PREVALENCE AND RISK FACTORS OF SUB CLINICAL MASTITIS AND COAGULASE NEGATIVE INTRAMAMMARY INFECTION IN JIMMA DAIRY HERDS

M.Sc. Thesis

BY

Lidya Shafi

June, 2012

Jimma University

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M.Sc. Thesis

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By

Lidya Shafi

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School of Graduate Studies

As research advisor, I hereby certify that I have read and evaluated this thesis prepared under my guidance, by **Lidya Shafi** , entitled Prevalence and Risk Factors of Subclinical Mastitis and Coagulase Negative Intramammary Infection in Jimma Dairy Herds .I recommend that it be submitted as fulfilling thesis requirements

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As member of *Board of Examiners of M.Sc Thesis Open Defense Examination*, we certify that we have read, evaluated the thesis paper prepared by **Lidya Shafi** and examined the candidate. We recommended that the thesis could be accepted as fulfilling the thesis requirement for the degree of Master of Science in veterinary Epidemiology.

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External examiner	Signature

DEDICATION

I would like to dedicate this work to my beloved families, especially to my father Ato Shafi Bushura and my mother W/ro Bekelech A/Mesekel.

STATEMENT OF THE AUTHOR

I declare that this thesis is my original work and the thesis is not submitted to any University for the awards of any academic degree or diploma. This thesis is submitted in partial fulfillment of the requirements for an Msc degree in veterinary Epidemiology at Jimma University College of Agriculture and School of Veterinary Medicine and this is deposited at the university library to be made available to borrowers under rules of the library. Permission must be obtained from the Author and advisors.

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BIOGRAPHICAL SKETCH

Lidya Shafi, the author, was born in Oromia regional state in Jimma town in 1985 G.C. She attends her elementary school in Kitto elementary school from 1-6, and from 7-8 in Jimma Setto - Semero primary and comprehensive secondary school and she studied from 9-12 in Jimma high school after she completed high school studies ,she joined Addis Ababa University Faculty of Veterinary Medicine in 2003 and graduated in Doctor of Veterinary Medicine in 2009. After graduation she joined Agricultural Offices in Jimma zone, Sigimo woreda. Then she started graduate studies in Jimma University College of Agriculture and Veterinary Medicine, School of Veterinary Medicine in Veterinary Epidemiology.

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ABBREVIATIONS

Blood Agar	BA
California Mastitis Test	CMT
Coagulase Negative Staphylococci	CNS
Central Statistic Agency	CSA
Deoxyribonucleic Acid	DNAse
European Union	EU
Food and Agricultural Organization	FAO
Gross Domestic Product	GDP
Intra Mammary Infection	IMI
Modified White Side Test	MWT
National Committee for Clinical Laboratory Standards	NCCLS
National Mastitis Council	NMC
National Metrological Service Agency	NMSA
Poly Morpho Nuclear cells	PMN
Somatic Cell Count	SCC
Sub Clinical Mastitis	SCM
United States	US
White Blood Cells	WBC

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ABSTRACT

A cross sectional study was conducted in Jimma dairy herds from June 2011 to December 2012 with the objectives of determining the prevalence and associated risk factors of sub clinical mastitis as well as isolating, identifying and determining the antibiotic sensitivities of Coagulase negative staphylococci infections of the mammary glands. Mastitis is a serious problem which affects the dairy cows all over the world and there are different microorganisms which are responsible for mastitis infection in dairy farms. California mastitis test was used to detect the presence of sub clinical mastitis. Identification of coagulase negative staphylococci species was carried out based on catalase reaction, Gram staining, reaction on DNAse media and coagulase test. Antimicrobial susceptibility test was performed using McFarland standards of disk diffusion method on Mueller Hinton Agar. A total of 264 lactating cows from 48 herds and 1056 quarters were used. The overall prevalence of bovine mastitis during the study period was 75.75%. Of this prevalence of sub clinical mastitis was 62.1% and clinical mastitis was 13.6% on quarter level in Jimma dairy farms. From total of 264 lactating cows, 72.7% of them were positive to subclinical mastitis at cow level and 95.8% on herd level. The prevalence of coagulase negative staphylococci was 23.1% , 22.72% and 72.9% on cow, quarter and herd level, respectively. No previous history of clinical mastitis and frequency of body washing of dairy cows have a significant association with the prevalence of sub clinical mastitis ($p<0.05$). Late stages of lactation have significant association with the prevalence of coagulase negative staphylococci infections ($p<0.05$). The prevalence of coagulase negative staphylococci were relatively higher on cows with teat injury and in primiparous ($p>0.05$). Antimicrobial sensitivity tests found that coagulase negative staphylococci is susceptible to all antimicrobials employed; Streptomycin (91.7%), Ampicillin (91.7%), Cefuroxime (91.4%), Tylosin (89.9%), Amoxicillin (91.7%), Trimetroprim (100%) and Tetracycline (80.6%).

Key words: *Prevalence, Sub clinical mastitis, Coagulase Negative Staphylococci, risk factor, Antimicrobial Susceptibility Test, Jimma dairy cows.*

1. INTRODUCTION

Ethiopia is a country with vast and diversified livestock resources. The livestock holding of the country comprises of 50.8 million heads of cattle, 25.9 million heads of sheep, 21.9 million heads of goats,42 million chickens, horses 1995,306, donkey 5,715,129, mules 365,584, camels 807,581 and bee hives 4,598,226 (CSA, 2009). A 9.6 million of the 50.8 national cattle holding in Ethiopia represents milk producing cows indicating huge potential for lucrative dairy production (FAO, 2005; CSA, 2009). However, widespread prevalence of diverse animal health problems affecting milk production is a serious concern for the dairy sector in Ethiopia.

Mastitis, inflammation of the mammary glands, is the single most important cause of losses in milk production (Halasa *et al.,* 2007). More than 130 species of bacteria have been associated to bovine mastitis, but *Staphylococcus aureus, Streptococci,* and members of the *Enterobacteriacae* represent the most common etiological agents for mastitis in cattle (Smith and Hogan, 2001).

Subclinical forms of mastitis is a frustrating, costly and extremely complex disease that results in a marked reduction in the quality and quantity of milk throughout the world (Harmon, 1994). Cows with sub clinical mastitis do not show visible changes in the appearance of the milk. However, milk production decreases by 10 to 20%, with undesirable effect on its constituents and nutritional value rendering it of low quality and unfit for processing (Holdway, 1992).

Among the different Staphylococci species that causes bovine mastitis, Coagulase Negative Staphylococci (CNS) are the pathogens most frequently isolated from mastitic milk samples, especially samples from first lactation cows and heifers (Harmon and Langlois 1995). Coagulase negative Staphylococci were long considered as minor pathogens isolated from both clinical and sub clinical intramammary infections. However, studies indicated that Coagulase negative Staphylococci are important mammary gland pathogens

and almost all Coagulase negative Staphylococci species have higher prevalence of ß-lactamase production as compared to *Staphylococcus aureus* (Devriese and Keyser, 1980; Barkema *et al*., 1998; De Vliegher *et al*., 2003; Bradley *et al*., 2007; Sampimon *et al*., 2007).

Coagulase negative staphylococci is a heterogeneous group of bacteria consisting of a number of different species having different virulence factors. The organisms mostly cause mild infections and sub clinical mastitis in many countries (Bradley *et al.,* 2007; Koivula *et al.,* 2007; Piepers *et al.,* 2007; Sampimon *et al.,* 2009). A higher prevalence of clinical mastitis has also been reported in some countries (Tenhagen *et al.,* 2006; Koivula *et al.,* 2007; Olde Riekerink *et al.,* 2008). Cows and heifers are infected with Coagulase negative staphylococci at the time of calving and the prevalence of infection caused by coagulase Negative Staphylococci is higher in heifers (Piepers *et al.,* 2010) and cows in their first lactation (Pamela, 2001). Coagulase negative staphylococci accounted for 67 % of total mammary tissue infections and infection persists to the end of lactation in 85% cases leading to a 6-7 % total milk loss in dairy farms (Timms and Schultz, 1987). The loss is attributable to their effect in increasing somatic cell count in milk which downgrades both the quantity and quality of milk production. The latter effect leads to further economic loss by forcing the culling of a dairy cow at unacceptable age (Fetrow, 2000).

It has been suggested that Coagulase negative staphylococci should be considered as emerging mastitis pathogens that need attention in dairy herds (Davidson *et al.*, 1992; Pyorala and Taponen, 2009; Paradise *et al.*, 2010). There are different reports regarding; prevalence of sub clinical mastitis and Coagulase negative staphylococci at the quarter and cow level and their impact on the quality and quantity of milk as well as their drug resistance patterns from different parts of the world. Reports are also abundant concerning the prevalence of staphylococcal mastitis and its drug sensitivity patterns. However, none of these previous works specifically address the issue of coagulase negative staphylococci. Researches concerning the prevalence of sub clinical mastitis, coagulase negative staphylococci, risk factors associated to Coagulase negative staphylococci infection and its antibiotics susceptibility pattern are also very limited in Jimma. Such knowledge would be

indispensable to the work of developing effective management practices to prevent and control bovine mastitis caused by coagulase negative staphylococci in the study area. There for the objectives of this study were:

- To isolate and to determine the prevalence of sub clinical mastitis using CMT and associated risk factors in selected dairy herds in Jimma.
- To isolate and to determine the prevalence of sub clinical mastitis caused by coagulase negative staphylococci from herds which are positive in CMT in selected dairy herds Jimma.
- To identify risk factors associated with coagulase negative staphylococci intramammary infection in the area.
- To test the susceptibility of identified coagulase negative staphylococci to different antibiotics agents and to recommend the best antibiotics agents.

