1113

ADDIS ABABA UNIVERSITY
FACULTY OF VETERINARY MEDICINE

BOVINE MASTITIS AND ANTIBIOTIC RESISTANCE PATTERNS OF MAJOR PATHOGENS IN SMALL HOLDER DAIRY FARMS IN THE CENTRAL HIGHLANDS OF ETHIOPIA

A thesis submitted to the school of Graduate Studies of Addis Ababa University in partial fulfillment for the degree of Master of Science in Tropical Veterinary Medicine

By
GETAHUN KASSAYE WOLDYES

JUN 2006
DEBRE ZEIT ETHIOPIA

BOVINE MASTITIS AND ANTIBIOTIC RESISTANCE PATTERNS OF MAJOR PATHOGENS IN SMALL HOLDER DAIRY FARMS IN THE CENTRAL HIGHLANDS OF ETHIOPIA

BY

GETAHUN KASSAYE WOLDYES

Board of Examiners **Signature**

Academic Advisers:

Dr. Kelay Belihu. _____

Dr. Merga Bekana. _____

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ANNEXES

LIST OF ABBREVATIONS

AADPA	Addis Ababa Dairy Producers Associations
ARDU	Arsi Rural Development Unit
CADU	Chilalo Agriculture Development Unit
CMT	California Mastitis Test
CNS	Coagulase Negative Staphylococci
ICSCC	Individual Cow Somatic Cell Count
IMI	Intra Mammary Inflection
LF	Left Front
LR	Left Front
MIC	Minimum Inhibitory Concentration
NCCL	National Committee for Clinical Standards
RF	Right Front
RR	Right Rear
SCC	Somatic Cell Count
SSA	Sub-Saharan Africa

ACKNOWLEDGEMENTS

First of all, I would like to extend my gratitude to my advisors Dr. Kelay Belihu and Dr. Merga Bekana, for their wise advice, guidance, and contributions towards the preparation, execution and realization of this thesis.

I gratefully acknowledge the Girar-Jarso Wereda Rural and Agricultural Development Office for offering me the sponsorship during my postgraduate study.

I would like also to thank the Sebeta National Animal Health Research Center, and the staff of microbiology department: Dr. Mesfin Sahile, Ato Melaku Somba, W/ro Letabirhan Yimesgen, Ato Africa Tefera and Ato Dereje Shegu for all technical assistance.

Above all, my profound thanks goes to the farmers for their willingness to provide their farms as research grounds. I would like also to express my gratitude to Dr.Bekele Werede, Ato Gizachew Degefu, from Mullo-Sullulta Rural and Agriculture Development Office, for their unlimited support to collect milk samples and conduct a questionnaire survey in the study area. I also thank Dr. Fekadu Regassa, and Dr. Ademe Zerihun, for technical and material assistance.

ABSTRACT

The study was conducted with the objectives of determining the prevalence of clinical and subclinical mastitis, identifying the major pathogens of bovine mastitis and testing antimicrobial resistance of the major pathogens isolated from quarter milk samples in smallholder dairy farms in the central highlands of Ethiopia. For this study, 109 smallholder farmers and 500 dairy cows were selected by a one-stage cluster sampling. Questionnaire survey was administered to the farm owners to collect data on cow and farm attributes. Clinical examination, CMT and bacteriological culturing were performed to diagnose the occurrence of clinical and subclinical mastitis. Data collected was analyzed using descriptive statistics and univariate and multivariate logistic regression. The results revealed that the prevalence of clinical mastitis at herd, cow and quarter level after culturing was 11%, 2.6% and 0.51%. The prevalence of subclinical mastitis at herd, cow and quarter level was 54.7%, 22.3% and 10.06%, respectively. The most important pathogens isolated from subclinical mastitis cases were *S. aureus* (46.6%), *S. epidermidis* (22.8%), *St. agalactiae* (11.1%) and *St. uberis* (10.1%), whereas those of clinical mastitis were *St. agalactiae* (30%), *St. dysgalactiae* (30%) and *S. aureus* (20%). The univariate logistic regression showed that, among the risk factors considered, presence of teat lesion ($p<0.01$), stage of lactation ($p<0.05$) and parity number ($p<0.05$) had significant effect on the prevalence of subclinical mastitis, whereas only presence of teat lesion ($p<0.01$) and stage lactation ($p<0.05$) had significant effect with multivariate logistic regression. None of the farm attributes considered as potential risk factors (barn floor status, milking hygiene and milking mastitic cows at last) had significant effect ($p>0.05$). In addition, the prevalence of subclinical mastitis at herd level was not significantly affected by all the factors considered (farm attributes). Regarding the antimicrobial susceptibility test, *St. intermidius* and St. dysgalactiae were the species, which showed high level of susceptibility for most of the antimicrobials, while the rest had different levels of resistance for almost all the antimicrobials. From the antimicrobials applied sulphonamides was the most effective drugs, while ampicilin was the least effective drug. From this study it can be concluded that the prevalence of subclinical and clinical mastitis

were low and moderate, respectively, in the study area. *S. aureus*, was found to be the major isolate and many of the pathogens showed resistance to commonly used drugs.

Key words: smallholder, dairy, clinical, subclinical, mastitis, prevalence, isolates, antimicrobials

1. INTRODUCTION

The world's human population is expected to increase from 5.4 billion in 1990 to approximately 7.2 billion in 2010. Most of the increase will be in tropical developing countries, where there will be a marked shift from rural areas to urban centers, with major shifts in patterns of food production, marketing and consumption (Sére *et al.*, 1996). Dairy production is a biologically efficient system that converts large quantities of roughage, the most abundant feed in the tropics, to milk, the most nutritious food known to man. Where there is access to a market, dairying is preferred to meat production since it makes more efficient use of feed resources and provides a regular income to the producer. It is also more labor intensive and supports substantial employment in production, processing and marketing (Walshe *et al.*, 1991). The challenge represented by the expanding demand for milk and dairy products in tropical countries is great, and the resultant opportunities for smallholders are large. However, low animal productivity, inappropriate technologies, inadequate research and extension support, poor infrastructure and unfavorable external conditions have contributed to the poor performance of the livestock sector in general, and of the dairy sub-sector in particular (Williams *et al.*, 1995).

The economic losses of dairy industry due to mastitis remain the major cause all over the world. According to the study carried out in England and Wales from 1979 to 1982, the average cost of a case of mastitis due to antibiotics used, milk discarded, reduction in quality and quantity of milk produced by a cow was estimated 60 pound for each case (Blowey, 1990). Based on the research works, most estimates show that on the average the affected quarter suffers a 30% reduction in productivity and affected cow estimated to loss 15% its production (Blood and Radostits, 1989).

Milk from mastitic animals is also of a great public health importance by serving as a vehicle in the spread of such diseases as tuberculosis, streptococcal sore-throat, scarlet fever, staphylococcal food poisoning and brucellosis (Blood and Radostits, 1989). Public health hazards associated with the consumption of antibiotic contaminated milk and milk

products cause allergic responses, changes in intestinal flora and development of antibiotic-resistant pathogenic bacteria (Thirapatsakun, 1999).

Ethiopia holds large potential for dairy development due to its large livestock population, the favorable climate for improved high-yielding animal breeds, and the relatively disease-free environment for livestock. Given the considerable potential for smallholder income and employment generation from high-value dairy products, development of the dairy sector in Ethiopia can contribute significantly to poverty alleviation and nutrition in the country. The first effort, initiated by the governments of Ethiopia and Sweden, was the establishment of the Chilalo Development Agricultural Unit (CADU), later named Arsi Rural Development Unit (ARDU), between 1970 and1980. The unit produced and distributed crossbred heifers, provided artificial insemination (AI) services and animal health service, in addition to forage production and marketing (Staal, 1995). Later on SDDPP (1981-1994) and SDDP (1995–1998) contributed for the development of the dairy sector in the central highlands of Ethiopia. The two projects distributed crossbred dairy cattle with a package of improved management technologies to smallholder dairy farmers and also promoted the establishment of small scale milk collection and processing centers which are connected well to market infrastructure. The dairy sector in Ethiopia is expected to continue growing over the next one to two decades given the large potential for dairy development in the country, the expected growth in income, increased urbanization, and improved policy environment. The shift towards market economy is creating large opportunity for private investment in urban and peri-urban dairying (Felleke and Geda 2001; Gebre Wold *et al.* 2000).

According to the reports of FAO (2003), the total annual national milk production in Ethiopia ranges from 797,900 to 1,197,500 metric tones raw milk equivalents. Out of total national milk production, between 85 and 89% is contributed from cattle. However, this amount is by far below the national demand for milk and milk products in the country. Many reasons could be ascribed for the low annual national milk yield among which mastitis is one of the most important factors. A number of reports indicated that mastitis is a serious problem in the dairy industry of Ethiopia. Nesru (1999) reported a mastitis prevalence rate of 85.6% and 81.2% using CMT and SCC, respectively.

According to the same report, out of the CMT positive animals, 37.2% did harbor a causal agent for mastitis. Biru (1989) reported a combined mastitis prevalence of 67.4% at cow level while Bishi (1998) reported a subclinical mastitis prevalence of 35.5% and 34.3% for small and large-scale farms, respectively. As for clinical mastitis prevalence, it was Bishi (1998) who reported 4.4% and 5.7% prevalence rates at cow level for small and large-scale farms, respectively. The productivity and financial losses due to mastitis in Addis Ababa milk-shed was estimated by Mungube (2001). The loss due to subclinical mastitis was 1.51 kg of milk per cow per milking and 3.01 birr per cow per day. In clinical mastitis, these lose was 4.4 kg milk per day and 176 birr in 20 days.

Although there are a number of reports on the prevalence, major pathogens and antimicrobial resistance pattern of mastitis in the different parts of the country (Werkineh *et al.*, 2002; Abayineh, 2001; Mungube, 2001; Nesru, 1999; Bishi, 1998; Biru, 1989), little has been done in the current study area which is located within the Addis Ababa Milk shed, currently supplying a considerable volume of milk to government and private milk processing plants located in or around Addis Ababa and having still higher potential for dairy production.

The objectives of this study were therefore:

- To estimate prevalence of subclinical and clinical mastitis at herd, cow, and quarter level in smallholder dairy farms;
- To assess the major risk factors associated with the occurrence of subclinical mastitis in smallholder dairy farms;
- To identify and isolate the major bacterial causes of clinical and subclinical mastitis and
- To conduct in-vitro antimicrobial susceptibility test on most frequently isolated bacteria

2. LITERATURE REVIEW

2.1. Definition of bovine mastitis

The term 'mastitis' is an inflammation of the mammary gland primarily resulting from invasion of pathogenic microorganisms through the teat canal (Erskine, 2001). There are two forms of mastitis:

Subclinical mastitis: characterized by the presence of pathogenic microorganisms in the milk and the inflammatory response detected only by screening test with out visible signs of the disease.

Clinical mastitis: characterized by visible signs of mastitis. In chronic clinical mastitis, the signs include flakes or clots in the milk and slight swelling of infected quarter. Swelling of the mammary gland with less systemic signs is seen in acute clinical mastitis. The per-acute form is characterized by abnormal milk secretion, hot and swollen quarter of udder, fever, rapid pulse, loss of appetite, dehydration and depression, death may occur.

2.2. Aetiology

Jubb *et al.* (1993) has shown that modern techniques of microbial classification have identified more than hundred species, sub species, and serovars isolated from the mammary gland. *Streptococcus agalactiae* and some types of S. *aureus* are obligate parasites of the gland and inevitable pathogens, but the great majority of infections are opportunistic. In addition, some viral diseases like Pseudo Cowpox, *Herpes mamilitis,* Cowpox, Papilloma, Foot and Mouth Disease and Vesicular stomatitis affecting the epithelium of the teat orifice are mentioned to result in or predispose to mastitis (Hillerton *et al.,* 2001). From the etiological point of view, the pathogenic microorganisms have been classified into two groups, namely, contagious and environmental pathogens based

on their distinct characteristics of distribution and interaction with teat and duct (Calvinho *et al.*, 1998). Within the two groups, there are two other sub-divisions as major and minor pathogens. Major pathogens are responsible for most severe cases of mastitis while the minor pathogens are rarely associated with marked leukocytosis and clinical manifestations (Rainard and Peutrel, 1988; Radostits *et al.*, 1994)

2.2.1. Major pathogens

S. aureus, S. *agalactiae*, and *Mycoplasma bovis* have been reported to be major pathogens causing contagious mastitis. These pathogenic microorganisms live and multiply on and in the cow's mammary gland and are spread from cow to cow primarily through milkiers' hands and udder wash clothes during milking. Major pathogens causing environmental mastitis are Coliforms (*E. coli, Klebseila spp*, *Enterobacter aerogens)*, and *St. uberis* (Watts, 1988; Calvinho *et al.*, 1998).

