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AEROBIC BACTERIAL ISOLATES, INCIDENCE RATE AND THE ASSOCIATED RISK FACTORS OF HEIFER MASTITIS IN AND AROUND DEBRE-LIBANOS WOREDA, NORTH SHOWA, OROMIA, ETHIOPIA

M.Sc THESIS

BY

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BISHOFTU, ETHIOPIA

AEROBIC BACTERIAL ISOLATES, INCIDENCE RATE AND THE ASSOCIATED RISK FACTORS OF HEIFER MASTITIS IN AND AROUND DEBRE-LIBANOS WOREDA, NORTH SHOWA, OROMIA, ETHIOPIA

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

By

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As members of the Examining Board of the final M.Sc open defense, we certify that we have read and evaluated the Thesis prepared by Daniel Fisseha entitled: Aerobic bacterial isolates, incidence rate and the associated risk factors in and around Debre-Libanos Woreda, North Showa, Oromia, Ethiopia and recommend that it be accepted as fulfilling the thesis requirement for the degree of: Masters of Veterinary Microbiology.

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STATEMENT OF AUTHOR

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LIST OF ABBREVIATIONS

A.pyogens:	*Actinomyces bovis*
a.s.l:	Above sea level
C.bovis:	*Corynebacterium bovis*
CI:	Confidence interval
CM:	Clinical mastitis
CMT:	California mastitis test
CNS:	Coagolase negative staphylococci
cwt:	Cow weight
DHI:	Daily herd improvement
DIM:	Days in milk
DNA:	Deoxyribonucleic acid
E.coli:	*Eschershia coli*
HF:	Holistein-fresian
IMI:	Intramammary infection
IMViC:	Indol, Methyl red, Voges-proscaur, Citrate
K.pneumoniae	*Klebsiella pneumoniae*
Kg:	Kilogram
ml:	Milliliter
mm:	Millimeter
OR:	Odds ratio
PMN:	Polimorhonuclear cell
S.aureus:	*Staphylococcus aureus*
S.chromogens:	*Staphylococcus chromogens*

S.hyicus: *Staphylococcus hyicus*

S.simulans: *Staphylococcus simulans*

SCC: Somatic cell count

St.agalactiae: *Streptococcus aureus*

St.dysagalaciae: *Streptococcus dysagalaciae*

St.uberis: *Strepyococcus uberis*

TSI: Triple sugar iron

WBC: White blood cells

ABSTRACT

Mastitis is a major disease in dairy cattle of Ethiopia. The objectives of this study were to identify aerobic bacterial pathogens, estimate the incidence of mastitis, identify the associated risk factors and assess drug resistance patterns of bacterial isolates in heifers mastitis. The study was conducted for eight months and a total of 124 quarters were examined to detect clinical mastitis and subclinical mastitis by udder physical examinations and the California mastitis test (CMT) respectively. California mastitis test positive milk samples were used to isolate and identify bacteria. The study revealed that the incidence rate of mastitis per gland month at risk was 39.45%. The incidence of mastitis was found to be affected by management system, udder hygiene, dry cow therapy and lactation stage ($p < 0.05$). The percentage of CMT positive glands that remained positive after a month and the percentage of CMT negative glands that remained negative after a month were 57.14% and 79.91% respectively. *Staphylococcus aureus* (25.11%), Coagulase negative *staphylococci* (CNS) [14.70%] and *Streptococcus* species (14.70%) were the predominant bacteria. In the present study, out of eight *in vitro* antimicrobials used; gentamycine (0%), kenamycin (0%), ampicillin (62%), penicillin G (89%), chloramphinicol (8%), Cefoxitin (16%), tetracycline (84%) and trimethoprim (8%), showed resistance to heifer mastitis pathogens. Gentamycine and chloramphinicol were the most effective antibiotics among all the tested ones. Finally due to the high resistance level detected in the study, it was believed that it is necessary to set up antimicrobial resistance surveillance programmes in the country. It was also concluded that the prevalence of *Staphylococcus aureus* warrants serious attention, the application of antibacterial agents earlier before calving may help to reduce the incidence of mastitis during the early lactation period.

Key words: Heifer mastitis, incidence rate, risk factors, bacterial isolates

1. INTRODUCTION

Mastitis is inflammation of the parenchyma of the mammary gland regardless of the cause. There is swelling, heat, pain and edema in the mammary gland in many clinical cases. However, a large proportion of mastitic glands are not readily detectable by manual palpation nor by visual examination of the milk using a strip cup; these quarters represent subclinical infections. Bovine mastitis is associated with many different infectious agents, commonly divided into those causing contagious mastitis; which spread from infected quarters to other quarters, cows and inhabit normal teat skin and those causing environmental mastitis, which are usually present in the cow's environment and reach the teat from that source. Pathogens causing mastitis in cattle are further divided into major pathogens (those that cause clinical mastitis) and minor pathogens (those that normally cause subclinical mastitis and less frequently cause clinical mastitis) (Radostits *et al*., 2006).

Mastitis is a common infectious disease in dairy cattle and is among the most economically damaging animal health problem producers face (Myllys, 1995). Traditionally heifers have been thought of as a group free of mastitis. Without appreciable lacteal secretion, there is reduced nutrient fluid available to support growth of intramammary pathogens (Fox, 2009). Most studies of intramammary infections (IMI) in heifers have been performed as single surveys to determine the prevalence of IMI at, or close to, parturition or have compared IMI several months before parturition with IMI at parturition (Fox *et al*., 1995; Myllys, 1995).

Mastitis in heifers was first recognized over 60 years ago (Palmer *et al*., 1941; Schalm, 1942). However, IMI in unbred and pregnant heifers was thought to be very low. Over 20 years ago, Oliver and Mitchell (1983) showed that a high percentage of pregnant heifer mammary glands will be infected during late gestation, at calving and during early lactation. Intramammary infections (IMI) in breeding age and pregnant heifers are much higher than previously thought. Many of these infections; can persist for long periods of

time, are associated with elevated somatic cell counts and may impair mammary development and affect milk production after calving (Trinidad *et al*., 1990a).

Mastitis in dairy heifers is recognized as a distinct problem from that in older cows because of its different pattern of disease (Barkema *et al*., 1998). Indeed, currently a high proportion of heifers freshen with an intramammary infection (IMI) (Fox, 2009) which is in flat contradiction with the once generally accepted thought that heifers' udders are sterile. Clinical and subclinical mastitis is a significant problem in primiparous dairy cattle (heifers) with a higher prevalence and incidence in heifers than cows, especially early in lactation (McDougall *et al*., 2009). The vast majority of those IMIs reveals itself as subclinical mastitis, but clinical mastitis is not uncommon either (Barkema *et al*., 1998; Compton *et al*., 2007; Nyman *et al*., 2007). Clinical mastitis is most commonly seen in lactating animals, particularly in early lactation, but may develop in the non-lactating gland also. Because the dry animal is rarely closely scrutinized and so some of these cases remain undetected until calving (Andrews *et al*., 2004).

High individual cow somatic cell counts and/or clinical mastitis in heifers result in lower production during their first lactation, long-term production loss (Woolford *et al*., 1983), increased susceptibility to clinical mastitis in the following lactation, and increased risk of premature removal from the herd (Myllys and Rautala, 1995; Rupp and Boichard, 2000; Rupp *et al*., 2000). The incidence risk of clinical mastitis, especially around the time of calving, has been shown to be higher in heifers than in cows, although the reasons for these are unclear (Hogan *et al*., 1988; Barkema *et al*., 1998; McDougall, 2009).

The prevalence of intramammary infections (IMI) in non-lactating and freshly calved heifers has been the focus of study in many countries (Myllys, 1995; Oliver *et al*., 2004). Subclinical mastitis in heifers has been detected as early as 9 months before calving (Trinidad *et al*., 1990b) and the incidence of clinical mastitis in dairy heifers during the first 14 days in lactation is high compared to older cows (Barkema *et al*., 1998). Heifers are the future milk producers of every dairy herd. Subclinical and clinical mastitis during development of the mammary gland and in early lactation are hypothesized to adversely affect their future milk production, udder health and longevity, leading to considerable financial losses for dairy farmers (Borm *et al*., 2006; Trinidad *et al*., 1990b). In Flanders,

Belgium, 35% of the heifers in early lactation are suspected to suffer from subclinical mastitis as evidenced by a composite milk somatic cell count (SCC) > 150,000 cells/ml between 5 and 14 days in milk (DIM) (De Vliegher *et al*., 2001). The early lactation period of dairy heifers is furthermore characterized by a high incidence rate of clinical mastitis (CM) as well (Barkema *et al*., 1998; Nyman *et al*., 2007; Olde Riekerink *et al*., 2008). More than 30% of the CM cases in heifers occur in the first two weeks of lactation which is substantially higher than in multiparous cows (Barkema *et al*., 1998).

In heifers, known risk factors for clinical mastitis include crosssuckling between heifers, blood in the milk (Waage *et al*., 2001), oedema of the udder and teat (Waage *et al*., 2001), leakage of milk at the time of calving (Myllys and Rautala 1995; Waage *et al*., 2001), and mechanical spread by flies (Owens *et al*., 2001).

Prevalence of IMI in breeding age and pregnant heifers is variable and can be caused by different mastitis pathogens at pre and postpartum (Fox *et al*., 1995; Waage *et al*., 1999). Generally, the pathogens that cause mastitis in heifers are the same as those that cause infections in the older cows. In all but one study reviewed, coagulase-negative staphylococci (CNS) are the most prevalent cause of subclinical intramammary infections in heifers (Fox, 2009). Majority of microorganisms that are responsible for mastitis and spoilage of milk could be *Staphylococcus aureus*, *Streptococcus agalactiae, Corynebacterium bovis*, *Mycoplasma* species, *Streptococcus uberis* (Erskine, 2001), coliforms (*Escherichia coli*, *Klebsiella* species and *Enterobacter aerogenes), Serratia, Pseudomonas, Proteus* species, environmental *Streptococci, Enterobacte*r species (Quinn *et al*., 2002). In many countries, IMI with *Streptococcus agalactiae* is still common (Zadoks and Fitzpatrick, 2009). *Staphylococcus aureus* is a common cause of mastitis on dairy farms (Olde Riekerink *et al*., 2008) and the most frequently isolated major pathogen in heifer mastitis (Borm *et al*., 2006). Prevalence of *Mycoplasma* mastitis has increased in several countries (Fox and Gay, 1993). *Mycoplasma* has been a major cause of clinical mastitis in large and expanding dairy farms in the southern United States (González and Wilson, 2003).

In Ethiopia, the total population of animals used for milk production is enormous. About 42 percent of the cattle population are milk cows managed by the private sector (ILCA, 1993), and the indigenous cattle are of poor genetic potential and their productivity is low. The largest proportion of the milk comes from these indigenous cattle while the exotic cattle and their crosses contribute small amount to the national output of milk and milk products (Alemu *et al*., 1998).In the livestock development policy to improve the per capita milk consumption, improvement of the genetic potential of the indigenous zebu through breeding with high-grade exotics is included (Asfaw, 1997). According to Lemma *et al* (2001) of the major diseases of crossbred cows in Addis Ababa milk shed mastitis will be the second most frequent disease next to reproductive diseases. The prevalence of clinical and sub clinical mastitis in Ethiopia range from 1.2 to 21.5% and 19 to 46.6%, respectively (Hussein *et al*., 1997; Kassa *et al*., 1999). However, there is lack of information on the status of heifer mastitis in the country.