2. LITERATURE REVIEW

2.1. Sub Clinical Mastitis

Sub clinical mastitis is the most serious type of mastitis. Infected cows do not show obvious symptoms and secrets apparently normal milk for a long time, at this time the organisms spread infection in a herd by different method (Harmon, 1994). Economical losses are due to loss in milk production, discarding of milk due to drug residue, degrading of milk quality, costs of drugs, veterinary services and, increased risk of subsequent mastitis, herd replacement, and problems related to antibiotics residues in milk and milk products (Harmon, 1994 and Barmely *et al.,* 1996).

2.1.1. Etiology

There are different organisms that are responsible for mastitis infections. Commonly mastitis begins as a result of penetration of the teat duct by pathogenic bacteria due to physical or chemical injuries. Among the bacterial pathogens S*taphylococcus aureus*, *Streptococcus agalactia*, Streptococcus species other than S*treptococcus agalactia*, coagulase negative Staphylococci species, *Escherichia coli*, Micrococcus species, *Cornybacterium* species, *Bacillus* species, *Pasteurella* species, *Klebsiella* species, Mycoplasma species and Nocardia species (Aaretrup *et al.*, 1995; Hussien *et al*., 1999; Wilson *et al*.,1999) . Also some viral pathogens plays an important role on predisposing a cow for mastitis for instance Pseudo cowpox, Herpes Mamillitis, Cowpox, Papilloma, Foot and Mouth disease and Vesicular Stomatitis affecting the epithelium of the teat orifice are mentioned to result in or predispose to mastitis (Hillerton *et al*., 2001). In addition of bacteria and virus pathogens there are about thirty species of yeast that can cause mastitis those are *cryptococuse neaoformans, Baciluscerus* and *Candidia species* and also some of them have zoonotic importance like *cryptococus neoformans, Norcar.*

2.1.2 Epidemiology of sub clinical mastitis

Sub clinical mastitis is serious and economically devastitating type of mastitis which affects the quality and quantity of milk (Ramachandrainh *et al.,* 1990). There are several reports regarding the prevalence of sub clinical mastitis and associated risk factors in Ethiopia. However, it still remains a major problem on many dairy farms in Ethiopia by causing culling of dairy cows at early stages. According to Kassa *et al* (1999) who were carried out a survey of mastitis in dairy herds in Ethiopian central high lands. Out of 10, 724 quarters examined from 2,681 cows, they found the prevalence of clinical mastitis, non-functional or blocked quarters and sub clinical mastitis were 1.2%, 3.8% and 38.9% on cow basis, respectively. The report by Hussein *et al.* (1999) on the prevalence of sub clinical mastitis was found to be 19% on cow basis and 7.4 % on quarter basis, shows in the central regions of Ethiopia. A study that was conducted at Repi and Debre-Zeit dairy farms, out of 186 lactating cows 38% were with sub clinically infected (Workineh *et al.*, 2002). Bishi (1998) reported mastitis prevalence rates of was 34.3% and 5.3% at cow level in Addis Ababa region, for sub clinical and clinical mastitis, respectively. In the same study area, Mungube (2001) reported an overall prevalence of 46.6% for sub clinical mastitis at cow level and 27.8% at quarter level. This great variation could result from differences in environment and management (Kerro and Tareke, 2003). Among the different risk factors stages of lactation are the common risk factor in the occurrences of coagulase negative staphylococci infections. The prevalence of coagulase negative staphylococci is higher during early lactation and it ranges from 5% to 6% and increased from 14% to 17% towards the end of lactation (Davidson *et al.,* 1992). Different researchers declared that an intramammary infections caused by coagulase negative staphylococci are important pathogens in cattle of all ages but the predominant coagulase negative staphylococci species causing infection is differ between age groups. *S. chromogenes* are the major coagulase negative staphylococci species which affects pre calving heifers and primiparous cows (Trinidad *et al.,* 1990; Schultz *et al.,* 2007; Taponen *et al.,* 2006). However *S. simulans* are frequently isolated from cows in later lactations (Taponen *et al.,* 2006). Cows

with multiparious generally become infected with coagulase negative staphylococci during later stages of lactation whereas primiparous cows usually already have the infection at the beginning of lactation (Grohn *et al.,* 2004; Taponen *et al.,* 2007). The prevalence of CNS mastitis is higher in primiparous cows than in multiparious cows (Matthews *et al.,* 1992; Poelarends *et al.,* 2001; Tenhagen *et al.,* 2006). Also coagulase negative staphylococci can colonize the mammary gland of pregnant heifers and CNS were isolated from the mammary gland and teat apices of heifers as young as 10 months old (Boddie *et al.,* 1987; White *et al.,* 1989; Myllys, 1995; De Vliegher *et al.,* 2003). Reports by (Waage *et al.,* 2000 ; Sol *et al.,* 2000) indicate that coagulase negative staphylococci intramammary infection is higher in high milk producing cows as compared with low milk producing cows.

2.1.3. Pathogenesis of sub clinical mastitis caused by coagulase negative staphylococci

The pathogenesis of mastitis refers to the process of infection where in an organism invades the udder and migrate up the teat canal and colonize the secretary cells. Then, colonizing organisms produce toxic substances which are harmful to the milk producing cell (Mac Donald, 1997). The main reservoirs of mastitis infection in herds are infected quarters and the skin of the udder and teat the presence of lesions on teat skin allows persistent colonization of the skin by mastitis causing pathogens. After the entrance of bacteria through teat orifice the bacteria can persist and multiply and colonize of the mammary gland. This is a preliminary step for the mammary gland to be infected and it seems that the closer the bacteria colonizing the teat canal the higher is the risk of mammary infection (Sutra and Poutrel, 1994). Commonly, the infection begins with penetration of the teat duct by pathogenic bacteria during milking time. The organisms present in the milk or at the teat end will enter to the teat canal, after the bacteria enters to cisternal area invasion of the teat will occur (MacDonald, 1997). After milking the teat canal remains open for 1-2 hours while the canal of a damaged teat may remain partially open permanently (Sutra and Poutrel, 1994). This makes easier for organisms from the environment or those found on injured skin to enter to the teat canal then the bacteria eventually enter to glandular tissues finally damage, toxin production and death will occur

by bacteria to milk secreting epithelial cells (MacDonald, 1997). The development of mastitis explained in terms of three stages: invasion, infection and inflammation. Invasion is the stage at which pathogens move from the teat end to the inside the teat canal (Sutra and Poutrel, 1994). Infection is the stage in which the pathogens rapidly multiplies and invades the mammary tissue then inflammation follows infection and represents the stage at which clinical mastitis occurs with varying degrees of clinical changes of the udder, then different systemic effects from mild to per acute, gross lesions and subclinical abnormalities in the milk appear (Sutra and Poutrel, 1994). Polymorpho nuclear leukocytes play a central role in the pathogenesis of bovine mastitis intramammary challenge with the pathogens (MacDonald, 1997).

2.1.4. Clinical signs and gross lesions of coagulase negative staphylococci

Commonly coagulase negative staphylococci bacteria regarded as minor pathogens that mostly affects dairy cows around calving (Piepers *et al.,* 2007). Mostly, they do not cause clinical signs and cause increases in the somatic cell count comparable to other mastitis causing pathogens, and disappear soon after parturition (Piepers *et al.,* 2007). It is generally held that in coagulase negative staphylococci mastitis has only mild local signs like slight swelling and changes in the milk appearance; however studies that have investigated thoroughly clinical characteristics of mastitis caused by coagulase negative staphylococci are scant (Simojoki *et al.,* 2007). According to Jarp (1991) report the reports that clinical signs of coagulase negative staphylococci mastitis most often were sub clinical or mild clinical, although severe clinical signs occasionally were recorded. The concentrations of different inflammation parameters in milk were 10 to 100 times lower than in an experimentally induced *Escherichia coli* (*E. coli*) mastitis, and the clinical signs were very mild (Simojoki *et al.,* 2007). Out of 72% of clinical cases the only clinical signs detected was changes in the milk appearance, like clots and flakes. According to the results obtained from the three studies on histopatologic changes of mammary tissue that was caused by staphylococci bacteria coagulase negative staphylococci infection causes a similar type inflammation but possibly less serious damage in the mammary gland than *staphylococcus aures* infection. Boddie *et al.* (1987) reported that of udder is infected with *staphylococcus*

aures, and three quarters naturally infected with coagulase negative staphylococci. The quarters infected with *Staphylococcus aureus* and coagulase negative staphylococci showed less alveolar, epithelial and luminal areas, more inter alveolar stroma and greater leukocyte infiltration compared with the uninfected quarters (Simojoki *et al.*, 2007).

