MASTITIS IN HEIFER: PREVALENCE, ASSESSEMENT OF RISK FACTORS AND ANTIMICROBIAL SENSETIVITY TESTS ON MAJOR BACTERIAL ISOLATES IN CENTERAL ETHIOPIA

M.Sc. Thesis

BY

FEYISA BEKUMA

June, 2012

Jimma University

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Feyisa Bekuma

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School of Graduate Studies

As thesis research advisor, I hereby certify that I have read and evaluated this thesis prepared under my guidance, by **Feyisa Bekuma**, entitled Heifer Mastitis: Prevalence, Risk Factors and Antibiotic Resistance Patterns of Major Pathogens in central Ethiopia. I recommend that it be submitted as fulfilling thesis requirement.

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As member of the *Board of Examiners of M.Sc. Thesis Open Defense Examination*, We certify that we have read, evaluated the thesis prepared by **Feyisa Bekuma** 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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Chairperson Signature

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DEDICATION

I dedicate this thesis to my family for nursing me with affections and love and their dedicated partnership for success in my life.

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 has been submitted in partial fulfillment of the requirements for MSc. degree at Jimma University College of Agriculture and School of Veterinary Medicine and it will be deposited at the University Library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate. Brief quotations from this thesis are allowable without special permissions provided that 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 School of Graduate Studies when in his or her judgment the proposed use of the material is in the interest of scholarship. In all other instances, however, permission must be obtained from the author.

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BIOGRAPHICAL SKETCH

The author was born in 1985 at Nekemte, East Wellega Zone, Oromia Region. He attended his elementary, junior and secondary school education at Filege Yordanos elementary school and Abiyot Kirs senior secondary school, respectively, in Addis Ababa. He then joined Addis Ababa University School of Veterinary Medicine in 2004 and graduated with a degree of Doctor of Veterinary Medicine (DVM) in 2009. Thereafter, he was employed at private collage in Nekemte as an instructor and served for six months. In March 2010, He joined Jimma University College of Agriculture and School of Veterinary Medicine for his post-graduate studies in the field of Veterinary Epidemiology.

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LIST OF ABBREVIATION

€	Euro
^{0}C	Degree Centigrade
CI	Confidence Interval
CM	Clinical Mastitis
CMT	California Mastitis Test
CNS	Coagulase Negative Staphylococci
CSA	Central Statistical Agency
DNA	Deoxyribonucleic Acid
EIAR	Ethiopia Institute of Agricultural Research Center
IDF	International Dairy Federation
IMI	Intramammary Infection
km	kilo meter
ml	Milliliter
mm	Millimeter
NCCLS	National Committee For Clinical Laboratory
NMC	National Mastitis Council
OR	Odds Ratio
SCC	Somatic Cell Count

ABSTRACT

Heifer mastitis causes significant economic losses to the dairy development sectors and the infection causes detrimental mammary gland development affecting the subsequent lactation stage, udder health and related culling hazard. A cross-sectional study was conducted from June 2011 to March 2012 on cross breed heifers in Debre-zeit and Sebeta towns to estimate the prevalence of heifer mastitis isolate bacteria causing mastitis and test their antimicrobial susceptibility. One hundred fifty eight heifers were able to include from 149 cooperative smallholder dairy farms during the study period. From the total of 158 heifers sampled, 46(29.1%) were positive for mastitis (9.5% clinical and 19.6% subclinical cases). Identification of the bacteria on primary culture was made on the basis of colony morphology, hemolytic characteristics, Gram stain reaction including shape and arrangements of the bacteria, catalase and oxidation and fermentation (O-F) tests and further differentiation within the species level were done by using selective media. The most frequently isolated bacteria from quarter milk samples for clinical and subclinical mastitis were 7(24.1%) and 22(75.9%) CNS, 7(26.91%) and 19(73.1%) Staphylococcus aureus and 4(22.2%) and 14(77.8%) E.coli respectively. Other bacterial isolates were Streptococcus agalactiae(1(11.1%) and 8(88.9%)), klebsiella pnumonia(3(37.5%) and 5(62.5%)), Bacillus cerus(1(16.7%) and 5(83.3%)), actinomycet pyogens(1(25%) and 3(75%)), Streptococcus dysagalactiae(0 and 3(100%)), Entroccoccus feacalise(0 and 3(100%) and Streptococcus uberis(0 and 3(100%) for clinical and subclinical mastitis respectively. The univariable logistic regression showed that among the risk factors considered, age, heifer status, mastitic milk fed to calves, body condition scoring, usage of waste disposal and udder hygiene had significant effect on the prevalence of sub-clinical mastitis. However, after multivariable analysis, only age(OR=2.1;CI,1.5-2.9), mastitic milk fed to calves(OR=2.3;CI,1.5-3.5), udder hygiene(OR=1.9;CI,1.4-2.5) and usage of waste disposal(OR=2.7;CI,1.6-4.4) had significant effect. The antimicrobial sensitivity test showed for the majority of bacterial isolates 75-100% susceptibility pattern. Among all isolates CNS and Streptococcus dysagalactiae were showed 100% susceptibility for all of the antimicrobials tested, while the remaining species had varying levels of susceptibility. Among isolates Staphylococcus aureus show relatively lower susceptibility for almost all antimicrobials used. Streptomycin and Erythromycin was the most effective antibiotic followed by Sulfisoxazole and Ampicillin. The presence of mastitis in heifer in early age indicates important economic losses. Therefore, awareness creation at the smallholder dairy farm on the economic significance of heifer mastitis, risk factors that plays vital role in establishment and flourishment of potential pathogen and use of dry cow therapy before calving will help in reducing mastitis in heifer. Moreover, further studies on what extent the causative pathogen and the host itself affect the persistence of intramammary infection during calving and early lactating heifers, and evaluation of other risk factors in depth will merits the dairy farms.

Key words: prevalence, heifer Mastitis, bacterial isolates, antibiotic, susceptibility, central Ethiopia.

1. INTRODUCTION

Ethiopia holds large potential for dairy development due to its large livestock population and the favorable climate for improved high yielding animal breeds. Thus, the contributions of the dairy sector especially Market oriented smallholder dairy development is one of the promising avenues to improve food security and livelihood of rural households in Ethiopia (Ahmed *et al*., 2004). Replacement heifers are critical to herd productivity as they represent the future milking and breeding stock in all dairy operations. Hence, In the long run the goal of dairy farm should be to provide an environment for heifers to develop full lactation potential at the desired age with minimal expense. Animal health and well-being play vital roles in achieving this potential, and mastitis was found to be one of the major diseases that can influence such future productivity in dairy farms (Nickerson and Owens, 2010). Mastitis is defined as any inflammatory process affecting the mammary gland (IDF, 1987). Though heifers have been thought to be free of mastitis by most producers compared to multiparous cattle, nevertheless heifer can suffer from mastitis the presence of mastitis is not observed until time of calving or until the first signs of clinical mastitis in early lactation (Nickerson and Owens, 2010. Moreover an animal may carry an intramammary infection (IMI) for a year or more before it is diagnosed with mastitis Boddie *et al*., 1987. Research made clear that heifers are at risk for developing both subclinical and clinical mastitis more often than previously assumed and at even early age before attaining breeding age. Louisiana researchers documented mastitis in heifers as young as 6 months of age, and subsequent investigations inbreeding age and pregnant heifers have shown that infection rates can be as high as 97%(Boddie *et al*., 1987; Nickerson *et al*., 1995). Examination of mammary secretions collected from prepartum heifers have shown that the mammary glands of many heifers harbor organisms that frequently cause mastitis (Schultze 1985; Fox *et al*., 1995; Aarestrup *et al.,* 1997). *Staphylococcus aureus*, *Streptococcus dysagalactiae*, *Arcanobacterium pyogenes*, *Escherichia coli*, and coagulase-negative staphylococci seem to be the most important organisms that cause clinical mastitis in heifers (Myllys, 1995). Heifer mastitis was reported to affect the economy of farmers through reduced milk production, high culling rate, additional costs for veterinarians and drug, discarding milk during treatment period and waiting days and extra labour (De Vliegher *et al*., 2005b). *Staphylococcus aureus* mastitis in heifers has shown to

cause significant production losses during the first lactation and if left untreated, they produced 10% less milk in early lactation than those receiving intramammary non lactating cow therapies during gestation (Owens *et al*., 1991). The greatest development of milk-producing tissue in the udder occurs during the first pregnancy, so it is important to protect the mammary gland from pathogenic microorganisms to ensure maximum milk production during the first lactation (Nickerson, 2009). Though heifer mastitis was found to be prevalent and economical significant in different parts of the world, there is no information available on heifer mastitis and associated factors in Ethiopia. Hence the study sites both Debre zeit and Sebeta are the known for their large number of smallholder dairy farms and due to the fact that they are the main suppliers of the high demand of dairy products in Addis Ababa such information might help farmers in ensuring future productivity of replacement heifer. Therefore, this study was undertaken with the following objectives:

- To determine the prevalence of heifer mastitis,
- To assess the major risk factors associated with the occurrence of heifer mastitis, and
- To isolate the major bacterial pathogens and test their antimicrobial susceptibility

2. LITERATURE REVIEW

2.1. 1ntramammary Infections in Dairy Heifers

In the previous century, many studies were performed on the prevalence of mastitis in lactating and dry cows and on how to prevent and control it. Until 1980 little was known on the prevalence and incidence of IMI in heifers. Heifers were thought to be free from the disease because their teats had not been challenged yet by the milking process which is considered one of the principal risk factors of mastitis and for most of the heifer's life, the mammary gland has been immature and it would seem less likely to be in close physical contact to the environment (Fox, 2009). Nevertheless, dairy heifers can suffer from mastitis. From the 1980ies on, research made clear that both subclinical and clinical mastitis occurred more often in dairy heifers than previously assumed (Fox *et al*., 1995; Nickerson *et al.,* 1995).