There are several works on the prevalence and risk factors associated with bovine mastitis in Ethiopia (Alemu *et al*., 1998). However, information on the incidence and risk factors of heifer mastitis is scarce. Knowledge about the types of heifer mastitis causing pathogens, incidence and risk factors associated with it are prerequisite for establishing control strategies. Furthermore, knowledge regarding the persistence of IMI with different pathogens is necessary to make decisions regarding antibiotic treatment.

Therefore, the objectives of this paper are as follows:

- ✓ To assess the incidence of heifer mastitis and determine causative bacteria species in dairy farms in and around Debre-Libanos woreda.
- ✓ To identify potential risk factors for heifer mastitis.
- ✓ To evaluate the sensitivity of the bacterial isolates for different antibacterial agents.

2. LITERATURE REVIEW

2.1. Definition of mastitis

When studying the literature on mastitis difficulties are constantly encountered because the concept “normal”, “udder infection”, subclinical” and” acute mastitis” are insufficiently delineated.

According to Schalm et al. (1942) the term “mastitis” is derived from the Greek word “mastos” meaning breast and the suffix “itis” meaning inflammation of. Thus mastitis means inflammation with in mammary gland. Detailed and comprehensive definition of mastitis is given by Faull and Hughes (1985) as:

Normal quarter is a quarter with no pathogens and few neutrophils in the milk and which feels normal.

Subclinical mastitis is a quarter with pathogens and many neutrophils in the milk, but milk looks normal and quarter feels normal.

Clinical mastitis:

Acute mastitis is when there are obvious signs of inflammation of the udder such as heat, pain and swelling. The milk is macroscopically abnormal and the animal may have feverish temperature.

Subacute mastitis is when there is no obvious change in the udder but when there are persistent clots especially in the foremilk.

2.2. Etiology of mastitis

Most commonly, mastitis begins as a result of penetration of the teat duct by pathogenic bacteria (Jabb and Kennedy, 1993).However, some viral diseases like Pseudo-cowpox, Herpes Mammillitis, Cow pox, Papilloma, Foot and mouth disease and Vesicular

stomatitis affecting the epithelium of the teat orifice are mentioned to result in or predispose to mastitis. Although variation exists on the type and isolation rate of mastitis pathogens from country to country, the most commonly incriminated and reported causes of mastitis include *Staphylococcus aureus, Streptococcus agalactiae,* Coagolase negative *staphylococci* species, *Escheichia coli, Micrococcus* species, *Corynebacterium* species, *Bacillus* species, *Pasteurella* species, *Klebsiella* species, *Mycoplasma* species and *Nocardia* species (Pearson *et al*., 1979, Sears *et al*., 1991; Aaerturp *et al*., 1995).

Among the bacterial species associated with bovine mastitis, two categories are distinguished. These are major pathogens which are responsible for most severe cases of mastitis and the minor pathogens, which are rarely associated with marked leukocytocis and clinical manifestations (Rainard and Poutrel, 1988; Radostitis *et al*., 1994a).

2.2.1. Major pathogens

Agreement is not yet reached to specify the identities of major pathogens in mastitis. Usually, a combination of high prevalence, the contagious nature of intramammary infection and costly effect per case are used to determine which major pathogens are or not (Wilson *et al*., 1997). In this group S.*aureus, St.agalactiae, St.dysagalctiae, St.uberis, Mycoplasma* species and *E.coli* are included (Rainard and Poutrel, 1998; Radostitis *et al*., 1994b; Wilson *et al*., 1997).

In a study carried out in Zimbabwe by Perry *et al*. (1987) *S.aureus* was the most frequently isolated bacterium from both clinical and subclinical mastitis. Sargent *et al*., (1998) also reported types and isolation rate as *S.aureus* (6.8%), *St.agalactiae* (0.7%), other *Streptococcus* species (14.1%), Coliforms (17.2%), *C.bovis* (1.7%) and other *Staphylococcus* species (28.7%) from mastitic milk samples collected from 834 clinical cases. This study showed that other than major pathogens, minor pathogens could also be a common cause of severe cases of clinical mastitis. However according to Miline *et al*. (2002) the most frequent isolates from clinical mastitis were major pathogens including *E.coli* (19.6), *S.aureus* (14.4%), *St.uberis* (11.9%) and *St.dysagalactiae* (8.9%).

2.2.2. Minor pathogens

Some organisms, particularly non-hemolytic CNS and *C.bovis* are almost ubiquitous inhabitants of the bovine mammary gland and are regarded as part of the normal flora (Jabb and Kennedy, 1993).

There is a considerable debate as to the significance of these organisms for mammary gland and for cow productivity. Minor pathogens have been credited with maintaining a higher than normal somatic cell count (SCC) and with increasing the resistance of the colonized quarter to invasion by major pathogens. There is evidence that long term intensive programmes of teat dipping and dry cow therapy can markedly reduce the prevalence of these minor pathogens (Radostitis *et al*., 1994b) which might increase the susceptibility to these major pathogens.

Rainard and Poutrel (1988) observed that quarters initially harboring minor pathogens were significantly less infected by new major pathogens than uninfected quarter by minor pathogens. However, Hogan *et al*. (1988) compared rates of environmental *Streptococci* and Coliforms infection among quarters priory infected with either *Corynebacterium* or CNS and those not infected. He found intramammary infection rate of environmental Streptococci was 3.9 times greater in *C.bovis* infected quarter than uninfected quarters. Similarly, the rate of environmental Streptococci infection was 2.6 times greater in quarters infected with Staphylococci species than uninfected ones. No difference was observed for Coliforms.

Rainard and Poutrel (1988) suggested that to envisage infections by minor pathogens as a biological tool for controlling bovine mastitis against major pathogens, it is necessary to evaluate the economical balance between the increase in milk production resulting from a reduction in prevalence of major pathogens and the economic loss as a result of minor pathogens.

2.3. Diagnosis

Clinical mastitis is recognized by the appearance of abnormal milk, gland swelling and illness. Subclinical mastitis is characterized by normal milk and hence requires indirect tests to detect.

2.3.1. Somatic cell count (SCC)

Somatic cells are composed of white blood cells (WBC) and occasionally sloughed epithelial cells. Cells found in normal cattle milk from uninfected glands include neutrophils (1.1%), macrophages (66.68%), lymphocytes (10-27%) and epithelial cells (0-7%) (Larsen, 2000).

When bacteria invade and colonize the mammary gland, the macrophages respond by initiating the inflammatory response that attracts polymorphonuclear cells (PMN) in to the milk to engulf and destroy bacteria. More than 90% of SCC in infected glands is composed of neutrophils. The cells can be counted by a direct microscopic method on stained milk smears. The most commonly used automated device for rapid determination of SCC in milk samples is the fossomatic milk cell counter. This instrument stains cells with a fluorescent dye and then counts the number of fluorescing particles (Schalm *et al*., 1971).

Monitoring udder health status is an important principle of mastitis control. A regular quantitative assessment of udder health status is available through the use of SCC data. The practical use of SCC data to determine cow infection status requires the selection of a threshold level (Radostitis *et al*., 1994b). Dohoo and Meek (1982), however, stressed that somatic cell counts are general indicators of udder health which are subject to many factors including age, stage of lactation, season, stress and management. Mean SCC decreased markedly soon after the commencement of lactation and increased during late lactation. The basic patterns of change over lactation remain the same in health or mastitic cows (Auldist *et al*., 1995). However, Harmon (1994) argued that marked increases in SCC are a result of cells being attracted to the mammary tissue because of direct mediators produced during a local infection; events that do not affect udder healthy are unlikely to have a direct or dramatic effect on SCC. According to him little evidence

exists other than normal diurnal variation any factor did not have a major influence on SCC in the absence of intramamary infection.

To determine specificity and sensitivity of SCC (for quarters, cow or bulk milk) several studies have been conducted. Larsen (2000) reported sensitivities ranging from 73-89% with corresponding specificities of 75-85% using threshold of 200,000 cells per ml taking culture as "gold standard". Sensitivity and specificity is affected by threshold (cut point for intramammary infection). Dohoo and Meek (1982) reported 250,000 and 300, 000 cells /ml threshold to quarter and cow, respectively. Emanuelson (1997) used a threshold of 200,000 cell/ml for cow level to monitor herd mastitis in Sweden. The thresholds for milk quality have no relation to the definition of udder health categorization. At present a threshold of 100,000 cells/ml can be assumed an internationally accepted definition of udder health (Hamann, 2003). Less than 200, 000 cells/ml for cow and less than 130,000 cells/ml for bulk tank milk were reported by Larsen (2000). To the knowledge of the reviewer, no information is available on this particular issue in Ethiopia.

2.3.2. California mastitis test (CMT)

The California Mastitis Test (CMT) remains the only reliable screening test for subclinical mastitis that can be easily used at the cow side (Schalm *et al*., 1971). 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., 1999). The test results are interpreted subjectively as either a negative, trace, 1 +, 2 + or 3 + inflammatory response based on the viscosity of the gel formed by mixing the reagent with milk (Radostits *et al*., 1994b). 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 15 seconds of mixing because weak reactions will disappear after that time. 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).

2.3.3. Culture

The microbiological examination of both individual cow and bulk tank culture are elements of mastitis control. Most mastitis control programs include the use of individual cow cultures to determine which mastitis pathogens are present on the farm. 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).

There have been a number of studies to improve culture quality in identification of intramamary infection. Comparisons were made on pre and post milking samples, pre culture incubation, pre culture freezing, increased plate inoculation volumes, frequency of sampling and centrifugation (Dinsmore *et al*., 1992).

Sears *et al*. (1991) using both pre-milking and post-milking positive results as definitive diagnosis ("gold standard"), found sensitivities of 92, 86% and 99% for *Staphylococcus aureus*, coagulase negative Staphylococci and for *Streptococcus* species other than *Streptococcus agalactiae* in pre-milking milk samples, respectively. Similarly, for post-milking samples the corresponding values were 96%, 98%, and 99%. The sensitivity was higher in pre-milking samples although multiple isolates were more common. This study suggested that unless specific advantages can be demonstrated for pre-milking samples, collection of post-milking samples is recommended in order to minimize the likelihood of contamination.

Dinsmore *et al*. (1992) compared pre-culture incubation, pre-culture freezing and increased plate inoculation volumes with standard culture techniques. They found that

pre-culture incubation and larger plate inoculation volumes (0.1ml) significantly increased the recovery rate of bacterial pathogens over the standard culture method. Recovery was enhanced significantly by this method for several organisms including environmental Streptococci and Coliform bacteria. Freezing milk before culture yielded a significantly higher positive culture rate. Sensitivity of bacterial culture could be increased by including second and third consecutive samples as this overcomes the problem of cyclic shedders (Sears *et al*., 1993).

The most widely accepted criterion for the diagnosis of intramamary infection is that it exists when the same organism is isolated from two samples or two of the three consecutive samples taken every other day.

2.4. Prevalence of IMI in heifers

Mastitis in heifers was first recognized over 60 years ago (Palmer *et al*., 1941; Schalm, 1942). However, IMI in unbred and pregnant heifers were thought to be very low. Over 20 years ago, Oliver and Mitchell (1983) showed that a high percentage of pregnant heifer mammary glands were infected during late gestation, at calving and during early lactation. During the last two decades, several additional studies on the prevalence of mastitis in heifers have been published. All of these studies suggest that IMI in heifers during the prepartum period occur frequently. However, marked herd variations in the rate of IMI and types of pathogens causing IMI have been reported (Aaerstrup and Jensen, 1997; Fox *et al*., 1995; Jonsson *et al*., 1991).