2.2. Diagnosis

The identification of sub clinical mastitis and different species of coagulase negative staphylococci is important to determine their pathogenicity and to develop specific management control practices to prevent bovine mastitis infections. The invisible changes in sub clinical mastitis is recognized indirectly by several diagnostic methods using California mastitis test (CMT), Modified white side test (MWT), SCC (Lesile *et al.*, 2002).

2.2.1. Tentative diagnosis of sub clinical mastitis

Based on history obtained from the client and symptoms observed on cows used as a good tool for examination of sub clinical mastitis. The tentative diagnosis is done by using the California Mastitis Test then by measuring the amounts of somatic cells in the milk (Radostits, 1994)

2.2.2 Bacteriological diagnosis

Most of the bacterial pathogens that causes bovine mastitis grows on sheep blood agar and Maconkey agar and any gram negative bacteria can grow on the medium (Radostits, 1994). The identification of type of bacteria is essential for the treatment as well as for the control and prevention of bovine mastitis in dairy farms. There are different types of media that are used for the growth of bacteria both for fastidious and non fastidious pathogens (Radostits, 1994. The isolation of the coagulase negative staphylococci bacteria is performed by gram staining, catalase test, culturing on DNAse media and coagulase test (Quinn, 1994).

2.2.3 Serological diagnosis

Serological tests, unlike culture and microscopic investigations, provide indirect evidence of infection by detecting bacterial antigens, or antibodies produced in response to them. Such tests are now widely used in microbiology because of their high specificity and sensitivity (Radostits, 1994). Individual milk samples and pooled milk samples can be used for serology and it helps to detect herd prevalence infections by milk testing. Bulk milk serology is widely used for control and surveillance of diseases (Niskanen, 1993).

2.2.4 Molecular diagnostic techniques

Molecular epidemiological analysis enables the genotyping of strains and has uses in the tracing and control of disease outbreaks. Pathogenic strains of bacteria have been identified by typing methods as belonging to distinct clonal lineages that demonstrate a unique combination of virulence alleles or genes (Radostits, 1994).Typing may enable the detection of certain genotypes that are associated with more severe or unusual disease signs. The isolates, or particularly those genotypes associated with low virulence, may be useful in vaccine development. Recently developed molecular typing methods are capable of distinguishing whether genetic variation is due to recombination or point mutation; they may also have applications for fundamental research as they can enable the definition and further understanding of microbial population structures and dynamics (Radostits, 1994). Diagnosis of coagulase negative staphylococcus caused mastitis done by using biochemical test and molecular techniques such as genotyping by using amplified fragment length polymorphism and Phage typing are used for identification of coagulase negative staphylococci bacteria (Sampimon *et al.,* 2009). The molecular techniques can be used to identify coagulase negative bacteria to the species level.

2.2.5 Differential diagnosis

It is essential to differentiate the presence of bovine mastitis in respect to its differential diagnosis. Rupture of suspensory ligament, oedema, heamatoma and early parturition and presence of blood in the milk is the differential diagnosis used for identifying bovine mastitis (Quinn, 1994).

2.3. Control and Prevention

In dairy farms prevention and control of intramammary infection is the key point to solve the problem. However more knowledge and experience is needed to find the most effective strategies for prevention of mastitis caused by different micro organisms (Sampimon *et al.*, 2007).

2.3.1 Prevention

For the prevention and controls of sub clinical mastitis in dairy farms it is necessary to practice a regular screening of cows by California Mastitis Test (CMT). There are different methods to prevent and control coagulase negative Staphylococci bovine mastitis like pre milking udder preparation which is the important way for minimizing bacterial contamination and avoiding disinfectant residues (Devriese and Dekeyser, 1980). Udder health management is an integral method for elimination of existing and prevention of new intramammary infections. To avoid these problems, teats should be clean and dry at the time of machine attachment. Control measures against contagious mastitis pathogens such as post milking teat disinfection reduce coagulase negative staphylococci infections in the herd (Hogan *et al.*, 1987; Djaber *et al.*, 2002). Culling of chronically infected cows is an effective way of eliminating mastitis in the herd. Pre partum intramammary antibiotic therapy for heifers reduced the number of coagulase negative staphylococci infections during first lactation (Oliver *et al.*, 2003; Middleton *et al.*, 2005; Borne *et al.*, 2006). External and internal teat sealants in pre partum heifers will reduce the risk of intramammary infections and clinical mastitis of post calving (Parker *et al.*, 2007). Good

management practice in the herd including good nutrition formulation reduces the prevalence of bovine mastitis in dairy farms. In order to prevent and control coagulase negative staphylococci infection control measures should be apply in lactation, in dry cows and also in breeder heifers (Nickerson and Boddie, 1994; Smith *et al.,* 1985). In order to control the infection there should be a separate individual pens for the heifers and do not allow them to suckle each other because this transmits the bacteria and causes persistent infections that become established early in the life of the animal. Do not feed lactating heifers with infected milk to avoid transmission of mastitis from the adult cows to young cows. Separate the heifers from the cows before calving and provide clean areas for the cows to calve (Nickerson and Boddie, 1994).

2.3.2. Treatment

Generally assumed that the spontaneous cure rate of coagulase negative staphylococci seems high as compared to other *staphylococci* bacteria (McDougall, 1998; Taponen *et al*., 2006). Reports on coagulase negative staphylococci bacteria suggest the spontaneous cure rates of coagulase negative staphylococci mastitis ranging between 16% and 70% but more than half of cases persist until the end of lactation (Timms and Schultz, 1987; McDougall, 1998; Wilson *et al.,* 1999; Deluyker *et al.,* 2005). Persistent coagulase negative staphylococci responded much better to antimicrobial therapy than *Staphylococcus aureus* does. Antimicrobial treatment at drying off remains a good tool, as cure rates of dry cow therapy are generally very high for coagulase negative staphylococci infections (Newton *et al.*, 2008). Treatment by intramammary therapy in the peripartum and drying is effective for controlling infections caused by coagulase negative staphylococci (Turgutoul *et al.*, 2006). Antimicrobial treatment for mastitis due to *B-lactamase* coagulase negative staphylococci is with penicillin G parenterally or intramammarily. Intramammary treatment comprised of a combination of penicillin G and aminoglycoside. Mastitis caused by *B-lactamase* positive cloxacillin or the combination ampicillin and cloxacillin used (Turgutoul *et al.,* 2006). Coagulase negative staphylococci tends to be more resistant to antimicrobials than *Staphylococcus aureus* and easily develop multi resistance for the drug (Taponen *et al.,* 2008). The most common resistance mechanism in *staphylococci* is *beta-*

lactamase production which results in resistance to penicillin G and amino penicillin (Taponen *et al.,* 2008). Considerable research has been carried out to determine the antibiotic resistance of coagulase negative staphylococci species. Among *staphylococci* species, 31 isolates of coagulase negative staphylococci found phenotypically resistant to methicillin, penicillin G, ampicillin, amoxycillin and cloxacillin and the resistance strain is more widespread among coagulase negative staphylococci than *Staphylococcus aureus* (Turutogalu *et al.,* 2006). Almost all coagulase negative staphylococci isolates from bovine mastitis show resistance to two or more antimicrobial agents, the most frequently observed pattern of multiple resistances to penicillin, ampicillin, gentamicin, kanamycin and combination resistance was found in 4.2% resistant strains. The reported percentage of penicillin resistance for coagulase negative staphylococci isolated from mastitis is 36% in Norway, 25% in Denmark, 41-61% in the Netherlands (Maran, 2004), and 32% in Finland (Pitkala *et al.,* 2004). Sub clinical mastitis caused by coagulase negative staphylococci in Finland 52% (Pitkala *et al.,* 2004). In clinical mastitis in Finland, the proportion of *B*-lactamase positive *Staphylococcus aureus* is 13% and that of *beta-lactamase* positive coagulase negative staphylococci is 23% (Nevala *et al.,* 2004). Resistance of coagulase negative staphylococci to macrolides and lincosamides reported 6–7% in Germany (Luthje and Schwarz, 2006). Resistance of coagulase negative staphylococci that were cows isolated from mastitis to oxytetracycline 9% in Finland (Pitkala *et al.,* 2004) and 16% in the Netherlands (Maran, 2004).

2.4. Impact of Sub Clinical Mastitis on Economy

Annual losses in the dairy industry due to mastitis was approximately 2 billions dollars in USA and 526 millions dollars in India, in which sub clinical mastitis are responsible for approximately 70% of these dollars losses (Varshney and Naresh, 2004). Sub clinical mastitis is a major problem affecting dairy animals all over the world. It causes enormous losses for breeders and consequently influences the national income of the country (Ramachandrainh *et al.,* 1990). In Ethiopia, there is a limited information on the economic loss due to mastitis either clinical or sub clinical mastitis. However, the few data available indicated that the loss is significant. Mungube (2001) estimated the economic loss from mastitis in the urban and peri urban areas of Addis Ababa were 210.8 birr per cow per

lactation. In this study, loss due to culling, milk loss, treatment, and withdrawal contributed 49%, 38.4%, 9.3% and 3.3% to the total mastitis losses, respectively. Milk production losses contributed 38.4% of the total losses, sub clinical mastitis contributing 94% and clinical mastitis 6% of the milk losses.