According to International Dairy Federation (IDF), 1987 mastitis is defined as any inflammatory process affecting the mammary gland: mastitis is an inflammation of the mammary gland in response to injury for the purpose of destroying and neutralizing the infectious agents and to prepare the way for healing and return to normal function National Mastitis Council (NMC), 1996. "Heifer mastitis" is referred to dairy heifers calving with infected quarters, likely resulting in damaging implications for the future productive life of this important group of animals (De Vliegher *et al.,* 2004). Louisiana researchers documented mastitis in heifers as young as 6 months of age, and subsequent investigations inbreeding age and pregnant heifers have shown that infection rates can be as high as 97% (Boddie *et al*., 1987; Nickerson *et al*., 1995).

The relevance of heifer mastitis was recently studied. De Vliegher *et al*. (2005a) found that dairy heifers with an elevated test-day SCC early in lactation had a significant loss in milk production in their first lactation. The risk of being culled was increased as well (De Vliegher *et al*., 2005b). In case of a high prevalence of heifer mastitis on his farm, the farmer will suffer severe economical losses caused by the decreased milk production, the higher rate of culling, additional extra labour. Huijps *et al*. (2009) estimated the cost of heifer mastitis per heifer present on a farm in the Dutch/Belgian dairy sector at € 31, ranging from € 0 to € 220. However, it was

recently suggested that the negative impact of heifer mastitis in early lactation for the heifers' future performances depended on the pathogen that was involved (Kirk *et al*., 1996; Piepers *et al*., 2010). Remarkably, CM in early lactation occurs more often in heifers than in older cows (Barkema *et al*., 1998). Heifers suffering from clinical mastitis in early lactation can have high production losses (Gröhn *et al*., 2004). Furthermore, the risk of being culled for these heifers is highly elevated and therefore production losses might be underestimated (Waage *et al*., 2001; Piepers *et al*., 2009).

2.2. Epidemiology

Mastitis is a worldwide problem and affects dairy cows. Mastitis is a multifactorial disease results when management and environmental factors interact to increase or reduce resistance and deposition of organisms into teat canal. (Radostitis *et al*., 2006)

2.3. Heifer Mastitis Risk Factors

The three determinant risk factors, which play an important role in epidemiology of bovine mastitis, are also important for heifer mastitis which includes the microbial factors, host factors and environmental factors (Quinn *et al.*, 2004*).*

2.3.1. Microbial Factors

Sources of infection for heifer may include bacteria that are the normal flora on udder skin, which are in an opportunistic position to colonize the teat end and enter the teat orifice; bacteria harbored in the oral cavities of calves, which suckle other calves; bacteria present in the heifers' environment (such as those found in soil, manure, and bedding materials) and bacteria present on biting flies that congregate on teat ends (Nickerson and Owens, 2010). However, to induce mastitis, a pathogen must first cause infection in sufficient amount, should have the ability to survive in the immediate environment of the animals, should able to colonize the teat duct, to adhere to mammary epithelium and not to be fleshed out with milk flow and should be able to resist phagocytosis, antibacterial substance in the udder and resistance to antibiotics are

considered as major characteristic of each pathogen (Quinn *et al*., 2004). Although other factors include prevalence of infection: the greater prevalence of the disease in the herd, the greater new infection rate (Radostitis *et al*., 2006). The internal environment of a normal mammary gland is ideally sterile, but saprophytic bacteria may be found as commensals in some normal mammary glands. If the internal environment of the gland is favorable to survival and multiplication of the invading bacteria, the products of bacterial growth and metabolism may irritate the delicate mammary tissue and induce an inflammatory reaction (Bachaye *et al*., 2005). Martin-Richard, (2001) found that the presence of *Staphylococcus aureus* and Mycoplasma species in the farm and presence of pathogens on heifer body sites (Roberson *et al*., 1998) plays a role as risk factors for heifer mastitis.

2.3.2. Host Factors

Genotype and age of the animal, increased age at first calving, and milk leakage (Waage *et al*., 1998) ; blood in the milk, udder and teat edema (Waage *et al*., 2001) and immunological factors such as level of local immunoglobulin (IgA), lactoferrin and phagocytes in the mammary gland are considered as host factors (Quinn *et al*., 2004). Breed influence on prevalence of mastitis could be attributed to the difference in certain physiological and anatomical characteristics of the host and the mammary gland such as length of the leg in proportion to the udder size and relative strength of the udder attachment are examples. Large, pendulous udders tend to exceed the capacity of the supporting ligaments, with a consequent of breakdown of the udder. This will subject the udder to more physical injuries and thus increases the incidence of mastitis (Schutz, and Pajor, 2001).

As age increase, body defense by cellular and humoral immunity against pathogen decreased and hence disease condition including mastitis increase as cows get older. It is also possible for the udder of the first calving heifer to be infected at the time of parturition (Khan and Khan, 2006). Teat lesions and leaking milk have an important value for the occurrence of mastitis. Cows with udder/teat injuries are at greater risk of getting the disease than those with no injuries. Although

leaking of milk between milking has been associated with risk of clinical mastitis (Erskine, 2001).

2.3.3. Environmental and Managements Factors

Several factors in the environment affect the exposure of a heifer to microorganisms. Among factors Season is the one in which heifer mastitis cases mostly occur during calving in the summer months. This is particularly true in housed cattle and commonly caused by environmental infections; especially if the season is wet. Seasonal difference between the prevalence of individual bacteria as an example Streptococcus species is common in all seasons except in winter while *Staphylococcus aureus* is seen throughout the year (Radostitis *et al.,* 2006).

Risk factors for heifer mastitis were feeding calves mastitic milk, contact among calves, absence of antibiotic therapy to heifers, contact with adult cows, inadequate milking practices, and poor housing conditions (Martin-Richard,2001). A number of management practices contribute to the lower cell counts, including the proper hygienic procedure and timing, removal of udder hair, ample bedding, clean milking parlors, efficiency of milking personnel, consistent dry cow treatment, fresh feed in bunks as cows return after milking and nutrient supplementation for springing heifers, dry and lactating cows (Radostitis *et al.,* 2006). If the above management practices are neglected the prevalence of environmental and contagious mastitis can be disastrous (Barkema *et al.,* 1998).

Others heifer mastitis risk factors identified include an increase in the incidence of clinical mastitis in a herd, and absence of fly control, since fly populations can rapidly increase to several thousand per animal under favorable conditions; the need for early fly control on dairy heifers is marked. Once scabs are obvious and fly populations are high, spread of new infections is likely. Prevention of initial high populations of flies on heifers is important to help reduce new infections (Oliver *et al*., 2004). Presence of IMI before calving increased risk of infection during lactation (Aarestrup and Jensen, 1997); IMI at calving increased the risk of clinical mastitis within the first week after calving, and mastitis prior to parturition and mastitis within the first

week after calving increased the risk of further cases of mastitis and culling during the first 45 days of lactation (Edinger *et al*., 1999).

2.4. Detection of Mastitis

The ideal means of dealing with mastitis is to prevent it from happening. However, even under the best prevention and control programs, mastitis will occur. Detection of mastitis is generally based upon some indicators of the inflammation. However, treatment of mastitis works best if there is some information on the particular bacterium causing the problem (Walter, 2010).

2.4.1. Approaches to Detection of Mastitis

2.4.1.1. Visualization and Palpation of the Udder

The initial diagnosis of clinical mastitis is made during the routine physical examination. In clinical mastitis the udder may turn hard, red, and hot to the touch. Palpation of the udder may be painful to the cow. These symptoms arise from the changes in vascularity and blood flow of the gland when inflamed (Radostitis *et al.,* 2006).

2.4.1.2. Detection of the Inflammation

The detection of the inflammation is based upon the response of the animal to the infection. Several significant changes occur in the tissue and in the milk in response to infection. These include infiltration of leukocytes (referred to as somatic cells) and increased vascular permeability, resulting in alterations in the chemistry of the milk resulting from hydrolysis of milk proteins by hydrolytic enzymes and oxidative substances released from phagocytes, alterations in milk pH and ionic solutes, and ingestion of milk components by phagocytes (Walter, 2010).

2.4.1.3. Visualization of the Milk

Gross changes in the milk may be observed at the time of milking such as the presence of flakes, clots or serous milk. This is the most common means of detection of clinical mastitis. Stripping the first few squirts of milk from each quarter into a strip cup at the beginning of milking is a preferred method of detecting flakes or clots in the milk (Walter, 2010).

2.4.1.4. Detection of Somatic Cells (California Mastitis Test)

A key response of the cow to infection by pathogens is localized entry of leukocytes from the blood vessels in the infected tissue into the tissue near the site of infection.Only the udder quarter that is infected will have a significant increase in concentration of leukocytes (SCC). Tests such as the California Mastitis Test offer a cow-side very rough estimate of the SCC for each quarter and allow for focusing treatment efforts on that quarter (Walter, 2010). The CMT was developed to test milk from individual quarters but also been used on composite and bulk milk samples. The CMT involves mixing and swirling equal parts of bromocresol violet reagent and milk in a plastic paddle with a compartment for each quarter (Quinn *et al*., 2004). Fresh unrefrigerated milk can be tested using the CMT for up to 12 hours. Reliable readings can be obtained from refrigerated milk for up to 36 hours. If stored milk is used, the milk must be thoroughly mixed prior to testing because somatic cells tend to segregate with milk fat. The CMT reaction must be scored within 25 seconds of mixing because weak reactions will disappear after that time (Radostitis *et al*., 2006). The degree of reaction between the detergent and the DNA of nuclei is a measure of the numbers of somatic cells in milk. The threshold for CMT scores depend on the objective of the study. If it is used to minimize the rate of false negatives, the test should be read as negative versus positive with trace scores regarded as/ recorded as positive. If the CMT is to be used in culling decisions, a threshold with a lower rate of false positives may be desirable (Larsen, 2000). The test results are interpreted subjectively as either a negative, trace, 1, 2 or 3 based on gel formation by mixture of the reagent with milk and read as negative (CMT = negative or trace) or positive (CMT = 1, 2 or 3) (Radostitis *et al*., 2006).