Trinidad *et al*. (1990a) demonstrated that the prevalence of IMI in unbred heifers and heifers during different stages of pregnancy was very high. Unbred heifers had a higher percentage (86.7%) of infected quarters compared with the overall mean for pregnant heifers (70%). *Staphylococcus* species were observed most frequently and 8 different species were isolated. The three most common species isolated from unbred and pregnant heifer mammary glands were *Staphylococcus chromogenes*, *Staphylococcus hyicus* and *Staphylococcus aureus*. Coagulase-negative *Staphylococcus* species (CNS) accounted for 67.4% of bacteria isolated. Mammary secretions from infected mammary glands had

significantly higher SCC than secretions from uninfected mammary quarters. In addition, tissue from mammary glands of unbred heifers infected with CNS exhibited greater leukocyte infiltration and increased connective tissue compared with tissue from uninfected mammary glands (Trinidad *et al*., 1990b). Thus, infection of heifer mammary glands by mastitis pathogens can occur at a very early age and some of these infections may impair mammary growth and development and influence future milk production.

Palmer *et al*. (1941) reported that approximately 46% of heifers and 19% of quarters were infected during early lactation based on duplicate samples obtained from 382 heifers within 3 days after calving. CNS was the most prevalent bacteria isolated and were found in 22.8% of heifers and 11.4% of quarters. Matthews *et al*. (1992) indicated that 35.5% of colostrum samples were positive for 7 different *Staphylococcus* species. Species isolated most frequently were *S.chromogenes, S.aureus* and *S.simulans. Staphylococcus* species were isolated from about 18% of heifer mammary glands weekly for the first 5 weeks of lactation. Oliver and Sordillo (1988) showed that 19.7% of heifer mammary glands (59 of 300) were infected at calving and CNS caused 71.2% of these IMI. During early lactation, 15.7% of heifer mammary glands (47 of 300) were infected and 48.9% were due to CNS. Thus, the number of mammary quarters infected with CNS decreased significantly from calving to early lactation suggesting that some CNS isolated from heifer mammary glands were either colonizing the teat duct and subsequently eliminated as a result of the milking procedure or that a high rate of spontaneous elimination occurred. Similar findings were reported by Harmon *et al*. (1986) and Oliver (1988) in multiparous cows.

Oliver *et al*. (1992) conducted a study to determine the prevalence of mastitis and types of pathogens causing IMI in pregnant Jersey heifers prior to calving and during early lactation. This study was conducted in a herd that was *Streptococcus agalactiae*-negative and had a low prevalence of *S. aureus.* This pattern of infection would be typical of many dairy herds that practice postmilking teat disinfection and antibiotic dry cow therapy. Heifers (n=115) were sampled 7 days before expected calving, and 3 (C+3) and 10 (C+10) days after calving. About 90% of heifers and 61% of quarters were infected during the prepartum period. The majority of IMI (243 of 279) were due to CNS. This is

higher than what we observed previously in a study conducted in another herd (Oliver and Mitchell, 1983; Oliver and Sordillo, 1988), but types of mastitis pathogens isolated were similar. Trinidad *et al*. (1990a) also observed considerable herd-to-herd variation both in prevalence of IMI and mastitis pathogens causing IMI in unbred and pregnant heifers. For example, in one herd, 44.3% of quarters were uninfected, 12.3% were infected with *S.aureus*, 41.5% were infected with CNS and 1.9% was infected with *streptococci* other than *St.agalactiae.* In another herd, 17.6% of quarters were uninfected, 23.1% were infected with *S.aureus*, 49.5% were infected with CNS and 9.9% were infected with *Streptococcus* species. In other studies (Oliver and Mitchell, 1983; Oliver, 1988; Oliver *et al*., 1992; Oliver *et al*., 1997; Oliver *et al*. 2003), CNS were isolated most frequently followed by environmental mastitis pathogens primarily *Streptococcus* species.

Fox *et al*. (1995) reported on a large survey of 28 dairies in four states to determine the prevalence of IMI in unbred and pregnant dairy heifers and to determine potential factors that influenced herd variation. Most IMI were due to CNS and *S. aureus*. Location, herd, season, and trimester of pregnancy significantly influenced prevalence of IMI in heifers. Heifers in the third trimester of pregnancy had the highest prevalence of IMI.

Myllys (1995) indicated that CNS were the most frequently (57.8%) isolated bacteria from mammary secretions obtained from 200 heifers with mastitis and from 65 non-mastitic control heifers followed by *S.aureus* (20.1%) and *Streptococci* (11.3%). *Staphylococcus simulans*, *Staphlococcus hyicus*, *Staphylococcus xylosus* and *Staphylococcus chromogenes* were frequently found in milk from heifers with clinical mastitis after calving, whereas other CNS were equally or more often found in non mastitic control heifers.

More recently, Waage *et al*. (1999) reported results of a one-year field investigation of clinical mastitis in heifers in Norway. The study included 1,361 cases of clinical mastitis in 1,040 heifers that occurred prepartum or within 14 days after calving. Mastitis pathogens isolated most frequently from mammary quarters with clinical mastitis were *S.aureus* (44.3%), *St.dysgalactiae* (18.2%), *S.aureus* together with *St.dysgalactiae* (1.2%), CNS (12.8%), *Arcanobacterium pyogenes* (3.5%), *A. pyogenes* together with *St.*

dysgalactiae (0.5%) or *S.aureus* (0.4%), and *Escherichia coli* (6.4%). Of the CNS, *S. simulans* (53.7%), *S.hyicus* (14.8%), and *S.chromogenes* (14.8%) were the most prevalent species. Except for a higher relative percentage of *A.pyogenes* in cases that occurred before parturition (8.2%) than in cases that occurred after parturition (2.7%), no significant differences were observed in the distribution of the various organisms among prepartum and postpartum cases. Regional variations were observed in the distribution of organisms. *Staphylococcus aureus* and *A.pyogenes* clinical mastitis were highest in late autumn and early winter, CNS clinical mastitis was lowest in late autumn and early winter, and *E. coli* clinical mastitis was highest in the summer.

2.5. Significance of heifer mastitis

Heifer mastitis is much more significant in that it is here that the udder starts to develop and become efficient in producing milk. And so mastitis here determines the future milk producing capacity of the udder. To fully understand mastitis in heifers is also important to apply its controlling measures so that the udder will fully develop with the future highest production capacity possible.

2.5.1. Udder health

Based on Dairy herd improvement (DHI) data from 30 herds, coffey *et al*. (1986) concluded that for heifers the initial rank of SCC classes in early lactation (<100,000, 100,000 to 400,000, and > 400,000 cells/mL) was maintained throughout the remainder of the first and subsequent lactations. Four weekly recorded DHI data were available to study the association between the early lactation SCC, measured between 5 and 14 DIM, and test-day SCC measured after 14 DIM (De Vliegher *et al*., 2001). An increase of the test-day natural log –transformed SCC later in lactation by 0.22 units.

Minor pathogen IMI in early lactation in heifers had no effect on SCC in the first 5 milk recordings (De Vliegher *et al*., 2001). This coincides with the fact that no association was found between culture of CNS out of one or more quarters within 5 DIM and the risk of the heifer having test-day SCC > 200,000 cells/ml within the same lactation (Compton *et*

al., 2007). In contrast, isolation of *S.aureus* or environmental pathogens from one or more quarters within 5 days postpartum increased the risk of the heifer having test-day SCC >200,000 cells/ml in that lactation (Compton *et al*., 2007).

2.5.2. Milk production

Heifers with a first test-day SCC <100,000, between 100,000 and 400,000, and 400,000 cells/ml produced 6452, 6050, and 5696 kg milk during first lactation, respectively (Coffey *et al*., 1986), indicating a likely effect of IMI at calving on future production. An increase of the natural log-transformed SCC measured between 5 and 14 DIM by one unit was, on average, associated with a decrease in milk yield of 0.13 kg/d later in lactation SCC of 50,000 cells/ml measured at 10 days in milk, was estimated to produce 119 kg and 155 kg more milk during its first lactation than heifers with an early lactation SCC of 500,000 cells/ml and 1,000,000 cells/ml, respectively.

Intramammary infections with CNS in heifers in early lactation had no effect on average milk production during early to mid-lactation, suggesting that IMI due to CNS could be considered harmless (De Vliegher *et al*., 2001). Compton *et al*. (2007) reported that milk yield at first production recording and average daily milk yield over the entire lactation were even higher in heifers with IMI due to CNS postpartum than in heifers with no pathogens isolated. A possible explanation could be that heifers infected with CNS are protected against clinical mastitis (Matthews *et al*., 1992), which may indirectly result in a higher milk production. Alternatively, high producing heifers may be more sensitive to IMI with CNS that low producing heifers.

2.5.3. Culling

Nearly 11% of heifers that were treated for clinical mastitis before calving or within the first 14 DIM were culled within one month after treatment (Waage *et al*., 2001). The main culling reason of 96% of these heifers was mastitis. Udder health problems were the culling reason for 10% of the culled heifers in the study from De Vliegher *et al*. (2001). For each unit increase in the log-transformed SCC in early lactation the culling hazard increased by 11%. Isolation of major pathogens between calving and 5 DIM was associated with a 60% increased risk of removal from the herd during first lactation

(Compton *et al*., 2007). Fertility problems were the most common reason for removal, whereas mastitis was given as a reason for removal for only 6 heifers.

2.6. Heifer mastitis risk factors

Several potential heifer mastitis risk factors have been identified. In an epidemiological survey on 171 dairy farms from five regions of Spain, Martin-Richard *et al*. (2001) found that risk factors for heifer mastitis were calving in summer, high herd SCC, presence of *S. aureus* and *Mycoplasma* species, absence of fly control, feeding calves mastitic milk, contact among calves, absence of antibiotic therapy to heifers, contact with adult cows, inadequate milking practices and poor housing conditions. Other heifer mastitis risk factors identified include an increase in the incidence of clinical mastitis in a herd, a decrease in the bulk tank SCC, an increase in herd mean milk yield, calving in late spring or early summer, 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 presence of pathogens on heifer body sites (Roberson *et al*., 1998). 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).

Studies have provided convincing evidence that the horn fly (*Hameotobia irritans*) is an important vector in the transmission of *S.aureus* mastitis in heifers. *Hameotobia irritans* can be colonized with *S.aureus* during feeding activities and can remain colonized for several days with substantial numbers of organisms present. When *S.aureus* colonized horn flies were allowed to feed on teats of uninfected dairy heifers, IMI with the same *S. aureus* DNA fingerprint subtype resulted (Gillespie *et al*., 1999). This indicates that the horn fly can transmit *S.aureus* to heifer teats if a sufficient source of organisms is present. That source was shown to be present in existing scabs on teat ends of heifers (Owens *et al*., 1998). High concentrations of *S. aureus* (>107 colony forming units/mg) were found in scab material present on heifer's teats. When uncolonized flies were allowed to feed on

this material they became colonized with *S.aureus* just as readily as flies that had fed on experimentally infected blood. Thus, a vector shown capable of transmitting infection is readily present. When a source of *S.aureus* exists such as scabs on heifer teats; the potential for passage of IMI from heifer to heifer via horn flies exists. The threshold number of flies needed to transmit IMI is unknown. However, since fly populations can rapidly increase to several thousand per animal under favorable conditions, the need for early fly control on dairy heifers is apparent. 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.

