Martin M. C. (2005): Cytotoxin and pyrogenic toxin superantigen profiles of *Staphylococcus aureus* associated with subclinical mastitis in dairy cows and relationships with macrorestriction genomic profiles. *J. Clin. Microbiol*., **43**:1278-1284.

Girma S., Mammo A. and Bogele K. (2012): Study on prevalence of bovine mastitis and its major causative agents in West Harerghe zone, Doba district, Ethiopia. Journal of Veterinary Medicine and Animal Health Vol., **4**(8): 116-123.

Girma T. (2001): Prevalence of mastitis at Alemaya University dairy farm. *J. Ethiopian Veterinary Association,* **5**: 17–21.

Goodyear C. S. and Silverman G. J. (2004): Staphylococcal toxin induced preferential and prolonged in vivo deletion of innate-like B lymphocytes. *Proc. Natl. Acad. Sci. USA*, **101**:11392-11397.

Gomez M. I., Lee A., Reddy B. and Muir G. (2004): *Staphylococcus aureus* protein A induces airway epithelial inflammatory responses by activating TNFR1. *Nat Med*., **10**:842-848.

G/Michael L., Benti D., Feyissa B. and Abebe M. (2013): Study on prevalence of bovine mastitis in lactating cows and associated risk factors in and around Areka town, Southern of Ethiopia. Vol., **7**(43), 5051-5056

Haftu R., Taddele H., Gugsa G. and Kelayou S. (2012): Prevalence, bacterial causes, and antimicrobial susceptibility profile of mastitis isolates from cows in large-scale dairy farms of Northern Ethiopia. *Trop. Anim. Health Prod*., **44**:1765-1771.

Hanselman B., Kruth A., Rousseau S. A., Low J., Willey D. E. and Mcgeer B. M. (2006): Methicillin- resistant *Staphylococci aureus* colonization in veterinary personnel. *Emerg. Infec. Dis.,* **12**: 1933-1938.

Harmon R. J. (1994): Symposium Mastitis and genetic evaluation for somatic cell count. *J. Dairy. Sci*., **77**(7): 2103-2112.

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. Clin Microbiol*., **48**: 2130-2139.

Heilmann C., Niemann S., Sinha B., Herrmann M., Kehrel B. E. and Peters G. (2004): *Staphylococcus aureus* fibronectin-binding protein (FnBP)-mediated adherence to platelets, and aggregation of platelets induced by FnBPA but not by FnBPB. *J. Infect. Dis*., **190**: 321-329.

Hilerton J. E. (1987): Summer Mastitis: Vector transmission or not? Parasitol. Today, **3**(4): 121-122.

Hori S., Sunley R., Tami A. and Grundmann H. (2002): The Nottingham *Staphylococcus aureus* population study: prevalence of MRSA among the elderly in a university hospital. *J. Hosp. Infect*., **50**: 25-29.

Horsburgh M. J. (2008): The response of *Staphylococcus aureus* to environmental stimuli. Staphylococcus molecular genetics. Lindsay, J. A. (ed), Caister Academic Press, Norfolk, pp 185-206.

Huber H., Koller S., Giezendanner N., Stephan R. and Zweifel C. (2010): Prevalence and characteristics of meticillinresistant *Staphylococcus aureus* in humans in contact with farm animals, in livestock, and in food of animal origin, Switzerland. *Euro Surveill.*, **15** (16): 540-542.

Hundera S., Ademe Z. and Sintayehu A. (2005): Dairy cattle mastitis in and around Sebeta, Ethiopia. *Intern. J. Appl. Res. Vet. Med*., **3**: 332-338.

Hussein N., Yehualashet T. and Tilahun G. (1997): Prevalence of mastitis in different local and exotic breeds of milking cows. Eth. *J. Agr. Sci*., **16**: 53-60.

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

Jayaraman S., Manoharan M., Ilanchezian S., Sekher R. and Sathyamurthy S. (2012): Plasmid analysis and prevalence of Multidrug resistant *Staphylococcus aureus* reservoirs in Chennai city, India, pp 211-276.

Jorgensen H. J., Mork T. D., Caugant A., Kearns A. and L. Rorvik M. (2005b): Genetic variation among *Staphylococcus aureus* strains from Norwegian bulk milk. *Appl. Environ. Microbiol*., **71**: 8352-8361.

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

Juhasz-Kaszanyitzky E., Janosi S., Somogyi P., Dan A., van der Graafvan Bloois L., van Duijkeren E. and Wagenaar JA. (2007): MRSA transmission between cows and humans. Emerg. *Infect. Dis*., **13**: 630-632.

Kamio Y., Rahman A., Nariva H., Ozawa T. and Izaki K. (1993): The two Staphylococcal bicomponent toxins, leukocidin and gamma-hemolysin, share one component in common. FEBS Lett., **321**:15-18.

Katayama Y., Ito T. and Hiramatsu K. (2000): A new class of genetic element Staphylococcus cassette chromosome *mec*, encodes methicillin resistance in *Staphylococcus aureus.* Antimicrob. Agents Chemother., **44**: 1549-1555.

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

Kloos W. (1997): Taxonomy and systematics of staphylococci indigenous to human's molecular basis of pathogenesis. In: The staphylococci in human disease. Crossley, K. B. and G. L. Archer (eds.). Churchill Livingstone, N. Y., p_p 113-137.

Kluytmans J., Ven Griiethhuysen A., Willemse P. And Van Keulen P. (2002): performance of CHROM agar selective media and Oxacillin resistance screening agar base for identifying *Staphylococcus aureus* and detecting methicillin resistance. *J. clin. Microbiol*., **40**: 2480- 2482.

Kuroda M., Ohta T., Uchiyama I., Baba T. and Yuzawa H. (2001): Whole genome sequencing of methicillin- resistant *Staphylococcus aureus*. Lancet., **357**:1225-1240.

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

Lee H., Jeong J., Park Y., choi S., Kim Y., Chae J. and Moon J. (2004): evaluation of the Methacillin resistant *Staphylococcus aureus (MRSA).*Screening latex agglutination test for detection of MRSA of animal orrigen. *J. clin. Microbial*, **42**: 2780-2782.

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

Linehan D., Etienne J. & Sheehan D. (2003): Relationship betweenhaemolytic and sphingomyelinase activities in a partially purified-like toxin from *Staphylococcus schleiferi*. FEMS *Immunol. and medical Microbiol*., **36**(1/2): 95-102.