2.4.1.5. Bacteriological culture

Culturing can be used in a targeted fashion for specific control programs such as segregation plans for contagious mastitis or for surveillance to detect the presence of new or emerging pathogen. Culturing is also used to evaluate treatment efficacy and to establish susceptibility patterns to aid in the development of rational treatment strategies (Larsen, 2000).

2.5. Pathogens Causing Heifer Mastitis

Intramammary infections in heifers are basically caused by the same pathogens as IMI in older cows (Fox, 2009). Mastitis causing pathogens are often grouped as major and minor pathogens. Major pathogens are considered to be more virulent, are more likely to cause clinical mastitis, and result in more pronounced milk yield losses (Timms and Schultz, 1987). Mastitis pathogens can also be classified as contagious (or "adapted") or environmental (or "opportunistic") pathogens, depending on their epidemiological behavior. A variety of different mastitis pathogens have been identified including Coagulase-negative staphylococci (CNS) *and Corynebacterium bovis* (minor pathogen) and *Staphylococcus aureus*, *Streptococcus dysagalactiae*, *Streptococcus agalactiae*, *Streptococcus uberis* ,*Escherichia coli* ,*Klebsiella pneumoniae*, *Arcanobacterium pyogenes*, *Peptococcus indolicus* and Mycoplasma species (Fox, 2009).

One common denominator of all studies conducted on heifer mastitis throughout the world is the high prevalence of CNS IMI. The prevalence of CNS in mammary secretions of primigravid heifers during the prepartum period has been reported to be as high as 50% of mammary quarters (0liver *et al*., 2005). CNS are not as pathogenic as the other principal mastitis pathogens and infection mostly remains subclinical (Piepers *et al*., 2010). However, CNS can cause persistent infections, which result in increased milk somatic cell count (SCC) and decreased milk quality (Kirk *et al*., 1996). CNS infection can damage udder tissue and lead to decreased milk production (Compton *et al*., 2007a). *Staphylococcus simulans* and *Staphylococcus chromogenes* are currently the predominant CNS species in bovine mastitis. *Staphylococcus hyicus* and *Staphylococcus epidermidis* have also frequently been isolated (Myllys, 1995).

CNS mastitis is not a therapeutic problem as cure rates after antimicrobial treatment are usually high (Thorberg *et al*., 2006). Based on current knowledge, it is difficult to determine whether CNS species behave as contagious or environmental pathogens. Control measures against contagious mastitis pathogens, such as post-milking teat disinfection, reduce CNS infections in the herd. Phenotypic methods for identification of CNS are not sufficiently reliable, and molecular methods may soon replace them. Knowledge of the CNS species involved in bovine mastitis is limited (Pyörälä and Taponen, 2007).

Staphylococcus aureus IMI often lead to strongly elevated SCC or clinical mastitis, even in dairy heifers (Waage *et al*., 1999). The milking process is considered to be the most important route of spreading from cow to cow (Bramley and Dodd 1984). Milk of infected cows and heifer body sites are believed to be the major sources for *Staphylococcus aureus* IMI in heifers (Roberson *et al*., 1998).

Streptococcus dysagalactiae spreads from cow to cow but the environment can just as well be the source of infection. In a Norwegian study, primiparous cows (heifers) with an IMI caused by *Streptococcus dysagalactiae* produced 1.1 kg of milk per day less than culture-negative animals of the same age (Whist *et al*., 2007). In Belgium and the Netherlands Due to the development and implementation of proper contagious mastitis control programs, *Streptococcus agalactiae* forms no longer a threat for the udder health of dairy cattle (Barkema *et al*., 2009). *Streptococcus uberis* is a common major mastitis pathogen and although cow-to-cow transmission is described (Zadoks *et al*., 2003), the environment is likely to be the major infective source (McDougall *et al*., 2004).

Coliforms can cause severe clinical mastitis in heifers. In a Norwegian study performed by Waage *et al*. (1999) 6.7% of milk samples collected from quarters with clinical signs from heifers was positive for coliforms. Coliforms are considered to be environmental pathogens. Two of the more important members are *Escherichia coli* and *Klebsiella pneumoniae* (Hogan and smith, 2003).*Corynebacterium bovis* is classified as a contagious pathogen (Fox *et al*., 1995) and is often isolated from cases of subclinical mastitis (Fox *et al*., 1995; Parker *et al*., 2007a). As

C. bovis rarely causes clinical mastitis, has a low impact on SCC and usually cures spontaneously, it is considered as a minor pathogen (Honkanen-Buzalski *et al*., 1984). Some studies have reported protective effects of IMI caused by minor pathogens against IMI with major pathogens (Lam *et al*., 1997). *Arcanobacterium pyogenes* and *Peptococcus indolicus* are frequently isolated from cases of "summer mastitis" (Shearer *et al.*, 1993). The pathogens can cause severe clinical mastitis in both dry cows and heifers on pasture and are spread by the fly *Hydrotaea irritans* (Yeomen and Warren, 1984).

Heifers as well as pluriparous cows can also suffer from mastitis caused by Mycoplasma species. The prevalence of Mycoplasma mastitis seems to increase in several countries. Furthermore, Mycoplasma mastitis might be under-diagnosed because identification of this group of pathogens requires 10 days of incubation under highly specific conditions (Fox *et al*., 2005). Diagnosing as well as successfully treating IMI caused by Mycoplasma is difficult. Mycoplasma spp. are hardly sensitive to antibiotics and therefore treatment is from an economical point of view not feasible (Bushnell, 1984). Mycoplasma bovis is likely to be the most prevalent Mycoplasma species causing IMI and is highly contagious (González and Wilson, 2003).

2.6. Prevention of Heifer Mastitis

Strategies to prevent and control mastitis in heifers should be based on risk or protective factors identified and tested through sound epidemiological research. Not all factors that have been identified as being associated with heifer mastitis can be implemented as prevention and control tools, either because they relate to animal specific aspects that cannot be altered (e.g., trimester of gestation) or because they relate to factors that do not lend themselves to the development of specific intervention strategies (e.g., location of a herd, season). Still, knowledge that, for example, heifers in late gestation are more likely to get infected should stimulate farmers to improve housing and comfort of this group of animals (De Vliegher *et al.*, 2012).

Heifer mastitis is a multi-factorial disease and studies have been conducted on how to prevent it. Several authors suggested prepartum antibiotic treatment as a way of controlling it. Oliver *et al*.

(2003) showed that heifers treated with a lactating cow product prepartum had a higher milk production and a lower SCC than untreated heifers and claimed therefore that the economic benefit cannot be doubted. Sampimon *et al.* (2009) proved similar positive effects by using a dry cow product and concluded that prepartum treatment of dairy heifers can offer a temporarily solution on farms with heifer mastitis problems. However, the results on the positive effect of prepartum antibiotic treatment of heifers before calving and the heifers' milk production and udder health during first lactation are not conclusive yet. Borm *et al*. (2006) observed no significant effect of treatment on milk production or SCC and questioned the usefulness of prophylactic antibiotic therapy as a control measurement. One should also be aware of the disadvantages of prophylactic pre-calving treatment with antibiotics. Appropriate withholding times are not known and the risk of antibiotic residues in food for human consumption is elevated (Compton and McDougall, 2008). Additionally, antimicrobial resistance can develop due to the latter practice (Rajala-Schultz *et al*., 2004). Besides antibiotic treatment, other treatments were suggested as well. For example, in a study in New Zealand, heifers under pastoral conditions sprayed with a commercial iodine-based teat sanitizer in the prepartum period were less likely to freshen with *S. uberis* IMI in the peripartum period compared to control animals (Lopez-Benavides *et al*., 2009). Parker *et al*. (2007a) found a decreased prevalence of both subclinical and clinical mastitis in the first 2 weeks postpartum when a bismuth sub nitrate teat-canal sealant was administered before calving. The efficacy of commercial vaccines with a potential protective effect against IMI with *Staphylococcus aureus* and CNS is still under debate (Middleton *et al*., 2009).

All the latter measurements to prevent heifer mastitis require individual treatment and can be costly. Changes in heifer management to prevent IMI are to be preferred above tools to cure existing IMI. Dystocia and udder edema are risk factors for heifer mastitis and therefore minimizing the incidence of these through optimization of the feeding and housing management can aid in the prevention of heifer mastitis (Compton *et al*., 2007b). Flies may act as vectors for several mastitis pathogens (Chirico *et al*., 1997; Gillespie *et al*., 1999). Hence, fly control can reduce the incidence of mastitis in heifers and pluriparous cows (Edwards *et al*., 2000). Mastitis pathogens can spread from older cattle to heifers making a physical separation between both age

groups advisable (Parker *et al*., 2007b). Furthermore, heifers with poor udder hygiene have a higher risk of IMI (Compton *et al*., 2007a).

Farms where clean calving pens are present and cubicles are cleaned more than twice daily are more likely to have a lower bulk milk SCC (Barkema *et al*., 1999). Therefore, hygiene should be optimal on farms to prevent heifer mastitis problems and mastitis problems in general. Finally, a lower incidence of IMI in early lactating heifers can also be achieved by increasing the genetic resistance against mastitis pathogens through selection. Several selection criteria are possible. Although dairy cattle is a typically out bred population complicating qualitative genetic research, several research groups have searched for candidate genes which might be of interest in the selection towards a lower mastitis susceptibility. Most of these genes are related to one or more components of the innate immunity and certain mutations in these genes have already been shown to be associated with mastitis susceptibility (Ogorevc *et al*., 2009).