Bishi (1998) he reported the economic losses from clinical and sub clinical mastitis to be approximately 270 Ethiopian birr per cow per lactation. The prevalence of sub clinical mastitis in dairy herds is often surprising to producers, moreover, sub clinically infected udder quarters can develop clinical mastitis and the rate of new infections can be high (Zdunczyk *et al.,* 2003).

There are no visible or palpable external changes (Blowey and Edmondson, 1995).The prevalence of sub clinical mastitis in dairy herds is often unexpected to the dairy producers, moreover, sub clinically infected udder , quarters can develop clinical mastitis and the rate of new infections can be high (Zdunczyk *et al.,* 2003). Cows with sub clinical mastitis are those with no visible changes in the appearance of the milk and/or the udder, but milk production decreases by 10 to 20% with undesirable effect on its constituents and nutritional value rendering it of low quality and unfit for processing (Holdway, 1992).

2.5. The Status of Sub Clinical Mastitis - Bovine mastitis in Ethiopia

In Ethiopia there are different reports on the prevalence of sub clinical mastitis and coagulase negative staphylococci bacteria and the prevalence varied from study to study. Although the objectives of the studies were not to study the status of coagulase negative staphylococci shown on Table 1.

Table 1.Prevalence of sub clinical mastitis in different part of Ethiopia

Study area	Isolation rate of subclinical mastitis (%)	Reference
Addis Ababa	46.6	Mungube *et al.*,(2004)
Asella	38	Lakew *et al.*,(2009)
Holeta	48.6	Mekbib *et al.*,*(*2009)
Sebeta	36.7	Sori *et al.*,(1997)
Jimma	35.6	Haile (2010)
Addis Abeba	34.3	Bishi (1998)
AddisAbeba	19	Hussein (1999)

3. MATERIALS AND METHODS

3.1. Study Area

The study was conducted in selected dairy farms in Jimma town, the capital of Jimma Zone, is located in Oromia Regional State about 355km south-west of capital city of Ethiopia. Jimma town lies between a latitude of 7°41'N and longitude of 36°50'E and having an elevation of 1750 metres above sea level. The area is characterized by a humid tropical climate of heavy annual rainfall that ranges from 1200-2000 mm per year. About 70% of the total annual rainfall is received during rainy season, which lasts from the end of May to September. The mean annual maximum 26.7°C and minimum 14.4°C temperature (NMSA, 2010).

3.2. Study Design

A cross sectional study was conducted to determine the prevalence and risk factors of sub clinical mastitis and coagulase negative staphylococci intramammary infection in Jimma dairy herds from June 2011to December 2012. There were 50 small holder dairy farms found in Jimma town. All of them were registered by dairy cooperative located in Jimma town and 50 small holder dairy farms were considered as a study frame for this study, of these 48 dairy farms were included in the study. The milk sample was collected from all volunteer farms found in the study frame and from all lactating dairy cows found in the dairy farms.

3.3. Study Animals

The study animals were dairy cows which is found in the study frames. There are about 800 dairy cows in Jimma dairy farms and it consists of both cross and local breeds of cows are found in the farms and also most of them are young and the age ranged from 3- 5. Almost all dairy farms found in Jimma town are under the same management systems.

3.4. Sample Size Determination and Sampling Methods

The sample size was determined from the 50 small holder dairy farms which are registered by dairy cooperatives in Jimma town. The sampling frame from the study site indicated that the dairy farms in Jimma were small holder dairy farms having an average of four lactating cows each. In addition, of the registered dairy farms all volunteer farms in Jimma were included in the study. Accordingly, all the lactating cows from the 48 dairy farms were considered for this study during the study periods which consisted of a total of 264 lactating cows and 1056 quarters milk were collected.

3.5. Study Methodology

3.5.1. Questionnaire survey

A questionnaire survey was conducted to obtain basic information on different potential risk factors that influence the occurrence of sub clinical mastitis and coagulase negative staphylococci induced intramammary infections. Regarding cow and farm attributes such as parity of cow, stage of lactation, age, and hygiene of teats, udder, flank and tails of dairy cows, breed teat injury, previous history of clinical mastitis and frequency of body washing; farm floor status (concrete, wood and soil (ground).

3.5.2. California mastitis test

The California mastitis test was used to detect the presence of sub clinical mastitis and it was carried out according to the procedures given by NMC (1990). After the milk sample arrived to the Jimma University College of Agriculture and veterinary medicine and school of Veterinary Medicine microbiology laboratory a squirt of milk about 2 ml from each quarter of the udder was placed in each of four shallow of California Mastitis Test paddle. Then an equal amount of the California mastitis test reagent was added to the California

Mastitis Test paddle and then a gentle circular motion for 15 seconds was applied in a horizontal plane to mix the milk. A gel formation within a few seconds was an indication of a positive test result, the result was scored 0 as negative and T, 1, 2, and 3 as positive based on the degree of gel formation. The determination of degree of California Mastitis Test was based on the degree of gel formation and the sample were categorized as negative if there was no gel or precipitation formation. If at least one quarter was positive for California mastitis test then the cow was considered as positive for mastitis infection.

3.5.3. Isolation and identification of coagulase negative staphylococci

3.5.3.1 Sample collection

Milk sample collection was carried out according to the procedures given by National Mastitis Council (1990). The udder and teats were washed thoroughly with tap water then the teat ends are swabbed with cotton soaked in 70% ethyl alcohol. Separate pledged cotton was used for each teat then allowed till it dries. Collection of milk sample was done before the cows were milked by the owners. As per the National Mastitis Council (1990) guidelines, after preparation of teats, the first 3-4 streams milk was discarded from each quarter. Then 25 ml of milk sample was collected from each quarter in to a sterilized and labeled screw capped test tubes. The collected milk samples were transported in icebox containing ice packs to Jimma University, College of Agriculture and Veterinary Medicine school of veterinary medicine Microbiology laboratory and cultured immediately or stored at 4 °C until cultured.

3.5.3.2. Isolation of coagulase negative staphylococci

After the California mastitis test the samples which are positive or not for sub clinical mastitis was kept for overnight in a refrigerator at 4°C then the milk sample was thawed for 3-5 hr at room temperature. A 0.1 ml of milk was inoculated aseptically in to sterile Blood Agar Plates enriched with 7% heparinized sheep blood. Then after the milk sample s were inoculated on blood agar, the media were incubated at 37°C for 24 hr under aerobic culture

conditions. After 24 hr the blood agar plates was examined for the presence of Staphylococcus colonies and hemolytic pattern on the surface of blood agar plates. Then gram staining was carried out to detect and characterize the isolated colonies. Then those colonies that were Gram positive cocci were sub cultured on nutrient agar plates and incubated at 37°C for 24 to 48 hrs. From the sub cultured pure bacterial colonies slide catalase test was applied.

3.5.3.3. Identification of coagulase negative staphylococci

The staphylococci bacteria inoculated on DNase media and incubated for 24hr, 37^{o}C under aerobic condition. The media was examined by adding 20% HCl on the DNAse media then the media was checked for the presence of dark clear zone produced by precipitation of bacterial DNase due to the reaction produced with the 20% HCl on the DNase media. After identifying the presence of staphylococci colonies by the above techniques, the tube coagulase test was carried out. In the coagulase test the tube coagulase test was performed in sterile test tubes by adding 0.5 ml of citrated rabbit plasma then staphylococc*i* colonies obtained from blood agar were inoculated in to tubes containing 0.5ml of citrated rabbit plasma then it was incubated at 37°C under aerobic conditions. Evaluation of clotting was taken at 30 min intervals for the first 4 hr of the test and then after 24 hr incubation period. the reaction were considered as positive if any degree of clotting from a loose clot to a solid clot that is immovable when the tube is inverted was visible within the tube and no degree of clotting was taken as coagulase negative staphylococci.

3.5.4. Antibiotic susceptibility test

The Antibiotic susceptibility test was performed by using McFarland standards of disk diffusion method on Mueller Hinton Agar. A bacterial colony that was obtained from the sub culturing on nutrient agar medium was incubated at 37ºC under aerobic conditions for 24 hrs. These pure colonies were used for the susceptibility testing. The McFarland standard 0.5 was fixed by mixing 1-2 bacterial colonies with 5ml of saline solution. After

bacterial colony was mixed in saline solution turbidity of the bacterial growth was measured at 0.5 McFarland. Then a loop of the suspension was inoculated on the Mueller Hinton agar plate by using cotton swabs until it covers the whole surface of agar. Using antibiotic applicator, antibiotic discs were dispensed on the already inoculated Mueller Hinton agar plate and incubated aerobically at 37ºC for 24 hr. Tetracyclin (80µg), streptomycin (100µg), ampicillin (33µg), amoxycillin (30µg), tylosin (150µg), trimetroprim (2-5µg), and cefuroxime (30µg) were antibiotics that were used for sensitivity test. The results were recorded as resistant and susceptible depending on the diameter of the inhibition zone. Digital caliper was used to measure the inhibition zone and interpretation of results was done in accordance of National council of clinical and laboratory standards (NCCLS).