Maira-Litran T., Kropec A., Goldmann D. and Pier G. B. (2004): Biologic properties and vaccine potential of the staphylococcal poly-N-acetyl glucosamine surface polysaccharide, Vac., **22**: 872-879.

McDougall S., Agnew K. E., Cursons R., Hou X. X. and Compton C. R. (2007): Parenteral treatment of clinical mastitis with tylosin base or penethamate hydriodide in dairy cattle.

Megersa B., Chala T., Abunna F., Regassa A., Berhanu M. and Etana D. (2010): Occurrence of mastitis and associated risk factors in lactating goats under pastoral management in Borana, Southern Ethiopia. *Trop. Anim. Health Prod*., **42**:1249-1255.

Mehndiratta L. and Bhalla P. (2012): Typing of methicillin resistance *Staphylococcus aureus*: a technical review. *Indian J. med. Microbial*., **30**: 16-23.

Mekebib B., Furgasa M., Abunna F., Megersa B. and Furgasa A. (2009): Bovine mastitis prevalence, risk factors and major pathogens in dairy farms of Holeta Town, Central Ethiopia. *Vet. World*, **13**(9): 397-403.

Mekibib B., Furgasa M., Abunna F., Megersa B. and Megersa A. (2010): Bovine Mastitis: Prevalence,Risk Factors and Major Pathogens in Dairy Farms of Holeta Town, Central Ethiopia. *Vet. World*, **3**(9): 397-403.

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

Mekuria A., Daniel A., Woldeamanuel Y. 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. Vol., **7**(27): 3501-3510.

Mifflin M. (2004): Bovine Mastitis- Definition of bovine mastitis in Medical Dictionary, the free dictionary by Farlex., p_p 15-20.

Morgan M. (2008): Methicillin-resistant *Staphylococcus aureus* and animals: zoonosis or humanosis? *J. of Antimicrobial Chemotherapy*, Vol., p_p 1181-1187

Mork T., Tollersrud T., Kvitle B., Jørgensen H. J. and Waage S. (2005): Comparison of *Staphylococcus aureus* genotypes recovered from cases of bovine, ovine, and caprine mastitis. *J. Clin. Microbiol*., **43**: 3979–3984.

Moussa M ., AL- qahtani A., Gassem A., Ashgan H., Ismail H., Ghazy I. and Shibl M. (2011): pulsed- field Gell electrophoresis (PFGE) as an epidemiological marker for typing Methicillin resistant *Staphylococcus aureus* recovered from KSA. Afr. *J. Microbial. Res*., **5**: 1492-1499.

Mullarky I. K., Su C., Frieze N., Park Y. H. and Sordillo L. M. (2001): *Staphylococcus aureus agr* genotypes with enterotoxin production capabilities can resist neutrophil bactericidal activity. *Infect. Immunol*., **69**: 45-51.

Muller A., Mauny F., Bertin M., Cornette C., Lopez-Lozano J. M., Viel J. F., Talon D.R. and Bertrand X.(2003): Relationship between spread of methicillin-resistant *Staphylococcus aureus* and antimicrobial use in a French University hospital. *Clin. Infect. Dis*., **36**: 971-978.

Mungube E.O., Tenhhagen B.B., Kassa T., Regassa F., kuyle MN., Garinar M. and Baumann MPO. (2004): Risk factors for dairy cows' mastitis in the central highlands of Ethiopia. *Trop. Anim. Health. Prod.,* **36** (5): 463-472.

Naimi T. S., Dell LE. and Corno-sabetti K. (2003): Comparism of community-and health careassociated MRSA infection. *J. Am. Med. Assoc.,* **290**: 2976-2984.

National Committee for Clinical Laboratory Standards (NCCLS). (1997): Performance standard for antimicrobial disk and dilution susceptibility test for bacteria isolated from animals and humans. Approved Standard, NCCLS document M 31-A, NCCLS, Villanova, PA.

NMC. (1990): Microbiological procedure for the diagnosis of bovine udder infection. 3rd ed. Arlington V A: National Mastitis Council, Inc.

Normanno G., LaSalandra G., Dambroise A., Quaglia NC., Corrente M., Parisi A., Firinu A., Crisetti E. and Celano GV. (2007): Occurrance, Characterization for enterotoxigenic *Staphylococcus aureus* isolated from meat and dairy product. *Int. J. Food Microbiol*., **115**: 290-296.

Nunang C. and Young R. (2007): MRSA in farm animals and meat: A new threat to human health. Soil association, USA.

Ogston A. (1984): On Abscesses-Classics in Infectious Diseases. *Rev. Infect. Dis*., **6**: 122-128.

Park P.W., Foster T.J., Nishi E., Duncan S.J., Klagsbrun M. and Chen Y. (2004): Activation of Syndecan-1 Ectodomain Shedding by *Staphylococcus aureus* -Toxin and -Toxin. *J. Biol. Chem*., **279** (1): 251-258.

Paterson K., Larson J., Harrisson M., Larson R., Morgan J., Peacock J., Parkhill J., Zadocks N. and Homes A. (2012): First detection of live stock- associated Methicillin- resisatance *Staphylococcus aureus* CC398 in bulk tank milk in the united king dom. Euro srveil., **17**(50): 20337.

Pinho M. G., Ludovice A. M., Wu S. and De-Lencastre H. (1997): Massive reduction in methicillin resistance by transposon inactivation of the normal PBP2 in a Methicillin-resistant strain of *Staphylococcus aureus*. *Microb. Drug Resist*, **3**: 409-413.

Pradeep V., Manoj K., Mohan N., Thirunavukkarasu A. and Kumar S.V. (2003): Phenotypic and Genotypic characterization of *Staphylococcus aureus* for biofilm formation. *Vet. Microbiol*., **92**: 179-185.

Prakash B., Veeregowda B.M. and Krishnappa G. (2003): Biofilms: A survival strategy of bacteria, Current Sci., **85**: 1299-1307.

Prevost G., Cribier B., Couppie P., Petiau P., Supersac G., Finck-Barbancon V., Monteil H. and Piemont Y. (1995): Panton-Valentine leucocidin and gamma- hemolysin from *Staphylococcus aureus* ATCC 49775 are encoded by distinct genetic loci and have different biological activities. *Infect. Immunol*., **63**: 4121-4129.

Projan S. J. and Novick R. P. (1997): The molecular basis of pathogenesis. In: The staphylococci in human disease. Crossley, K. B. and G. L. Archer (Eds.). Churchill Livingstone, N. Y., p_p 55-81.

Pyorala S. and Taponen S. (2009):coagulase-negative staphylococci-emerging mastitis pathogen. *Vet. microbial*. **134**: 3-4.