The predominant infectious agent of mammary gland in most countries is *S. aureus*, followed by *St. agalactiae* (Radostits *et al.*, 1994). In a study carried out in Zimbabwe by Perry *et al.* (1987) *S. aureus* was the most frequently isolated bacteria from both clinical and subclinical mastitis. Sargeant *et al.* (1998) reported isolation rates *of* 6.8%, 0.7%, 14.1%, 17.2%, 1.7% and 28.7% for *S. aureus, St. agalactiae,* other *Streptococci* species, Coliforms, *Corynebacterium bovis* and other *Staphylococcus* species, respectively, from 834 mastitic milk samples collected from clinical cases.

2.2.2. Minor pathogens

In the minor pathogens are included; coagulase negative *Staphylococcal* species, *Actinomyces bovis*, *Bacillus cereus*, and *Serratia marcescens* (Brooks *et al.*, 1983). Coagulase negative Staphylococci (CNS) consisting of a variety of *Staphylococcus spp (S. hyicus, S. simulance, and S. epidermidis),* and *C. bovis* are contagious minor pathogens. The agents simply colonize the teat streak canal but do not cause a clinical

disease (Harmon *et al.*, 1986; Radostits *et al.*, 2000). Nocardia *asteriodes, Serratia marcescens, Prototheca zopfii, Leptospira interrogen (serovar pomona) and Pseudomonas aerugenosa* are environmental minor pathogens of bovine mastitis (Quinn *et al.*, 1999).

2.3. Risk factors associated with bovine mastitis

Mastitis is a multifactorial disease and results when management and environment factors interact to increase exposure, to reduce udder resistance and to aid deposition of organisms into the teat canal (Phillpot, 1984). However, many researchers on the disease complex have only restricted the disease causation to microbial infection, ignoring the other important epidemiological players like environmental and management factors (Radostits *et al.*, 1994).

2.3.1. Host factors

Grommers (1988) has suggested that host characteristics are important risk determinant in the pathogenesis of mastitis. These factors are associated with the development of specific immunity and with none specific host defense mechanisms (teat sphincter and keratin). The resistance by the host relates to genetic predisposition, anatomical characteristics, nutritional status, stage of lactation and parity. Host defense mechanisms are critical affecting the susceptibility of the dairy cow to udder infection (Nickerson, 1987; Radostits, 1994; Erskine, 2001).

2.3.2. Agent factors

The microbial factors which affect mastitis occurrence are the ability to survive in the immediate environment of the animal, the ability to colonize the teat duct, the ability to adhere to mammary epithelium and not be flushed out with milk flow, the degree of invasiveness (*Streptococci* cause little pathological change to secretory cell while

Staphylococci initiate degenerative changes), the ability to resist phagocytosis and anti bacterial substance in the udder, including resistance to antibiotics (Quinn *et al.*, 1999).

2.3.3. Environmental factors

Radostits *et al.* (2000), has stated that the quality and management of housing for dairy cattle has a major influence on the types of mastitis pathogens, which can infect the mammary gland and the degree of infection pressure. Bedding type such as organic bedding and other housing conditions that provide nutrients to bacteria, like damp and wet conditions will promote greater exposure of cows' teat to pathogens and result in higher incidences of mastitis (Nickerson, 1987). Increased herd size makes manure disposal and sanitation tasks difficult and hence increases exposure to environmental pathogens (Bartlett *et al.*, 1992).

Dorgent-Melina *et al.* (1988) has suggested that udder preparation both before and after milking influences the rate of mastitis in a given herd. It has been established that farmers who use a common close (sponge) for drying teats after cleaning the udder have a greater odds of having a high prevalence on infection than herds using individual paper towels. Feeding a cow after it is milked is necessary since the cow remains standing for a while. Lying down immediately after milking can be a risk factor as the teats are still open. This gives the teats time to switch back to their normal anatomical shape other wise the risk of acquiring environmental pathogens is very high (Peeler *et al.*, 2000; Radostits *et al.*, 2000). In contrast to other species, particularly, the mammary glands of dairy cows are commonly predisposed to invasion by pathogenic organism due to high incidence of trauma to the orifice, sphincter, or streak canal of the teat (Jones *et al.*, 1997).

2.4. Pathogenesis

Infection of intramammary gland always occurs via the teat canal. However, the development of mastitis is more complex. The most well clarified stages are invasion,

infection, and inflammation. Following invasion, a bacterial population may be established in the teat canal, which multiplies and extended into mammary gland tissue where infection occurred frequently or occasionally (Radostits *et al.,* 1994). The invasion by microorganisms results in involvement of macrophages against bacterial pathogens, which upon stimulation produce interleukin-1, which leads to release of prostaglandin and leukotrines. Leukotrine B_4 is a powerful attractant of neutrophils. The biological function of soluble CD14 was shown to modulate humoral and cellular immune responses by interacting with both T and B lymphocytes (Filipp *et al.*, 2001). Lee *et al.* (2003) reported that CD14 is involved in a spectrum of biological and immunological responses. It has been well documented that milk CD14 expressed on leukocytes is the key molecule in recognizing invading pathogens and eliciting a cascade of inflammatory responses. On the other hand, CD14 has been shown to prevent death induced by 'septic shock,' inhibit dissemination of pathogens, stimulate lymphocyte proliferation, and facilitate phagocytosis of bacteria. It has also been indicated that bacterial infection in the udder increases permeability and alters the plasma contents in milk, which in turn alters ion balances. The alveolar epithelium is damaged by inflammation or by pressure from adjacent inflammatory process resulting in secretion of less milk (Jubb *et al.*, 1970).

Jones *et al.* (1997) has suggested that trauma to the teats may cause breakdown of natural barrier and render the cow more susceptible to infection. Other resistance factors in addition to physical barrier that imposed by smegma in the teat canal, include humeral and cellular components in milk that inhibit microbial growth or enhance clearance of invading organisms by phagocytosis, such substance are lactoferin, immunoglobulines, lysozyme, lactoperoxidase, perturbations any or all of these defense mechanisms may increase susceptibility to mastitis.

2.5. Diagnosis of bovine mastitis

2.5.1. Screening tests

Clinical examination and use of strip-cup

Clinical mastitis may be detected by examining the udder for warm, swollen quarters, which are indicative of acute mastitis, or misshapen, hard, atrophied and fibrotic quarters, indicating the permanent damage caused by chronic mastitis (IDF, 1987). Proper examination of the milk requires the use of strip cup, preferably one that has a shiny, black plate permitting the detection of discoloration as well as clots, flakes, and pus. In this method, Milk is drawn on to the plate in pools and comparisons made between the milk of the different quarters (Blood and Radostits, 1989).

Direct microscopic count (Modified Breed's Smear)

The direct microscopic count has been used to count leukocytes in the milk directly. A volume of 0.01 ml of milk is spread over a microscope slide, defatted and then stained by methylene blue based stain. The microscope is calibrated, and then, leukocytes are counted on about 50 fields to calculate the number of leukocytes per ml of milk as described previously (Quinn *et al*, 1999).

Individual indirect cell counts (CMT)

The California Mastitis Test is based on an anionic detergent, Na-lauryl sulphate (SDS), which dissolves cell membranes and nuclei. Consequently DNA is released and it forms a transient gel with the detergent. The more DNA there is in the sample, the higher the viscosity of the gel. Equal volumes of milk and 3% Na –lauryl sulphate (2-3 ml of both) are mixed on the test plate having wells reserved for each of the four quarter. As most of

the DNA in milk originates from somatic cell, CMT reflects the somatic cell content of milk (Sandholm, 1995).

Electronic somatic cell counters

Electric somatic cell counting of suspicious milk is another screening test for the diagnosis of mastitis based on cell counts. The two most commonly used are the Coulter Milk Cell Counter, which counts particles as they flow through an electric field, and the Fosomatic Milk Cell Counter, which stains cells with a fluorescent dye and then counts the number of fluorescing particles (Dohoo and Meek, 1982).

Indirect chemical tests to detect mastitis

Some tests are based on the increase in sodium and chloride ions in mastitic milk that have higher content of sodium and chloride than normal milk. The bacterial infection in the udder alters the cell wall permeability and permits an increased flow of sodium and chloride into the milk (Kitchen *et al.*, 1994).

2.5.2. Microbiological test

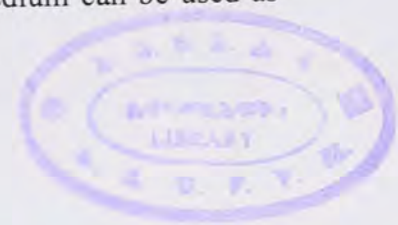
Microbiological diagnosis is sought in case of elevated somatic cell counts as given by CMT, direct or electronic somatic cell counts to differentiate the microorganisms involved and also to act as a confirmatory test (Erskine, 2001).

Direct Microscopy

The milk collected as sample can be centrifuged and a stained smear made from the deposit. A Gram stain is used routinely to detect gram-positives such as *Staphylococci, Streptococci, Bacillus spp.*, and also yeasts such as *Candida albicans* that stain deeply by crystal violet. A modified Ziehl-Nielsen-stained smear can be made if *N. asteroids* is suspected and a Ziehl- Nelson-stained smear is prepared when rare cases such as *Mycobacterium fortuitum* or *M.bovis* are suspected (Quinn *et al.*, 1999).

Culture

The incriminated milk samples are cultured on ox or sheep blood agar, which supports growth of mastitis pathogens. *Candida albicans* and *Aspergillus fumigatus* are able to grow on blood agar at 37°C in 2-3 days, if there is no competition from the faster growing bacteria. MacConkey agar plate is streaked in parallel to detect *E. faecolis* and any gram-negative bacteria that is able to grow on the medium. Edward's medium can be used as selective media for streptococci suspected milk (Quinn *et al.*, 1999).



2.6. Control of bovine mastitis

Successful treatment of clinical mastitis requires a history of the herd, identification and susceptibility pattern of the bacteria involved, as well as relevant information on the milking management. Intramammary drug formulation is a suitable route for administration of long – acting antimicrobial preparation at " dry off " as part of mastitis control programme (Quinn *et al.*, 1999).

Erskine (2001) has suggested that the prevalence of *St. agalactiae* intramammary infection can be reduced rapidly by "blitz" treatment. With this method an entire herd, or more economically, all the culture positive cows in a herd, are treated with antimicrobial. The most efficacious and cost effective regimen is to use intramammary β – lactam therapy. Cure rates can often be from 75% to 90% and are economically beneficial (Eriskine *et al.*, 1996). Studies of treatment efficacy of *S.aureus* mastitis have found cure rates of 25% to 55% of infected quarters in experimental infections that were evaluated for 21 to 60 days after infection (Owens *et al.*, 1998). However, natural infection is usually of longer duration before therapeutic intervention is used and thus more refractile to therapy. Intramammary cefoperzon for the treatment of clinical *S. aureus* mastitis led to bacteriologic cures for only 39% of the cases, as measured 14 days after treatment (Wilson *et al.*, 1986).

In Ethiopia, Nesru *et al.* (1997) reported that out of five isolates of *S.aureus* four were sensitive to penicillin and streptomycin, all to erythromycin, three to chloramphenicol and ampicilin but most isolates (four) were less sensitive to tetracycline. Therefore, intramammary therapy may have the best probability of success by including parenteral administration. Preferentially the therapy should be administered for periods long enough to insure drug levels above minimal inhibitory concentration (MIC) to allow effective killing of the pathogens (Erskine, 2001).

2.7. Bovine mastitis in Ethiopia

2.7.1. Prevalence of bovine mastitis

A number of epidemiological studies of bovine mastitis were carried out in Ethiopia. Nesru *et al.* (1997) reported the prevalence of clinical and subclinical mastitis to be 5.3% and 19% on cow basis, respectively and 1.9% and 7.4% on quarter basis, respectively, in central Ethiopia. Bishi (1998) reported an the over all prevalence of to be 30.2% and 5.5% for subclinical and clinical mastitis, respectively, in a study conducted in urban and peril-urban dairy production systems in and around Addis Ababa. According to Kassa *et al.* (1999) a survey carried out on mastitis in dairy herds of the Ethiopian central highland, the prevalence of clinical mastitis and subclinical mastitis on cow basis to be 1.2%, and 38.9%, respectively. Mungube (2001) reported an over all prevalence of 40.6% for subclinical mastitis at cow level and 27.8% at quarter level, in peri urban and urban areas of Addis Ababa. In another study in the same area, Mekonen *et al.* (2001) reported that clinical mastitis was the second most frequent disease next to reproductive diseases. Variation of the prevalence of mastitis due to differences in environment and management were reported by Odessa (1997).

There are also some limited reports on the incidence of mastitis. A survey on bovine mastitis in milking cows at Alemaya University Dairy Farm by Tefera (2001) for 6 years (from 1993-1998) revealed an over all clinical mastitis incidence of 34 cases/100 cows

per year. Nesru, (1999) reported incidence rates of 1.33% and 11.2% per cow per month for clinical and subclinical mastitis, respectively in urban and peri-urban dairy farms in Addis Ababa.

2.7.2. Economic losses

The economic loss due to mastitis in general in Ethiopia is not known, however, some research works indicate that the loss is significant. Bishi, (1998) reported that the economic losses from clinical and subclinical mastitis in Addis Ababa milk-shed to be approximately 270 birr per lactation. In another study conducted by Mungube (2001) in the same study area, losses due to mastitis (milk production losses, treatment cost, withdrawal losses and culling losses) were estimated to be 210.8 birr per cow per lactation from which milk production losses contributed 38.4%. The contribution of subclinical mastitis (94%) to the losses was by far higher than that of c linical mastitis (6%).