2.7. Economics of Mastitis

Mastitis is one of the most costly diseases in dairy industry. Mastitis problems don't only occur in older lactating cows, heifer mastitis is also a well-known problem. Intramammary infections in dairy heifers in late gestation or early lactation may have a negative effect on the development of the mammary gland and on heifers' future milk production, udder health and related culling hazard (Piepers *et al*., 2009).Heifer mastitis potentially causes economic losses, caused by an elevated somatic cell count (SCC) at calving, and subclinical and/or clinical mastitis cases throughout lactation, which result in a decreased milk production, treatment costs, culling costs, and extra labour Oliver *et al*. (2003).

2.8. Public Health Significance

Milk and milk products have the potential to transmit pathogenic organisms to humans. All the nutritional components that make milk and milk products are important parts of the human diet also support the growth of pathogenic organisms raw (unpasteurized) milk has been found to participate in spreading out of illnesses caused by *Listeria, Campylobacter, Yersinia, Salmonella,*

Staphylococci species, and *E. coli.* With severe clinical *mastitis*, abnormalities of milk are easily observed and milk is discarded by the producer. Such milk normally would not enter the food chain. But when milk of cows with sub-clinical *mastitis*, *i.e*. with no visible changes, is accidentally mixed into bulk milk, it enters food chain and can be dangerous to humans. Although pasteurization is likely to destroy all human pathogens, there is concern when raw milk is consumed or when pasteurization is incomplete or faulty (Karima Galal *et al*., 2006).

Milk and other dairy products are frequently infected with *Staphylococcus aureus.* According to Gilmour and Harvey, (1990) milk of infected animals is the main source of enterotoxigenic *Staphylococcus aureus* of animal origin. For example certain *Staphylococcus aureus* strains produce heat-resistant enterotoxins, which cause nausea, vomiting and abdominal cramps when ingested by humans and are responsible for staphylococcal food poisoning outbreaks (Kluytmans *et al.,* 1997).

3. MATERIALS AND METHODS

3.1. Study areas and Population

The study was conducted from June 2011 to March 2012 in smallholder dairy farms found in Debre Zeit and Sebeta. Debre Zeit town is located at 45 km southeast of Addis Ababa and situated at a latitude and longitude of 8°44′N and 38°38′E, respectively. The area has an altitude of 1900 meter above sea level and experiences a bimodal rainfall pattern with a long rainy season from June to September and a short rainy season from March to May. The area receives an average annual rainfall of 1100 mm with respective average maximum and minimum temperatures of 28.3^{o}C and 8.9^{o}C (EIAR, 2012).

Sebeta is located 25 km southwest of Addis Ababa and situated at a latitude and longitude of 8°55′N and 38°37′E, respectively. It has an elevation of 2356 meters above sea level. The area is classified as temperate Highland with an annual rainfall of about 1650 mm. The mean annual minimum and maximum temperature is 8^{O}C and 19^{O}C, respectively. Sebeta is the administrative center of Alem Gena Woreda. Based on the report of Central Statistical Agency (CSA, 2008) Sebeta town has an estimated total human population of 56,131 of which 27,862 were males and 28,269 were females.

Debre-zeit and Sebeta have the potential for both crop and livestock production, which is mainly undertaken by smallholder farmers. There are also a relatively growing number of commercial farms and agro-processing industries operating in the area. The district agricultural potential and the infrastructure and institutional arrangements have encouraged the emergence of private service providers such as animal feed factory, private animal health institutions, agro processors and private livestock farms. There were more than 900 and 700 market oriented smallholder dairy farms (MOSH) which were milk suppliers for ada'a cooperatives and Sebeta agro industry (MAMA), with an average herd size of three animals. The majority of such dairy farm holders were organized under dairy cooperatives. The majority of the smallholders keep their animal in door. The types of antibiotics used in the study areas were Alamycin (Oxytetracycline), pen-strep (Penicillin and Streptomycin combination), intramammary infusions, procaine penicillin and

intertrium (Trimethoprim and Sulfonamide combination), Pen-strep (Penicillin and Streptomycin combination) and oxytetracycline were the most widely used drugs to treat mastitis and other infectious diseases.

3.2. Study Design

A cross sectional study type was carried out from June 2011 to March 2012 to investigate the prevalence of mastitis, assess the risk factors associated with the prevalence of mastitis, isolate bacterial pathogens and estimate their antimicrobial susceptibility patterns to the commonly used antimicrobial agents.

3.3 Sampling Technique and Sample Size

Non probability sampling method was used to determine the number of heifers to be sampled. The study sites were selected purposively due to the availability of large number of smallholder and commercial dairy farms in the areas also due to the fact that they are the main suppliers for the high demand of dairy products in Addis Ababa. List of households were obtained from milk collectors in the study sites (ada'a cooperatives from Debre zeit and Sebeta agro industry from Sebeta) and through the help of veterinary experts. Unfortunately heifers were only taken from households which were willing to cooperate. Therefore, a total of 158 heifers 85 from Debre zeit and 73 from Sebeta were included in the study.

3.4 Study Methodology

3.4.1 Detection of clinical mastitis

The udder of selected heifers was first examined by visual inspection and then by palpation to detect the presence of visible injuries, atrophy, swelling of the supra-mammary lymph nodes, fibrosis and cardinal signs of inflammation and appearance of milk secretion from each quarter was examined for the presence of abnormalities such as clots, flakes and blood (Radostitis *et al*., 2006).

3.4.2 Detection of sub-clinical mastitis

Subclinical mastitis was diagnosed based on California Mastitis Test (CMT) results and the nature of coagulation and viscosity of the mixture (milk and CMT reagent), which show the presence and severity of the infection, respectively (Walter, 2010). Before sample collection for bacteriological examination, milk sample was examined for visible abnormalities and screened by the CMT according to Quinn *et al*., (2004). From each quarter of the udder, a squirt of milk samples were placed in each of the cups on the CMT paddle and an equal amount of CMT reagents were added to each cup and mixed well. Reactions were graded as 0 and Trace for negative, 1, 2 and 3 for positive results according to (Radostitis *et al*., 2006). The interpretation for each result is shown in Annex 2. The CMT and milk electrical conductivity are not good predictors of intramammary infection for Holstein heifers in the last 2 weeks precalving. These is because the negative predictive value at quarter and heifer levels to identify IMI caused by major pathogens are high, a negative CMT or milk conductivity results could be used precalving to identify heifers or quarter not infected(Jean-Philippe *et al*., 2009). Therefore, precalving subclinical mastitis were diagnosed through direct culturing method.

3.4.3. Bacteriological examination of milk samples

3.4.3.1. Preparation of udder and teats

The udder, especially the teats was cleaned or washed with tap water and dried before milk sample collection. Dust, particles of bedding and other filth were also removed by brushing the surface of the teats and udder with a dry towel. Then the teats were swabbed with cotton, soaked in 70% alcohol (NMC, 1990). To prevent recontamination of teats during scrubbing with alcohol, teats on the far side of the udder was scrubbed with alcohol first, then those on the near side.

3.4.3.2. Milk sample collection, handling and storages

Udder secretion were collected from heifers that are at last month of pregnancy particularly at the last weeks of pregnancy and in their first 3 weeks of lactation after calving with strict and proper restraining method. Udder secretion was collected by a standard milk sampling techniques (NMC, 1990). Udder quarter secretions were collected aseptically to reduce contamination of the teat ends during sample collection. The near teats were sampled first followed by the far once. Then, samples were placed in racks for ease of handling and transported in an ice box to the microbiology laboratory of Addis Ababa University, school of veterinary medicine. Samples were then either stored at 4^0C for a maximum of 24 hours until inoculated on a standard bacteriological media or frozen at -20^0c for further delay (NMC, 1990).

3.4.3.3. Bacteriological isolation and characterization

Bacteriological culture was performed on all quarter udder secretion samples. Out of the 632 quarters examined, 11 were found blocked and hence, udder secretion samples were collected and cultured from the remaining 621 functional quarters. Identification of mastitis pathogens was carried out following microbiological procedures for diagnosis of bovine udder infection described in National Mastitis Council, NMC (1990). For Milk samples that had been refrigerated, dispersion of bacteria and fat were accomplished by warming the samples at room temperature (25^0C) for about an hour and then mixed by shaking. The samples were allowed to stand for a while for the foam to disperse and just before inoculation the tube was inverted gently. One standard loop (0.01ml) of milk sample was streaked on 7% blood agar. The inoculated plate was incubated aerobically at 37 ^{0}C. The plates were checked for growth after 24, 48 and 72 hours to rule out slow growing microorganisms such as Corynebacterium species. For primary identification, colony size, shape, color, hemolytic characteristics, Grams reaction and catalase production were used. The procedures followed for the identified pathogens are presented in Annex 3. Interpretation was made according to National Mastitis Council, (NMC, 1990).

3.4.4. Antibiotic Susceptibility Test

Antibiotic susceptibility test was undertaken to determine the resistance pattern of heifer mastitis causal bacteria to commonly used antimicrobials in the study area to provide information to concerned stakeholders. Agar disc diffusion (Kirby - Bauer method) was used as described in (Quinn *et al*., 2004). The procedures for the preparation of inoculum, inoculation to the Mueller - Hinton agar and disc application are presented in Annex 4. For Streptococcus species blood was added to Mueller - Hinton agar. After measuring the zone of inhibition, isolates were classified into sensitive and resistant. National Committee for Clinical Laboratory Standard (NCCLS) breakpoints was used to interpret the inhibition zone adapted from in Quinn *et al.* (2004). The following antimicrobial discs with their corresponding concentration (Oxoid, Basing Stoke, UK) were used: Sulfisoxazole (300µg), Tetracycline (TE)(30µg), Erythromycin(ERY) (15µg), Ampicillin (AMP) (10µg), Chloramphenicol (C30)(30µg), Polymixin B(PB) (300µg) and Streptomycin (S)(10µg).