2.7. Antimicrobial susceptibility

Considerable evidence suggests that IMI in pregnant heifers occurs frequently and that some infections may be detrimental to mammary gland development and influence subsequent lactational performance. Methods of controlling mastitis in heifers may eliminate or markedly reduce the deleterious effects of prepartum infections. One common denominator of all studies on heifer mastitis is the high prevalence of CNS IMI. Trinidad *et al*. (1990d) demonstrated that 90% of 311 *Staphylococcal* isolates (primarily CNS) from heifer mammary glands were susceptible to antibiotics *in vitro*. Some variability to antimicrobial susceptibility of bacteria obtained within and among herds was noted; however, in general, bacteria were highly susceptible to all antibiotics evaluated. Oliver *et al*. (2003) determined minimum inhibitory concentrations of penicillin, cloxacillin, cephapirin, ceftiofur, novobiocin, enrofloxacin, erythromycin and pirlimycin against 1494 microorganisms isolated from heifer mammary glands. The majority of *Staphylococcus* species were susceptible to the antimicrobial agents evaluated. However, antimicrobial susceptibility was variable for *Streptococcus* species and poor against Gram-negative enteric organisms. These data suggest that antibiotic therapy may be an effective means of eliminating *Staphylococcus* species IMI that have been shown to cause the majority of IMI of heifer mammary glands.

2.8. Strategies for controlling heifer mastitis

As explained earlier heifer mastitis is much more significant than mastitis in cows in that it is here that the udder starts to develop and become efficient in producing milk. And so mastitis here determines the future milk producing capacity of the udder. Therefore it is mandatory to develop heifer mastitis controlling strategies.

2.8.1. Antibiotics

Peripartum treatment of heifer mammary glands with antibiotics involves treatment of heifers at a time when animals are not generally worked with, would be an additional step in the management of heifers, and also might require restraining facilities for safe treatment that may not be readily available. A more "user friendly" approach would likely benefit the majority of producers that are interested in controlling mastitis in heifers, and this might enhance adoption of techniques shown to be effective for controlling mastitis in heifers.

Evaluation of a more "user friendly" approach was conducted to determine efficacy of intramammary antibiotic therapy of heifer mammary glands following the first milking after parturition on mastitis during early lactation and to determine if antibiotic treatment influenced lactational performance of heifers (Oliver *et al*., 2003). Jersey (n = 43) and Holstein heifers (n = 36) from two dairy research herds were assigned to one of three treatment groups: 1) no intramammary infusion following the first milking after parturition, 2) intramammary infusion of all mammary glands with pirlimycin hydrochloride following the first milking after parturition, and 3) intramammary infusion of all mammary glands with novobiocin sodium plus penicillin G following the first milking after parturition. Almost 93% of Jersey heifers (40 of 43) and 73.1% of quarters (125 of 171) were infected at the first milking after parturition. Of the mammary quarters infected at parturition, 76.7% (33 of 43) were cured following treatment with pirlimycin, 61.8% (21 of 34) were cured following treatment with penicillin-novobiocin, and 39.6% (19 of 48) were uninfected in the untreated negative control group. Significantly fewer infections were observed in pirlimycin or penicillin-novobiocin treated mammary glands of Jersey heifers during early lactation than in untreated control mammary glands.

Almost 89% of Holstein heifers (32 of 36) and 52.8% of quarters (76 of 144) were infected at the first milking after parturition. Of the mammary quarters infected at parturition, 57.1% (12 of 21) were cured following treatment with pirlimycin, 41.4% (12 of 29) were cured following treatment with penicillin-novobiocin, and 23.1% (6 of 26) were uninfected in the untreated negative control group. Significantly fewer infections were observed in pirlimycin treated mammary glands of Holstein heifers during early lactation than in untreated control mammary glands. However, no significant differences in infections during early lactation were observed following penicillin-novobiocin treatment of Holstein heifers after the first milking of lactation compared to untreated control quarters. Coagulase-negative staphylococci and *Streptococcus* species, primarily *St.uberis* and *St.dysgalactiae*, were isolated most frequently in heifers from both herds. The SCC score of milk from Jersey heifers during the first 90 days of lactation was significantly lower in pirlimycin treated heifers than in untreated controls. However, no significant difference was observed in the SCC score of milk from penicillin-novobiocin treated heifers when compared to untreated controls. In addition, there was no evidence of an antibiotic treatment effect on milk production in Jersey heifers during the first 90 days of lactation. Thus, a more "user friendly" approach for controlling mastitis in heifers based on intramammary treatment following the first milking after parturition did not appear to be as effective as prepartum antibiotic treatment of heifer mammary glands. Differences are likely due to the timing of antibiotic treatment, persistence of antibiotics in mammary tissue and secretions, and the time when new IMI occur in heifers during the periparturient period.

Another strategy that has received considerable research attention is based on intramammary treatment of heifer mammary glands with a dry cow antibiotic formulation during different trimesters of pregnancy (Owens *et al*., 1991; Owens and Ray, 1996; Trinidad *et al*., 1990c). Mammary quarters of 35 breeding-age and primigravid Jersey heifers were infused with a non-lactating cow antibiotic formulation containing penicillin/streptomycin. Thirty-eight breeding age and primigravid Jersey heifers served as untreated controls (Trinidad et al., 1990c). Of the 35 treated heifers, 34 (97.1%) were infected at time of treatment. In the untreated control group, all 38 heifers (100%) were infected at treatment time. At parturition, prevalence of IMI in treated heifers decreased

to 40%, whereas prevalence in the control group remained about the same (97.4% of heifers). Prevalence of *S.aureus* mastitis in treated heifers was reduced from 17.1% to 2.9% after treatment. In the control group, prevalence of *S.aureus* mastitis decreased from 26.3% to 15.8%. Heifers treated during the second trimester of pregnancy had the greatest reduction in prevalence of mastitis. Results of this study suggest that intramammary treatment of primigravid heifers during pregnancy was effective in reducing prevalence of mastitis and SCC at parturition.

However, efficacy of prepartum antibiotic therapy at 7 or 14 days prior to expected calving (Oliver *et al*., 1997; Oliver *et al*., 2003) was considerably higher than that reported by Trinidad et al. (1990c). This could be due, in part, to the time when heifers were treated with antibiotics, differences in the pathogens causing IMI, and the time when IMI occur. In support of this hypothesis, Fox *et al*. (1995) indicated that the prevalence of heifer IMI was highest during the last trimester of pregnancy. Thus, methods of controlling mastitis in heifers would likely be more effective if administered during the last trimester of pregnancy as opposed to early gestation.

In another study, a non-lactating cow antibiotic formulation containing cephapirin benzathine was evaluated in pregnant and non-pregnant Jersey heifers for its effect on experimentally induced *S.aureus* mastitis (Owens *et al*., 1991). Cephapirin was detectable in mammary secretion of non-pregnant heifers for up to 5 weeks and in tissue for 1 week after intramammary infusion. *Staphylococcus aureus* was not detectable in tissue and secretion of treated quarters at 1 and 3 weeks but was not eliminated from two quarters of one heifer tested at 6 weeks after treatment. Histologic evaluation of mammary tissue from non-pregnant heifers revealed significant differences in leukocytosis between uninfected and *S.aureus* infected mammary quarters but no differences in epithelium, lumen, and stroma, indicating no difference in secretion potential or glandular development. Pregnant Jersey heifers (n=25) were experimentally infected in two mammary quarters with *S.aureus* 12 to 14 weeks prepartum. After 1 to 3 weeks, 13 heifers were infused in 21 *S.aureus*-infected mammary quarters with a commercial cephapirin formulation approved for use in non-lactating cows. Nine infected mammary quarters were left untreated. All treated mammary quarters were bacteriologically

negative both at calving and through 2 months after calving. Of the 9 infected mammary quarters not treated prepartum, 1 spontaneously cured and 2 became non-functional. The remaining quarters were treated at calving with a commercial cephapirin formulation approved for use in lactating cows. Of these, 3 cured and 3 failed to resolve. Heifers with cured *S.aureus* IMI produced 16.4 kg of milk per day while heifers that remained infected with *S.aureus* produced 14.5 kg of milk per day, or 11% less (Owens *et al.,* 1991).

In a much larger study, 233 dairy heifers were treated 0 to 90, 90 to 180, or 180 to 270 days prepartum with one of five different antibiotic formulations for use in non-lactating cows to determine the best time to treat and the most effective product to use (Owens *et al*., 2001). At the initial sampling, 56.5% of mammary quarters were infected and 15.4% of mammary quarters were infected with *S.aureus*. Treatments included a commercially available cephapirin dry cow product, a commercially available penicillin-novobiocin dry cow product, a commercially available penicillin-streptomycin dry cow product, an experimental dry cow product containing tilmicosin, and a cephalonium dry cow product not available in the United States. Cure rates for the five antibiotic products were equally effective against *S. aureus* and all were significantly more effective than the spontaneous cure rate observed in untreated control mammary quarters. Furthermore, no differences in efficacy were observed due to the different treatment times prepartum. However, fewer new *S. aureus* infections occurred after treatment in the third trimester of pregnancy. Fox *et al*. (1995) indicated that the prevalence of heifer IMI was highest during the last trimester of pregnancy. Thus, methods of controlling mastitis in heifers would likely be more effective if administered during the last trimester of pregnancy as opposed to early gestation.

2.8.2. Vaccinations

Vaccination as a method to control mastitis in heifers also been conducted, however, results of those studies are equivocal. Nordhaug *et al*. (1994) used 108 heifers in a placebo-controlled multicenter study to evaluate an experimental *S.aureus* mastitis vaccine containing whole, inactivated bacteria with pseudocapsule, alpha and beta toxoids, and a mineral oil as adjuvant. Heifers were injected in the area of the supramammary lymph nodes twice before calving. None of the vaccinated animals

developed clinical *S.aureus* mastitis, and 8.6% developed subclinical *S.aureus* mastitis. Conversely, 16.0% of control heifers developed clinical or subclinical *S.aureus* mastitis. Mean SCC in vaccinated and control heifers were the same throughout lactation. In the statistical analyses, when cow was used as the unit of concern, no significant differences occurred between groups. However, when all parameters on udder health were considered together, results indicated a potential protective effect of this vaccine during the entire lactation. More recently, Nickerson *et al*. (2000) suggested a positive effect of vaccination with a polyvalent *S.aureus* vaccine by increasing anti-staphylococcal antibody titers and in preventing new *S.aureus* infections when the program was initiated at an early age in heifers from a herd with a high exposure to *S.aureus*.

A placebo-controlled field study was performed to evaluate the effect of a herd-specific vaccine against *S.aureus* on IMI, SCC, and clinical mastitis (Tenhagen *et al*., 2001). Heifers in the vaccination group (n = 164) were vaccinated twice at 5 and 2 weeks before expected calving. Heifers in the control group (n = 157) received the same treatment with a placebo containing no bacterial antigen. The prevalence of *S.aureus* in quarter milk samples taken at calving and 3 to 4 weeks after calving did not differ significantly between the vaccine and control group. Incidence of clinical mastitis during the first 3 months after calving and the prevalence of *S.aureus* in quarter milk samples taken before the onset of treatment did not differ significantly between groups. The SCC was lower in vaccinated than in control heifers. However, the difference was only significant on the third milk test day. Use of a herd-specific vaccine against *S.aureus* did not prove to be effective on this farm.