3. MATERIALS AND METHODS

3.1. Study area

The study was conducted in Sellale area, North Shoa Zone of Oromiya Regional State, which is located northwest of Addis Ababa within about 190 km radius. This area is part of the central highlands of the country. Geographically, the Zone is situated between 9^0 – $10^024'$ North-latitude and $37^057'$- $39^033'$ East latitude (NSDAD, 2001). The total area coverage of the Zone is 11,607 km^2 while the altitude of the Zone is ranging from 1000 - 3500 masl (CSA, 2003). The high land, mid land, and low land cover 54%, 28% and18% of the total land mass, respectively (EASE, 2003). The mean annual rainfall of the Zone is 1026mm and the daily mean maximum and minimum temperature are 20.68^0c and 11.23^0c, respectively. The human population of the zone is estimated to be 1,420, 571 with crude population density of 124 persons per km^2. The average family size is 4.7 persons per household. The specific study area from the Zone is Mullo-Sullulta woreda.

3.2. Study population

According to the census result of CSA (2003), the total cattle population of Oromiya Regional State and North Shoa Zone is estimated to be 18,035,686 and 1,173, 543, respectively. The majority of cattle population is indigenous breed (99.26%) with the exception of 106,225 (0.58%) hybrid and 27,220 (0.15%) exotic breeds. The total cattle population in Mullo-Sullulta woreda is 186,136 out of which 6,593 heads of cattle are crosses of Holstein and indigenous breeds. The study population comprises therefore all crossbred cows in the Mullo-Sullulta woreda.

A total of 109 smallholder dairy farms including 500 lactating cows with the average herd size of 4.44 and originating from 11 peasant associations were randomly selected from the sampling frame, in order to determine the status of the prevalence of bovine mastitis and to identify the major intramammary infection in the study area.

3.3. Study design

3.3.1. Study type

A cross sectional type of study was carried out from September 2005 to March 2006 in Mullo-Sullulta woreda of the North shoa Zone of Oromiya Regional State.

3.3.2. Sampling procedure

All farmers' associations in the Mullo-Sullulta woreda, where smallholder dairying is practiced using crossbred cows were considered as the sampling frame. The list of all smallholder dairy cattle farms found in the selected farmers' associations was prepared and simple random sampling was carried out to select farms. All dairy cows in the selected farms were sampled as a cluster. The total number of animals sampled from the study area is calculated using the formula for one stage cluster sampling (Thrustfield, 1995). The considerations during the sample size determination included: 95% confidence interval, 5% precision and 60% of prevalence from previous studies in similar study area (Workineh *et al.*, 2002). Therefore, the sample size needed was determined as

$$g = 1.962^{*}/ nVc + Pexp (1-Pexp) nd^2,$$

Where g = number of clusters to be sampled,

Pexp = expected prevalence,

d = desired absolute precision

n = number of animals per cluster,

Vc = between cluster variance,

Accordingly the number of herds to be sampled was calculated to be 109. Thus 109 dairy cattle herds were sampled as a cluster. All dairy animals in the selected herds (500 in number) that were likely to have mastitis were included in the sample.

3.4. Data collection

3.4.1. Screening tests

Clinical examination

Clinical cases were recorded at the time of milk sampling. Clinical mastitis was diagnosed on the basis of manifestation of visible signs of inflammation. A quarter, which was warm and swollen and had pain upon palpation was considered to have acute clinical mastitis and misshaped, atrophied, hard and fibrotic quarters were considered to have chronic mastitis (I DF, 1987).

California Mastitis Test

The California Mastitis Test (CMT) was carried out as screening test for subclinical mastitis and for selection of samples for culture from the cows under study. A squirt of milk, about 2ml from each quarter was placed in each of four shallow cups in the CMT paddle. An equal amount of the commercial reagent was added to each cup. A gentle circular motion was applied to the mixtures, in a horizontal plane for 5 seconds. The reaction was interpreted based on the thickness of the gel formed by CMT reagent and milk mixture, and the test results were scored as negative (o), trace (T), + (weak positive), ++ (distinctive positive), and +++ (strong positive) according to the Quinn *et al.* (1994) (Annex 2). Quarters with CMT score of + or above were judged as positive. Cows were considered positive for CMT, when at least one quarter turned out to be positive for CMT. A herd was considered positive for CMT, when at least one cow in a herd was tested positive with CMT.

3.4.2. Milk sample collection procedure

Procedures for collecting milk sample were conducted according to Quinn *et al.* (1994). Strict aseptic procedures were used when collecting milk samples in order to prevent contamination with microorganisms present on the skin of cow's flanks, udder and teats,

on the hands of the sampler and in the barn environment. Udders and especially teats were cleaned and dried before sample collection. Each teat end was scrubbed vigorously with a pledged of cotton moistened (but not completely wet) with 70% ethyl alcohol; scrubbing the teats on the far side of the udder first, then those on the near side to avoid recontamination of teats during scrubbing. A separate pledged cotton was used for each teat. The time chosen for milk sample collection was before milking. This is because the cell count is elevated immediately after milking and for at least six hours thereafter. Teats towards the sampler were sampled first and then the far ones. The first 3-4 streams of milk were discarded. The collecting universal vial was hold as near and horizontal as possible and by turning the teat to a near horizontal position 5ml of milk was collected into the vial. After collection, the sample was placed and held in an icebox for transportation to the National Animal Health Research Center at Sebeta and samples were stored at 4°C until processing for bacteriological examination.

3.4.3. Bacterial isolation

Bacteriological examination of the milk was carried out following standard procedures (Quinn *et al.*, 1994). One standard loop (0.01ml) of milk sample was streaked on 5% blood agar. The inoculated plate then was incubated aerobically at 37 ^{0}C for about 24 to 48 hours. The plates were examined for growth and morphologic features; that is colony size, shape, color and hemolytic characteristics. Gram staining was used to classify bacteria as gram positive or negative and as cocci, rods and pleomorphic rods. Based on the above results, suspected colonies were isolated on to blood agar plates for further investigation. This was followed by examination of biochemical or metabolic properties of the organism in question as presented in Annex 3. Interpretation was made as provided by National Mastitis Council (NMC) (1990). The bacteriological culture was considered as negative if no growth occurs after 72 hours of incubation. Isolation of two or more colonies from a quarter sample was considered as contaminated and the result was disregarded.

3.4.4. Antimicrobial sensitivity test

In-vitro antibiotic sensitivity test (Kirby-Bauer disk diffusion method) (Carter *et al.* 1991) was carried out on the isolates using the following antimicrobial drugs: ampicilin 10, (AMP 10), penicillin G10 (PEN 10), kanamycin (K30), erythromycin (ERY15), polymixin B (PB300) streptomycin (S10), Oxytetracyclin (OXTE 30), and sulfonamide 300 (SULPHA 300) sensitivity discs (NCCLS 1997). Mueller-Hinton agar for less fastidious bacterial isolates, and 5% sheep blood agar for Streptococci species isolates were used as plating medium. Antibiotic impregnated paper discs and plates were incubated at 37^0c for 8 - 12 hours. The diameter of zones of growth inhibition was measured in millimeters and reported as susceptible, intermediate or resistant (Annex 14 and 15).

3.4.5. Questionnaire survey

A structured questionnaire with the primary objective of elucidating the multifactorial background of bovine mastitis was conducted. Information about cow and farm attributes was collected. Among the information collected the cow attributes include parity status and stage of lactation while the farm attributes include herd size, barn floor status, washing of milker's hand, washing udder before milking, use of separate towel for individual cow and sequencing milking of mastitic cows (Annex 1).

3.5. Statistical analysis

In this study, the occurrence or non-occurrence of clinical and subclinical mastitis at herd, cow, and quarter level as defined by the CMT score and bacteriological result was the dependent variables. Independent variables at the cow levels included parity, stage of lactation herd size and presence of teat lesion. The independent variables at herd level included barn floor status, milking hygiene and milking mastitic cows at last. Stage of lactation was classified into three in such a way that beginning of lactation referred to the first two months of lactation period, middle of lactation referred to the next five months

period and end of lactation referred to the last weeks of lactation. A farm was considered to have good barn floor status, if the floor is made of concrete and bad if the floor is muddy. A farm was regarded as having good milking hygiene, if it practiced either two of the practices including washing of hand before milking, use of separate towel for each lactating cow and drying of udder after washing.

Collected data was entered and stored in Microsoft Excel (2000). Descriptive statistics were estimated using SPSS for Windows (release 11.5.0, 2002). Analyses of associations between the prevalence of subclinical mastitis at cow and herd level with risk factors were estimated by univariate and multivariate logistic regression of Intercooled Stata 7.0 (2000).

4. RESULTS

4.1. Prevalence study

Among the total of 2000 quarters of the 500 lactating cows, 45 quarters (2.25%) belonging to 37 cows were blind and 17 quarters (0.87%) belonging to 16 cows were affected by clinical mastitis. From cows having blind quarters, 30 (81.1%) cows had only one blind quarter, 6 (16.2%) cows had two blind quarters and 1 (2.7%) cow had three blind quarters. With regard to the location of the blind quarters, 15 (33.3%), 11(24.6%), 10 (22.2%), and 9 (20.0%) were at the right rear, right front, left rear and left front positions, respectively.

4.1.1. Prevalence of clinical mastitis

As can be seen in Table 1, a total of 14 herds (12.8%), 16 cows (3.2%) and 17 quarters (0.87%) had clinical mastitis based on CMT. Out of the 17 quarters, 4 (23.52%) and 13 (76.42%) were acute and chronic form of mastitis, respectively. Mastitis causing bacteria were isolated only in 12 herds (11%), 13 cows (2.6%) and 10 quarters (0.51%) (Table 1). Regarding the locations of quarters, which had culture isolates, 1 (10%) was right rear, 4 (40%) were right front, 3 (30%) were left rear and 2 (20%) were left front.

Table 1. Prevalence of clinical mastitis at herd, cow and quarter levels based on CMT and culture

Observation level	N	Prevalence	
		CMT (n)	Culturing (n)
Herd level	109	12.80 (14)	11.00 (12)
Cow level	500	3.20 (16)	2.60 (13)
Quarter level	1955	0.87 (17)	0.51 (10)

N= number of observation, n=number of positives

4.1.2. Prevalence of subclinical mastitis

The prevalence rates of subclinical mastitis at herd, cow and quarter level based on CMT and bacteriological culturing are presented in Table 2. From a total of 77 (70.6%) herds, 147 (30.4%) cows and 264 quarters, which were CMT positive, mastitis causing bacteria, were isolated from 59 (54.7%) herds, 108 (22.3%) cows and 195 (10.06%) quarters.

Table 2. Prevalence of sub-clinical mastitis at herd, cow and quarter level after CMT and culturing

Observation level	N	Prevalence	
		CMT (n)	Culturing (n)
Herd level	109	70.60 (77)	54.70 (59)
Cow level	484	30.40 (147)	22.30 (108)
Quarter level	1938	13.63 (264)	10.06 (195)

N= number of observation, n=number of positives

From the CMT negative quarters, 1604 (95.8%) were without any reaction while 70 (4.2%) were with trace reaction. From the 13.63% prevalence of subclinical mastitis with CMT, 4.12%, 4.03%, and 5.41% of the CMT positive quarters were week (+), moderate (++), and strong (+++) reactors. As to the locations of quarters with culture isolates, 71 (26.9%) were right rear, 67 (25.4%) were right front, 67 (25.4%) were left rear and 59 (22.3%) were left front.

4.2. Bacterial isolates

The list, number and proportion of the bacterial isolates from a total of 109 cows and 281 quarters are presented in Table 3. A total of 199 isolates were found of which 10 (5%) were found from milk samples collected from clinically mastitic quarters. The most important isolates from the clinically mastitic quarters were *St. dysgalctiae* (30%) and *St. agalactiae* (30%).

About 95% (n=189) of the isolates were found from milk samples originating from cows without any apparent clinical signs of mastitis. The most commonly found isolates in a decreasing order of frequency are *S. aureus* (46.6%), *S. epidermidis* (22.8%), *St. agalactiae* (11.1%) and *St. uberis* (10.1%). Others isolates include *S. intermidius*, *St. dysgalctiae*, *Micrococcus species*, *B.cerus*, *A. pyogenes*, *E. coli*, and *C. bovis*. Of the total isolates from subclinical cases, 185 (97.9%) showed single growths while 4 (2.1%) had mixed growths.