3.5.5. Data management and analysis

The Data were entered and managed MS-Excel 2007. All the data analysis was done by statistical package for social science (SPSS) soft ware version 16.0. Explanatory variables were categorized as follows: lactation 1-90 days in milk, 90-180 days in milk and greater than 180 days in milk, and parity is categorized as primiparious for parity one and multiparious cows for more than one parity. The prevalence of coagulase negative staphylococci and sub clinical mastitis and the possible association of the disease with the different risk factors like stage of lactation, parity, age, farm facilities, milking procedures, and others were analyzed by descriptive statistic, and odds ratio, univariable and multivariable logistic regression were performed. The logistic regression was used to determine whether there is a significant difference association between the probabilities of occurrence of CNS and subclinical mastitis in response to the risk factors in one or more categories. The difference was statistically significant if the p-value was less than 0.05 ($p<0.05$).

4. RESULT AND DISCUSSION

4.1. Prevalence of Sub clinical mastitis and Associated Risk Factors

From the total of 1056 quarters that were examined 38 (3.59%) were blocked. In Jimma small holder dairy farms, almost all dairy farms used udder preparation before milking and washing of their hands prior to milking but none of them uses separate towel for drying of teats of cows after milking. Although 99 % of the owners have an idea about the clinical mastitis mammary infections but none of them knew about the presence of sub clinical mastitis except looking for clinical mastitis cases. The overall prevalence of bovine mastitis observed in Jimma small holder dairy farms was 75.75% at quarter level. Of this the total quarter prevalence of sub clinical mastitis and clinical mastitis was 62.1% and 13.6% respectively at quarter level. A total of 264 lactating cows examined 72.7% them found positive to sub clinical mastitis at cow level and 95.8% the 48 herds also positive for subclinical at herd level. Injured teats have a greater risk of getting sub clinical mastitis as compared to non injured teats and the distribution of sub clinical mastitis among quarter positions is summarized table 2 and their association was statistically insignificant ($p>0.05$).

Table 2. Prevalence of sub clinical mastitis on quarter positions in Jimma dairy farms.

Quarters of udder	Status of sub clinical mastitis			Univariable analysis		
	No of examined	Negative	Positive	P-value	CI (95%)	OR
Left Front	256	84	172(67.2)	0.53	0.786-1.600	1.1
Left Rear	255	100	155(60.8)	0.37	0.602-1.210	0.8
Right Rear	255	91	164(64.3)	0.98	0.692-1.399	0.9
Right Front	252	87	165(65.5)	0.50	1	1
Teat Injury						
Yes	24	7	17(70.8)	0.87	0.436-2.030	0.9
No	994	355	639 (64.3)			

In this study, the prevalence of sub clinical mastitis was higher on cross breed cows than local zebu breeds of dairy cows. Higher prevalence of sub clinical mastitis was observed in primiparous cows as compared to multiparious cows, and the difference was statistically is insignificant ($p>0.05$). Prevalence of sub clinical mastitis was high in animals with dirty teats, udders, flank and tail hygiene compared with clean teats, udders, flank and tail hygiene and their association were statistically insignificant ($p>0.05$) as shown on Table 3.

Table 3. Prevalence of sub clinical mastitis and cow associated risk factors

Cow factor	Status of sub clinical mastitis			Univariable analysis		
	No of examined	Negative	Positive (%)	P-value	CI (95%)	OR
Age						
3-5	827	279	548 (66.3)	0.00	0.458-0.850	0.6
>5	191	83	108 (56.5)			
lactation						
1-90	282	99	183 (64.9)	0.58	0.800-0.748-	1.0
91-180	315	111	204 (64.8)	0.98	1.346-11.489	1.0
>180	421	152	269 (63.9)	0.84	1	1
Breed						
Local	64	25	39 (60.9)	0.60	0.690-1.902	1.1
Cross breed	954	337	617 (64.7)			
Parity						
Primiparious	342	104	238 (69.6)	0.00	0.515-1.884	0.7
Multiparious	676	258	418 (61.8)			
Teat hygiene					0.690-1.147	
Clean	459	210	249 (54.2)	0.36		0.9
Dirt	559	152	407 (72.8)			
Udder hygiene	277	98	179 (64.6)	0.24	0.895-1.554	1.2
Clean	741	264	477 (64.4)			
Dirt						
Flank hygiene						
Clean	286	110	176 (61.5)	0.30	0.657-1.140	0.8
Dirt	732	252	480 (65.6)			
Tail hygiene						
Clean	306	113	193 (63.1)	0.64	0.716-1-231	0.9
Dirt	712	249	463 (65.0)			

Dairy cows were managed on soil (ground) flooring systems had the higher prevalence of sub clinical mastitis (75%) than dairy cows that were managed under wood (70.2%) and concrete floor types (62.5%). This association were statistically insignificant with the prevalence of sub clinical mastitis ($p>0.05$). The usual practice of body washing of the dairy cows in dairy farms had effect on the prevalence of sub clinical mastitis. Lower prevalence of sub clinical mastitis were observed in dairy cows that were washed every week than those cows washed every 15 days with more than 30 days intervals, and the association was statistically significant ($p<0.05$). The current finding show the prevalence of subclinical mastitis was higher on those dairy cows which do not showed clinical mastitis infection before as compared to clinically infected dairy cows and their association were statistically significant with prevalence of subclinical mastitis ($p<0.05$) as shown on table 4.

Table 4. Prevalence of sub clinical mastitis and related herd risk factors on management attributes in Jimma dairy farms

herd factor	Status of sub clinical mastitis			Univariable analysis		
	No of examined	Negative	Positive (%)	P-value	CI (95%)	OR
Floor						
Concrete	789	296	493 (62.5)	0.19	1	1
Wood	181	54	127(70.2)	0.93	0.367-1.231	0.6
Soil	48	12	36 (75.0)	0.05	0.500-1.894	0.9
Body washing						
7days	273	116	157 (57.5)	0.00	1	1
15-30days	599	215	384 (64.1)	0.00	0.284-1.671	0.5
>30 days	146	31	115 (78.8)	0.00	0.383-0.843	0.4
History of clinical mastitis				0.00	0.187-0.344	3.6
Yes	223	138	85 (38.1)			
No	795	224	571(71.8)			

4.2 Cultural Characteristics

The cellular morphology of staphylococci was found in bunch of grapes and having purple colors. The color and colony morphology depends on the types of bacterial isolates. Coagulase negative staphylococci do not have heamolysis on blood agar compared to staphylococcus *aures* bacteria. Coagulase staphylococci bacteria had a hemolysis on blood agar media. The coagulase negative staphylococci had white colony characteristics when it growth on blood agar. Of the total 1056 quarter cultured on blood agar 974 quarter was culture positive on blood agar medium. Among the total isolates of coagulase negative staphylococci all the isolates were culture positive.

4.3 Identification Using Biochemical Tests

The identification of coagulase negative staphylococci was performed by using biochemical tests like catalase test, growth on DNase agar medium and by coagulase tests. There are about two hundred forty coagulase negative staphylococci bacteria was isolated during the study period, as well as 10 coagulase positive staphylococci bacteria was isolated biochemical tests using the. All staphylococci bacteria was positive for catalase test ,they produce foam with in a few seconds after applying staphylococcal colony on slides containing two droops of Catalase enzymes.

4.4 Bacteriological Findings

Using biochemical tests 240 coagulase negative staphylococci were isolated. Along with coagulase negative staphylococci isolates, different other types of bacterial pathogens were isolated from bovine milk involved in the causes of intramammary infections in Jimma dairy herds. Among the different species of bacteria which is responsible for mastitis infections in dairy cows *Bacillus species* was the second frequently isolated pathogens followed by *Cornybacterium species, Staphylococcus aures, S. dysagalactia* and *S. agalactia*.

4.5. Prevalence and Associated Risk Factors of Mastitis Caused by CNS

From the total of 1056 quarter of 264 lactating dairy cows that were examined 240 coagulase negative staphylococci bacteria were isolated and identified on quarter level during the study period. The overall isolation rate of coagulase negative staphylococci at the quarter level was 22.72%, 23.1% on the cow level and 72.9% on the herd level. Among the total cows that were examined for the presence of coagulase negative staphylococci bacteria at quarter and cow level on clinical mastitis cases were 2.8% and 3.03% was positive isolation rate respectively. The prevalence of coagulase negative staphylococci from sub clinical mastitis cases were 19.9% and 20.07% at quarter and cow level respectively. Isolation rate of coagulase negative staphylococci in age groups, parity and stages of lactations were summarized on table 5. The association of ages, parity, teat, udder, flank tail hygiene and teat injury with the isolation rate of coagulase negative staphylococci at cow level was statistically insignificant ($p>0.05$). Animal with previous history of clinical mastitis has isolation rate of coagulase negative staphylococci intramammary infection 9.3% whereas in those cows with no previous history of clinical mastitis has 24.8% as shown in table 6.