The selection of the types of antimicrobial agents was made based on clinical considerations including frequent use of the drug in the study area and availability. Representative was taken for those antibiotics for which prediction is possible by the result of a representative (that is individual members within the group are related closely enough to assume cross-resistance). Tetracycline, Sulfisoxazole, Erythromycin and were used as a representative to predict the result against all other Tetracycline's, Sulfonamides and Macrolids respectively while Streptomycin, Chloramphenicol and Polymixin B because these individual members within each group are not related closely enough to assume cross-resistance thus they were tested separately.

3.5. Data Collection

3.5.1. Clinical Examination and Subclinical Examination

The selected smallholder dairy farms were visited at one time and two for some cases when the samples taken were not reliable. Crossbred heifers (precalving and post calving) were clinically examined for of mastitis. Clinical mastitis was diagnosed and data were recorded on the basis of

visible signs of inflammation on udder secretion and on the udder (present/absent). A quarter, which was warm, swollen and had pain and upon palpation, misshaped, atrophied, hard and fibrotic quarter was considered to have clinical mastitis. Clinical mastitis were also detected in quarters that have water secretions with clots or flakes compared to those with thick, honey-like secretions in pre-fresh normal heifers (Hallberg *et al*., 1995) and appearance of milk sample from each quarter was examined for the presence of abnormalities such as clots, flakes and blood in post calving heifer (Quinn *et al*.,2004).

The California Mastitis Test (CMT) was carried out only in post calving heifers as procedure described by Quinn *et al*. (2004) for screening sub-clinical mastitis. Heifers were considered positive for clinical and subclinical, when at least one quarter turned out to be positive for clinical examination and CMT. A herd was considered positive for CM and SCM, when at least one cow in a herd was tested positive with clinical examination and CMT.

3.5.2. Questionnaire survey

Questionnaire was compiled to collect data of potential risk factors for mastitis. Data on each sampled heifer was collected in a properly designed format (Annex 2). The factors were categorized into heifer factors (age, heifer status (before calving and after calving), body condition scoring (Category: 1 to 5(Edmonson *et al*.,1989) and presence of udder or teat injury (Yes versus No) herd factors (udder hygiene (1 to 4) (Nigel and Douglas, 2007)), floor type (concrete versus soil), milking practices after calving(Yes versus No), close contact among calves(Yes versus No), contact between heifer and adult cow(Yes versus No),separate calving(Yes versus No), frequency of heifer body washing(frequent ,moderate and not at all), mastitic milk fed to calves(Yes versus No), and usage of waste disposal method (Biogas versus 'fig'(a dried dung used as fire wood and fertilizer)).

3.6. Statistical Analysis

All data collected were stored and prepared in Microsoft office Excel. Prevalence was calculated for clinical and subclinical mastitis at herd, heifer and quarter level as defined by clinical manifestation the CMT score and bacteriological result. The prevalence of sub-clinical mastitis was the dependent variable while age, heifer status, body condition scoring and presence of teat or udder injury were independent variables considered at heifer level. The association between dependent and independent variables were tested initially by using univariable logistic regression ($p<0.05$) then those factors which were significant at $p<0.15$ were fitted to multivariable logistic regression model and tested statistically by using SPSS statistical package version 16.0.

4. RESULT AND DISCUSSION

4.1. Socio-demographic Characteristics of Smallholders

Majority of respondents in these study were male (61.9%). Almost three forth (70%) of the respondents were illiterate, whereas the remaining 30% were literate with educational level ranging from elementary to diploma (Table.1). The dominance of male households headed (80%) as compared to female was similarly reported in study done in peri-urban area of Addis Ababa (Mekonnen *et al*., 2010). In addition Kassa (2007) has reported in study 93.6% male owners and 52.5% of the households were illiterate in Fogera woreda of north Gonder zone. Also Eshetu, (2008) reported 52.7% of the respondent was male in central Ethiopia. The literacy can provide scope for an informative interface between farmers, extensionists, researchers and development agents (Chinogaramombe *et al*., 2008). However, the high levels of illiterate in this study might provide challenge for informative interface. The majority (77.2%) of livestock keepers depend solely on livestock herding, while the rest (22.8%) were keeping livestock as additional activities. These people are retired (10.7%) or civil servant (12.1%) involved in livestock keeping.

Table 1.Demographic structure of the smallholders in the study area.

Variable	Group	Number	Percentage (%)
Sex	Female	58	38.9
	Male	91	61.9
Level of education	Illiterate	104	70
	Literate	45	30
Occupation	Livestock keeper	115	77.2
	Civil servant	18	12.1
	Retired	16	10.7

4.2. Prevalence

Out of 158 heifers examined, an overall 29.1% prevalence of mastitis was recorded based culture results from which 9.5% was clinical mastitis and 19.6% subclinical mastitis. In Ethiopia, regarding the prevalence of heifer mastitis there is no information available so far. Even though, finding of scientific papers were not available there are some studies done with the objective of bovine mastitis mentioning the prevalence of mastitis at different level of parties. Prevalence of mastitis at first parities was reported by Bitew *et al*. (2010) and Gethaun *et al.* (2008) with 23.7% and 19.8% respectively. However, the prevalence reported were lower than this study probably their objectives were not targeting heifers like this study. A Similar study done stated the percentage of heifers with one or more subclinical mastitis infections was on average 27.2% per farm and clinical mastitis was recorded in 8.1% of the heifers with an average of 0.191 cases per 365 heifer days at risk (Bart *et al*., 2007). Nickerson *et al*. (1995) also reported higher clinical mastitis (15%) in heifer than present study. Oliver and Sordillo, (1988) and Pankey *et al*. (1991) also reported that approximately 46% of heifers and 19% of quarters were infected at calving and during early lactation which is higher than the present study.

The prevalence of subclinical and clinical mastitis and the distribution of the causative bacteria vary among studies. The magnitude of their effect is most likely related to the virulence of the causative pathogen, the persistence of the infection when milk production has started, the time of onset of infection, the ability of the animals to cope with the disease, and the response of the dairy manager to control the disease through management changes (De Vliegher *et al*. 2012). in this study, the relative increased proportion of subclinical mastitis observed might be due to similar fact as it is in bovine mastitis farmers specially smallholders were not well informed about the existence of subclinical mastitis (Hussein, 1999). Minimizing Subclinical and clinical mastitis during development of the mammary gland and in early lactation through awareness creation about heifer mastitis, subclinical mastitis and their importance might ensure future milk production, udder health and longevity and saves additional costs for veterinarians and drug.

CMT screening was only done on 403 of 632 quarters. From 403 quarters screened by CMT, 93(23.6%) them were CMT positive. Hundred (5.6%), 100 (5.4%), 101 (5.8%) and 102 (5.8%) of the CMT positive quarters were found in the left front, left rear, right rear and right front quarters, respectively and they were statistically not significant p>0.05 (Table.2).

Table 2.Prevalence of subclinical mastitis by using CMT test at quarter levels

Quarter	Number examined	Prevalence (%)	OR(95%CI)	p-value
LF	100	5.6	1.0(0.6-1.7)	0.998
LR	100	5.4	1.04(0.6-1.8)	
RR	101	5.8	1.04(0.6-1..8)	
RF	102	5.8		

In the current study out of 632 quarters examined 11 quarters (1.7%) belonging to 10 heifers were blind of which 9 (90%) heifers had only one blind quarter, 1 (10%) heifer had two blind quarters. The blind quarters were at the left front 4 (36.4%), left rear 3 (27.3%), and right rear 4 (36.4%) whereas, none blind quarter observed on right front positions. The occurrence of blind mammary quarters has a direct influence on milk production with a subsequent impact on food security, signifies the importance of the problem. Lack of screening and treatment of subclinical mastitis and inadequate follow-up of clinical and chronic cases coupled with persistent challenges of the mammary glands by microbial pathogens could be the main predisposing factors to quarter (Radostitis *et al*., 2006). This hidden and gradual destruction of the mammary tissues would end with non-functional quarters (Biffa *et al*., 2005).

Because mastitis is a complex disease involving interactions of several factors, mainly of management, environment, and factors relating to animal and causative organisms, its prevalence is expected to vary from place to place. This study also showed difference in prevalence of mastitis between the study sites (5.7% clinical and 10.8% subclinical in Debre zeit whereas 3.8 % clinical and 8.9% subclinical mastitis for Sebeta). Despite of the fact that it did not show statistical significance between study sites (p>0.05) (Table.3).

Table 3. Heifer level Prevalence of clinical and subclinical mastitis at study site

Types of mastitis	Study sites	Number examined	Prevalence (%)	OR(95%CI)	p-value
Clinical	Debre zeit	85	9(10.6)	1.1(0.6-1.9)	0.659
	Sebeta	73	6(8.2)		
Subclinical	Debre zeit	85	17(20)	1.2(0.7-1.6)	0.658
	Sebeta	73	14(19.2)		

The prevalence of subclinical mastitis at heifer and herd level are also shown in Table 4 and Table 5. Heifer status and age were found statistically significant ($p<0.05$) with heifer level prevalence of sub clinical mastitis whereas udder hygiene, mastitic milk fed to calves, and usage of waste disposal were statistically significant at herd level prevalence of sub clinical mastitis ($P<0.05$).