Table 3. Type of mastitis pathogens isolated from CMT positive milk samples

Type of isolates	Clinical		Subclinical	
	N	Proportion from Total (%)	N	Proportion from Total (%)
S. aureus	2	20	88	46.6
S. epidermidis	1	10	43	22.8
S. intermidius	1	10	2	1.1
Micrococcus spp	-	-	5	2.6
St. agalactiae	3	30	21	11.1
St. dysgalctiae	3	30	1	0.5
St. uberis	-	-	19	10.1
B. cerus	-	-	4	2.1
A. pyogenes	-	-	4	2.1
E. coli	-	-	1	1.1
C. bovis	-	-	1	1.1
Total isolates	10		189	

Based on their importance, 80% (n=8) of the isolates from the clinical cases were major pathogens while the rest were minor pathogens. In the case of subclinical mastitis, 134 (70.9%) isolates were major pathogens while 55 (29.1%) were minor pathogens. Among the major pathogens the predominant isolate was *S. aureus* (65.3%) (Figure 1) and that of minor pathogens was *S. epidermis* (77%) (Figure 2).

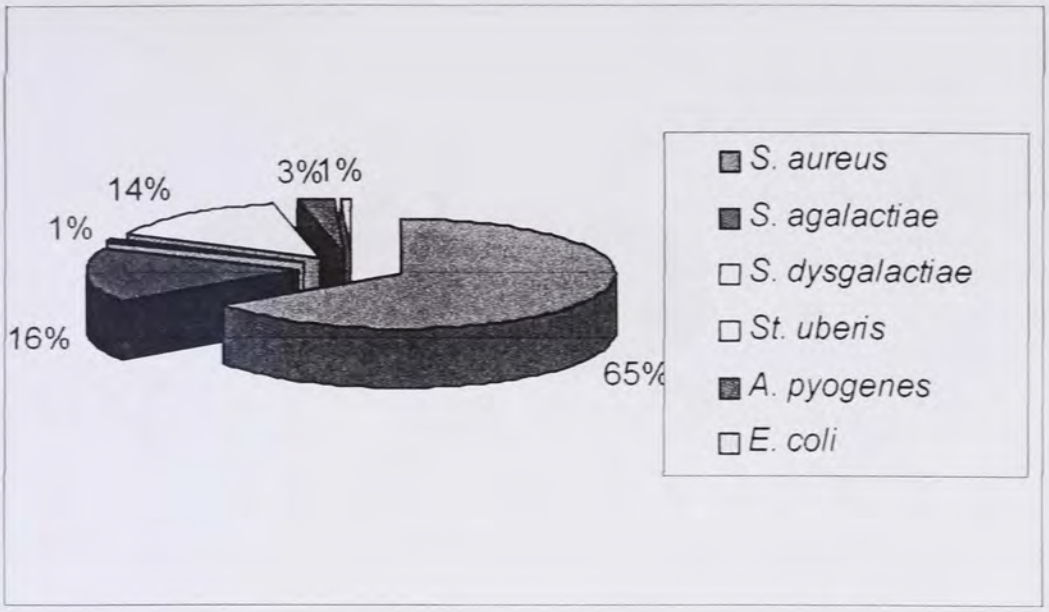


Figure 1. Proportion of different bacterial isolates from subclinical cases classified as major pathogens

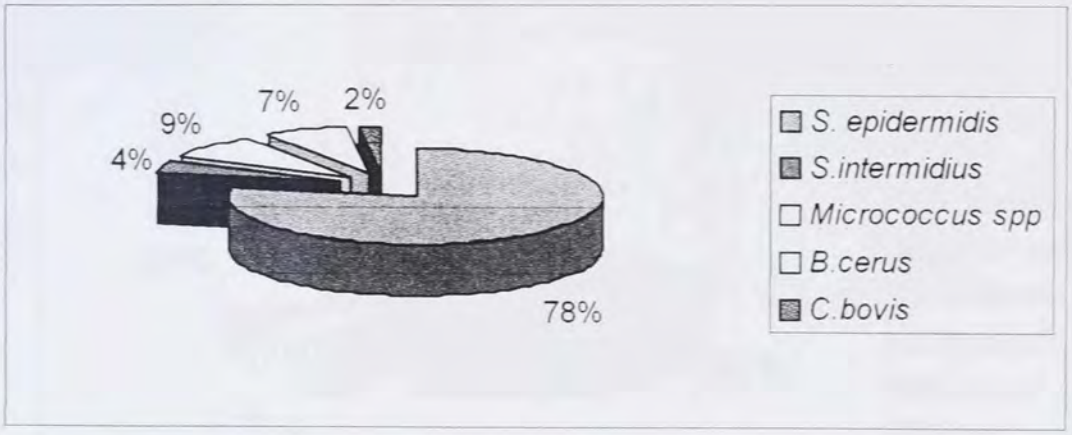


Figure 2. Proportion of different bacterial isolates from subclinical cases classified as minor pathogens

When the isolates are classified based on their origin, 80% (n=8) of the isolates from the clinical cases were contagious pathogens while the rest were environmental pathogens. In the case of subclinical mastitis, 115 (60.8%) isolates were contagious pathogens while 74 (39.2%) were environmental pathogens. Among the contagious pathogens the predominant isolate was *S. aureus* (77%) (Figure 3) and that of environmental pathogens was *S. epidermis* (58%) (Figure 4).

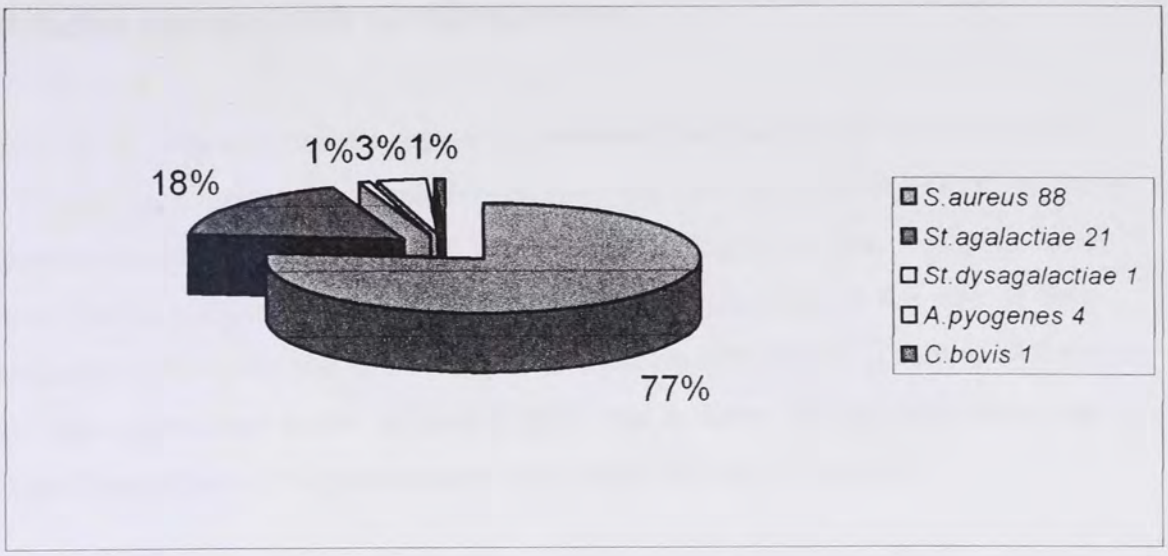


Figure 3. Proportion of different bacterial isolates from subclinical cases classified as contagious pathogens

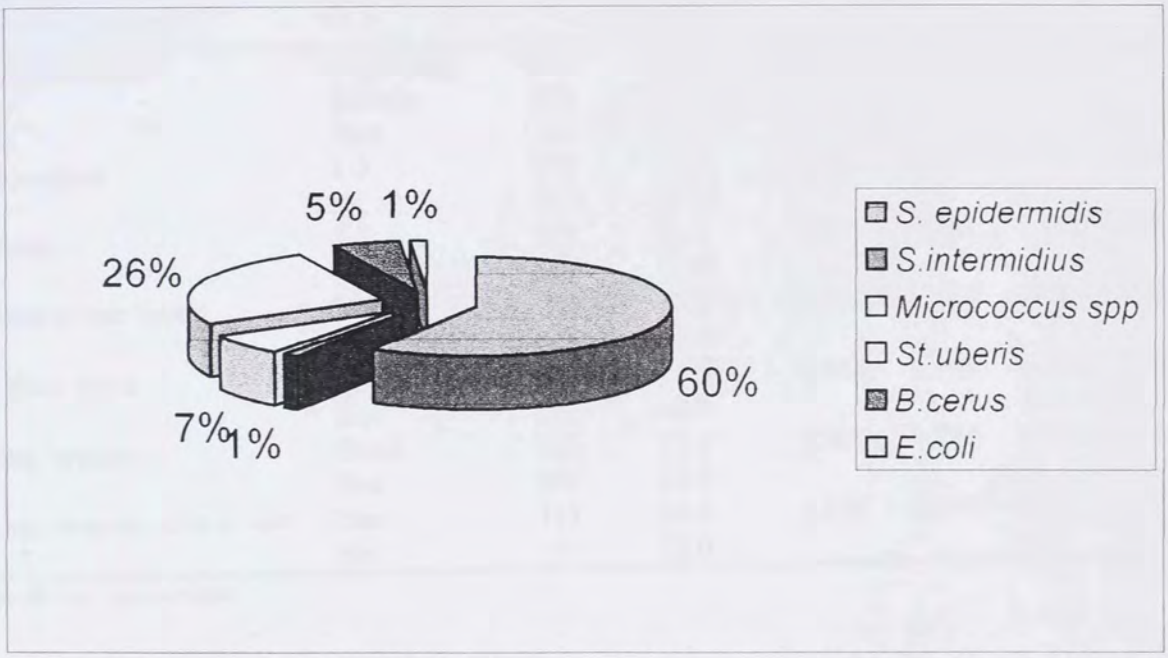


Figure 4. Proportion of different bacterial isolates from subclinical cases classified as environmental pathogens

4.3. Risk factors associated with subclinical mastitis

The results of a univariate logistic regression revealed that stage of lactation ($p<0.05$), parity ($p<0.05$) and presence of teat lesion ($p<0.01$) had significant effect on the prevalence of subclinical mastitis at cow level. The prevalence rate was higher in cows with more than 3 parities number (OR=1.58), in cows that were at the end of their lactation period (OR=1.48) and in cows with teat lesions (OR=6.44) (Table 4). All the farm attributes (barn floor status, milking hygiene and milking of mastitic cows at last had no significant effect of the prevalence of subclinical mastitis at cow level.

Table 4. Association of risk factors with the prevalence of sub-clinical mastitis at individual animal level (univariate logistic regression)

Risk factors	Groups	N	Prevalence (%)	P-value	OR	CI
Stage of lactation	Beginning	86	16.3	0.016	1.481	1.076-2.039
	Middle	242	20.2			
	End	156	28.8			
Parity number	1-3	334	19.8	0.045	1.579	1.010 - 2.468
	>3	150	28.0			
Herd size	1-3	115	26.1	0.267	0.759	0.467-1.234
	> 3	369	21.0			
Presence of teat lesion	Yes	11	63.6	0.003	6.446	1.850-22.452
	No	473	21.4			
Barn floor status	Good	332	22.3	0.984	0.995	0.627-1.578
	Bad	152	22.4			
Milking hygiene	Good	240	22.1	0.904	0.974	0.635-1.494
	Bad	244	22.5			
Milking mastitic cows at last	Yes	193	22.8	0.835	1.047	0.677-1.619
	No	291	22.0			

N= Number of observations

Risk factors that had significant effect on the prevalence of subclinical mastitis at cow level with univariate logistic regression were fitted in a multivariate model. The results showed that the effects of stage of lactation ($p<0.05$), and presence of teat lesion ($p<0.01$) were significant (Table 5). According to the results, there was a positive association between prevalence of mastitis and stages of lactation and presence of teat lesion. In

addition, the prevalence was also higher in cows with teat lesion than those without teat lesion.

Table 5. Association of risk factors with the prevalence of sub-clinical mastitis at individual animal level (multivariate logistic regression)

Risk factors	Groups	N	Prevalence (%)	P-value	OR	CI
Stage of lactation	Beginning	86	16.3	0.020	1.473	1.064-2.041
	Middle	242	20.2			
	End	156	28.8			
Parity number	1-3	334	19.8	0.079	1.503	0.953-2.371
	>3	150	28.0			
Presence of teat lesion	Yes	11	63.6	0.004	6.218	1.765-21.902
	No	473	21.4			

Moreover, the effects of the potential risk factors at herd level were analyzed using univariate logistic regression and the results revealed that non of the factors considered had significant effect the prevalence of subclinical mastitis at herd level ($p>0.05$) (Table 6).