Table 5. Prevalence of coagulase negative staphylococci on cows related risk factors

Cow factor	Groups	Status of coagulase negative staphylococci		Univariable analysis		
		No examined	CNS positive (%)	P-value	CI (95%)	OR
Age	3-5	827	197(23.8)	0.70	0.638-1.352	0.9
	>5	191	43(22.5)			
Parity						
	Primiparous	342	86(25.1)	0.40	0.648-1.189	0.8
	Multiparious	676	154(22.8)			
Breed						
	Local	64	11(17.2)	0.78	0.513-1.653	0.9
	Cross breed	954	229(24.0)			
Lactation	1-90	282	55(19.5)	0.16	1	1
	91-180	324	80(24.7)	0.05	0.317-1.016	0.5
	>180	412	105(25.5)	0.01	0.245-0.856	0.4
Hygiene	Clean	401	93(23.2)	0.81	0.717-1.299	0.9
of teats	Dirt	617	147(23.8)			
Hygiene	Clean	299	68(22.7)	0.68	0.680-1.289	0.9
of udder	Dirty	719	172(23.9)			
Hygiene	Clean	290	69(23.7)	0.79	0.757-1.437	0.7
of flank	Dirty	728	171(23.5)			
Hygiene	Clean	306	72(23.5)	0.98	0.727-1.366	0.9
of tail	Dirty	712	168(23.6)			
Teat	Yes	24	8(33.3)	0.25	0.694-3.886	1.6
injury	No	994	232 (23.3)			

Herd level risk factors associated with the prevalence of coagulase negative staphylococci like floor type, the infection of coagulase negative staphylococci were increased on cows kept under soil floor types as compared to concrete and wood floor types and not significantly associated ($p>0.05$) with coagulase negative staphylococci mammary infection shown on table 6.

Table 6. Prevalence of coagulase negative staphylococci and herd related risk factors in Jimma dairy farms

Herd level	Groups	Status of coagulase negative staphylococci		Univariable analysis		
		No examined	CNS positive (%)	P-value	CI (95%)	OR
Type of Floor	Concrete	789	181(22.9)	0.41	1	1
	Wood	181	44 (24.3)	0.19	0.351-1.421	0.6
	Soil	48	15 (31.3)	0.33	0.348-1.233	0.7
History of clinical mastitis	Yes	223	43(19.3)	0.08	0.501-1.049	0.7
	No	795	197(24.8)			
Body washing	7days	273	60(21.9)	0.68	1	1
	15-30days	610	147(24.1)	0.88	0.724-1.712	0.9
	>30 days	135	33(24.4)	0.62	0.596-1.562	1.1

Risk factors associated with the prevalence of coagulase negative staphylococci and sub clinical mastitis from the univariable analysis were offered to the final multivariable analysis for risk factors with (p -value < 0.15) shown on table 7.

Table 7**.** Risk factors offered to the final multivariate logistic regression analysis.

Variables	Category	Multivariable logistic regression analysis		
		P -value	CI (95%)	OR
Previous history of clinical mastitis	Yes	0.00	0.195-0.373	0.3
	No			
Body washing frequency	7days	0.03	1.012-1.548	1.3
	15-30days			
	>30 days			
Stages of lactations	1-90	0.04	1.006-1.836	1.3
	91-180			
	>180			

4.6. Antibiotic Susceptibility Test

Coagulase negative staphylococci bacteria isolated from intramammary infection cases of Jimma dairy herds were tested for susceptibility against seven common antimicrobial drugs. Coagulase negative staphylococci were (100%) sensitive to Trimetroprim followed by streptomycin (91.7%), ampicillin (91.7%), amoxycillin (91.7%), cefuroxime (91.7%), tylosin (89.9%) and tetracycline (80.6%) shown on table 8.

Table 8. Antibiotic susceptibility patterns of coagulase negative staphylococci bacteria for different antimicrobial agents

Antimicrobial agent	Drug content	Range of disk diffusion inhibition zone		
		Susceptible (%)	Resistant (%)	Standards of inhibition zone
Tetracycline	80µg	29 (80.6)	7 (19.4)	19-28mm
Streptomycin	100µg	33 (91.7)	3 (8.3)	14-22mm
Ampicillin	33µg	33 (91.7)	3 (8.3)	27-35mm
Amoxycillin	30µg	33 (91.7)	3 (8.3)	28-36mm
Tylosin	150µg	32 (89.9)	4 (11.1)	19-29mm
Trimetroprim	2-5µg	36 (100)	0	24-32mm
Cefuroxime	30µg	33 (91.7)	3 (8.3)	15-27mm

Sub clinical mastitis is the most frequently encountered forms of bovine mastitis in several dairy farms as it indicated in previous study in Ethiopia (Kassa *et al*., 1999; Hussein, 1999; Workineh *et al.*, 2002; Kerro and Tareke, 2003). This finding is also in line with the above authors. This might be attributable to the prevailing lack of knowledge and attention among dairy producers, shortage of proper management interventions of subclinical mastitis. The current finding showed that the prevalence of blind quarter were 3.59 %. The occurrences of blind quarters may be due to lack of screening and treatment of sub clinical mastitis and also inadequate follow up of clinical and chronic cases together with persistent challenges of the mammary glands by microbial pathogens which could be the main predisposing factors to quarter blindness.

Higher quarter level of sub clinical mastitis (62.1%) was recorded in the present study. This is nearly comparable with the 62.9% reported by Kerro and Tareke (2003) in Southern Ethiopia. However, this finding was higher than those reported from the central highlands of Ethiopia 22.3% (kelay *et al.*, 2008), 19% (Nesru *et al.*, 1997); 34.6% (Abaineh, 1997) and 36.7% (Sori *et al.*, 2005). This variation may be attributable to poor husbandry practices and other risk factors prevailing among Jimma dairy herds. In Tanzania the prevalence of sub clinical mastitis in small holder farms reported by Kivaria *et al*. (2004) were 90.3 at cow and 84.5 at quarter level. The prevalence was higher than the prevalence of sub clinical mastitis that was obtained present study in Jimma small holder dairy farms. The differences in results could be due to differences in management systems between dairy farms and also variation on breed of dairy cows.

Frequency of body washing of cows in dairy farms was significantly associated with the prevalence of sub clinical mastitis ($p<0.05$) and (OR=1.3). Poor farm sanitary or drainage conditions results in contamination of cows' environment by wastes. When a cow lays down and makes a direct contact with the environment, the teats, udder, flank and tail becomes dirty. Consequently, pathogens originating from the environment may get an access to colonize the teat canals and to cause infection. Keeping cows and farm in good hygienic conditions can help decrease to the incidence of intramammary infections caused by environmental pathogens as well as contagious pathogens.

Cow with no previous history of clinical mastitis had a high prevalence of sub clinical mastitis than cows with previous history of clinical mammary infections and their association was statistically significant ($p<0.05$). This may be due to the rise of new mastitis infections in dairy farms, lack of regular screening of dairy cows for sub clinical mastitis test, poor husbandry system in the farms as well as absence of treating dairy cows during dry periods may be attributable for increased the prevalence of subclinical mastitis on previously non clinically infected cows. Compared to wooden and concrete floor types, soil floor types were found to be associated to increased prevalence of sub clinical mastitis ($p>0.05$) in this study. Soil floor types are much difficult to clean and hence pose greater risk of contamination. Dirty and wet floors tends to harbor a variety of infectious agents, which may contaminate the udder and the teats and cause intramammary infection.

Injured teats had a high prevalence of sub clinical mastitis in contrast with non injured teats ($p>0.05$). Functional closure of the teat keratin as it was determined by Williamson (1995) he reported that it has a strong protective effect against new infection caused by both minor and major pathogens. When a teat gets injured it may lack this protective effect as a result of this it may increased in the prevalence of sub clinical mastitis. The association to sub clinical mastitis prevalence of other cow, quarter and herd level risk factors (age; parity; stage of lactation; teat injuries; breed; hygiene of teats, udder, flank and tail; quarter positions) was found to be insignificant ($P >0.05$) in the present study. This is in contrast to the significant association reported by Almaw (2004); Biffa *et al*, (2005) and Kelay (2008). This may be due to disparities on age, parity, lactation stages and herd size.

Coagulase negative staphylococci were long considered minor pathogens (Pyorala and Taponen, 2009). However, many studies from the developed world revealed coagulase negative staphylococci to be a highly prevalent cause of mammary infection having significant impact on dairy farms; by increasing the bulk tank somatic cell count as well as drug resistance pattern (Schukken *et al.*, 2003).