Table 4.Effect of risk factors of the prevalence of subclinical mastitis at heifer level

Risk factors	Group	N	Prevalence (%)	Univariable analysis	
				OR(95%CI)	P value
Heifer status	Before calving	54	12.9	1.7(1.1-2.8)	0.012
	After calving	104	23.1		
Age	≤3	88	14.8	1.9(1.2-2.9)	0.000
	3-4	56	23.2	3.2(1.7-6.1)	0.030
	>4	14	35.7	1	0.000
Udder/teat injury	Present	15	0	4.5(0)	0.997
	Absent	143	21.8		
Body condition score	Poor	11	9.1	2.6(0.9-7.4)	0.072
	Moderate	147	19		

Table 5. Effect of risk factors of the prevalence of subclinical mastitis at herd level.

Risk factors	Group	N	Prevalence (%)	Univariable analysis	
				OR(95%CI)	P value
Floor type	Earth type	38	21.1	1.3(0.58-1.9)	0.275
	Concert type	111	18.9		
Udder hygiene	Slightly dirty	44	13.6	2.7(1.6-4.6)	0.010
	Moderately dirty	71	16.9	1.2(0.7-2.0)	0.000
	Dirty	34	32.4	1	0.000
Heifer washing	Frequent	12	16.6	1.1(0.6-1.9)	0.795
	Moderate	137	19.7		
Mastitis milk fed to calves	Yes	59	23.7	1.9(1.2-2.8)	0.001
	No	90	16.6		
Separate calving house	Yes	20	15	1.01(0.6-1.6)	0.985
	No	129	20.15		
Milking practices after calving	Use towel to dry	10	10	1.1(0.5-2.1)	0.802
	Not use	139	20.1		
Contact among calves	Yes	136	18.9	1.1(0.5-1.9)	0.827
	No	13	19.7		
Contact between heifer and adult cow	Yes	137	19.7	1.01(0.5-1.8)	0.988
	No	12	16.6		
Usage of Waste disposal	Biogas	22	18.2	1.8(1.1-2.9)	0.027
	Fig	127	27.3		

4.3. Risk factors Associated with Sub-clinical Mastitis

Fifteen risk factors were considered as potential risks for the occurrence of subclinical mastitis in this study. By using univariable logistic regression analysis, heifer status, age, udder hygiene, mastitic milk fed to calve, waste disposal method, and body condition score were found to be significant ($p<0.05$). Herd attributes such as; floor type, milking practices after calving, close contact among calves, contact between heifer and adult cow, separate calving and frequency of heifer body washing and host factor (udder or teat injury) had no significant effect ($p>0.05$) on the prevalence of subclinical mastitis. All risk factors that had significant effect in univariable analysis with p-value less than 0.15 were fitted in to a multivariable logistic regression model and age, udder hygiene, mastitic milk fed to calves, and usage of waste disposal show a significant effect ($p< 0.05$) and taken as a final model (Table.6).

Table 6. Multivariable logistic regression analysis for the potential risk factors.

Risk factors	OR	95.0% C.I. for OR		p-value
		Lower	Upper	
Age	2.1	1.5	2.8	0.00
Udder hygiene	1.9	1.4	2.5	0.00
Mastitic milk fed to calve	2.3	1.5	3.5	0.00
Waste disposal practice	2.7	1.6	4.4	0.00

Heifers that are older at calving have an increased risk of mastitis, particularly from environmental sites. In some herds, it appears that the level of infection tends to increase with age as the heifers approach calving (Kirk, 1996). It has been demonstrated that yield from the subsequent lactation increased as age at first calving increased (Khan and Shook, 1996) and yield is evidently related to the degree of development of the udder at calving. A relationship between the susceptibility of heifers to mastitis and the degree of udder development is comprehensible.

Hence, in this study also indicated that heifer mastitis was more likely to occur in heifer that are above four years with 35.7% prevalence (OR=2.1; 95% CI, 1.5-2.9). The effect of age at first calving on subsequent risk of mastitis or IMI is not clear (De Vliegher *et al*., 2012).

Heifers become exposed to mastitis pathogens through several routes and consumption of mastitic milk at calf age is considered as one means (Nickerson *et al.,* 1995). In the present study the farms fed mastitic milk to calves were 2.3 times higher at risk of mastitis than those farms did not (OR=2.3;CI,1.5-3.5). Until recent knowledge, this risk factor has never been reported for other pathogens only for *Streptococcus agalactiae* (Nickerson *et al.,* 1995) and from an udder health point of view there is little risk of feeding mastitic or high SCC milk to calves when they are maintained in individual pens (Barto *et al*., 1982). In addition, heifers fed mastitic milk as calves suffered no more udder problems than did their mates that received other liquid feed (Kesler, 1981; Roberson *et al*., 1994a). Nevertheless, other concerns have been raised associated with feeding mastitic milk, including potential violative antibiotic residues in calf tissue (Musser *et al*., 2001) or transfer or induction of antibiotic resistance in the intestinal flora of calves (Langford *et al*., 2003). Additionally, transfer of other pathogens such as *Mycobacterium avium* subspecies *paratuberculosis* may occur (Ridge *et al*., 2005). For these reasons feeding mastitic milk to calves appears to be contra-indicated. The transfer of mastitis-causing bacteria through cross-suckling of calves fed mastitis milk can be prevented by housing calves in individual hutches.

Higher prevalence of subclinical mastitis were recorded in dirty udder heifers as compared with slightly dirty and moderately dirty udder heifers ($p<0.05$; OR=1.9; CI, 1.4-2.5). Compton *et al*. (2007a) also reported that heifers with poor udder hygiene have a higher risk of IMI. For the herd attribute the livelihood of subclinical mastitis were higher in heifers in those farms use the farm waste to produce ‘fig’ (2.7 times) than those whom uses to produce biogas. Poor udder hygiene and absence of immediate removal of waste in case of herds that use the dung to produce ‘fig’ might indicate that the potential pathogen to cause mastitis were given the immediate environment to flourish inevitably.

Most of the herd attributes considered in the current study (floor type, milking practices after calving, separate calving house, contact among calves, contact between heifers and adult cows and frequency of heifer body cleaning) and heifer factors (such as heifer status (precalving/post calving), udder /teat injury and body condition score) did not have significant effect on the prevalence of sub-clinical mastitis. However, the importance of these farm attributes in determining the prevalence of mastitis was indicated by Waage *et al*. (2001); Bassel *et al*. (2003) and Oliver *et al*. (2005). The homogeneity of the production environment under smallholder's condition and the little difference in farm hygienic practices could have contributed for the lack of significant effect of the farm attributes.

4.4. Bacteriological Isolate

A total of 109 bacteria were isolated from which 24 isolates were from clinical cases whereas, 85 isolates were from subclinical cases. Out of 85 subclinical cases isolates 71 were from CMT positive quarter while 14 isolates were obtained direct through culturing from pre partum heifer.

Table 7. Bacterial isolates from clinical and subclinical mastitic milk samples in smallholder crossbred heifer, in study area.

Bacteria isolated	Clinical (%)	Subclinical (%)	Total (%)
Actinomycet pyogen	1(25)	3(75)	4(4.2)
Bacillus cerus	1(16.7)	5(83.3)	6(5.5)
CNS	7(24.1)	22(75.9)	29(26.6)
E.coli	4(22.2)	14(77.8)	18(16.5)
Entroccoccus feacalise	0(0.0)	3(100.0)	3(2.75)
Klebsiella pnumonia	3(37.5)	5(62.5)	8(7.34)
Staphylococcus aureus	7(26.9)	19(73.1)	26(23.9)
Streptococcus uberis	0(0.0)	3(100.0)	3(2.75)
Streptococcus agalactiae	1(11.1)	8(88.9)	9(8.3)
Streptococcus dysagalactiae	0(0.0)	3(100)	3(2.75)
Total	24(22)	85(78)	109(100)

The result of various bacterial species isolated from the clinical and subclinical cases are shown in (Table.7). The most frequently isolated bacteria from quarters milk sample were CNS 29 (26.6%), *Staphylococcus aureus* 26 (23.9%) and *E.coli* 18(16.5%). Other bacterial isolates were *Streptococcus agalactiae* 9(8.3%), *klebsiella pnumonia* 8(7.3%), *Bacillus cerus* 6(5.5%), *actinomycet pyogens* 4(4.2 %), *Streptococcus dysagalactiae* 3(2.75%), *Entroccoccus feacalise* 3(2.75%) and *Streptococcus uberis* 3(2.75%) with decreasing order of frequency. Most *CNS* species were isolated from subclinical cases.

The prevalence of subclinical and clinical mastitis and the distribution of the causative bacteria vary among studies, but a common denominator is the high proportion of subclinical and clinical mastitis cases caused by coagulase negative staphylococci (CNS) (Myllys, 1995; Waage *et al*.,1999). In the current study, the quarter level prevalence for CNS was in agreement with (Fox

et al., 1995; Aarestrup and Jensen, 1997; Piepers *et al.,* 2010) who stated Coagulase-negative staphylococci (CNS) to be the most frequently isolated pathogens from dairy heifers suffering from subclinical mastitis. In the practice area of the Faculty of Veterinary Medicine, University of Helsinki, Finland, more than 20% of bacterial isolates from milk samples from clinical mastitis were CNS Nevala, (2004). CNS has been considered as normal skin flora which as opportunistic bacteria can cause mastitis. Some CNS isolated from mastitis may be opportunists from the environment, but it is very likely that at least the main species infecting bovine mammary gland are specialized for udder environment Oliver *et al*. (2004). The increased prevalence of clinical cases caused by CNS could indicate either an increased virulence of some species or strains or an increased susceptibility of the animal to these infections. However, because most routine laboratories do not differentiate between species and only report presence of CNS as a group, it is not clear yet how to proceed in practice (Smith and Hogan, 2001). Opinions are divided on CNS importance for udder health. Recent studies even found higher milk yield in CNS-infected cows than in culture-negative cows (Schukken *et al.,* 2009). Taponen *et al*. (2006) on the contrary claimed that CNS infections might be more harmful than assumed and that certain species can persist for a long time causing severe damage to the infected quarter. In solving CNS mastitis problems, focus should therefore be on the heifers, environment, feeding and management before calving. Welfare and comfort of heifers may be significant factors for good udder health (Pyörälä and Taponen, 2007)