Table 6. Association of risk factors with the prevalence of sub-clinical mastitis at herd level (univariate logistic regression)

Risk factors	Groups	N	Prevalence (%)	P-value	OR	CI
Herd size	1-3	45	48.9	0.301	1.505	0.693-3.710
	> 3	61	59.0			
Barn floor status	Good	65	58.5	0.409	1.468	0.627-1.578
	Bad	41	48.8			
Milking hygiene	Good	46	60.9	0.706	1.180	0.634-1.494
	Bad	60	50.0			
Milking mastitic cows at last	Yes	38	60.5	0.369	1.500	0.619-3.631
	No	68	51.5			

N=Number of observations

4.4. In-vitro antimicrobial susceptibility results

The results of antimicrobial sensitivity test for the different isolates are presented in Table 6. Isolates of *S. aureus* showed moderate to very high resistance for ampicilin (53.4%), penicillin 45.3%, streptomycin (63.35%) and polymixin B (97.7%). *S. epidermidis* isolates were highly resistant to polymixin (95.2%), oxytetracyclin (83.3%) and ampicilin (70.8%). Except to sulfonamides, isolates of *S. intermidius* were susceptible to all the antimicrobials applied. *St. agalactiae* isolates showed the highest resistance for kanamycin (53.8%). *St. dysgalctiae* isolates were strongly susceptible for all antimicrobials except kanamycin and erythromycin. Substantial isolates of *St. uberis* were susceptible for most of the tested antimicrobials except kanamycin. All the isolates of *B. cerus* were resistant to most of the isolates except for ampicilin, penicillin and polymixin. Except for penicillin, polymixin and sulfonamide, isolates of *A. pyogenes* showed high degree of resistance for the other drugs.

Table 7. In-vitro antimicrobial susceptibility test results

Isolates	N	Responses to application of antimicrobial disks (%)															
		AMP 10		PEN 10		KAN 30		ERY 15		POLY B 300		STR 10		OXY 30		SULPH 300	
		R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S
S. aureus	85	53.4	45.3	45.3	53.4	3.4	96.5	4.7	95.3	97.7	2.3	63.35	36.04	22.9	76.74	37.2	61.32
S. epidermidis	24	70.8	29.1	45.8	54.1	4.2	95.8	8.3	91.7	95.2	4.8	45.8	54.1	83.3	16.7	17.8	82.2
S. inermidius	3	-	100	-	100	-	100	-	100	-	100	-	100	-	100	25.0	75
St. agalactiae	13	15.5	84.5	15.4	84.6	53.8	46.2	7.4	92.5	7.6	92.4	15.4	84.6	28.5	70.5	7.6	92.3
St. dysgalactiae	3	-	100	-	100	33.3	66.6	33	66.6	-	100	-	100	-	100	-	100
St. uberis	19	36.8	63.2	34.5	65.5	64.5	31.5	-	100	-	100	7.4	92.3	7.5	92.5	30.2	69.8
B. cerus	4	100	-	100	-	25.0	50.0	25.0	50	50.0	25.0	25.0	75.0	25.0	75.0	25.0	75.0
A. pyogenes	3	66.6	33.3	-	100	100	-	100	-	33.3	66.6	100	-	100	33.3	33.3	66.6

N=Number of observations, S = susceptible, R = resistance

5. DISCUSSION

5.1. Prevalence of clinical and subclinical mastitis

The overall prevalence of clinical mastitis based on both CMT and culturing at cow level in this study (2.6%) is very close to the reports of Bishi (1998) (4.4%) and Mungube (2001) (3.6) both of which worked in Addis Ababa and the surroundings. In the case of subclinical mastitis, the overall prevalence after culturing (22.3%) is in agreement with the reports of Nesru *et al.*, (1997) (19%) for central parts of Ethiopia, but lower than those of Abayineh (2001) (34.6%) and Workineh *et al.* (2002) (45.4%) for Addis Ababa and the surrounding and higher than the reports of Klaustrup and Halliwell (1997) (17-19%) for smallholder dairy farms in Malawi. The similarity of the results of this study with the works of Bishi (1998), Mungube (2001), Nesru *et al.* (1997) is due to the similar nature of agroecology, herd size and management systems. The lower prevalence rate found in this study as compared to the works of Werkineh *et al.* (2002) and Abayineh (2001) is due to the fact that the two previous reports were from farms with larger herd size than those of the present study.

5.2. Bacterial isolates

The isolation of substantial number of staphylococcal (70.5%) and streptococcal (21.7%) species as causes of subclinical mastitis in this study is in agreement with the findings of Werkineh *et al.* (2002) for the same area and Mdegela *et al.* (2005) in smallholder dairy herds in urban and per urban area of the Dodema, Tanzania. The most important isolate found in this study was *S. aureus* (46.6%). Nesru et al. (1997) and Vaarst and Envolden (1997) also emphasized the leading role of *S. aureus* in subclinical mastitis. In addition, Pyorala (1995) reported that S. aureus was responsible for 30-40% of subclinical mastitis cases. The high prevalence *S.aureus* may be attributed to wide distribution of the organisms in side the mammary gland and the skin of teats and udder and its frequent

colonization teats (McDonald, 1997). The role of *S. aureus* as a cause of clinical mastitis (20%) in the present study is within the range reported by Pyorala (1995) (20-30%). The findings of this study have given highlight on the overt proportional isolation of major pathogenic microorganisms (70.8.%) in the occurrence of subclinical mastitis including *S. aureus, St. agalactiae, St. uberis, A. pyogenes, and E.coli.*

5.3. Effects of different risk factors

In this study farm attributes including barn status, milking hygiene and milking mastitic cows at last did not have significant effect on the prevalence of subclinical mastitis. This result is not in agreement with previous reports, which indicated that intramammary infection is transferred from cow to cow by cleaning towel and milkers' hands (Esa, 1995). In addition, Atwill *et al.* (1995) asserted that the probability of the occurrence of subclinical mastitis in an individual cow depends on not only the cow risk indicators, but also on the herd, in which the cow lives. The small herd size of the selected farms in this study could be one reason why the effects of farm attributes were insignificant.

The significant effect of stage of lactation on prevalence of subclinical mastitis in this study is in line with the reports of Nesru (1999) and Mungube (2001). The later author reported higher prevalence of subclinical mastitis for cows in late stage of lactation and Nesru (1999) reported higher prevalence in cows at 4-6 months of lactation. The same trend of association was also indicated by Radostits *et al.* (2000) who pointed out that the prevalence of subclinical mastitis caused by contagious pathogens such as *S. aureus.* increases as stage of lactation progresses. This is caused by lack of mastitis control efforts to limit cow-to-cow transmission of organisms rather than by physiological effect. In this study, prevalence of mastitis increased significantly with parity number. The same pattern of effect of parity was reported by Duniel *et al.*, (1988) and Schukken *et al.* (1989). The authors indicated that younger animals have a decreased susceptibility through a more effective host defense mechanism while older cows, especially after four calving are more susceptible to mastitis. The finding in this study regarding the effect of teat lesion was

also supported by Pyorala (1995) who reported by that damage on cows udder and teat skin is the most important risk factor of infections.

5.4. Antimicrobial susceptibility

According to the results of antimicrobial drug susceptibility test, isolates of *S.aureus* were more susceptible to penicillin, kanamycin, erythromycin, oxytetracyclin and sulphonamides but all isolates of *S.aureus showed strong resistance to polymixin B.* The same kinds of susceptibility pattern results were reported by Nesru (1997) and Mdegela *et al.*, (2005). It has been indicated by previous reports that the repeated use of antimicrobials against pathogens causing subclinical mastitis increases selection pressure for development of drug resistant bacterial strains (Pyorala and Vessa 1995).

7. REFERENCES

Abayineh, D., and Sintayehu, A. (2001): Treatment trial of subclinical mastitis with the herb Persicaria senegalense. *J. Trop. Anim. Hlth* Prod. **33**, 511 – 519

Atwill, E. R., Mohammed, H.O., Scarlet, J. M. and McCulloch, C.E. (1995): Extending the interpretation and utility of mixed effects logistic regression models, *Prev. vet. Med.* **24,** 187-201.

Bartlet, P. C., Miller, G. Y., Lance, S. E. and Heider, L. E. (1992): Environmental and managerial determinates of somatic cell counts and clinical mastitis incidence in Ohio Dairy Herds. *Prev. Vet. Med.* **14,** 195-207

Biru, G. (1989): Major bacteria causing bovine mastitis and their sensitivity to common antibiotics. *Eth. J. Agric. Sci.* **11,** 47-54

Bishi, A. B. (1998): Cross-Sectional and Longitudinal Prospective Study of Bovine Clinical, Subclinical Mastitis in Peri-urban and Urban Dairy Production System in Addis Ababa Region. Faculty of Veterinary Medicine, Addis Ababa University, Debre-zeit, Ethiopia, MSc Thesis.

Blood, D. C. and Radostits, O. M. (1989): Mastitis. In: Veterinary Medicine, A Text Book of the Disease of Cattle, Sheep, Goat, and Horses, 7th ed. Bailliere Tindal. London. Pp 501-599.

Blowey, R. W. (1990): Mastitis and Conditions of the Udder. In: A Veterinary Book for Dairy Farmers. 2nd ed. Pp 181-229.

Brooks, B. W., Barnum, D. A. and Meek, A. H. (1983): An Observational study of *Corynebacterium bovis* in selected Ontario Dairy Herds. *J. Comp. Med.* **47,** 73-78.

Brooks, B. W., Barnum, D. A. and Meek, A. H. (1983): An Observational study of *Corynebacterium bovis* in selected Ontario Dairy Herds. *J. Comp. Med.* **47,** 73-78.

Calvinho, L. F, Almeda, R. A., Oliver, S. P. (1998): Potential virulence factors of *Streptococcus dysgalctiae* associated with bovine mastitis. *Vet. Microb.* **61,** 91-110.

Carter, G. R. and Chengappa, M. .M. (1991): Essentials of Veterinary Bacteriology.and Mycology.4th ed. USA, Lea and Febiger.pp 81-94

Carter, J. R. (1984): Diagnostic Procedures in Veterinary Bacteriology and Mycology. 4th ed. USA: Charles C. Thomas Publisher.pp 367-395.

CSA (2003): Statistical Report on Livestock and Farm implements. In: Federal Democratic Republic of Ethiopia. Central Agricultural Census Commission, Ethiopian Agricultural Sample Enumeration, 2001/2002.

Dohoo, I. R and Meek, A. H. (1982): Somatic cell counts in bovine milk. *Can. Vet. J.* **23,** 119-123.

Dorgent-Mellna, P., Searlett, J., Pollock, R. V. H., Sears, P. (1988): Herd-level risk factors for *Staphylococcus aureus* and *Streptococcus agalactetia* intramammary infections. *Prev. Vet. Med.* **6,**127-142.

Dulien, A.M., Paape, M.J., Nickerson, S.C. (1988): Comparison of phagocytosis and chemiluminescence by blood and mammary gland neutrophilis from multiparous and nulliparous cows. *J.Am.Vet. Res.* **49**, 172 – 177.

Erskine, R. J., Bartlett. C., and Johnson, G.I. (1996): Intramuscular administration of ceftiofur sodium versus intramammary infusion of penicillin/novobiocin for

treatment of streptococcus agalactiae mastitis in dairy cows. *J. Am. Vet. Med. Assoc.* **208,** 258-260

Erskine, R. J., Eberhart, R. J., Spencer, S. B. and Cambell, M. A. (1988): Incidence and types of clinical mastitis in dairy herds with high and low somatic cell counts. *J. Am. Med. Assoc.* **192,** 761-765

Erskine, R. J. (2001): Mastitis control in dairy herds. In: Herd Health: Food Animal Production Medicine. 3rd ed, W. B. Sounders Company, Philadelphia. Pp 397-435.

Esa, M. (1995): Effect of milking and milking machine on udder health. In: Sandholm, M., Honkanen- Buzalski, T., Kaartinen, L., Pyorala (eds). In: The bovine udder and mastitis. Finland : Gummerus Kirjapaino Oy. Juvaskyla. Pp 235 – 245

FAO, (2003): Livestock Sector Brief. Livestock Information, Sector Analysis and Policy Branch April 2003. 1-15.

Felleke, G. and G. Geda. (2001): The Ethiopian dairy development policy: a draft policy document. Addis Ababa, Ethiopia: Ministry of Agriculture/ AFRDRD/AFRDT Food and Agriculture Organization/SSFF.

Filipp, D. K., Alizadeh – Khiavi, C., Richardson, A., Palma, N., Paredes, o., Takeuchi, S., Akira, and Julius, M. (2001): Soluble CD 14 enriched in colostrums and milk induces B cell growth and differentiation. Procc. *Natl. Acad. Sci.* **98,** 603 – 608.

Gebre Wold, A., M. Alemayehu, S. Demeke, S. Dediye, and Tadesse .A. (2000): Status of dairy research in Ethiopia .In: The *role of village dairy co-operatives in dairy development.* Smallholder Dairy Development Project (SDDP) Proceeding, Ministry of Agriculture (MoA). Addis Ababa, Ethiopia.

Grommers, F. J. (1988): Host Resistance Mechanisms of the Bovine Mammary Gland: an Analysis and Discussion, *Nether, Milk Dairy*. **42**,43.

Harmon, R. J. and Langloris, B. E. (1988): Prevalence of Minor Pathogens and Associated Somatic Cell Count. In proc. 25th annual management. National. Mastitis Counsel. Columbus, OH. Natl. Mastitis council, Inc., Arlington, VA. 11

Hass. De, Y. Veerkamp, H.W. Barkema, V.T Grohn, and Schukken, Y. H. (2004): Association between pathogen – specific cases of clinical mastitis and somatic cell count patterns. *J. Dairy Sci*. **85**, 1-16

Hillerton, J. E, Morgan W. E., Farnsworth, R., Neijenhuis, F., Bains, J. R., Main, E. A.,.Reineman, D. J. and Timma, L. (2001): Evaluation of bovine teat condition in commercial dairy herds, infectious and non-infections. In: Proceedings of the 2nd International Symposium on Mastitis and Milk Quality, Compton, United Kingdom. Pp 352-356

IDF (1987): Bovine mastitis, Definition and Guidelines for Diagnosis Bulletin of the International Dairy Federation, No. 211

Jones, T. C., Hunt, R, D., King, N.W. (1997): Infection of Mammary Gland, In: Veterinary Pathology. 6th (ed). Philadelphia, Lippincot Williams and Wilkins, pp1190-2000.