Thus, based on the few studies that have been reported, data are equivocal regarding efficacy of vaccination for the prevention of mastitis in heifers. One significant advantage of strategies based on vaccination is that this is a non-antibiotic approach for controlling mastitis and potential problems associated with antibiotic residues and antibiotic resistance are avoided. One important disadvantage, however, is that vaccination is pathogen specific. Since mastitis in heifers is caused by many different pathogens, vaccination against a single pathogen will not eliminate IMI caused by pathogens not targeted in the vaccine.

2.8.3. Peripartum teat disinfection

Another approach for controlling mastitis in heifers was based on prepartum teat disinfection with a germicide barrier teat disinfectant (Edinger *et al*., 2000). The effect of teat dipping with a barrier teat dip prior to parturition on IMI and clinical mastitis during the first 5 days postpartum was investigated in a split udder trial in 149 Holstein-Frisian heifers. Two mammary quarters were dipped three times weekly with a barrier teat disinfectant containing 0.1% polyvidon iodine from day 260 of gestation until parturition, and the remaining mammary quarters served as untreated controls. Bacteria were isolated from 52.2% of quarter milk samples collected immediately after parturition prior to first machine milking. *Staphylococcus aureus* and CNS were isolated most frequently (29.2 and 35.6% of the positive samples, respectively). At parturition, 6.7% of heifers showed signs of clinical mastitis and another 27.5% developed signs of clinical mastitis during the first five days of lactation. No significant differences in IMI and clinical mastitis were found between mammary quarters dipped in the barrier teat dip prior to parturition and undipped control quarters. Authors concluded that teat disinfection prior to parturition in primigravid dairy heifers did not improve udder health in this trial (Edinger *et al*., 2000).

2.9. Economic implications of antibiotic treatment

Prepartum antibiotic treatment to reduce the rate of mastitis in Jersey heifers during early lactation was economically beneficial (Oliver *et al*., 2003). Actual milk production averaged 11,429 pounds (5,195 kg) for untreated heifers and 12,597 pounds (5,726 kg) for antibiotic treated heifers. Multiplying the increase in actual milk production (1168 pounds, 531 kg) in prepartum antibiotic-treated heifers by a milk price of $18.50/cwt ($0.407/kg) yielded a $216.24 per-heifer increase in gross revenue. Treatment costs of $15.60 were as follows: teat hygiene ($0.10) which included the cost of a pre-milking teat disinfectant, barrier postmilking teat disinfectant and disposable paper towel; antibiotics ($10.00); and labor ($2.50). Another cost that may arise is the cost of testing for antibiotic residues in milk of heifers that calve too soon after treatment that we estimated to be $3.00. Subtracting the cost of treatment ($15.60/heifer) from gross revenue resulted

in a net revenue increase of $200.64 per heifer. These net revenue figures included the cost of testing for antibiotic residues for all antibiotic-treated heifers.

Break-even analysis indicated that it would be profitable to treat heifers before calving as long as the milk price was above $0.013 per pound or $1.30/cwt ($0.029/kg). Milk price would not likely fall low enough to make treatment of prepartum heifers unprofitable. A similar relationship between the increase in net revenue and the hourly wage rate of labor was determined. Given a milk price of $0.185/pound ($0.407/kg), net revenue is equal to zero where the hourly wage rate of labor equals $812.56 per hour. This suggests that treating heifers with antibiotics before calving would be profitable for wage rates below $812.56/hour. The relationship between net revenue increases and the increase in pounds (kg) of milk produced due to treatment, given a wage rate of $10.00/hr and a milk price of $0.185/pound ($0.407/kg) was determined also. Treatment would be profitable as long as the increase in milk production is greater than 84 pounds (38.2 kg; Oliver *et al*., 2003).

3. MATERIALS AND METHODS

3.1. Study Area

Debre-Tsige is a town of Debre-Libanos Woreda located 89 km from Addis Ababa. Debre-Libanos is part of the Semien Shewa Zone bordered on the North West by Gerar jarso, on the south west by Yaya Gulele, on the south and south east by Wuchale and on the north east by Amhara Region. The administrative center of Debre-Libanos is Debre-Tsige. Debre-Libanos District is located 9^{o} 48N and 38^{o} 44E at an altitude of 1500-2700m a.s.l; its minimum and maximum temperature vary from 19^{o}C to 23^{o}C. It gets bimodal rain fall that ranges from 800-1200mm and the predominant soil types are black soil (56%) and red soil (38%).Two agro ecologies are found in the area and mixed agricultural activities are performed. According to Debre-Libanos woreda Livestock office, there are about 80,796 head of cattle, 84,507 goats, 23,723 sheep, 10,899 equines, 1,894 camels and 75,305 poultry in the districts. All of these livestock species are reared mainly by smallholder farmers under intensive, semi intensive and extensive production system (Debre-Libanose Woreda Livestock Production, Marketing and Health Office, 2013).

The study was also conducted in Fitche/Selale, North Shewa zone of Oromia region, Ethiopia. Fitche/Selale is the town of North Shoa zone as well as Girar Jarso woreda. The woreda lies along the highway to Amhara National Regional State in the North Western direction at a distance of 112 km from Addis Ababa. It shares border with Amhara Region in the North, Yaya Gullalle woreda in the East, Debre-Libanos woreda in the South and Degem woreda in the West. Girar Jarso woreda has an altitude of 1300 to 3419 meters above sea level and astronomically the woreda occupies 9035'-10000'N latitude and 38039'-38039'E longitude. The high land areas of the woreda have a bimodal pattern of rainfall with a long rainy season from June to September and a short rainy season from March to April. According to Fiche Station meteorological data the average rainfall amount of the woreda is about 1200 mm and maximum and minimum rainfall is about 1115mm and 651mm, respectively. Temperature of the woreda ranges

from a minimum of 11.50c to a maximum of 35^0C (Girar Jarso Woreda Livestock Production, Marketing and Health Office, 2013).

Mukaturi is a town of Wuchale Woreda located 78 km north-west of Addis Ababa.Wuchale is bordered on the south by Berehna Aleletu, on the west by Mulona Sululta, on the northwest by Yaya Gullalle and Debre-Libanos on the northeast by the Amhara Region, on the east by Abichu gne'a, and on the southeast by Kembibit. Agro-ecologically, the woreda is categorized into three: Dega, Woina-Dega and Kolla constituting 87%, 11% and2% of the total area of the woreda respectively. Its altitude ranges from 1000-3000 m.a.s.i. The annual mean rain fall reaches 1028mm. The mean maximum and maximum temperatures are 11.23^0C and 20.86^0C, respectively. The climate of the area is favorable for crop and livestock production like the neighboring woredas (Wuchale Woreda Livestock Production, Marketing and Health Office, 2013).

3.2. Study design

A longitudinal type of study in which heifers were followed in three physiological status; before calving, at first lactation and second lactation stage, was conducted from November 2013 up to May 2014 to determine the incidence of heifer mastitis, identify the associated risk factors, isolate and identify bacterial species and evaluate antimicrobial sensitivity in the study area.

3.3. Study animals

The study animals were 31 pregnant heifers that were left with few weeks to give birth and were followed for the first two lactation stages after calving. Two breeds of heifers from small holder and commercial dairy farms (HF and jersey crosses) in and around Debre-Libanos District were included in the study. Twenty of them were from Debre-Libanos woreda while 6 and 5 heifers were from Wuchale woreda and Grar Jarso woreda,

respectively. The heifers were managed intensively by small holder dairy farms while they were managed semi-intesively under commercial farms.

3.4. Sample size and Sampling method

Purposive sampling technique was used in this study and 31 pregnant heifers left with few weeks to calve were included in the study. Numbered ear tags or local names were used to identify the pregnant heifers in each herd. The heifers were restrained and secretion samples for microbiological tests were taken from open udder quarters. The assessment of open or closed udder quarters was done using the method described by Oliver *et al*. (2003). It involved applying light pressure to the teat sinus by using a gentle milking action with the thumb and index finger, allowing the contents to slip upward within the teat. When the pressure applied by this action resulted in a drop of secretion at the orifice, the teat was classified as being 'open', otherwise as 'closed'. Only open quarters were sampled. Due to the small volume of secretion presented in most glands, foremilk secretion was not discarded strictly before collection of the sample *ante partum.* After calving samples were taken twice representing the first and the second stage of lactation. During sampling foremilk secretion was discarded before taking the samples.

3.5. Data collection

3.5.1. Questionnaire survey

Semi-structured questionnaire were developed and pre-tested, and all information relating to the study objectives were recorded. Data were collected on potential risk factors for the occurrence of mastitis in heifers based on observation and by interviewing the farm owners. The animal level factors such as herd size, presence of teat lesion, teat blindness, body condition and breed difference were recorded. The farm level factors such as housing types, farm hygiene, barn floor status, type of milking method, use of towels,

milking sequences and hygiene were also recorded. Udder and milk abnormalities (injuries, blindness, tick infestation and indurations, swelling, milk clots, abnormal secretion, etc.) were also recorded.

3.5.2. Physical examination of the udder

Heifers with clinical mastitis were identified first by examining the udder visually and then through palpation to detect possible fibrosis, inflammatory swellings, visible injury, tick infestation, atrophy of the tissue, heat, pain and/or hardness and swelling of supramammary lymph nodes (Biffa *et al.,*2005). Viscosity and appearance of milk secreted from each mammary quarter were examined for the presence of clots, flakes, blood and watery secretions. Rectal temperature of those cows with clinical mastitis was taken to check systemic involvement (Andrews *et al.,* 2004).

3.5.3. California Mastitis test

The California Mastitis test (CMT) was carried out as screening test for subclinical mastitis and for selection of samples for culture. Subclinical mastitis was diagnosed based on 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. In case of subclinical mastitis, a squirt of milk sample was placed in each of the cups on the CMT paddle from each quarter of the udder and an equal amount of 3% CMT reagent was added to each cup and mixed well. Reactions were then graded as 0 and trace for negative samples, or +1, +2 and +3 for positive ones. Quarters with CMT score ≥+1 were judged as positive (Quinn *et al*., 1999). Heifers were considered positive, when at least one quarter is screened out to be positive using CMT. But during the first sampling, CMT was found to be not effective method to detect mastitis as the sampling volume from pregnant heifers was very minute and the content (keratin like) was not milk and all the samples at this stage of sampling were directly cultured and culture positive samples were recorded as CMT positive.

3.5.4. Milk sample collection

Milk samples were collected according to the procedure recommended by Quinn *et al.* (1994). Strict aseptic procedures were followed when collecting milk samples in order to prevent contamination with microorganisms present on the skin udder and teats, on the hands of samplers and on the barn environment. Teat ends were cleaned and disinfected with ethanol (70%) before sampling. Strict foremilk (first jets) was discharged to reduce contamination of teat canal (Quinn *et al.,* 1999). Sterile universal bottles with tight fitting cups were used to collect milk sample. The universal bottles were labeled with permanent marker before sampling. To reduce contamination of teat ends during sample collection, the near teats were sampled first and then followed by the far ones. During sample collection the collecting universal bottle was held as horizontal as possible and by turning the teat to the near horizontal position; about 10 ml of milk sample was collected from each quarter of the udder into the universal bottle. Then the samples were properly packed and transported in an ice box to Addis Ababa University, Collage of Veterinary Medicine and Agriculture's Microbiology laboratory. Samples that were not immediately processed were refrigerated at +4°C for 24-72 hours.