Quinn O. K., Carter M.E., Markey B. and Carter G.R. (1999): Clinical Veterinary Microbiology. USA, Elsevier Limited, p_p 35-65.

Quinin P.J., Carter M.E., Markey B.K. and Carter G.R. (1994): Clinical Veterinary Microbiology, 6th edition, Wolfe Publishing, p_p 21-67.

Quinn P.J., Carter ME., Markey B. and Carter GR. (2002): Veterinary Microbiology Microbial Diseases- Bacterial causes of Bovine Mastitis, 8th edition. Mosby international Limited, London, p_p 465-475.

Rabello R. F., Moreira B. M., Lopes R. M., Teixeira L. M., Riley L. W. and Castro A. C. (2007): Multilocus sequence typing of *Staphylococcus aureus* isolates recovered from cows with mastitis in Brazilian dairy herds. *J. Med. Microbiol*., **56**:1505-1511.

Radostits D. M. (2003): Herd health, food animal production medicine, 3rd edition. W. B. Saunder, Philadelphia, p_p 397-428.

Radostitis O. M., Blood D.C. and Gay C.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 O. M., Gay C. C., Hinchcliff K. W. and Constable P. D. (2007): Mastits In Veterinary Medicine: A Text book of disease of cattle, sheep, pigs, goats, and horses 10th edition, Ballier, Tindall, London, p_p 674-762

Raimundo O., Deighton M., Capstick J. and Gerraty N. (1999): Molecular typing of *Staphylococcus aureus* of bovine origin by polymorphisms of the coagulase gene. *Vet. Microbiol*., **66**: 275–284.

Rainard P. and Riollet C. (2003): Mobilization of neutrophils and defense of the bovine mammary gland. *Reprod. Nutr. Dev*., **43**: 439-57.

Reller B., Weinstein M., Jorgensen H. and Ferraro J. (2009): Antimicrobial susceptibility testing; a review of general principles and contemporary practice *clin. Infect. Dis*., **49**(11): 1749-1755.

Roberson J. R., Fox K. L., Hancock D. D., Gay J. M. and Besser T. E. (1994): Ecology of *Staphylococcus aureus* isolated from various sites of dairy farms. *J. Dairy Sci*., **77**:3354- 3364.

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

Salasia S. I., Kushnan Z., Lammler C. and Zschock M. (2004): Comparative studies on pheno- and genotypic properties of *Staphylococcus aureus* isolated from bovine subclinical mastitis in central Java in Indonesia and Hesse in Germany. *J. Vet. Sci.,* **5**: 103-109.

Salgado C.D., Farr B. M. and Calfee D. P. (2003): Community-acquired methicillin-resistant *Staphylococcus aureus*: a meta-analysis of prevalence and risk factors: *Clin. Infect. Dis*., **36**: 131-139.

Sandholm M., Kaartinen L., Hyvonen P., Veijalainen K. and Kuosa P. L. (1989): Flotation of mastitis pathogens with cream from subclinically infected quarters. Prospects for developing a cream-rising test for detecting mastitis caused by major mastitis pathogens. Zentralbl. Vet., **36**: 27-34.

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

Schukken Y. H., Leslie K. E., Barnum D. A., Mallard B. A., Lumsden J. H., Dick P. C., Vessie G. H. and Kehrli M. E. (1999): Experimental *Staphylococcus aureus* intramammary challenge in late lactation dairy cows: quarter and cow effects determining the probability of infection. *J. Dairy Sci*., **82**: 2393-2401.

Seedy E., El-Shabrawy F. R., Hakim M., Dorgham A. S., Ata S.M., Nagwa S., Bakry M.A. and Osman N.M. (2010): Recent Techniques used for isolation and characterization of *Staphylococcus aureus* from mastitic cows. *Am. J. Sci*., **6**:1-8.

Sinha B., Francois P. P., Nusse O., Foti M., Hartford O. M., Vaudaux P., Foster T. J., Lew D., Herrmann P. M. and Krause K. H.. (1999): Fibronectin-binding protein acts as *Staphylococcus aureus* invasin via fibronectin bridging to integrin alpha 5 beta1. Cell *Microbiol*., **1**: 101-117.

Smith T. H., Fox L. K. and Middleton J. R. (1998): Outbreak of mastitis caused by one strainof *Staphylococcus aureus* in a closed dairy herd. *J. Am. Vet. Med. Assoc*., **212**:553-556.

Sori T., Zerihun A. and Abdicho S. (2005): Dairy Cattle Mastitis in and around Sebeta, Ethiopia. *Int. J. Appl. Res. Vet. Med*., **3**(4): 332-338.

Sori T., Jemal H. and Molalegn B. (2011): Prevalence and Susceptibility Assay of *Staphylococcus aureus* Isolated from Bovine Mastitis in Dairy Farms of Jimma Town, South West Ethiopia, P_P 745-749.

Sutra L. and Poutrel B. (1994): Virulence factors involved in the pathogenesis of bovine intramammary infections due to *Staphylococcus aureus*. *J. Med. Microbiol*., **40**: 79-89.

Takele S. (1987): A study of the prevalence of bovine mastitis in different cooperative dairy farms in chilalo awraja, Arsi region. D.V.M. theses. Faculty of Veterinary Medicine, Addis Ababa University, Ethiopia.

Tambekar D., Dhanorkar H., Gulhane D. V., Khandelval S. R. and Dudhane M. N. (2006): Antibacterial Susceptibility of some Urinary Tract Pathogens to commonly used antibiotics. *African J. Biotechnology*. **5**: 1562-1565.

Tamirat T. A. (2007): Comparison of clinical trials of bovine mastitis with the use of honey, MSc. thesis, Addis Ababa University, Ethiopia, p_p 14-30.

Thien F. M. and O' toole G.A. (2001): Mechanism of biofilm resistance to antimicrobial agents, Trends in *Microbiol*., **9**: 34-39.

Tolla T. (1996): Bovine Mastitis in Indigenous Zebu and Borena-Holstein Crosses in Southern Wollo. Thesis, Debre Zeit: Faculty of Veterinary Medicine, Addis Ababa University: Ethiopia, p_p 25-27.

Tsegaye A. (1988): Study on bovine mastitis in and around Holeta. Addis Ababa University, Faculty of Veterinary Medicine, Debere Zeit, Ethiopia, (Unpublished DVM thesis).