The second leading bacteria were *Staphylococcus aureus* 26 (23.9%) these report were comparable with Trinidad *et al*., 1990; Myllys, 1995 who reported 23.1% and 20.1% respectively. Fox, (2009) indicate the quarter prevalence of *Staphylococcus aureus* between 1 and 4 DIM was slightly higher than the average prevalence at calving of 2.3% across different studies. Although prevalence of *Staphylococcus aureus* IMI in heifers is generally lower compared with CNS, its importance should not be underestimated as this bacterium is one of the most difficult mastitis pathogens to control (Barkema *et al*., 2006). The higher incidence of the bacteria can most likely be attributed to the wide distribution of the organism is the infected udder of lactating heifers and cows which is the major reservoir site but this bacterium also colonizes teat skin, vagina, muzzle, and other body sites, as well as bedding, feedstuffs, air, and equipment (Boddie *et al*., 1987; Roberson *et al*., 1994, 1998). The bacteria usually establish

chronic, subclinical infections and are shed in the milk, which serves as a source of infection for other healthy cows and heifers during the milking process. Transmission among cows increase whenever there is lack of effective udder washing and drying, post- milking teat dip and drying, inter-cow hand-washing and disinfection, washing clothes and milking machine cups (Radostitis *et al*., 2006).

Coliforms are considered to be environmental pathogens. Two of the most important members are *Escherichia coli* and *Klebsiella pneumoniae* (Hogan and smith, 2003). In this study the prevalence of mastitis caused by *E.coli* and *Klebsiella pneumoniae* were 16.5% and 7.3% respectively. Waage *et al*. (1999) reported in a study performed in Norwegian 6.7% of milk samples collected from quarters with clinical signs from heifers was positive for Coliforms. Environmental mastitis pathogens will likely be the predominant pathogens isolated in heifer mammary glands when herds are with an environmental mastitis problem. The number of hours dairy cows kept indoor is also a factor that will increase the possibility of contact of teats with the environmental pathogens according to Saloniemi (1991).Poor hygiene of the calving area is, not surprisingly, associated with an increased prevalence and higher odds of being infected with environmental mastitis pathogens shortly after parturition. In early lactation the susceptibility of dairy cows to mastitis is increased, probably due to slow leukocyte recruitment to the mammary gland during the periparturient period and because of a negative energy balance and stress during early lactation (Suriyasathaporn *et al*., 2000). Also severity of mastitis is a result of interaction between immune defense of the host and bacterial characteristics. Burvenich *et al*. (2003) concluded in their review that cow factors rather than specific features of the bacterial strain mainly determine the severity of *E. coli* mastitis.

In one study 8 to 10% of heifer mammary glands were infected by environmental mastitis pathogens, primarily *Streptococcus* species, which was consistent with the pattern of IMI in lactating cows in the herds (Oliver, 2005). In the present study also *Streptococcus* species such as *Streptococcus agalactiae* (8.3%), *Entroccoccus feacalise* (4.2%), *Streptococcus dysagalactiae* (2.75%), and *Streptococcus uberis* (2.75%) were reported. Reasonable hypothesis is that heifers from herds with a high prevalence of contagious mastitis will likely be infected predominantly

by contagious mastitis pathogens and whenever there is lack of effective udder washing and drying, post- milking teat dip and drying, inter-cow hand-washing and disinfection, washing clothes and milking machine cups (Radostitis *et al*., 2006).The current study also identified a low prevalence *Bacillus cerus* 5.5%, *actinomycet pyogens* 4.2 %, *Streptococcus dysagalactiae* 2.75%, *Entroccoccus feacalise* 2.75% and *Streptococcus uberis* 2.75%.

4.5. Antimicrobial Sensitivity Test

As described in Table.8 , only 46 of the isolates were exposed to antimicrobial susceptibility these was based on the available antimicrobial disc and the isolates were Staphylococcus *aureus* 8(17.4%), CNS 10(21.7%), *Streptococcus agalactiae* 4(8.7%), *Streptococcus dysagalactiae* 3(6.5%), *E.coli* 7(15.2%), *klebsiella Pneumoniae* 5(10.9%), *Entroccoccus feacalise* 2(4.3%), *Actinomycet pyogen* 2(4.3%), *Streptococcus uberis ,* 2(4.3%) and *Bacillus cerus* 3(6.5%) were tested for susceptibility to seven antibiotics. The antibiotics were Sulfisoxazole, Tetracycline, Erythromycin, Ampicillin, Chloramphenicol, Polymixin B and Streptomycin. Susceptibility rates for all antibacterial product indicated that all were effective (Range: 70-100%) against every isolate .When comparing the overall efficacy on all isolates Streptomycin and Erythromycin (95.6%) was the most effective antibiotic followed by Sulfisoxazole (93.5%) and Ampicillin (93.5%).In contrast Tetracycline, Polymixin B and Chloramphenicol show relatively weak efficacy with 89.1%, 89.1% and 84.7% respectively.

Table 8. In vitro antimicrobial susceptibility test result of bacterial isolates.

Isolates	N	Response to application of antimicrobial discs (susceptibility in No. and %)						
		C	STR	AMP	PB	E	SXT	TE
Staphylococcus aureus	8	6(75)	7(87.5)	6(75)	6(75)	7(87.5)	8(100)	7(87.5)
Streptococcus agalactia.	4	3(75)	3(75)	4(100)	4(100)	4(100)	4(100)	4(100)
Streptococcus dysagalactiae	3	3(100)	3(100)	3(100)	3(100)	3(100)	3(100)	3(100)
E.coli	7	6(85.7)	7(100)	7(100)	6(85.7)	6(85.7)	7(100)	7(100)
klebsiella pnumonia	5	5(100)	5(100)	5(100)	4(80)	5(100)	4(80)	4(80)
CNS	10	10(100)	10(100)	10(100)	10(100)	10(100)	10(100)	10(100)
Entroccoccus feacalise	2	2(100)	2(100)	2(100)	2 (100)	2(100)	2(100)	1(50)
Actinomycet pyogen	2	2(100)	2(100)	2(100)	1(50)	2(100)	1(50)	1(50)
Bacillus cerus	3	2(66.6)	3(100)	2(66.6)	3(100)	3(100)	2(66.6)	3(100)
Streptococcus uberis	2	0	2(100)	2(100)	2(100)	2(100)	2(100)	1(50)
Total	46	39(84.7)	44(95.6)	43(93.5)	41(89.1)	44(95.6)	43(93.5)	41(89.1)

N = Number of observations; AMP= Ampicillin; E= Erythromycin; PB= Polymixin B; STR= Streptomycin; TE= Tetracycline; SXT= Sulfisoxazole.

CNS isolates were more susceptible to all sorts of antibiotics with 100% efficacy. *Staphylococcus aureus* isolates shows 100% susceptibility to Sulfisoxazole. *Staphylococcus aureus also* 87.5% susceptibility to Erythromycin, Streptomycin and Tetracycline. It also had relatively less effectiveness to Chloramphenicol, Ampicillin and Polymixin B with 75% susceptibility. *Staphylococcus hyicus* were 100% and 50% resistance for Chloramphenicol and Tetracycline respectively.

From Streptococcus species *Streptococcus agalactiae* were the most frequently isolated pathogens that showed (100%) susceptibility to all drugs where as *Streptococcus dysagalactiae* with the exception of Chloramphenicol and Streptomycin with 75% susceptibility the remaining antibiotics shows 100% susceptibility. *Entroccoccus feacalise* shows 100% susceptibility for all except for tetracycline (50%). For the gram negative bacteria's *E.coli* and *klebsiella pnumonia*

the drug susceptibility test result shows 80-100% susceptibility reaction to all of the selected antibiotics.

The antimicrobial sensitivity test showed in this particular study is almost all milk bacterial isolates including the major pathogens had shown 75-100% susceptibility pattern. This was in agreement with Watts *et al.* (1995) showing the testing of various staphylococcal isolates obtained from heifers for susceptibility to antibiotics commonly incorporated into mastitis infusion tubes has shown that antibiotic resistance is usually low. Greater than 90% of mastitis-causing *staphylococci* species are generally killed by the drug preparations used based on in vitro sensitivity testing using zone diffusion analysis. From a practical standpoint, neither subcutaneous nor intramuscular injections of drugs have been found to cure IMI in heifers because sufficient antibiotic does not pass into the mammary gland to be bactericidal. Thus, intramammary infusion is the route of choice. Therefore the treatment of heifers known to be at risk for developing IMI is an option and is advantageous because the cure rate is much higher than that obtained when treating infections during lactation (Owens *et al.,* 2001). Reasons for this high cure rate are unclear, but the relatively small secretory tissue area of heifer mammary glands compared with mature cows might allow for greater drug concentrations in the udder of the heifer. Similarly, histological studies have demonstrated less scar tissue and abscess formation in the mammary glands of heifers compared with older cows (Trinidad *et al.,* 1990), a condition which would allow for better drug distribution and greater contact with colonized bacteria.