3.5.5. Bacteriological isolation and characterization

Milk samples were bacteriologically examined according to the procedures employed by Quinn *et al*. (1999). In refrigerated milk samples, bacteria may be concentrated in the cream layer and held with in clumps of fat globules (Quinn *et al.,* 1999). Hence dispersion of fat and bacteria was accomplished by warming the samples at 25°C for15 minutes and shaking before plating on a standard bacteriological media. A loopful of milk sample collected from each infected quarter was inoculated separately on to MacConkey agar and blood agar base enriched with 7% defibrinated sheep blood. The inoculated plates were then incubated aerobically at 37°C for 24 to 48 hours. When growth was not observed after incubation for 24 to 48 hours, the quarter's milk sample was inoculated on an enriched tryptone-soya broth to amplify the bacterial growth. 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 O-F tests. *Staphylococci* were identified based

on catalase test, growth characteristics on Mannitol salt agar and purple agar and slide coagulase test. Identification of *Streptococci* were made only according to growth characteristics on blood agar, gram reaction, cellular arrangement and catalase test due to unavailability of Edward`s media. Gram negative isolates grown on MacConkey agar were identified based on growth characteristics on MacConkey agar, Oxidase reaction, catalase test, triple sugar iron (TSI) agar and the "IMViC" (indole ,methyl red, Voges-Proskaur and Citrate) test (Quinn *et al*., 1999).

3.5.6. Antimicrobial sensitivity testing

The antimicrobial resistance profiles of the bacterial isolates were determined using Kirby-Bauer-disk diffusion method (Quinn *et al.,* 1994). The antibiotic impregnated disks that were used for the test are Trimethprim, Ampicillin, Chloramphenicol, Cefoxitin, Kanamycin, Penicillin, Gentamycin and Tetracycline. Disks were stored under refrigeration at 4ºc to ensure maintenance of their potency. Single bacterial colonies of the same morphologic type were inoculated into 7 ml of Tryptophan soya broth and incubated at 37 ºC for 8 hours until turbidity was seen and which was compared to the 0.5 McFarland standards. Müeller-Hinton Agar for less fastidious bacterial isolates and 5% sheep blood added Müeller-Hinton Agar for *Streptococcus* species isolates were used as planting medium. Fifteen minutes after inoculation of the plates, the antibiotic impregnated disks were applied on the surface of inoculated plates with sterile forceps. All the disks were gently pressed down onto the agar with forceps to ensure complete contact with the agar surface. The plates were inverted and then incubated aerobically for 18 hours at 37 ºC. The diameters of the zone inhibition were measured to the nearest whole millimeter using the transparent rule. Zone of inhibition for individual antimicrobial agents were translated into susceptible, intermediate and resistant categories by referring the recommended Quinn *et al.* (2005) interpretative standard.

3.5.7. Data storage and analysis

All the research findings were stored in Microsoft Excel and prepared for analysis. The incidence rates were estimated at gland level and expressed in terms of gland month at risk according to Thrusfield (Thrusfield, 2007). Chi-square test and univariate logistic

regration were used to evaluate risk factors. The data was analyzed using Epi Info ™ (version 3.5.1., center for disease control, USA). Statistical significance was set at a P value of ≤ 0.05.

4. RESULTS

4.1. Gland infection dynamics

A total of 31 heifers were sampled at three stages to estimate the incidence rate; the overall incidence rate of sub clinical mastitis per gland month at risk was 37.33% while incidence rate of subclinical mastitis per gland month at risk at the first and second lactation stage was 52.63% and 21.62% respectively (Table 1).

Table 1: Incidence of subclinical mastitis per gland month at risk by lactation stage

Lactation stage	**Gland month**	**Number of new infection**	**Incidence Rate (%)**
First	38	20	52.63
second	37	8	21.62
Total	75	28	37.33

The percentage of CMT positive glands that remained positive after a month and the percentage of CMT negative glands that remained negative after a month were 57.14% and 79.91%, respectively (Table 2).

Table 2: Changes in gland infection status between CMT positive and negative quarters

Preceding CMT results	**No. of quarters**	**Gland status after a month**	
		No. of positive (%)	No. of negative (%)
Positive	35	20 (57.14)	15 (42.86)
Negative	89	25 (28.08)	50 (79.91)

The occurrence of new infection in heifers was not significantly affected by gland position, tick infestation, teat lesion and breed ($p>0.05$) but by management system, lactation stage, udder hygiene and dry cow therapy ($p<0.01$) (Table 3).

Table 3: Potential risk factors associated with the incidence of subclinical mastitis.

Risk factors	**Categories**	**Gland months**	**Incidence rate**	**X^2**	**P- value**
Gland position	Front	60	18	0.21	0.66
	Rear	24	6		
Management	Intensive	94	53	5.15	**0.005**
	Semi intensive	40	21		
Lactation stage	First	38	**52**	7.15	**0.007**
	Second	37	21		
Udder hygiene	Clean	84	34	6.25	**0.006**
	Dirty	33	5		
Dry cow therapy	Yes	75	15	7.18	**0.005**
	No	28	4		
Tick infestation	Yes	80	22	0.28	0.650
	No	48	15		
Teat lesion	Yes	66	20	0.13	0.719
	No	62	17		
breed	HF-Cross	33	8	1.33	0.513
	Jersy cross	59	20		

The incidence rate was about four times higher in heifers kept under intensive management system than heifers under the semi intensive management system (RR=3.94; 95% CI= 1.29 – 11.97) and it is two times higher in first lactation stage than mid lactation

stage (RR= 1.89; 95% CI= 1.02 – 6.89).Furthermore, incidence rate was two times higher in heifers that were not prophylactically injected with dry cow regimen than heifers that were not injected (RR= 2.02; 95% CI = 1.28 – 9.87).The incidence rate was also three times higher in heifers with dirty udder hygiene than in heifers with clean udder hygiene (RR = 3.02; 95% CI = 2.01 - 8.06) (Table 4).

Table 4: Univariate logistic regression of risk factors for mastitis at heifer level

Factors	**Categories**	**No**	**No.of +ve**	**p-value**	**OR**	**CI**
Gland position	front	11	8(72.72%)	0.143	2.34	0.333 - 0.589
	rear	20	10(50%)			
management	intensive	22	18(81.81%)	**0.032**	3.94	1.29 – 11.97
	Semi-intensive	9	2(22.22%)			
Lactation	First	19	14(73.68%)	**0.005**	1.89	1.02 – 6.89
stage	second	12	8(66.66%)			
Udder hygiene	Clean	15	7(73.68%)	**0.001**	3.02	2.01 – 8.06
	dirty	16	10(62.5%)			
Dry cow	Yes	9	3(33.33%)	**0.007**	2.02	1.28 – 9.87
therapy	No	22	14(63.63%)			
Tick	Yes	18	12(66.66)	0.28	4.97	1.68 – 4.56
infestation	No	13	6(46.15)			
Teat lesion	Yes	22	14(63.63%)	0.078	3.34	0.098 -1.08
	No	9	11.11%)			
Breed	HF-cross	22	14(63.63%)	0.164	2.84	1.96 - 2.68
	Jersy cross	9	3(33.33%)			

4.2. Bacterial species isolated from heifer mastitis

The result of bacteriological culture of milk samples collected from 31 heifers showed the involvements of 231 bacteria, belonging to 9 genera, in heifer mastitis. Both contagious and environmental bacteria were isolated from milk samples collected from the heifers during pre-calving, first lactation and second lactation stages. The predominant bacteria were *Staphylococcus aureus* (25.11%) followed by *Streptococcus* species (14.70%), *CNS* (14.70%). From both clinical and sub clinical heifer mastitis cases *S.aureus,* CNS and *Streptococcus* species were predominant bacteria (Table 5).

Table5: Summary of bacterial isolates from heifers with clinical and subclinical mastitis

Organisms	**Number of isolates**			
	Clinical mastitis	SubClinical mastitis	number	proportion
Staphylococcus aureus	11(4.76)	47(20.34)	58	25.11%
CNS	6(2.59)	28(12.12)	34	14.70%
*Streptococus*species	6(2.59)	28(12.12)	34	14.70%
*Micrococcus*species	-	24(10.38)	14	10.38%
Klebsiella pneumonae	-	23(9.95)	23	9.95%
Escherichia coli	5(2.16)	25(10.82)	30	12.98%
Corynebacterium Species	-	13(5.62)	13	5.62%
Enterobacter aerogens	-	10(4.32)	10	4.32%
*Bacillus*species	-	10(4.32)	10	4.32%
Total	28	203	231	100%

4.3. *In vitro* Antimicrobial susceptibility profiles

Antimicrobial sensitivity tests were done for all the isolates (Table 6). *S.aureus isolates were* highly sensitive to gentamycine (100%), trimethoprim (92%), kenamycin (90%) and Cefoxitin (80%); whereas the bacteria were resistant to penicillin G (89%), ampicillin (62%) and teteracycline (84%). *Streptococcus* species were sensitive to gentamycine (96%), chloramphenicol (94%), Cefoxitin (92%) and kanamycin (73%) while they were resistant to ampicillin (82%), teteracycline (81%) and penicillin G (72%). *CNS* was sensitive for chloramphinicol (100%), teteracycline (93%), gentamycine (92%) and Cefoxitin (70%) while it was resistant to trimethoprim (80%), kanamycin (72%) and ampicillin (72%). *E.coli* was sensitive to chloramphenicol (100%), gentamycine (92%), Cefoxitin (90%) and kenamycine (75%) while it was resistant to penicillin G(79%), ampicillin (75%) and teteracycline (65%). *Enterobacter aerogens* was almost sensitive to all antimicrobial disks applied. *Corynebacterium*species on the other hand was also sensitive to almost all antimicrobial disks except for Cefoxitin (33%) and trimethoprim (16%). *K.pneumonae* was also sensitive to almost all antimicrobial disks applied except for ampiciliin (35%) and trimethoprim (25%). *Bacillus* species was susceptible to gentamycine (100%), kanamycin (85%), ampicillin (71%) and tetracycline (71%) while it was resistant to penicillin G (80%) and Cefoxitin (54%). The last isolate,*Micrococcus* species was susceptible to kenamycine (100%), chloramphenicol (75%), Cefoxitin (75%) and gentamycine (72%) while it was resistant to ampicillin (75%), trimethoprim (65%) and tetracycline (62%) (Table 6).