Vanderhaeghen W., Cerpentier T., Adriaensen C., Vicca J., Hermans K. and Butaye P. (2010): Methicillin-resistant *Staphylococcus aureus* (MRSA) ST398 associated with clinical and subclinical mastitis in Belgian cows. *Vet. Microbiol*., **144**: 166-171.

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

Verhoef J. (1997): In: The staphylococci in human disease. Crossly, K. B. and G. L. Churchill Livingstone, N. Y., p_p 213-232.

Weese J. S. (2010): Methicillin-Resistant *Staphylococcus aureus* in Animals. *ILAR J*., **53**: 233-244.

Workineh S., Bayleyegn M., Mekonnen H. and Potgieter L.N. (2002): Prevalence and Etiology of Mastitis in cows from two major Ethiopian Dairies. Kluwer Academic Publishers, Netherlands.

Wulf M.W., Markestein A., van der Linden F.T., Voss A., Klaassen C. and Verduin C. M. (2008): First outbreak of methicillin-resistant *Staphylococcus aureus* ST398 in a Dutch hospital, June 2007. Euro Surveill., **13**: 8051-8059.

Yohannes A. (2003): Clinical and subclinical mastitis in primiparous dairy heifers in Jordan. Msc thesis, Faculty of Agricultural Technology. Al- Salt University College, Al-Salt Jordan.

Zadoks R. N., Allore H. G., Barkema H. W., Sampimon O. C., Wellenberg G. J., Grohn Y. T. and Y. H. Schukken. (2001): Cow-and quarter-level risk factors for *Streptococcus uberis* and *Staphylococcus aureus* mastitis. *J. Dairy Sci*., **84**: 2649–2663.

Zerihun T. (1996): A study on bovine sub clinicalmastitis at Stela dairy farm. Thesis, Debre zeit: Faculty of Veterinary Medicine, Addis Ababa University: Ethiopia, p_p 25-27.

8. APPENDICES

Appendix I: Questionnaire format for mastitis incidence

1. Farm Identification

Animal code: _____ Farm name: _____

Owner's name: _____

Address: _____

2. Dairy Cattle Status

2.1. Age: less than 4 year ☐ 4 – 7 years ☐ 7 – 10 years ☐ other ☐

2.2. Lactation: 1st ☐ 2nd ☐ 3rd ☐ 4th ☐ 5th ☐ other ☐

2.3. Parity: Few ☐ Moderate ☐ Many ☐

2.4. Breed: local ☐ Cross ☐ Exotic ☐

2.5. CMT score: HR ☐ HL ☐ FR ☐ FL ☐

3. Farm Description

3.1. Owner/manager educations status

Illiterate ☐ Read and write ☐ Elementary school ☐

High school graduate ☐ Professional ☐

If professional: Related to animal production ☐ Unrelated to animal productio ☐

3.2. Herd size: dairy cattle ☐

4. Management Data

4.1. Dairy cattle care takers (attendants): Owner (family member) ☐ Hired help ☐

4.1.1. Experience of dairy cattle care takers: Less than 5years ☐ Greater 5 years ☐

4.1.2. Education of dairy cattle care taker: Elementary school ☐ High School ☐

College graduate ☐ Professional ☐

4. Housing

4.1. Housing: Separate pen ☐ Together with cows in the same barn ☐ other ☐

4.2. How often the bedding is cleaned : > once a week ☐

Once a week ☐ < Once a Week ☐

4.3. Types of floor: Concrete ☐ Soil ☐ Other ☐

5. Antibiotic Use

Treatment recorded: Yes ☐ No ☐

6. Feeding Type and Milking Methods

6.1. Feeding type: CON. ☐ ORG. ☐

6.2. Milking methods: Machine ☐ Hand ☐

6.3. Udder hygiene: Washing/drying ☐ washing only ☐ Not at all ☐

7. Experience on dairy cattle health problem, prevention and control of the problems

7.1. Previous history of mastitis: Yes ☐ No ☐

7.2. Tick infestation: Yes ☐ No ☐

7.3. Major health problems for the farm_____

7.4. Measures taken to prevent disease problem_____

Appendix II: Flow chart for isolating *S. aureus* form CMT+ milk sample

1. CMT test on the fresh milk sample

2. Collection of milk sample from CMT+ quarter

3. Sample inoculation on blood agar base enriched with sheep blood

   - Incubation at 37 ^{o}c for 24-48 hour
   - Colony morphology observation

4. Gram staining

5. Subculture on nutrient agar and incubate for 24 hour

6. Primary identification test

   - Catalase test
   - O-F test
   - Coagulase test

7. Secondary identification tests

   - Growth on manitol salt agar and purple agar base
   - Biofilm production and drug sensitivity test

Appendix III: CMT and Format used for recording the results

California mastitis test

1. Put ½ teaspoon of CMT Solution in paddle cup, with equal amount of fresh milk. Always use as much CMT solution as milk.

2. Brief rotary motion of paddle brings out the reaction.

3. Degree of thickening shows reduction in lactose, casein and rennet coagulation properties, and increase in corpuscular protein, chlorides, catalase and spontaneous free fatty acids.

When using bulk milk, slight to medium thickening indicates that chronic mastitis is present in the herd.

Test should not be used on colostrums milk. Foremilk is preferable to stripping. Discard the first stream or two of milk.