Jubb, K. V. F. and Kennedy P. C. (1970): Bovine mastitis. In: Pathology o f Domestic Animals. 2nd edition, Academic Press, INC., New York, San Francisco, London, pp 430-435

Jubb, K. V., Kennedy, F. and Palmer, P.C. (1993): The Mammary glands. In: Pathology of Domestic Animals, 4th ed., Academic *Press*. Inc. San Diego, Pp 455-471

Kassa, T, Gazaheng, W. and Azage, T. (1999): Survey of mastitis in dairy herds in the Ethiopian Central Highlands *Ethi. J. Agr. Sci.* **22,** 291 – 301.

Kitchen, B. J, Kwee, W.S, Midleton, G, and Anderson, R. G. (1994): Relation between the level of N-acetyl-β-D-glucosaminidase (NAGase) in bovine milk and the presence of mastitis pathogens. *Dairy Res.* **51,**11-16

Klapstrup, N.O. and Halliwell, R.W. (1997): Prevalence of bovine subclinical mastitis in Malawi, Nordisk Veterinaermedicin. **29.** 331 – 336

Lam, T. J., Schukken, H.Y., Gummers, F.G., Smit, and Brand, A.(1993):Within - herd and between - herd variation in diagnosis of clinical mastitis in cattle. *J. Am. Vet.Med. Assoc* **202,** 938- 942.

Lee, T.W., Elsasser, T.H., Zhao, X. (2003): Elevated milk soluble CD 14 in Bovine Mammary Glands challenged with *Escherichia coli* Lipopolysacharide. *J. Dairy Sci.* **86,** 2382 -2389.

McDonald, J .S. (1997): Streptococcal and staphylococcal mastitis. In: Jarret, J. (ed): The veterinary clinics of North America. Large animals practice. Symposium on bovine mastitis. W.B. Sounders Co, pp 269 –285.

Mdegela, H. (2005): Mastitis in smallholder dairy and pastoral cattle herds in the urban and peri - urban areas of the Dodema municipality in Central Tanzania. *J. LLRD.* **17,** 1-9

Mekonen, L, Kassa, T, and Azage, T. (2001): Clinically manifested major health problems of crossbred dairy herds in urban and peri-urban production systems in the Central Highlands of Ethiopia. *Trop. Anim. Health and Prod.* **33,** 85-89

Mungube, E. O. (2001): Management and Economics of Dairy Cow Mastitis in the Urban and Peri-urban Areas of Addis Ababa Milk Shed. Faculty of Veterinary Medicine, Addis Ababa University, Debre-zeit, Ethiopia, MSc. Thesis.

NCCLS (1997): Performance standards for for antimicrobial disk and dilution susceptibility test for bacteria isolated from animals: Tentative standards, NCCLS document, Pennsylvania, USA, **17,** 11. 7 – 8

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

Nesru, H., Yohualashet, T. and Tilahun, G. (1997): Prevalence of mastitis in different local and exotic breeds of milking cows. In: IAR proceeding, **4,** 256-262

Nickerson, S. C. (1987): Resistance mechanisms of the bovine udder: New implications for mastitis control at the teat end. *J. Am Vet. Med. Assoc,* **1991,** 1484 -1494.

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

NSDAD (2001): Basic data on agricultural resources, development potential and constraints. North Shoa Department of Agricultural Development. Fitchee.

Odessa, K. (1997): A Study on Bovine Mastitis in S ome Selected Areas of Southern Ethiopia. Faculty of Veterinary Medicine, Addis Ababa University, Debre zeit, Ethiopia, DVM Thesis.

Omare, A. (1996): Epidemiology and economics of mastitis in the smallholder dairy sector of Kiambu district, Kenya, University of Nairobi, PhD Thesis.

Owens, W.E., Watts, J.L., and Boddie, R L. (1998): Antibiotic treatment of mastitis comparison of Intramammary plus intramuscular therapies. *J. Dairy Sci.* **71,** 3143-3147.

Peeler, E. J., Green, M. J., Fitzpatriek, J. L., Morgans, K. L. and Greens, L.E. (2000): Risk factors associated with clinical mastitis in low cell count British dairy herds. *J. Dairy Sci.* **83,** 2464 – 2472.

Perry, B. D., Carter, F. W., Hill, M. E., G. and Milne, A. C. (1987): Factors associated with occurrence, cause and outcome of clinical mastitis in dairy cattle. *Veterinary Record.* **105,** 456 – 463

Philpot, W. N. (1984): Economics of mastitis control. In: Jarret, J. A (ed.), Symposium on Bovine *Mastitis. Vet. Clinics of North America.* ***6, 233-245.***

Pyorala, S., and Vesa, M. (1995): Resistance of bacteria to antimicrobials. In: Sandholm, M., Honkanen- Buzalski, T., Kaartinen, L., Pyorala. (eds), In: The bovine udder and mastitis. Finland , Gummerus Kirjapaino Oy. Juvaskyla, Pp 235 – 245

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

Quinn, P. J., Carter, M. E., Morley, B., Carter, G. R. (1994): California Mastitis Test (CMT). In: Clinical Veterinary Microbiology. 1st ed. Wolfe publishing, London, Pp 333-334.

Radostits, O. .M., Blood, D. C. and Gay, C. C. (1994): Mastitis. In: Veterinary Medicine: Text Book of the Diseases of Cattle, Sheep, Goat and Horses. 8th. ed. Baillier Tindal, London, Pp 563-614.

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

Rainard, P and Peutrel, B. (1988): Effect of naturally occurring intra-mammary infections by minor pathogens on new infections by major pathogens in cattle. *Am. J. Vet. Res.* **49,** 327.

Sandholm, M. (1995): Detection of inflammatory changes in milk. In: Sandolm, M., Honkanen- Buzalski, T., Kaartinen, L., Pyorala .(eds), In: The bovine udder and mastitis. Finland, Gummerus Kirjapaino Oy. Juvaskyla, pp 89 – 104.

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

Schukken, Y. H., Grommers, F. J., Van de Geer, Brand, A. (1989): Intramammary infections and risk factors for clinical mastitis in herds with low somatic cell counts in bulk milk. *Veterinary Record,* 125, 393-396

Sére, C., Seinfeld, H., Goenewold, J. (1996): World Livestock Systems. Current Status, Issues and Trends. Animal Production and Health Paper no. 127, FAO, Rome, 81.

Staal, S. J. (1995): Peri urban Dairying and Public Policies in Ethiopia and Kenya, A comparative Economic and Institutional Analysis. University of Florida, Gainesville, Florida. PhD. Dissertation.

Tefera, G. (2001): Prevalence of mastitis at Alemaya University Dairy Farm, *J. Eth. Vet. Assoc.* **17,** 21.

Thirapatsakun, T (1999): Mastitis management. In: Smallholder Dairying in the Tropics. In: Hunt and Chantalakhana. (eds). ILRI, Kenya, Nairobi, Pp299-339.

Thresfeild, M. (1995): Veterinary epidemiology. 2nd ed. Blackwell Science, Pp 182-190.

Vaarst, and Enevoldsen. C. (1997): Patterns clinical mastitis manifestation in Danish organic dairy herds. *J.Dairy Res*. **64**,23-27.

Walshe, M. T., Grindle, T., Nell, A. and Baschaman, M. (1991): Dairy Development in Sub Saharan Africa: A study of Issues and Options, World Bank Technical Paper, 135, Africa Technical Department series, Washington D.C., The World Bank.

Watts, J. L., Yancey, R. J. (1994): Identification of veterinary pathogens by the use of commercial identification system and new trends in antimicrobial susceptibility testing of veterinary pathogens. *J. Clin. Microb*. Rev. **7,** 3346.

Watts, J. L. (1988): Etiologic agents of bovine mastitis. *Vet. Microb.* **16,** 41-46.

Werkineh, S., Bayleyeng, M., Mekonnen, H., Potgieter, L. N.D. (2002): Prevalence and aethiology of mastitis in cows from two major Ethiopian dairies. Tropical Animal Health and Production, **34,** 19-25.

Williams, T. O., DeRosa, D. A., Badiane, O.(1995): Macroeconomic, international trade and sect oral policies in livestock development: an analysis with particular reference to low income countries. In: Wilson, R. T., Ehui, S, and Mack, S. (eds). Livestock Development S trategies for low income Countries. Proceeding of the Joint FAO/ILR Roundtable on Livestock Development Strategies for low income Countries, ILRI, Addis Ababa, Ethiopia, 27 February 02 March 1995. 45-68.

Wilson, C.D., Ager, N., Gilbert, G. A. (1986): Field trials with cefoperzon in the treatment of bovine mastitis. *Veterinary Record*. **118,**17-23.

8. ANNEXES

Annex 1. Questionnaire Format

Owners' Name _____Address_____Date of sampling_____

1. Cow History:

Breed_____Age_____Last date of calving_____

Parity_____Mastitis history_____(Y/N)

Teat lesion: present_____absent_____Tick infestation_____

Gross milk quality: watery_____bloodtinged_____flakes_____normal_____

Sample collected from: RR___RF___LF___LR___

CMT score: RR___RF___LF___LR___

2. Milking hygiene practice:

Do you wash your hand before milking? Yes____ No_____

Do you wash teat udder before milking? Yes____ No_____

Do you dry the wet udder with separate towel? Yes____ No_____

Do you practice milking mastitic cows last? Yes____ No_____

3. House floor status:

Floor type: concrete____sloppy/level, Hardcore_____ sloppy/level, Soil/Muddy

Roof: metal sheet ____ grass_____

Wall: concrete_____mud_____othrs_____

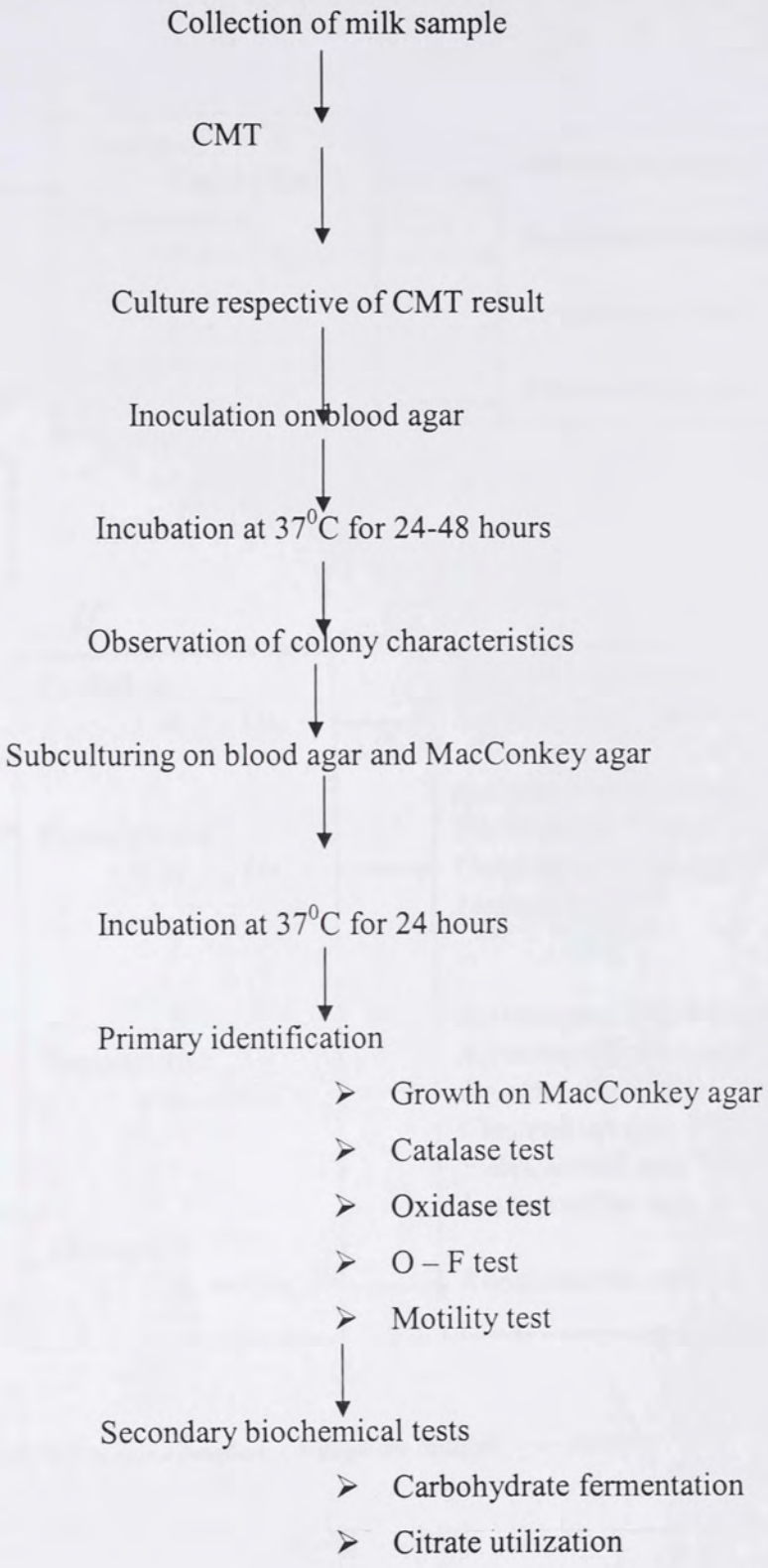
Manure disposal: dailly____weekly_____monthly_____others_____