5. SUMMARY AND CONCLUSION

Considerable evidence suggests that Mastitis in dairy heifers in late gestation or early lactation occurs more frequently than previously assumed and some infections may be detrimental to mammary gland development, influence subsequent lactation performance, udder health and related culling hazard. The overall prevalence of mastitis in heifer at the current study was 29.1% with 9.5% clinical and 19.6% subclinical mastitis. Although mastitis in this study seems to be less prevalent than mastitis in older cows during lactation, it is still a significant prepartum and postpartum heifer disease. The most frequently isolated bacteria from quarters sample were CNS 29 (26.6%), *Staphylococcus aureus* 26 (23.9%) and *E.coli* 18 (16.5%). Other bacterial isolates were *Streptococcus agalactiae* 9(8.3%), *klebsiella pneumoniae* 8(7.3%), *Bacillus cerus* 6(5.5%), *actinomycet pyogens* 4(4.2%), *Streptococcus dysagalactiae* 3(2.75%), *Entroccoccus feacalise* 3(2.75%) and *Streptococcus uberis* 3(2.75%) with decreasing order of frequency. Coagulase-negative staphylococci cause the majority of heifer mastitis in this study. These organisms seem to have a minor impact on the future milk production and udder health, although there is a difference in virulence and persistence among CNS species. The longer infection exist and the longer they persist into lactation, and as in this study with the involvement of contagious pathogen such as *Staphylococcus aureus* the larger the impact on heifers' future udder health and milk production will be. The potential risk factors which influenced the prevalence of subclinical mastitis in the study were age, udder hygiene, mastitic milk fed to calves, and usage of waste disposal. Thus it is essential for the smallholder dairy owners in the study area to monitor the udder health, to practice adequate hygienic condition of dairy environment, good milking procedure, good animal health service and giving proper attention to health of the mammary gland status regularly and implement control strategies as required. Awareness should also be created among smallholder farmers about the economic impacts and benefits of controlling mastitis.

The antimicrobial sensitivity test showed for the majority of bacterial isolates including the major pathogens had 75-100% susceptibility pattern.CNS and *Streptococcus dysgalactiae* were the species, which showed 100% susceptibility for all of the antimicrobials tested, while the remaining species had varying levels of susceptibility (50-100%). Among isolates

Staphylococcus aureus show relatively lower susceptibility for almost all antimicrobials used. Streptomycin and Erythromycin was the most effective antibiotic followed by Sulfisoxazole and Ampicillin. The antimicrobial sensitivity test pattern indicates that treatment of pre-freshen heifer for mastitis is an option.

Therefore based on the above conclusive remarks the following recommendations are forwarded

- The transfer of mastitis-causing bacteria through cross-suckling of calves fed mastitis milk can be prevented by housing calves in individual hutches therefore farm owners should be advised to do so.
- In order to preserve overall herd health and productivity, smallholder farm owners should be advised in evaluating udders health and improving udder and farm hygiene long before heifer's calves so that it will not be too late to effectively treat the infection.
- Treatment with Both dry cow and lactating cow products have to been evaluated frequently in smallholder farms in different part of the country before practical use of antibiotics as prophylactic and control of heifer mastitis .
- Minimizing Subclinical and clinical mastitis during development of the mammary gland and in early lactation through awareness creation about heifer mastitis, subclinical mastitis and their importance might ensure future milk production, udder health and longevity and saves additional costs for veterinarians and drug.
- Risk factors related to the feeding and other management factors should be evaluated more in depth as they could be valuable in optimizing the immunity around calving, and in enhancing the natural resistance and the bacterial clearance during this period of immune suppression.

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7. APPENDICES

Appendices .1. Questionnaire format

I. Demographic structure of the farm owner (interview)

1. Date of interview_____

2. Region_____Zone_____Wored_____PA_____

3. Code of the farmer_____

4. Gender of farmer _____

5. Age _____

6. Education of household head_____

7. Occupation? _____

II. Animal factor (observable data)

8. Age _____

9. Types of breed a) local b) exotic c) cross

10. Have you ever seen an udder and teat edema? a) Yes b) No

11. Body condition score a) Poor b) Moderate c) Good d) Fat e) Extremely fat (obese)

12. Heifer status a) before calving b) after calving

13. Quarter tested LF_____LR_____ RR_____ RF_____

14. CMT score LF_____LR_____ RR_____ RF_____

15. udder/teat injury and abnormality a) present b) absent

III .Herd Factors

16. Have you ever feed mastitic milk fed? a) Yes b) No

17. Floor type? a) Concert type b) earth type

18. How often did you clean the floor? a) Once a day b) twice a day c) other

19. Udder hygiene? a) Clean b) Slightly dirty c) Moderately dirty d) Dirty

20. How often did you wash the heifers (cleaned up)? a) Once a day b) Twice a day c) other

21. Does the farm have a separate calving house? a) Yes b) No

22. How many cattle do you own? _____

23. How many cows are currently milked? _____

24. How many cows are currently not milked? _____

25. How many cows are currently pregnant? _____

26. How many calves _____, heifer_____ and oxen_____ did you have?

27. Do you use separate towel to dry udder after washing? a) Yes b) no

28. Is their close contact among calves? a) Yes b) no

29. Is their contact between heifer and adult cow? a) Yes b) no

30. Useage of animal waste disposal ? a) "fig" production b) bio gas

Appendices .2 Interpretation of CMT findings

Appendices Table 1. CMT test score and their interpretation

CMT score	Interpretation	Visible reaction	Total cell count
0	Negative	Milk fluid is normal	0-200,000 (0-25% neutrophils)
T	Trace	Slight precipitation	150,000-500,000 (30-40% neutrophils)
1	Weak positive	Distinct precipitation but not gel formation	400,000-1,500,000 (40-60% neutrophils)
2	Distinct positive	Mixture thickens with gel formation	800,000-5,000,000 (60-70% neutrophils)
3	Strong positive	Strong gel that is cohesive with a convex surface	$\geq$ 5,000,000 (70-80% neutrophils)

Source: Quinn *et al*. (2004)

Appendices .3. Procedures for the identification of mastitis pathogens

Appendices Table 2.Differential tests for isolation and identification of Mastitis Causing Staphylococcus aureus, CNS and Micrococcus species.

Tests	*Staphylococcus aureus*	CNS	Micrococcus
Catalase	+	+	-
Coagulase	+	-	-
Heamolysis	+	-	-
Manitol/f	+	-	-
Maltose/f	+	V	-
Glucose/f	+	-	-

V=variable +=positive -=negative

Source: (Quinn *et al*., 2004)

Appendices Table 3. Differential tests for isolation and identification of Streptococci species.

Species	Asculine hydrolysis	Growth on MacConkey agar	Camp test	Catalase test	Gram stain
Str.agalactiae	-	-	-	-	+
Str.dysagalactiae	-	-	-	-	+
Str.uberis	+	-	-	-	+
Ent.fecalis	+	+	+	-	+

Str.=Streptococcus ent.=Entrococcus +=positive -=negative

Source: (Quinn *et al*., 2004).

Appendices Table 4. Differential tests for the isolation and identification of *klebsiella pneumonia* and E.*coli*.

Species	Growth on MacConkey agar	Indole production	Heamolysis	Color on MacConkey agar
Klebsiella pneumoniae	+	-	-	Pink yellowish mucoid
E.coli	+	+	V	Pink dry colony

+= Positive - =negative e. coli= Escherichia coli v=variable

Source: (Quinn *et al*., 2004).

Appendices Table 5. Differential tests for the isolation and identification of pathogens causing mastitis.

Species	Shape	Catalase	Heamolysis	Growth on MacConkey agar	Colony morphology	Gram stain
Pseudomonas aeroginosa	Rod	+	+	+	Greenish blue pigment	G -
Actinomyces pyogen	Rod	-	+	-	Pinpoint colony	G+
Bacillus species	Rod	+	+	-	Gray	G+
Corenebacterium bovis	Rod	+	+	-	Small granular	G+

+ =positive - =negative G-=Gram negative G+=gram positive

Source: (Quinn *et al*., 2004)

Appendices .4 .Procedures to conduct antibiotic susceptibility test

I. Preparation of the inoculums

Inoculation of 6 to 7 distinct colony in to 5ml of saline was made first. Then the turbidity is compared with 0.5 Macfarland standard. This standard was prepared by adding 0.5 ml of 1 %(11.75g/litre) $Bacl_2$. $2H_20$ to 99.5ml of 1 % (0.36N) H_2SO_4. Inoculation to Mueller-Hinton agar

For slow growing bacteria, Streptococci and Corynebacterium species, 7% whole blood added Mueller-Hinton Agar was used. A sterile 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.

II. Disc application

Within 15 minutes (time used to dry the inoculum) after the plates were inoculated, antibiotic impregnated discs were applied to the surface of the inoculated plates by hand using a sterile forceps. All discs gently pressed down on to the agar with forceps to ensure complete contact with the agar surface. The discs were no closer than 1.5 cm to the edge of the plate and they were rest 3 cm apart from each other.

III. Incubation

The plates were incubated inverted aerobically for 24 hours at 37^0C

IV. Interpretation

Inhibition zone was measured in millimeters using a transparent ruler on the under surface of the Petri dish. For measuring purpose, the end was taken as complete inhibition of growth as determined by naked eye. The result was interpreted according to the Table presented below taken from Quinn *et al*. (2004).

Appendices Table 6. Zone size interpretive chart for antimicrobials (Inhibition Zone Diameter (mm).

Antimicrobial agent	Disc potency	Resistance	Intermediate	Susceptible
Streptomycin S10	10µg	≥11	12-14	≤15
Tetracycline TE30	30µg	≥14	15-18	≤19
Erythromycin E15	15µg	≥13	14-17	≤18
Penicillin G10 for staphylococci	10U	≥20	21-28	≤29
Penicillin G10for other microorganisms	10U	≥11	12-21	≤22
Gentamycin CN 10	10µg	≥12	13-14	≤15
Chloamphinicol C30	30µg	≥12	13-17	≤18
Polymyxin B PB30	300U	≥8	9-11	≤12
Novobiocin*	30µg	≥17	18-21	≤22
cloxacillin	30µg			
Kenamycin K30	30µg	≥13	14-17	≤18
Oxacillin	1µg	≥10	11-12	≤13

*Not applicable to media that contain blood.

Source: (Quinn *et al*., 2004).