Table for Data collection format

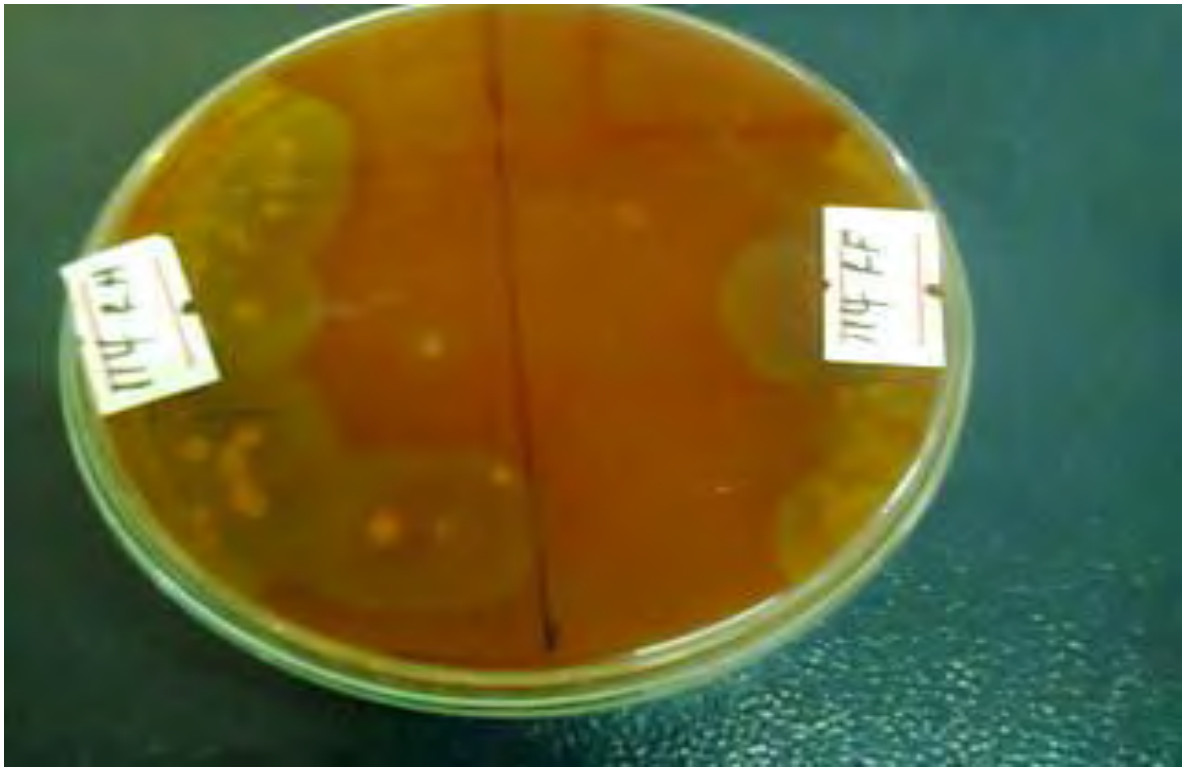
Q	FR		FL		HR		HL	
M.T	cli.	Subcli.	Cli.	Subcli.	Cli.	Subcli.	Cli.	Subcli.

Table for CMT result and interpretation

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

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

Appendix IV: Primary and secondary identification test

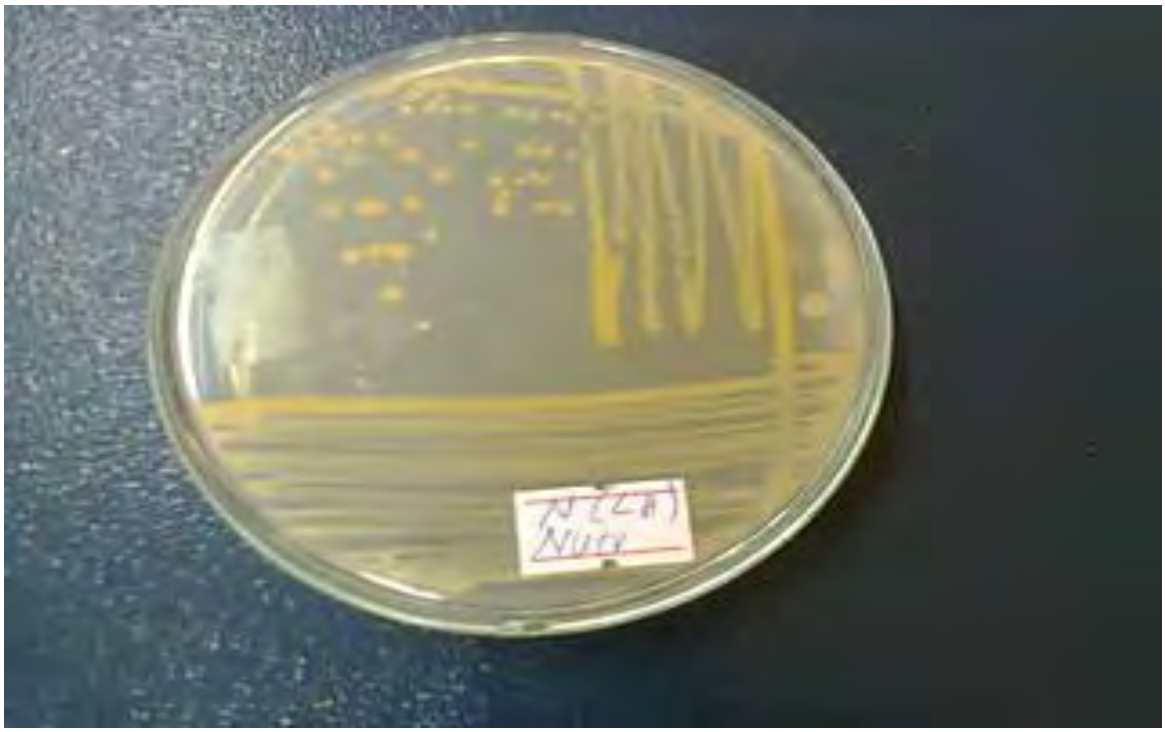


Colony growth on blood agar base and hemolysis formation

Gram staining

Procedure:

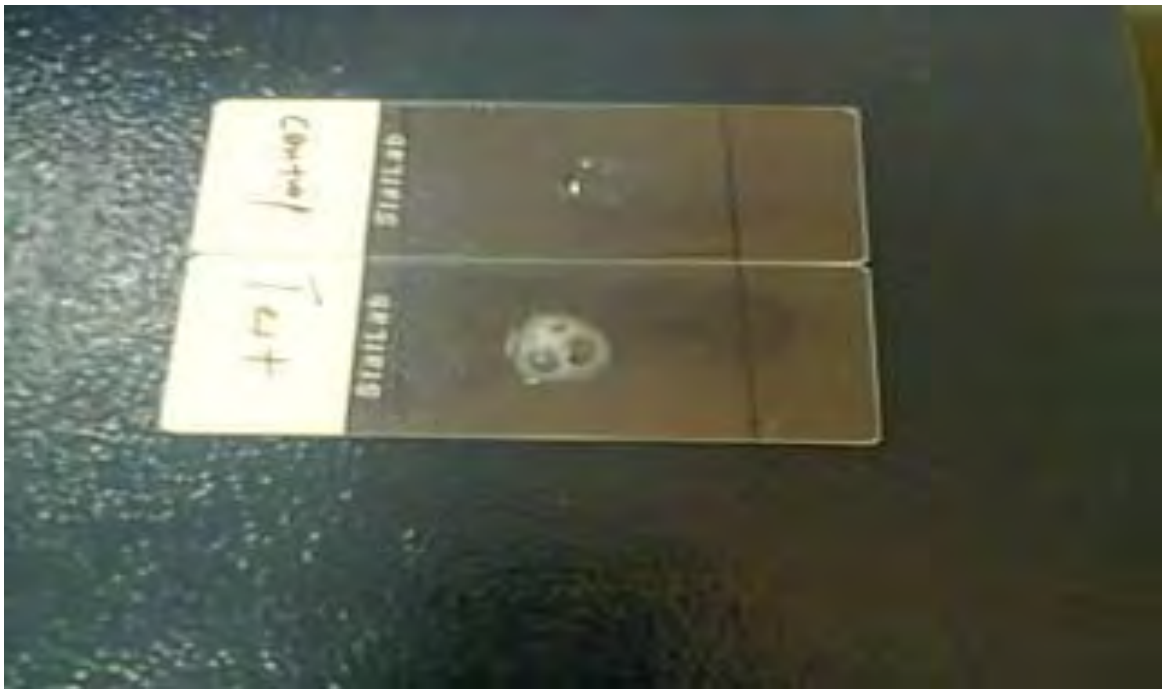
- Applying a primary stain (crystal violet) for 60 second to a heat-fixed smear of a bacterial culture. Then wash off with tap water.
- Addition of iodide which remain for 60 second. Then wash off with tape water.
- Rapid decolorization with ethanol or acetone for only 15-30 second. Then wash off with tape water.
- Counterstaining with safranin for 60 seconds. Then wash off with tap water and dried with bloating paper.