This study finding showed the isolation rate of coagulase negative staphylococci in Jimma dairy herds were 22.72% at the quarter level 23.1% at the cow level and 72.9% at the herd level. This finding is nearly in agreement with reports of Haile (2010) 23.16%. But higher than the findings of Sori *et al.,* (2010), 18.8%; Lakew *et al.*,(2009),17.3%, and 19% by Mekonen *et al.*,(2011). This may be due to the higher occurrence of injured teats, lack of treating cows at the time of dry period and poor husbandry system in dairy farms. On the contrary, the current quarter level prevalence was lower than those reported from Bahir-Dar (Bishi *et al.,* 1998) 54% and the Addis Ababa milk shed (Mekbib *et al.,* (2009), 30.06%; Bitew *et al*.(2010), 51.9%: Hussein *et al.*,(1999), 42%: Almaw (2004),49.63% and 26.9% Mungube (2001) in Addis Ababa milk shed. The variation observed between the prevalence of coagulase negative staphylococci on many researches as compared with the present findings could be due to disparities of parity and stages of lactations and poor husbandry practices in the dairy farms as well as presence of teat injury in farms. Many studies on coagulase negative staphylococci bacteria stated that coagulase negative staphylococci are opportunistic pathogens and when ever there is a lowered immunity of mammary glands there will be an increase in the prevalence of mammary gland infections by this agent. The present finding was lower than the finding in developed countries in Finland (Taponen *et al.,* 2008). This could be due to the variation with the herd size and difference in lactation stags and parity of dairy cows.

The difference among the isolation rate of coagulase negative staphylococci among different studies might be due to absence in post teat dipping, lack dry cow therapy and absence of proper udder health and hygiene. But the isolation rate of coagulase negative staphylococci at the cow level in present finding were higher than Haltia *et al*.(2006) in Estonia 4.5% in cow level and lower than 34.4% Sampimon *et al.*,(2010). This variation may be due to several factors like management practices in dairy farms and other factors may play a role. Late stage of lactation were a risk factors associated with the prevalence of coagulase negative staphylococci intramammary infections. There was ($p<0.05$) statistically significant. This report agrees with Timms and Schultzh (1984), Oliver Leavens *et al.* (1997), Chaffer *et al.,* (1999) and Taponen *et al.,* (2007) reports. During early lactation lack of proper feed for the dairy cows may result lowered migration of

neutrophils and delay in neutrophil migration may results in increase in prevalence of intramammary infections. lack of post milking teat disinfection, absence of treatment of clinical cases during lactation period, absence of treating dairy cows during dry and peripartum periods also has a great contribution for increased the isolation rate of coagulase negative staphylococci. Relatively the prevalence of coagulase negative staphylococci were increased in Primiparious cows as compared to more than one parity or multiparious cows ($p>0.05$). The variation in exposure rate may be associated with loss of premature keratin plug in teat canals are common in heifers and in primiparious cows, keratin plugs are physical protective barrier for mastitis causing pathogens and loss of protective effect of keratin plugs may results with infection caused by coagulase negative staphylococci and absence of treating dairy cows during dry periods and few weeks before parturition may increase infections of coagulase negative staphylococci than more than one parity (Hogan and Smith, 1988; Dingwell *et al.,* 2004). Present finding revealed that, coagulase negative staphylococci intramammary infection more frequently occurred in subclinical mastitis than mild clinical mastitis cases. These finding agrees with reports by Jarp (1991) and Taponen *et al.* (2006) and attributed to the fact that coagulase negative staphylococci causes mild udder infection.

Cows having teat injury (OR=1.6) had a risk of getting coagulase negative staphylococci infection as compared to non injured teats. Teat canal keratin provides an important protective effect by physically blocking the teat canal space and by producing antibacterial factors (Hogan *et al.,* 1988; Dingwell *et al.,* 2004). Injured teats may lack this protective effect against pathogens. When it gets injured teats skins may serve as harboring mastitis causing pathogens and the teat canal may remain open as the result of physical damage on the teat and coagulase negative staphylococci bacteria then easily penetrate the mammary tissue. The prevalence of coagulase negative staphylococci was moderately increased on dairy cows managed under soil floor types as compared to concrete and wood floor types this may be due to soil (ground) could have a higher risk of contamination of cows from the environments by wastes as compared to wood and concrete floor types. According to different reports some species of coagulase negative staphylococci freely lives on the environments (Harmon *et al.,* 1995). Other risk factors such as cow, quarter and herd level

risk factors (age, parity, stage of lactation, teat injuries, hygiene of teats ,udder ,flank and tail) was found to be insignificant (P >0.05) in the present study. This is in contrast to the significant association reported by Taponen (2008); Schukken (2003) and Sampimon (2010). The disparities on age, parity stages as well as herd size may contribute in significant effect on listed risk factors.

This study shown that coagulase negative staphylococci isolates were highly susceptible for Trimetroprim drugs (100%) followed by Streptomycin (91.7%) Ampicillin (91.7%) Amoxycillin (91.7%) Cefuroxime (91.4%) Tylosin (89.9%) and Tetracycline (80.6%).The susceptibility of coagulase negative staphylococci bacteria to tetracycline was different as compared with Almaw (2004) who reported susceptibility to tetracycline at 90.9% and to Streptomycin 81.81%. He reported for Streptomycin is lower as compared to the findings of the present study 91.4% as well as from that of Sori *et al.* (2010) who reported 100%. Sampimon *et al.* (2009) reported higher the resistance of coagulase negative staphylococci for penicillin. Hawari and Fowzi (2008) reported that the sensitivity of coagulase negative staphylococci for tetracycline was 52.8% lower than the present findings. Least sensitivity of CNS was reported to Ampicillin (Dhakal *et al.*, 2008; Kumar and Sharanu, 2002). According to Basappa *et al.* (2001) the susceptibility of CNS for Ampicillin and Amoxacillin were 36.7% and 29.4%, which was lower than the current findings. This might be due to the development of *B*-lactamase by coagulase negative staphylococci bacteria due to ubiquitous miss use of drugs. Turgutol (2006) reported the susceptibility of Trimetroprim and oxytetracycline were 62.2% and 68.45 respectively lower than present finding. In general our finding showed, susceptibility patterns of coagulase negative staphylococci to trimetroprim, ampicillin, streptomycin, tetracycline, tylosin, cefuroxime, and amoxicillin was higher.

5. SUMMERY AND CONCLUSION

The present study showed that sub clinical mastitis and intramammary coagulase negative staphylococci infections are a major problem in Jimma dairy herds. The prevalence of sub clinical mastitis is much higher than that of clinical mastitis. Floor type and Cow washing practice were found to be important herd attributes determining occurrence of sub clinical mastitis. Cows managed under soil (ground) flooring types and cows washed at less frequent intervals had a high risk of getting sub clinical mastitis. Among the cow related attributes, age, parity, no previous history of clinical mastitis and teat injury were found to increase risk of sub clinical mastitis. On the other hand, the occurrence of intramammary coagulase negative staphylococci infections was found to be higher among sub clinical mastitis cases as compared to clinical cases. Prevalence of intramammary coagulase negative staphylococci infections was affected by some cow (parity, stage of lactation, history of clinical mastitis and teat injury) and herd (floor type and washing practice) attributes. Prevalence of coagulase negative staphylococci increased with soil based floor types, advancing stages of lactation and with incidence of injured teats. On the contrary, prevalence was reduced by previous history of clinical mastitis, advancing parity and increased frequency of cow washing. In this study, coagulase negative staphylococci showed high sensitivity to trimetroprim followed by streptomycin, ampicillin, amoxycillin, cefuroxime, tylosin and tetracycline. In order to be able to reduce the prevalence and impacts of sub clinical mastitis and intramammary coagulase negative staphylococci infections among the Jimma dairy herd, the following measures are worth consideration

- Further detailed studies should be conducted to elucidate the risk factors, pathological mechanisms and impacts of intramammary coagulase negative staphylococci infections

- It is essential to improve the awareness of the smallholder dairy producers regarding the presence of sub clinical intramammary infections and important risk factors in the study area.

- Dairy owners should be advised and trained on; improved sanitation, udder and teat health monitoring, effective use of teat dipping and effective use of dry period therapy.

- Treatment of sub clinical mastitis cases associated to coagulase negative staphylococci should be conducted in accordance to findings of antibiotic sensitivity tasting and common drugs like trimetroprim, cefuroxime, tylosin, amoxicillin, ampicillin and streptomycin can be effective in managing such infections

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7. APPENDICES

Appendices.1. Questioner format

1. Name of the owner _____ sex_____ age_____

2. Educational level of owner of the farm_____

3. Do you have any idea about what mastitis infection is?

Yes ☐ No ☐

4. If your answer is yes say something about its impact on the dairy herds?

_____

5. Animal factor

5.1 Age of the animal _____

5.2 Breed _____

5.3 Amount of milk production of the cow per day (lit) _____

5.4 Stage of lactation _____

5.5 parity_____

5.6 Health of the udder _____

5.7 Does the animal infected with mastitis before?

Yes ☐ No ☐

5.8 If the answer is yes, what measures undertaken to control the infection?

_____

5.9 At which period of time the infection of mastitis is commonly happen in your farm?

During lactation ☐ during dry off time ☐

At the time of parturition ☐

6. Farm factors

6.1 Type of the farm _____

6.2Type of barn

Open ☐ closed ☐

6.3 Number of stalls in the diary farm_____

6.4 What is flooring system of the farm?

Soil ☐ concrete ☐ wood ☐

6.5 Does the farm use a separate house for calving?

Yes ☐ No ☐

6.6 Cleaning of calving pen after each calving

Yes ☐ No ☐

6.7 Frequency of cleaning of cows environment day once a day ☐ Twice a day ☐ Three times a day ☐ other ☐

6.8 Frequency of body washing of the cow?

Every week ☐ 15-30 days ☐ >30 ☐

6.9Does the farm use concentrates for feeding of the cow?