Table 6: Antimicrobial susceptibility patterns of bacterial isolates in percent

Bacterial isolates	No	Kanamycin			Ampicillin			Penicillin G			Chloramphinicol			Cefoxitin			Tetracycline			Trimethoprim			Gentamycine		
		R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
S. aureus	58	0	10	90	62	16	22	89	6	5	8	0	92	16	4	80	84	0	6	8	0	92	0	0	100
E. coli	30	25	0	75	75	0	25	79	0	21	0	0	100	5	5	90	35	0	65	65	0	35	8	0	92
CNS	34	72	8	2	72	0	28	50	0	50	0	0	100	30	0	70	0	7	93	80	3	17	8	0	92
Streptococcus species	34	24	3	73	82	0	18	72	0	18	6	0	94	8	0	92	81	3	16	65	0	35	4	0	96
Enterobacter aerogens	10	0	0	100	25	0	75	10	0	90	0	0	100	25	0	75	0	0	100	25	0	75	0	0	100
Corynebacterium species	13	17	0	83	10	7	83	33	0	67	33	0	67	70	7	33	17	0	83	80	4	16	50	0	50
Klebsilla pneumonia	23	4	0	96	65	0	35	25	0	75	0	0	100	20	0	80	11	0	89	75	0	25	0	0	100
Bacillus species	10	15	0	85	29	0	71	80	6	14	37	0	63	54	0	46	20	9	71	40	19	41	0	0	100
Micrococcus species	38	0	0	100	75	0	25	75	0	25	25	0	75	25	0	75	62	7	31	65	0	35	28	0	72
Mean (%)		18	3	79	55	3	42	57	2	41	12	0	88	28	2	70	35	3	62	56	3	41	11	0	89

No-number of isolates; R-resistant; I-intermediate; S-susceptible

5. DISCUSSION

5.1. Gland infection dynamics

The effort made to determine the incidence rate of heifer mastitis in the present study revealed that the incidence rate of mastitis was higher in heifers kept under the intensive management system compared to heifers under semi- intensive management system. In support of this observation higher risks of mastitis with restricted exercise (Waage *et al*., 1998; Gustafson, 1993) and incombined housing management of heifers with multiparous cows (De Vliegher *et al*., 2001) were reported. This could be due to restricted exercise of animals that may facilitate the transmission of pathogens from infected cows to primiparea as animals were housed together.

Incidence rate of mastitis was also higher in heifers at first lactation stage than in heifers with mid lactation stage. This result was agreeable with a report made by De Vliegher *et al*. (2001) who reported that the occurrence of mastitis is twice likely in the first lactation than the second lactation stage. This could be due to increase in number of immature neutrophils in bovine blood and decreasing of mature neutrophils in the blood and mammary secretions resulting in to relaxed intramammary defence mechanisms. Neutrophils populations also exhibit impaired function of major defense-related activities, such as phagocytosis, respiratory burst activity, superoxide anion production, random cellular migration and chemotaxis around parturition (Kehrli *et al.*, 2004). Absence of dry cow therapy regimen could possibly be the major factor contributing to higher incidence at early lactation associated with delayed diapedesis of neutrophils into the mammary gland (Kehrli *et al.*, 2004).

Heifers that were not treated during dry period were more affected by mastitis than those treated at dry period and there was statistical difference between treated and untreated groups. This agrees well with a report made by Fox *et al.* (2009) who reported that incidence of mastitis is twice higher in untreated heifers than the treated ones. This difference could be associated with low bactericidal and bacteriostatic quality of milks and mammary gland 15 days before and 15 days after calving. Moreover, the capacity of

the quarter to provide phagocytic and bactericidal activity generally diminishes during this period (Ismail *et al*., 2005).

The other risk factor found in this study to increase incidence of heifer mastitis was udder hygiene. Heifers with dirty udder hygiene had higher incidence of mastitis than heifers with clean udder hygiene. This agrees well with Waage *et al*. (2001) who reported higher incidence of mastitis (three times) in animals with dirty udder hygiene than in animals with clean udder hygiene. This may be due to increased exposure and transmission of pathogens from the dirt material during milking (Kivaria *et al*., 2004).

Negative quarters could remain negative for a relatively longer period compared to positive quarters. The likelihood that quarter not infected in the preceding month could remain negative in the subsequent month was about twice higher than the likelihood that an already infected gland could be free of infection. The result is suggestive evidence that the application of antibacterial agents before the expected date of calving could help to reduce the incidence of mastitis during the early lactation period. The application of antimicrobials approximately two months peripartum was reported to have reduced the incidence of intramammary infections by 59% and treatments in the third trimester generally reduced the occurrences of new peripartum intramammary infections and persisted upto calving with cure rates ranging from80-100% for *Staphylococci* and *Streptococci* (Nickerson *et al*., 1995).

In this study subclinical mastitis has been found to be higher in heifers than clinical mastitis. This could be attributed to the little attention given to subclinical mastitis while treating clinical cases. Moreover, farmers in Ethiopia are not well informed about the silent cases of mastitis (Kassa *et al*., 1999). A similar observation of dominance of subclinical mastitis was observed by several studies both in heifers and older cows (Nickerson *et al*., 1995; Workineh *et al*., 2002; Kerro and Tarek, 2003; Sori *et al.,* 2011).

5.2. Bacterial isolates

The result obtained from bacteriological analysis of the samples revealed that the predominant organisms isolated from heifer milk samples were *Staphylococcus aureus* followed by *CNS* and *Streptococcus* species. This is comparable with the observations of Waage *et al*. (1999).The predominance of these bacteria is due to frequent colonization of teats as they are commensals of the skin. They can easily get access to the teat canal during milking or suckling and can be transmitted from quarter to quarter and from cows to heifers during milking practices. Their ability to exist intracelullarly; especially *Staphylococcus aureus* and *Streptococcus* species, and localize within micro-abscessation in the udder and hence, resistant to antibiotic treatments (MacDonald, 1997) could also be important factor contributing to the predominance of these organisms. However the proportion of *Streptococcus* species in this study is greater than that of Waage *et al*. (1999) and Miline *et al*. (2002) who found *Streptococcus* species to be 53.55% and 45.5%, respectively. The result of *CNS* in the current study is much lower than the findings of Oliver *et al*. (1997) (42%) and is comparable to the result of Miline *et al.* (2002), which was reported as 10%.CNS are regarded as minor pathogens and normal inhabitants of bovine mammary gland and usually are mentioned in association with a slight increase in SCC (Rainard and Poutrel, 1988). However, recently increased proportion of CNS was isolated from bovine and other dairy animal's mastitic milk samples (Ameh *et al.*, 1993; Boscos *et al.*, 1996). Some studies indicated that CNS could be pathogenic and even cause more mastitis than *S. aureus* (Almaw and Molla, 2000).

The other predominant bacterial species isolated were the coliforms. This finding is comparable with the results of Oliver *et al*. (1997), in which the coliforms accounts for 28.85% and were the third predominant pathogens from dairy cows in southern Ethiopia. *Escheishia coli* were the predominant bacteria among the coliforms in this study which is in consent with the observation of Waage *et al*. (1999) and Miline *et al*. (2002) who reported 14.6% and 13.14%, respectively. In this study, *Klebsiella pneumniae* is the second predominant bacteria isolated among the coliform which is in agreement with Waage *et al*. (1999) who reported a proportion of 11.32%. Furthermore, the prevalence of *Entrobacter aerogens* in the present study may be due to poor hygienic conditions in the

herds and this infection is becoming more and more frequent and tends to follow the infection of S*taphylococcus* species (Waage *et al*., 1999).

The prevalence of *Bacillus* species in the present study is comparable with a result of Waage *et al*. (1999) who reported prevalence of 5.36%. Udder infection as a result of *Bacillus* species could be due to environmental factors like soil water and manure; these are the main source of bacteria when animals are exposed to the above environmental factors, bacteria like *Bacillus* enter via teat canals.

5.3. *In vitro* antimicrobial sensitivity test

The *in vitro* antimicrobial sensitivity test showed that gentamycine is the most effective drug followed by chloramphinicol and kenamycine in the study area. Actually these antibiotics are the least frequently used drugs; especially in large animal practice in Ethiopia. According to Rediet *et al.* (2012), the development of antibiotic resistance nearly always has followed the therapeutic use of antimicrobial agents. Similar results have been published by Rediet *et al*. (2012) in support of this study who reported both gentamycine and chloramphinicol to be the drug of choice in Adama town, Ethiopia consecutively. Similar findings have been reported by Sumahti *et al*. (2008) in which gentamycin was found to be most effective antibiotic. The effectiveness of gentamycine detected in this study also agrees with the findings of Quinn *et al*. (2005) who asserted that gentamycine is effective drug against *S.aureus, E.coli* and environmental *Streptococcus* species.

In contrast to these findings, it has been reported that 64% of the isolates, from mastitis milk were sensitive to teteracycline and 52.8% to ampicillin by Nesru *et al*. (1999) which is different from the present study's finding. Recently on the other hand, it has been reported that the highest number of *S.aureus* and *CNS* isolated from bovine mastitis were susceptible to ceftriaxone by Sumathi *et al*. (2008). However, *Staphylococcus aureus* and *Streptococcus* species showed the existence of resistance to penicillin G and tetracyclinein this study. In contrast to gentamycine and chloramphinicol, these two antibiotics are the most widely used in many parts of Ethiopia in large animal practice.

They are sometimes the only available and affordable antibiotics in many veterinary clinics. It might be this wide use of these drugs and inappropriate administration that contributed to the development of resistance by the predominant bacterial agents in the area.

6. CONCLUSIONS AND RECOMMENDATIONS

The present study was conducted with the aim of determining the incidence rate of heifer mastitis at different stages of lactations and it is recognized that high incidence of heifer mastitis occurred in the first lactation than in mid lactation stage. *S.aureus* and *Streptococcus* species have been identified as the predominant causes of heifer mastitis in the study area. Though, *CNS* and environmental pathogens also played a significant role as causative agents. Udder hygiene, lactation stage, dry cow therapy and management system have been found as a potential risk factors of heifer mastitis in the study area. Most frequently used and sometimes the only available drugs in veterinary medicine such as tetracycline and penicillin G have been found to be resisted by different bacterial isolates while drugs that are not usually used in veterinary medicine; gentamycine and chloramphinichol, have been found to be effective. Based on the above conclusions the following recommendations are forwarded:

- Farmers' education should be conducted on basic farm and animal health management systems like milking procedures, housing and animal hygiene, dry cow therapy and early peripartum udder health which is essential for control and prevention of heifer mastitis should be conducted.

- Due to the high level of resistance detected in the present study, it is believed that it is necessary to implement permanent resistance surveillance programmes in the country.

- The prevalence of *Staphylococcus aureus* warrants serious attention and the application of antibacterial agents earlier before calving that may help to reduce the incidence of mastitis during the early lactation period should be followed.

- Appropriate use of antibiotics and other options when available must be followed by the veterinarians.

- The availability of antibiotics variety should be increased in the country's veterinary medicine practice so that first line of drug of choice would be used as one way of minimizing antibiotics resistance.

- Antibiotics found to be effective should further be studied to know their optimum dosage and to change them into an appropriate intamammary infusion drug form.

- Further research on heifer mastitis must be done in other study areas.

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8. ANNEXES

Annex 1. List of some medias and their preparation used for bacterial isolates.

1.MacConkey Agar (MCA) (HiMedia, India)

Ingredients g/l: Peptic digest of animal tissue 20.00 gm, Lactose 10.00 gm, Bile salt 5.00 gm, sodium chloride 5.00 gm, neutral red .07 gm, Agar 15.00 gm, distilled water 1000.00 ml, Final pH (at 25^{o}C) 7.5 + 0.2.

Preparation: Suspended 55.07 gm of dehydrated MCA in 1000 ml distilled water and sterilized by autoclaving at 15 psi pressure, 121^{o}C for 20 minutes. The molten medium was cooled to about 50^{o}C temperature and poured into sterile Petri plates.

2.Brilliant Green Agar (BGA) (Titan Biotech Ltd., Bhiwadi, India)

Ingredients (g/l): Proteose peptone10.000, Yeast extract, 3.000, Lactose, 10.000 Sucrose10.000, Sodium chloride, 5.000, Phenol red, 0.080, Brilliant green, 0.0125, Agar20.000, Final pH (at 25°C)6.9±0.2.