Colony growth on nutrient agar

Nutrient agar

Prenciple**:** The colony that were suspected as *S.aurues* by colony morphology and gram stianing streaked on nutrient agar for further identification tests.



Positive result for catalase test

Catalase test (Quinn *et al.,* 2002).

Principle**:** the breakdown of hydrogene pereoxide in to oxygen and water is medieted by the enzyme catalase.

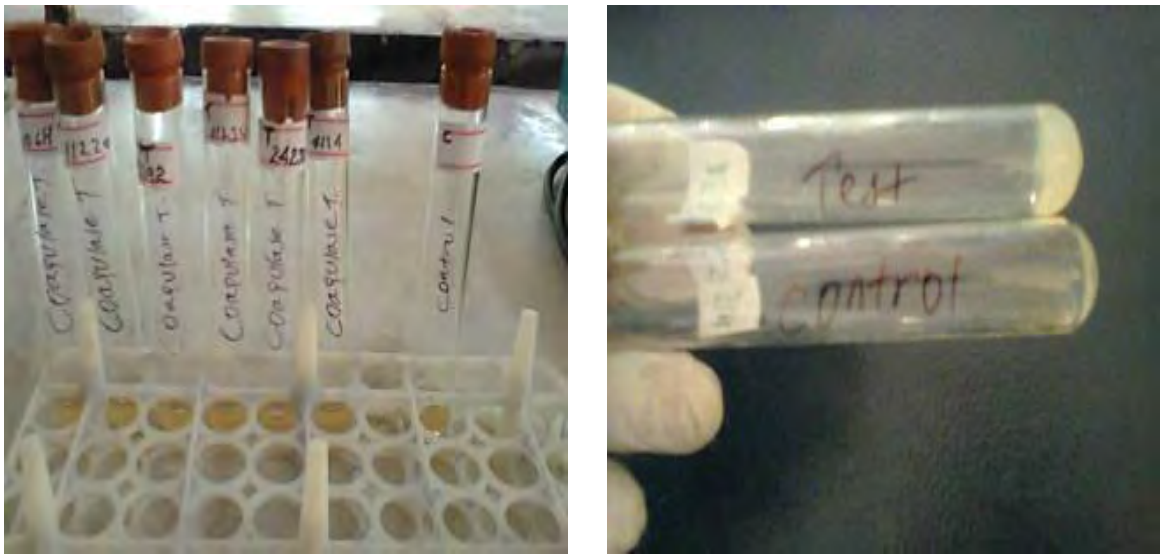
Procedure**:** a loop of bacterial growth is taken from nutrient agar medium.then the bacterial cell placed on a clean microscopic slide and a drop of 3% hydrogen peroxide is added. An effectiveness of oxygen gas, with in a few seconds, indicates a positive reaction.

O-F test

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

Principle**:** This test is used to determine the oxidative or fermentative metabolism of a carbohydrate by the bacterium. The medium is semi-solid and usually contains glucose as the test agar and bromothymol blue as P^H indicator.

Procedure**:** Two tubes of the O-F medium were heated in a beaker of boiling water immediately before use. Each tube of sample was inoculated with unknown organism using a straight needle and stabbing the medium to the bottom of the tube. One tube of each pair was covered with 1cm layer of sterile mineral oil. The other tubes were slightly closed and both tubes were incubated at 37^0C and the result was recorded after 24 hours incubation.