Annex 2. Interprétation of CMT findings

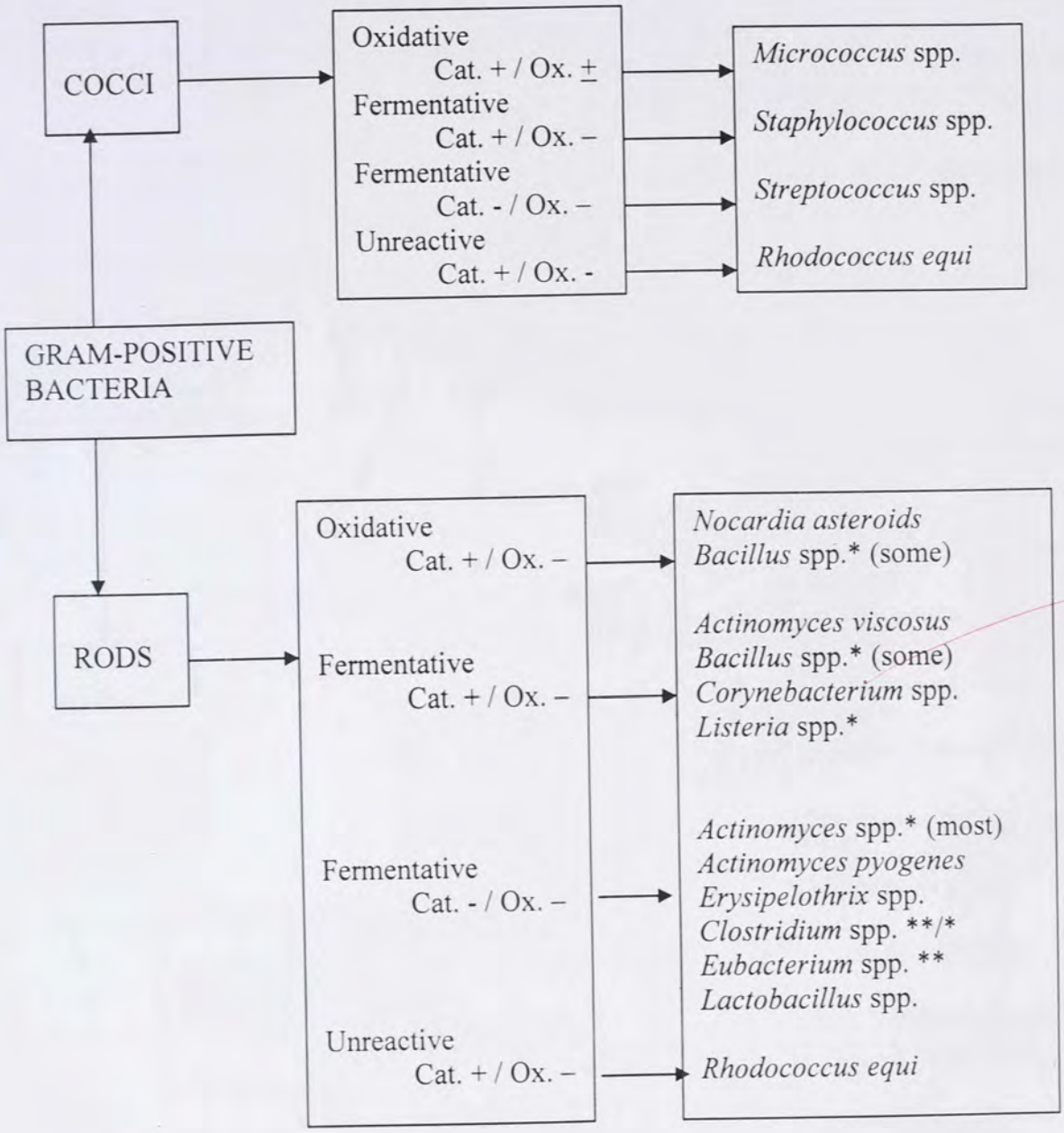
Score	Interpretation	Visible reaction
0	Negative	Milk fluid and normal
T	Trace	Slight precipitation
1	Weak positive	Distinct precipitation but no gel formation
2	Distinct positive	Mixture thickens with gel formation
3	Strong positive	Viscosity greatly increased, strong gel i.e. cohesive with a convex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

Source: Quinn *et al* (1994)

Annex 3. Flow chart for isolation and identification of bacteria from milk

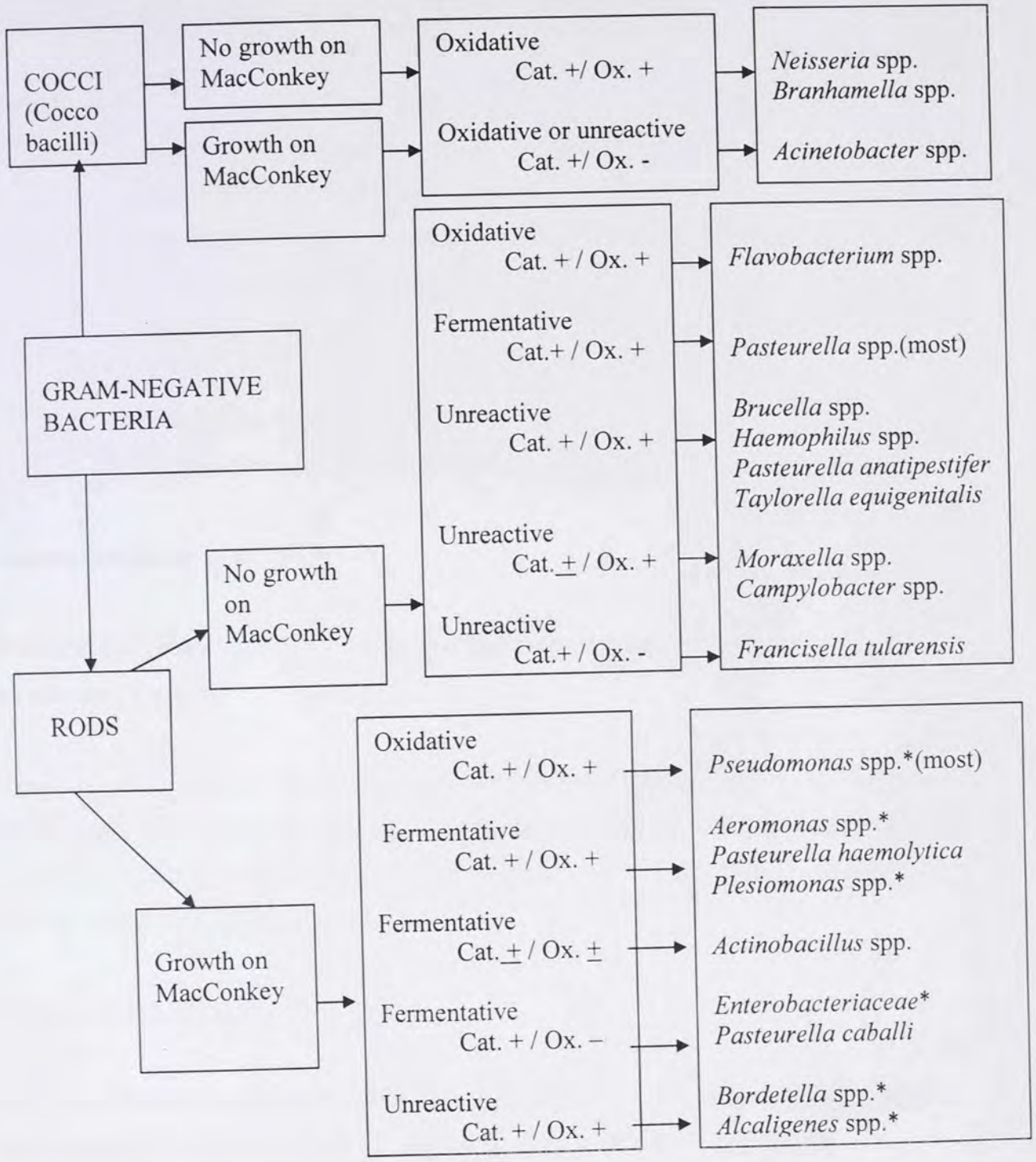


Annex 4. Primary identification of gram-positive bacteria



(Cat. = Catalase; Ox. = oxidase; + = positive reaction; - = negative reaction; ± = variable; *= motile; ** = anaerobic)

Annex 5. Primary identification of gram-negative bacteria



(Cat. =Catalase; Ox. =oxidase; += positive reaction; - = negative reaction; ± = variable, *= motile)

Source: (Quinn et al., 1999)

Annex 6. Primary identification tests

Gram's stain (Carter, 1984)

Procedure:

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

Catalase test (Quinn *et al*, 1999)

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

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

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

Principle: The cytochrome oxidase enzyme is able to oxidize the substrate tetramethyl-p-phenylenediamine dihydrochloride, forming a coloured end product, indophenol.

Procedure: Prepare a solution of 1 % tetramethyl-p-phenylenediamine dihydrochloride, then a piece of filter paper is moistened in a Petri dish with fresh reagent and the test

bacterium is streaked firmly across the filter paper with a glass rod. A dark purple colour along the streak line with in 10 seconds indicates a positive reaction. *Pseudomonas aerugenosa* can be used as a positive control organism

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

Procedure: Prepare O-F base medium and when the O-F base has cooled to 50 ^{0}C add 20 ml of sterile glucose solution into 200 ml of O-F base, for a final concentration of 1 % glucose and dispense in to tubes. Two tubes of the O-F medium are heated in a beaker of boiling water immediately before use to drive off any dissolved oxygen and the tubes are then cooled rapidly under cold running water. Both tubes are stab-inoculated with the bacterium and a layer of sterile paraffin oil is layered on top of one of the tubes (sealed tube) to a depth of about 1cm and the tubes are incubated at 37^{0}C and examined in 24 hours and then daily for up to 14 days.

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

Procedure: SIM medium (BBL) was used to detect motility and the medium was stab inoculated using a straight wire. Then the tube is examined for motility after 24 and 48 hours. If there is a diffuse growth throughout the medium, the bacterium is motile. The growth of a non-motile bacterium is confined to stab line. To interpret the results, hold the tube against a good light and compare the inoculated tubes with un inoculated one.

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

Procedure: A culture of the *Staphylococcus aureus*, with a wide zone of partial haemolysis (beta-haemolysin) is streaked across the center of a sheep or ox blood agar plate. A streak of the suspect Group B streptococcus is made at right angles to, and taken to with in 1 to 1.5 mm of staphylococcal streak. The plate is incubated at 37^{0}C for 18 – 24 hours. An arrow–head of complete haemolysis, indicates a positive CAMP test. The group B streptococci produce diffusible metabolites that complete the lysis of the red cells, only partially haemolysed by the beta –haemolysin of the staphylococcus.

Annex 7. Secondary identification tests

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

Principle: Indole positive bacteria possess an enzyme tryptophanase which converts tryptophan to indole.

Procedure: Stab inoculate SIM medium with test bacterium and incubate at 37 ^{0}C for 18 to 24 hours. Then add Kovac's reagent (0.2 ml) to tube and stand for 10 minutes.

Interpretation: The formation of dark red ring indicates positive reaction while in negative reaction a yellow ring is formed.

Methyl red (MR) test (Quinn *et al.*, 1999)

Principle: It is a quantitative test for acid production, requiring positive organisms to produce strong acids (lactic, acetic, and formic)

Procedure: Inoculate MR-VP broth with pure culture of test organism and incubate at 37^{0}C for two days, then add 5 drops of MR solution in to the media.

Interpretation: Production of red colour indicates a positive result and yellow colour negative in methyl red test.

Voges-Proskauer (VP) test (Quinn *et al.*, 1999)

Principle: Some organisms produce acetoin as the chief end product of glucose metabolism and form less quantity of mixed acids.

Procedure: Inoculate MR-VP broth with pure culture of the test organism and incubate at 37^{0}C for 2 days. Then aliquot 1 ml of broth to a clean test tube and add 0.6 ml of 5 % α-

naphthol followed by 0.2 ml of 40 % KOH. Shake the tube gently to expose the medium to atmospheric oxygen and allow the tube to remain undisturbed for 10 to 15 minutes. Interpretation: A pink colour indicates a positive reaction.