Yes ☐ No ☐

6.10Sources of watering for the animal's _____

7. Does the farm use pre milking udder preparation?

Yes ☐ No ☐

7.1 If the answer is yes what type of udder preparation

Washing of the udder by tap water ☐ disinfecting the udder by alcohol ☐

Other ☐

7.2 Does the owner of the farm use a separate towel after milking?

Yes ☐ No ☐

7.3 Does the milker wash and disinfect his or her hand before milking

Yes ☐ No ☐

7.4 Does the farm use post milking teat disinfection or teat dipping?

Yes ☐ No ☐

7.5 Frequency of milking of cow?

Once a day ☐ Twice a day ☐ 3x a day ☐

7.6 Do you have an idea about the presence of sub clinical mastitis?

Yes ☐ No ☐

Appendices .2.Checklist information format for cows

ID	Breed	Age	parity	lactation	Hygienic score				Presence of		
					Teat	Udder	Flank	Tail	injury	Tick	History CM

Appendices.3. Interpretation of CMT scorings

CMT score	Interpretation	Visible reaction
0	Negative	Milk fluid and normal
Trace	Trace	Slight precipitation
1	Weak positive	Distinct precipitation but no gel formation
2	Distinct positive	Mixture thickens with gel formation
3	Strong positive	Viscosity greatly increased when there is formation of strong gel

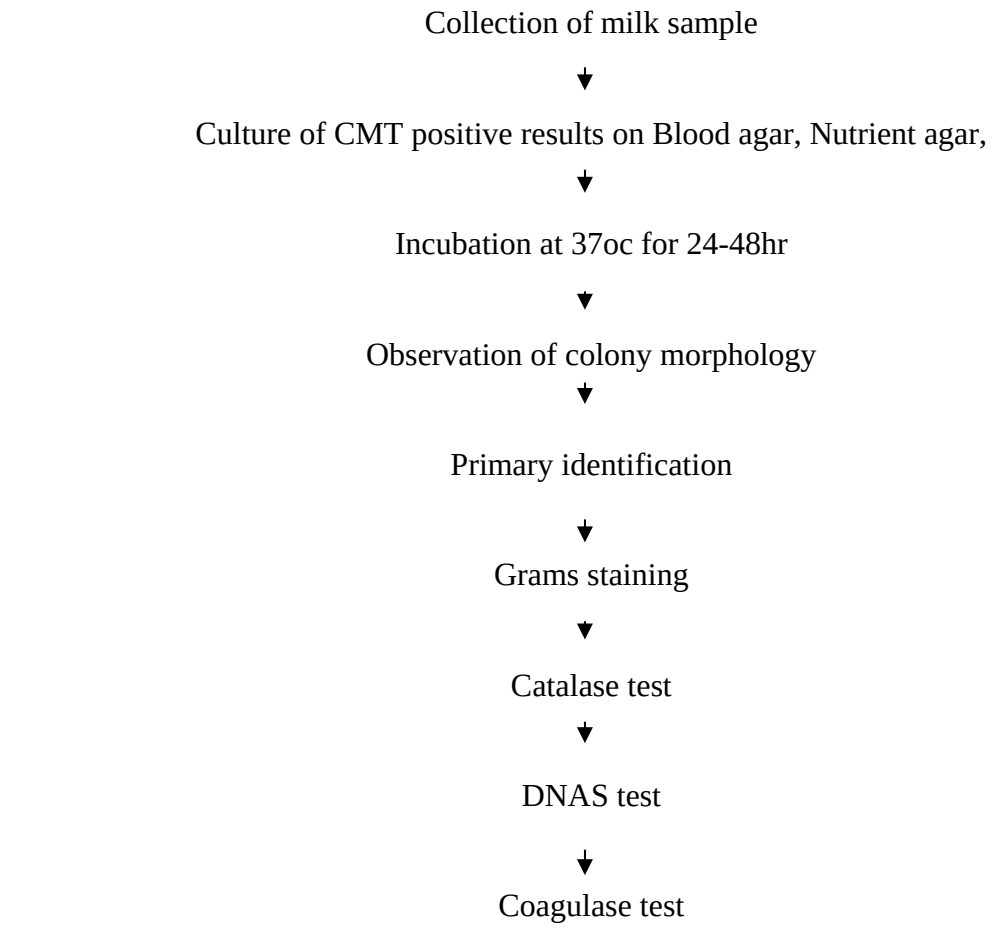
Source: NMC (1990)

Appendices.4. Gram Staining

A gram stain test was done for the identification of staphylococci bacteria. Primarily a thin smear or film then allow a thin smear to dry in air then flood the slide with crystal violate for one minute after this wash the slide and pour grams iodine wait for one minute and wash the slide and decolorize with acetone or alcohol then wash the decolorizer and pour with counter stain wait for one minute. After that see the prepared smear under microscope at 100x magnification power

Interpretations: Gram positive = purple and Gram negative = red

Appendices.5. Flow chart for isolation and identification



Interpretations: Coagulase positive if there is any visible clot formation

Coagulase negative if there is no clot formation

Sources: Quinn, 1994

Appendices.6. Isolation of bacteria using different test

Catalase test

Principle: Staphylococci bacteria converts hydrogen per oxide in to oxygen and water

Procedure: A loop full of bacterial growth from nutrient agar is taken, the bacterial colony placed on a clean microscopic slide and a drop of 3% H2O2 is added and presence of foam formation indicates a positive reaction and absence of foam formation regarded as negative for catalse test (Quinn, 1994).

Coagulase test

Principle: The coagulase enzymes converts fibrinogen in to fibrin

Principle: Staphylococci colony grown on an overnight incubated on blood agar media and a 0.5 ml of rabbit plasma in a sterilized small tube were mixed. The fibrinogen in rabbit plasma is converted to fibrin by coagulase, interpretations: when there is clot formation its coagulase positive *staphylococci* and if there is no formation of clot, regarded as coagulase negative *staphylococci* (Quinn, 1994).

Appendices.7. Media that used for isolation and identification of bacteria

1. Blood agar base, 500 g (oxoid, UK).

Composition :Heart muscle ,infusion from (solids)2;0 pancreatic digest of casine 13,0;yeast extract 5,0;sodium chloride 5.0;agar15.0.

Preparation :- Suspended a 40.0g of powder in 1 lit of distilled water ,mixed thoroughly and heated with frequent agitation and boiled for 3 min to completely dissolved the powder ,autoclaved at 121 °C for 15 min and cooled the base to 45-50°C and added 7% sterile defibrinated sheep blood.

2. Nutrient agar 500 g (Himedia, India).

Compositions: -Peptic digest of animal tissue5.00, beef extract, 1.50, yeast extract1.50, sodium chloride 5.00, agar 15.0.

Preparation: - Suspend a111.02 g of powder in 1 lit of distilled water, mixed thoroughly heated with frequent agitation and boiled for 3 min to completely dissolved the powder, autoclaved at 121 °C for 15 min and cooled the base to 45-50 °C.

3. DNase agar 500 g (oxoid, UK).

Compositions : -Enzymatic Digest of Casein 15 g, Enzymatic Digest of Animal Tissue5 g, Sodium Chloride 5 g, Deoxyribonucleic Acid 2 g, Agar 15.

Preparation:- Suspend a 39 g of powder in 1 lit of distilled water ,mixed thoroughly heated with frequent agitation and boiled for 3 min to completely dissolved the powder ,autoclaved at 121 °C for 15 min and cooled the base to 45-50 °C.

4. Muller Hinton agar 500 g (oxoid, UK).

Compositions: - Casien acid hygrolysate 17, Starch 1.5 g, beef infusion 300.00g, starch, 1.5g, agar, 17.0g.

Preparation: - Suspend a 30 g of powder in 1 lit of distilled water, mixed thoroughly heat ed with frequent agitation and boiled for 3 min to completely dissolved the powder, auto claved at 121°C for 15 min and cool the base to 45-50 °C. Then using a steriled cotton swab on a wooden applicator stick was used to transfer the diluted bacterial suspension to a plate, excess fluid was squeezed out by rotating the swab against the sides of the tube. The plate was seeded uniformly by rubbing the swab against the entire agar surface in three different planes.

Disc application: With in 15 minutes (time used to dry the inoculum) after the plates were inoculated, all discs gently pressed down on to the agar with forceps to ensure complete contact with the agar surface. The discs were no closer than 1.5 cm to the edge of the plate and they were rest 3 cm apart from each other. Incubation the plates were incubated aerobically for 24 hours at 37 °C.

Appendices.8. Differentiation of *Staphylococcus aureus* and CNS by different tests

Test	*S. aures*	CNS
Growth on BA	+	+
Heamolysis on blood agar	+	-
Catalase	+	+
Coagulase	+	-
DNAse agar	+	-

Sources: Quinn *et al*., 1994

Appendices.9 Teat and udder hygiene scorings

Hygiene score	Category
1	Clean
2	Slightly clean
3	Moderately clean
4	Dirty

Sources: (Nigel and Douglas, 2007).