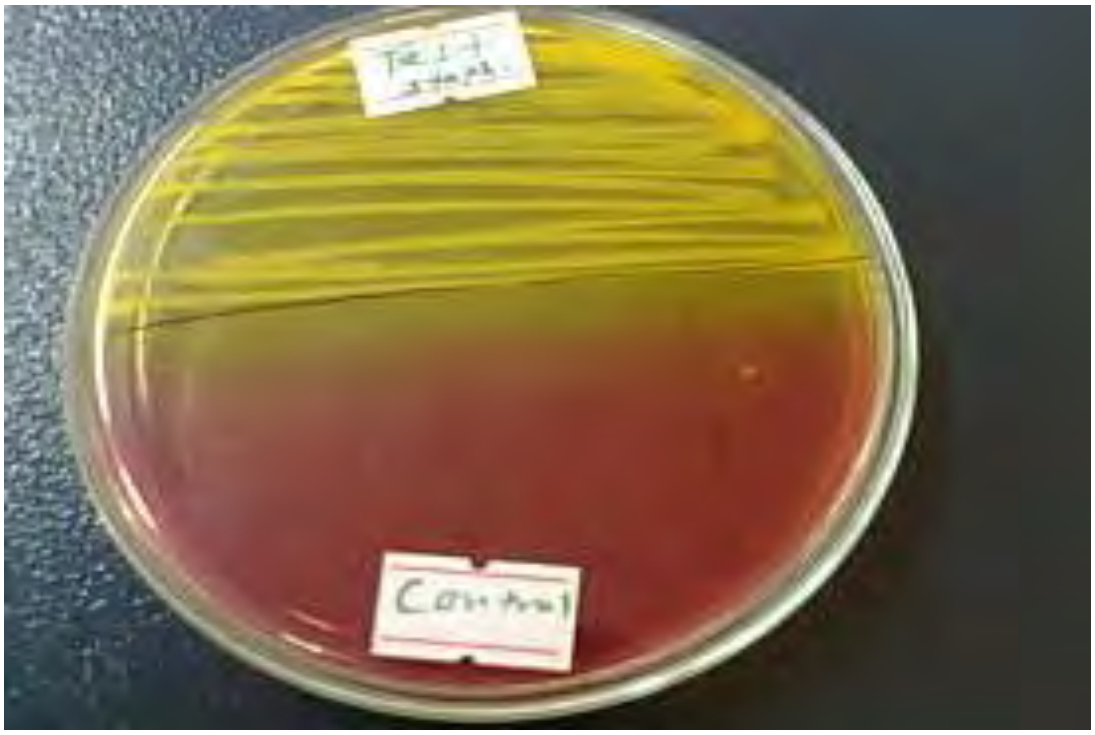


Coagulase test

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

Principle**:** The free coagulase secreted by *S. aureus* reacts with coagulase reacting factor (CRF) in plasma to form a complex, which is thrombin. This converts fibrinogen to fibrin resulting in clotting of plasma.

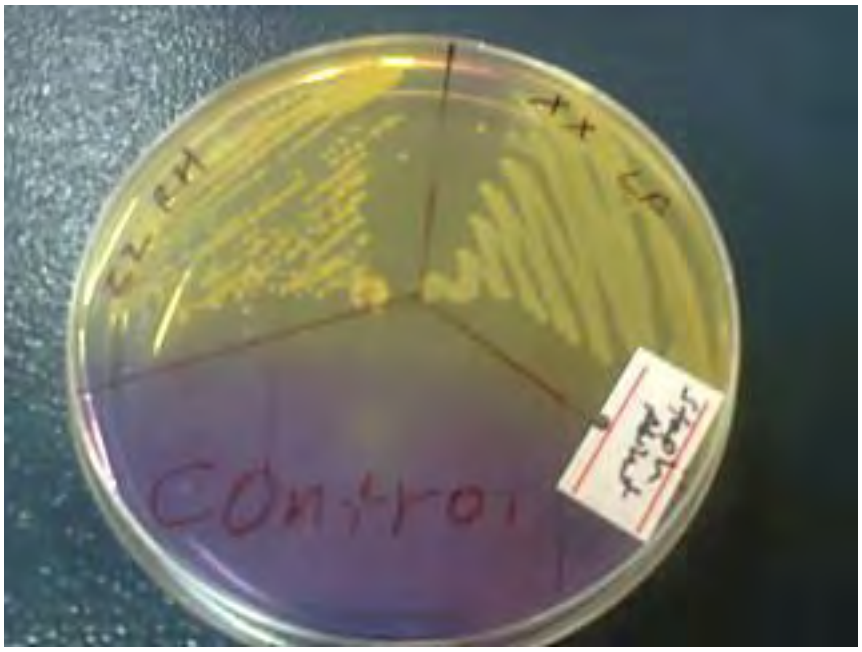
Procedure**:** 0.5 ml of oxalated or citrated rabbit plasma were added to a tube and mixed in colonies from a plate or add 0.5 ml of broth culture to the tube. The tube was covered to prevent evaporation and incubated at 37^{o}C. The tests were read by slowly tilting the tube. A positive test results in a highly viscous clot formation in the plasma. Once a coagulum, no matter how small, has formed the test is considered positive (usually within 4 hours). A negative test results in the plasma remaining free flowing with no evidence of a clot, were incubated overnight before a test is called negative, but prolonged incubation (over 24 hours) may result in the dissolution of a formed clot.



Colony growth on manitol salt agar

Manitol salt agar

Procedure**:** All the colonies that were collected through the necessory identification tests(catalase, O-F and coagulase test) were streaked on manitol salt agar which is selective media for members of Staphylococci and the bacterium were incubated at 37^{o}c for about 24 hr. A positive result showing growth and a clear media change from red to yellow.



Colony growth on Purple agar base

Purple agar base test

Principle**:** Purple agar base contains maltose as a substrate and bromocresol purple indicator. Used to identify bacterium that can ferment maltose.

Procedure**:** Purple agar base was pepared and the bacterium was inoculated and placed in the incubator at 37^{o}c for 24 hour. A yellow color (acid) is a positive reaction for fermentation of the carbohydrate incorporated into the medium. Bubbles in the inverted fermentation vials are an indication of gas production.

Table for Summary on mian difference and characterestics of gram positive cocci.

Species	catalase	O-F test	Coagulase	Manitol salt agar	Purple agar base
S. aureus	+	F	+	+(Yellow colony)	+(yellow colony)
Staphylococci (Non pathogenic)	+	F	_	+(white colony)	_
Micrococci	+	O	_	_	_
Streptococci	_	F	_	_	_
Entrococci	_	F	_	_	_

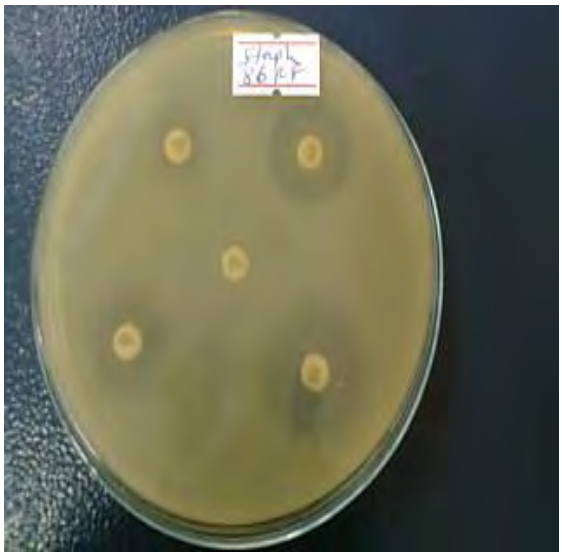
(+) = positive: (-)= negative: (F)= fermentative: (O)= oxidative

Red colonies on Modified Congo Red Agar

Modified congo red agar test (Freeman *et al.,* 1989).

Prenciple**:** Modified Congo Red Agar contains Congo red dye as indicator for black colony production if the bacterium is a biofilm producer.

Procedure**:** The pure isolated *S. aurues* inoculuted on a Modifeied Congo Red Agar which is composed of blood agar base, glucose and Congo red dye. Positive biofilm production represented by black colonies with dry consistency, while none biofilm producer isolate remain and stained as red colonies.