Preparation: Suspend 29.045grams in 500 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. AVOID OVERHEATING. For more selectivity, aseptically add rehydrated contents of 1 vial of Sulpha Supplement (FD068). Mix well before pouring into sterile Petri plates.

3. Xylose-Lysine Deoxycholate Agar (XLD Agar) (Titan Biotech Ltd., Bhiwadi, India)

Ingredients (g/l) Yeast extract**,** 3.000L-Lysine**,** 5.000**,** Lactose7.500**,** Sucrose**,**7.500**,** Xylose**,** 3.500**,** Sodium chloride**,** 5.000**,** Sodium deoxycholate**,** 2.500**,** Sodium thiosulphate**,** 6.800**,** Ferric ammonium citrate**,** 0.800**,** Phenol red**,** 0.080**,** Agar**,** 15.000**,** Final pH (at 25°C)7.4±0.2.

Preparation: Suspend 56.68 grams in 1000 ml distilled water. Heat with frequent agitation until the medium boils. Do not autoclaveor overheat. Transfer immediately to a

water bath at 50°C. After cooling, pour into sterile Petri plates. It is advisable notto prepare large volumes that will require prolonged heating, thereby producing precipitate

4. Nutrient Agar (Oxiod, England)

Ingredients (g/ l): Peptone, 10.000, Beef extract 10.000, Sodium chloride 5.000 Agar, 12.000, pH, after sterilization 7.3±0.1

Preparation: Suspend 28.0 grams in 1000 ml purified/distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

5. Triple Sugar Iron Agar (TSIA) (HiMedia, India)

Ingredients (g/l): Beef extract 3.000 Peptones (Casein and Beef) 20.000, Yeast extract 3.000, Lactose monohydrate 10.000 Sucrose, 10.000 Glucose monohydrate 1.000, Ferric ammonium citrate 0.300, Sodium chloride, 5.000, Sodium thiosulphate, 0.300 Phenol red, 0.025, Agar, 12.000 pH after sterilization (at 25°C) 7.4±0.2

Preparation: Suspend 64.0 3 grams (the equivalent weight of dehydrated medium per liter) in 1000 ml purified /distilled water. Heat to boiling to dissolve the medium completely. Mix well and distribute into test tubes. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes or as per validated cycle allow the medium to set in sloped form with a butt about

6. Phenol Red Agar (Difco, France)

Ingredients (g/l) Proteose peptone 10.000, Beef extract 1.000, Sodium chloride 5.000, Phenol red 0.018 Final pH (at 25°C) 7.4±0.2.

Preparation: Suspend 16 .02 grams in 1000 ml purified/ distilled water,mix well. Heat if necessary to ensure complete solution. Distribute in fermentation tubes (tubes containing inverted Durham's tubes). Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Aseptically add filter sterilized or autoclave sterilized carbohydrate solution to sterile basal medium.

7. MR-VP Medium (HiMedia, India)

Ingredients g/l: Buffered peptone, 7.000, Dextrose,5.000, Dipotassium phosphate, 5.000Final pH (at 25°C)6.9±0.2.

Preparation: Suspend 17 grams in 1000 ml of distilled water. Heat if necessary to dissolve the medium completely. Distribute in test tubes in 10 ml amounts and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

8. Mueller Hinton agar (Oxoid, England)

Ingredients g/l: Casein acid hydrolysate 17.50, Beef heart infusion 2.00, Starch, soluble 1.5, Agar 17.00 gm, Final pH (at 25°C) 7.3 +0.2.

Preparation: Suspended 38 gm in 1000 ml distilled water. Sterilized by autoclaving at 15 psi pressure, 121oC for 20 minutes. The molten medium was cooled to about 50oC temperature and poured into sterile Petri plates.

Annex 2. Questionnaire format in English

1. The respondent's identification data

1.1. Heifer`s owner name…….

1.3. Address:kebele……house no……

1.4. Date of interview…….

1.5. Name of interviewer…….

1.6. Questionnaire no……

2. Heifer`s history

2.1. Heifer ID or local name…….

2.2. Breed: Holiestain cross…..jersey cross….others (specify)……

2.3. Age of the heifer…….

2.4. Body condition score: poor…..good…..very good…..

2.5. Herd size of the small holder farm……

2.6. Average milk yield of the herd per day……

3. Milking procedures

3.1. Udder preparation: whole udder wash….teat wash…..none…..

3.2. Hand preparation: with soap wash……without soap…..none…..

3.3. Udder towel: no towel…..individual…..common…..

3.4. Mastitic cow milking: at last…..in between…..first…..

3.5. Heifer milking: first…..middle…..last…..

3.6. Pre and post teat dipping: present…..absent…..

4. Management

4.1. Farming system: intensive…..semi-intensive…..extensive…..

4.2. Type of housing: closed…..open…..

4.3. Barn floor type: concrete…..rammed soil…..stone…..

4.4. Bedding: yes…..no…..

4.5. Separate calf unit: yes…..no…..

4.6. Separate feeding area: yes…..no…..

4.7. Separate milking area; yes…..no…..

4.8. Feeding calves mastitic milk: yes…..no…..

5. Hygiene of barns and heifers

5.1. Cleanliness of barns: very clean…..clean…..dirty…..

5.2. Heifer with dirt: udder…..perineum…..clean….

5.3. Teat disinfection before heifer calves: yes…..no…..

5.4. Presence of excessive number of flies: yes…..no…..

6. Clinical inspection

6.1. Udder or teat lesion: yes…..no…..

6.2. Udder consistency: normal…..fibrotic…..

6.3. Tick infestation: present…..absent…..

6.4. Gross milk quality: watery…..blood tinged…..clot/flakes…..normal…..

6.5. Dry cow therapy of the heifer: yes…..no…..

6.6. Heifer`s milk leakage: yes…..no…..FR, FL, HR, HL

6.7. CMT result: FL…..FR…..HL….. HR…..

6.8. Reproductive disorders on the heifer such as retained foetal membrane,endometritis,pyometra : yes…..no….

Annex 3. Questionnaire format in the local language (Oromiffa)

1. Tesoo gaafiwaan dihatee kan debiisu

1.1. Maqqaa abaa radaa…….

1.2. Tesoo: Ganda…….Lakk. mana……..

1.3.Guyyaa gaafii rawatee…….

1.4. Maqqaa gaafii dhiyeesse…….

1.5. Lakk. Gaffanoo……..

2.**Seena radaa**

2.1. Maqqaa ykn. Lakka radaa……..

2.2. Gossa radaa: kan biyya alla……..kan biyaa kessa……

2.3. Ogaa ukn. Umrii radaa…….

2.4. Qamma radaa: Olaana……bayyee ollana……Gadii anaa……..

2.5. Bayyaiina loonii…….

2.6. Omiisha ananii gidu gallessa sa`a tokko irra. Lita/day……

3. Tartiiba anaan itii elmmamu

3.1. Qophii harmma:muchaa hundaa qulqqullessu……fixee harmaa qoffa……..

Homma osso immichiin ukn hinqqulqullessea elemu……

3.2. Qophi harkka: samuna dhan diqachuu……..bishaniin qoffa….osso hin diqqatiin……

3.3. Huchuu(foxaa) kan harmma itiin qulqqulessu:kan fayadamu…….tokko tokkon sa`af

Fayyadamu……..waliitii kan fayyadamu…..

3.4. Dhibee muchaa sa`aa qabamee Eelmu:dhummara….gidugallessa….jalqqabaa…..

3.5. Ra`ada Eelmmamtu:jalqqabaa irra…gidugallessa irra…..dhummara…….

3.6.Eelmma dhuraa fi Eelmma boda muchaa ishee chemicallan chubuu:jirra….hin jiiru…..

4. Kununssa lonii

4.1. Malla kununssa horssissa loonii sana:manatii hidanii kununssu…..mana fi allatii

Kununssu……..allatii quffa olchuu……

4.2. Gossa manaa:chuffa…..banaa…..

4.3. Gossa laffa manichaa:leshoo…..biyyoo….dakka……..

4.4. Affaa laffa: jiira….hinjiiru…….

4.5. Jabeellen iddo horiin Eelmamtu:qaba….hinqabu…….

4.6. Anaan dhukubaa harmmatiin falamee jabiif ni fayadammu…..hin fayyadamu……..

5. Qulqquliina mana fi radda

5.1. Qulqquliina mana loonii: bayye qulqulu…… qulqulu…..xura`a…….

5.2. Qulqquliinaa radaa:muchaa irra……milla irra……qulqqullu…….

5.3. Osso hindalliin dura qullqquliin radaa muchii ishee ni efamma: Eeye…lakii…….

5.4. Tassisnii bayyiinaan ni argama….ykn hin argammu……

6. Bu`aa qorannoo

6.1. Madaa muchaa irra ykn fixaa muchaa irra jirra……hinjiiru……

6.2. Muchii diytaa: qaba……..hin qabu

6.3. Silmii: qaba……hinqabu……

6.4. Qulqquliina ananii:bishan fakata…..diqaa makata….wal. qabaata…..inititaa……

6.5. Radnii Eergaa omiishaa dhabdee boda yalii muchaa ni godamma:Eeye…..lakke…..

6.6. radni anaan dagallassu: jira…….hinjiru……yoo jiratee kan fundura mirgga….

Kan fundura bitta….kan dubaa mirgga…..kan dubaa bitaa…..

6.7. Qoranooananii(CMT):kan fundura mirgga….Kan fundura bitta….

kan dubaa mirgga…..kan dubaa bitaa…..

6.8. Rakko da`umssa irra jiruu :jira……hinjiiru…….

Annex 4.Check list format used for recording data

Heifer IDNo_____owner's name _____ Breed_____ age _____

First visit	FR		FL		HR		HL		remark
Date	Clinical	Subclinical	Clinical	Subclinical	Clinical	Subclinical	Clinical	Subclinical	
2^{and} visit	FR		FL		HR		HL		remark
Date	Clinical	Subclinical	Clinical	Subclinical	Clinical	Subclinical	Clinical	Subclinical	
3rd visit	FR		FL		HR		HL		remark
Date	Clinical	Subclinical	Clinical	Subclinical	Clinical	Subclinical	Clinical	Subclinical	
4th visit	FR		FL		HR		HL		remark
	Clinical	Subclinical	Clinical	Subclinical	Clinical	Subclinical	Clinical	Subclinical	

Annex 5. Procedures to conduct antibiotic susceptibility test

Source: Quinn et al . (2005)

Preparation of the inoculum

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 20to 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.

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.

Incubation

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

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 . (1999).

Zone size interpretive chart for antimicrobials

Antimicrobial agent	**Disk potency**	**Resistant ≤**	**Intermediate**	**Susceptible ≥**
Gentamycin	10 µg	12	13-14	15
Kanamycin	30 µg	13	14-17	18
Penicillin G for Staphylococcus	10 units	20	21-28	29
Penicillin G for others	10 units	11	12-21	22
Teteracycline	30 µg	14	15-18	19
Sulphamethoxazon	10 µg	10	11-15	16
Chloramphinicol	30 µg	12	13-17	18
Cefoxitin	30 µg	14	15-20	21
Ampicillin for staphylococcus	10 µg	28	29-31	32
Ampicillin for others	10 µg	15	16-19	20

*Not applicable to media that contain blood