Staphylococcus aureus showing resistant to cefoxitin (centre disc) and other antibiotics

S. aureus susceptible to cefoxitin (center disc)

Appendix V: Antibiotic sensetivity test

Inoculums Preparation

At least three to five well-isolated colonies of the same morphological type were selected from an agar plate culture. The top of each colony was touched with a loop, and the growths were transferred into a tube containing 4 to 5 ml of a suitable broth medium, such as tryptic soy broth. The broth culture was incubated at 35-37°C until it achieves or exceeds the turbidity of the 0.5 McFarland standards (usually 2 to 6 hours). The turbidity of the actively growing broth culture was adjusted with sterile saline or broth to obtain turbidity optically comparable to that of the 0.5 McFarland standards. This standard is prepared by adding 0.5 ml of 1% (11.75g/liter) $Bacl_2$ $2H_2O$ to 99.5 ml of 1% (0.36N) H_2SO_4.

Inoculation of Test Plates

Optimally, within 15 minutes after adjusting the turbidity of the inoculums suspension, a sterile cotton swab was dipped into the adjusted suspension. The swab were rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This will remove excess inoculums from the swab. The dried surface of a Mueller-Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure is repeated by streaking two more times, rotating the plate approximately 60° each time to

ensure an even distribution of inoculums. As a final step, the rim is left 3 to 5 minutes, but no more than 15 minutes, to allow for any excess surface moisture to be absorbed before applying the drug impregnated disks.

Application of Discs to Inoculated Agar Plates

The predetermined battery of antimicrobial discs was dispensed onto the surface of the inoculated agar plate. Each disc was pressed down to ensure complete contact with the agar surface. Whether the discs are placed individually or with a dispensing apparatus, they must be distributed evenly so that they are no closer than 24 mm from center to center. Ordinarily, no more than 12 discs should be placed on one 150 mm plate or more than 5 discs on a 100 mm plate. Because some of the drug diffuses almost instantaneously, a disc should not be relocated once it has come into contact with the agar surface. Instead, place a new disc in another location on the agar. The plates were inverted and placed in an incubator set to 35°C within 15 minutes after the discs are applied.

Reading Plates and Interpreting Results

After 16 to 18 hours of incubation, each plate was examined. Zones were measured to the nearest whole millimeter, using sliding calipers or a ruler, which was held on the back of the inverted petri plate. The petri plate were held a few inches above a black, nonreflecting background and illuminated with reflected light. 24 hours of incubation are required for vancomycin and oxacillin, but other agents can be read at 16 to 18 hours.

Table for zone of inhibition interpretation chart for antimicrobials (NCCLS, 1997).

		In millimeter		
Antimicrobial agents	Disc potency	Resistance	Intermediate	Susceptible
Streptomycin	10µg	≤11	12-14	≥15
Compound sulphonamides	300 µg	≤12	13-16	≥17
Oxytetracycline	30 µg	≤19	_	≥28
Ceftriaxone	30 µg	≤13	14-20	≥21
Cefoxitin	30 µg	≤21	_	≥22
Kanamycin	30 µg	≤13	14 -17	≥18
Amoxacilin	30 µg	≤19	_	≥20
Nalidixic acid	30 µg	≤10	11-12	≥13

Appendix VI: Medias used for bacterial identification

1. Blood agar base (oxoid,England)

Composition(g/l): hear muscle, infustion from(solid)2.0; pancreatic digest of casein13; yeast extract 5.0; sodium chloride 5.0; agar 15.0.

Direction. Sespend 40g of powder in 1 liter of distled water. Mix throughly and heat with frequent agitation and boil for 1 minute to completely disolve the powder. Autoclave at 121 ^{o}c for 15 minute. Cool the base to 45-50 ^{o}c and 5-7 % sertile defiberinated sheep blood.

2. Grams reagent
   - Crystal violet
   - Iodine (mordant)
   - Ethanol 95%
   - Counter-stain (sufranin)

3. Nutrient agar (oxoid, England)

Composition(g/l): lab-lemco powder 1.0; yeast extract 2.0; pepton 5.0; sodium chloride 5.0; Agar 15; p^{H}: 7.4 ± 0.2.

Direction: suspend 28 g in 1 liter of disteled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121 ^{0}c for 15 minute.

4. O-F basal medium (himedium, india)

Composition(g/l): sodium chloride 5.0; casein enzymatic hydrolysate 2g; Agar 2g; dipotassium phosphate 0.3g; bromothymol blue 0.08g.

Direction: dissolve 9.4g in 1000ml disteled water. Gently heat to dissolve the medium completely. Sterilize by autoclaving at 121 ^{0}c for 15 minute. Add 10% sterile glucose solution. Mix and dispense in 5ml amounts in sterile tube in duplicate for aerobic and anearobic fermentation solution.

5. Mannitole salt agar(oxoid, England)

Composition (g/l): lab-lemco poweder 1.0; peptone 10.0; mannitol 10.0: sodium chloride 75.0; phenol red 0.025; agar 15.0 pH: 7.5± 0.

Direction: suspend 111 g in 1 liter of disteled water. Bring to the boil to disolve completely sterilze by autoclaving at 121^0c for 15 minute.

6. Purple agar base

Composition (g/l): Peptone, special 10.000; Beef extract 1.000;Sodium chloride 5.000; Bromo cresol purple 0.020; Agar 15.000 ; Final pH (at 25°C) 6.8±0.2.

Direction: Suspend 31.02 grams in 1000 ml distilled water. Add 5 - 10 grams of the carbohydrate to be tested. Heat to boiling to dissolve the medium completely. Dispense in tubes as desired and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Alternatively sterilize the basal medium prepared using 900 ml distilled water and add 100 ml separately sterilized 5 - 10% solution of the desired carbohydrate to it.

7. Modified Congo Red Agar

Composition (g/l): blood agar base (hear muscle, infustion from(solid) 2.0; pancreatic digest of casein13.0; yeast extract 5.0; sodium chloride 5.0; agar 15.0) 40.00; glucose 5-10%; Congo red dye 0.8.

Direction: suspend 40 grams blood agar base in 1000 ml distled water.Add 5-10 grams of glucose to be heated. Bring to the boil to disolve completely sterilze by autoclaving at 121^0c for 15 minute. Gently add Congo red dye (0.8%) to sterile media before dispensing.

8. Muller hinton Agar (oxoid, England)

Composition (g/l): beef extracts 2; acid hydrolysate of casein 17.5;starch 1.5; agar 17.

Direction: suspend 38g of the powder in 1 liter of distled water. Mix throughly, heat with frequent agitation and boil for 1 minute to completely dissolve the powder autoclave at 121^0c for 15 minute.