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

Principle: Urease is an enzyme possessed by many species of microorganism that can hydrolyze urea with the formation of ammonia (alkaline).

Procedure: The surface of the agar slant is streaked with the test organism and incubated at 37^0C for 18 to 24 hours.

Interpretation: Organisms that hydrolyze urea rapidly may produce positive reaction with in 1 or 2 hours. Red (pink) colour through out medium indicates positive reaction

Annex 8. Differentiation of mastitis causing Staphylococcus spp and Micrococcus spp

Test	*S. aureus*	CNS	*Micrococcus*
Catalase	+	+	+
Coagulase	+	–	–
Haemolysis	+	–	–
Manitol (A)	+	–	–
Maltose (A)	+	V	–
Glucose (A	+	+	–

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

Annex 9. Differentiation of mastitis causing streptococcus species

Species	CAMP Test	Growth on MacConkey	Easculin hydrolysis	Other Confirmatory Tests
Str. agalactiae	+	-	-	-
Str. uberis	±	-	+	Manitol +(A)
Str. dysgalactiae	-	-	-	Salicin +(A)
Str. faecalis	-	+	+	salicin +,Manitol +
Str. pyogens	-	-	-	Salicin (-)
Str. pnuemoniae	-	-	+	Manitol (-)

+ = Positive reaction, - = Negative reaction, ± =Positive or Negative

Annex 10. Differential test used for Bacillus spp.

Species	Citrate	Arabinose	Manitol	Voges Proskauer
B.steariothermophilus	–	v	–	–
B.cerus	+	–	–	+
B.pumilus	+	+	+	+
B.brevis	d	–	d	–
B.coagulans	d	d	d	d

V=variable reaction, d=11-89% strains are positive

Source: Carter (1990)

Annex 11. Differential tests used for Corynebacterium and Actinomyces spp

Species	Catalase test	Haemolysis	Glucose	Lactose	Maltose	Trehalose
C. ulcerans	+	V	+	-	+	+
C.bovis	+	-	-	-	-	-
C.pseudotuberculosis	+	+	+	+	+	+
A.pyogenes	-	+	+	+	+	V

V=Variable reaction,

Source: Carter (1984), Quinn *et al.* (1999)

Annex 12. Differential test used for Gram – negative rods

G-ve bacteria	1	2	3	4	5	6	7	8	9
E.coli	+	+	-	-	(+)	-	***Y/Y, Gas+, H_2S^-***	-	+
K. pneumoniae	-	-	+	+	+	+	Y/Y, Gas+, H_2S^-	-	+
C. freundi	-	+	-	+	-	+	Y/Y, Gas+, H_2S^+	-	+
P. aeuroginosa	-	-	-	+	-	-	R/R, Gas+, H_2S^-	+	+
P. mirablis	-	+	-	±	-	+	Y/Y, Gas^+, H_2S^+	-	+
P. vulgaris	+	+	-	D	-	+	Y/Y, Gas^+, H_2S^+	-	+
E. aerogenes	-	-	±	D	+	-	Y/Y, Gas^+, H_2S^-	-	+
E. agglomerans	+	-	+	+	D	D	Y/Y, Gas^+, H_2S^-	-	+
P. multocida	+	-	-	-	-	-	H_2S^+	±	-

1=indole test, 2=Methyl red test, 3=VogesProskauer test, 4=Citrate utilization, 5=lysine decarboxylase test, 6=urase test, 7=TSI test, 8=oxidase test, 9=Growth on MacConkey agar D = 26-75% of strains positive, (+)=76-89% 0f strains are positive

Annex 13. Media used for isolation and identification of bacteria

1. Blood Agar Base (BBL®, Becton Dickinson, USA)

Composition (g/l): Heart muscle, infusion from (solids) 2.0; pancreatic digest of casein 13.0; Yeast extract 5.0; sodium chloride 5.0; agar 15.0

Preparation: Suspend 40.0g of the powder in 1 liter of distilled water. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder, Autoclave at 121°C for 15 minutes. Cool the base to 45 to 50°C and add 5% sterile defibrinated blood.

2. MacConkey Agar (Oxoid, Hampshire, England)

Composition (g/l): Peptone 20.0; lactose 10.0; bile salts No.3 1.5 ; sodium chloride 5.0; neutral red 0.03; crystal violet 0.001; agar 15.0

Preparation: Suspend 51.5g in 1 liter of distilled water. Bring to boil completely. Sterilize by autoclaving at 121°C for 15 minutes.

3. SIM Medium (BBL®, Becton Dickinson, USA)

Composition (g/l): Pancreatic digest of casein 20.0; peptic digest of animal tissue 6.1; ferrous ammonium sulfate 0.2; agar 3.5

Preparation: Suspend 30g of the powder in 1 liter of distilled water. Mix thoroughly. Heat with frequent agitation and boil for 1minute, Autoclave at 121°C for 15 minutes.

4. O-F basal medium (Merck, Darmstadt, Germany)

Composition (g/l): Peptone from casein 2.0; yeast extract 1.0; sodium chloride 5.0; Di-potassium hydrogen phosphate 0.2; bromothymol blue 0.08; agar-agar 2.5.

Preparation: Suspend 11g in 1 liter of distilled water by heating in a boiling water bath or in a current steam, autoclave (15 minutes at 121°C); at approximately 50°C mix in 100ml/ liter of filter sterilized 10% solution of D (+) glucose, lactose, sucrose, or other carbohydrates; dispense in to tubes to give a depth of approximately 5cm.

5. Phenol-red broth base (Merck, Darmstadt, Germany)

Composition (g/l): Peptone from casein 5.0; peptone from meat 5.0; sodium chloride 5.0; phenol red 0.018.

Preparation: Suspend 15g in 1liter of distilled water; dispense into tubes and insert fermentation tubes; if necessary; autoclave (15 minutes at 121°C). At less than 60°C add the reactants (final concentration 5 to 10g / liter) as sterile solutions.

6. Mannitol Salt Agar (Difco, Detroit, USA)

Composition (g/l): Proteose peptone No.3 10.0; Bacto-beef extract 1.0; D-mannitol10.0; sodium chloride 75.0; Bacto-agar 15.0; phenol red 0.025

Preparation: Suspend 111g in 1 liter distilled water and heat to boiling to dissolve completely. Sterilize in the autoclave for 15 minutes at 15 pounds pressure (121°C). Cool to 45 to 50°C and dispense in to Petri dishes.

7. Nutrient agar (Oxoid, Hampshire, England)

Composition (g/l): "Lab-Lemco" powder 1.0; yeast extract 2.0; peptone 5.0; sodium chloride 5.0; agar 15.0.

Preparation: Suspend 28g in 1 liter of distilled water. Bring to boil to dissolve completely. Sterilize by autoclaving at 121^{0}C for 15 minutes.

8. Triple sugar iron agar (Merck, Germany)

Composition (g/l): Peptone from casein 15.0; peptone from meat 5.0; meat extract 3.0; sodium chloride 5.0;lactose 10.0; sucrose 10.0; D (+) glucose 1.0; ammonium iron (III) citrate 0.5; sodium thiosulfate0.5; phenol red0, 0024; agar –agar 12.0.

Preparation: Suspend 65g in 1 liter of distilled water by heating in a boiling water bath or in a current steam; dispense in to tubes; autoclave (15 minutes at 121^{0}C). Allow to solidify to give agar slants.

9. Simmons Citrate Agar (BBL®, Becton Dickinson, USA)

Composition (g/l): Ammonium dihydrogen phosphate 1.0; dipotassium phosphate 1.0; sodium chloride 5.0; sodium citrate 2.0; magnesium sulfate 0.2; agar 15.0; bromothymol blue 0.08.

Preparation: Suspend 24.2g of the powder in 1 liter of distilled water. Mix thoroughly, heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Autoclave at 121^{0}C for 15 minutes. Cool tubed medium in a slanted position for slants.

10. Edwards medium, modified (Oxoid, Hampshire, England)

Composition (g/l): "Lab-Lemco" powder 10.0; peptone 10.0; aesculin 1.0; sodium chloride 5.0; crystal violet 0.0013; thallus sulphate 0.33; agar 15.0.

Preparation: Suspend 41g in 1 liter of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 115^{0}C for 20 minutes. Cool to 50^{0}C; add 5 to 7% of sterile bovine or sheep blood. Mix well and pour plates

11. Eosin Methylene Blue Agar (modified) Levine (Oxoid, Hampshire, England)

Composition (g/l): Peptone10.0; lactose 10.0; Dipotassium hydrogen phosphate 2.0; eosin Y 0.4; methylene blue 0.065; agar15.0.

Preparation: Suspend 37.5g in 1 liter of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121^{0}C for 15 minutes. Cool to 60^{0}C and shake the medium in order to oxidize the methylene blue (i.e. restore its blue colour and to suspend the precipitate which is an essential parts of the medium.

12. MR-VP medium (Oxoid, Hampshire, England)

Composition (g/l): Peptone 7.0; Glucose 5.0; Phosphate buffer 5.0

Preparation: Suspend 17g in 1 liter of distilled water. Mix well, distribute in to final containers and sterilize by autoclaving at 121 ^{0}C for 15 minutes.

13. Urea Agar Base (BBL®, Becton Dickinson, USA)

Composition (g/l): Pancreatic Digest of Gelatin 1.0; Dextrose 1.0; Sodium Chloride 5.0; Potassium Phosphate 2.0; Urea 20; Phenol red 0.012.

Preparation: Suspend 29g of the powder in 100 ml of distilled water. Mix thoroughly and Sterilize by filtration. Suspend 15g of Agar in 900 ml distilled water, autoclave at 121^{0}C for 15 minutes. Cool to 50^{0}C and add 100 ml of urea agar base. Mix thoroughly and dispense aseptically in sterile tubes. Cool tube medium in a slanted position so that deep butts are formed.

14. Muller Hinton Agar (BBL®, Becton Dickinson, and USA Composition (per litter-purified water): Beef extract 2gm, Acid Hydrolysate of casein 17.5gm, Starch 1.5gm, Agar 17gm.

Preparation: Suspend 38gm of the powder in 1 litter of purified water. Mix thoroughly. Heat with frequent agitation and boil for 1 min. to completely dissolve the powder. Autoclave at 121^0C for 15 minutes. Do not over heat.

nex 14. Procedures to conduct antibiotic susceptibility test

Preparation of the inoculum

Inoculation of a distinct colony in to 5ml of nutrient broth, and incubat at 35-37^{0}C for about 5 hours. Then the turbidity is compared with 0.5MacFarnand standard. This standard is prepared by adding 0.5 ml of 1 %(11.75g/litre) $Bacl_2.2H_20$to 99.5ml of 1 % (0.36N) H_2SO_4.

- Inoculation to Mueller-Hinton Agar

Mueller-Hinton Agar cooled to 50^{0}C and poured in to a sterile Petri dish on a level surface to a depth of 4 mm. this is equivalent to 60ml in a 15cm plate and about 25 ml in a 10 cm plate. For slow growing bacteria 5% defibrinated whole blood could be added. Then a sterile cotton swab on a wooden applicator stick is used to transfer the diluted bacterial suspension to a plate; excess fluid must be squeezed out by rotating the swab against the sides of the tube. The plate is seeded uniformly by rubbing the swab against the entire agar surface in three different planes roughly 60 degrees to each other.

- Disc Application

Within 15 minutes (time used to dry the inoculum) after the plates are inoculated, antibiotic impregnated discs are applied to the surface of the inoculated plates by hand using a sterile forceps. All discs gently pressed down on to the agar with forceps to ensure complete contact with the agar surface. The discs should no closer than 1.5 cm to the edge of the plate and they should rest 3 cm apart from each other. The large Petri dishes easily accommodate 9 discs in outer ring and three in the center, where as no more than 8 should be placed in small plates (10 cm plates).

- Incubation

Incubate the plates invented aerobically for 16 to 18 hours at 35^{0}C but not 37^{0}C

- Interpretation

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

Zone Size Interpretive Chart for Antimicrobials

Inhibition Zone Diameter (mm)

Antimicrobial agent	Disc potency	Resistance	Intermediate	Susceptible
Streptomycin S10	10μg	≤ 11	12-14	≥15
Oxytetracyclin OXTE 30	30μg	≤14	15-18	≥19
Erythromycin E15	15μg	≤ 13	14-17	≥ 18
Penicillin G10 for staphylococci PenicillinG10 for other microorganisms	10 IU 10 IU	≤ 20 ≤ 11	21-28 12-21	≥ 29 ≥ 22
Sulphanomide S 300	300μg			
Streptomycin S 10	10 IU			
Polymixin B PB30	300μg	≤ 8	9-11	≥ 12
Kanamycin K30	30μg	≤ 13	14-17	≥ 18

CURRICULUM VITAE

1. Personal Data

Name	Getahun Kassaye Woldyes
Date of Birth	29 / 9/ 1963
Place of Birth	Addis Ababa, Ethiopia
Nationality	Ethiopian
Marital status	Married
Profession	Veterinarian
Permanent address	Addis Ababa, Ethiopia. getahunkassaye@yahoo. Comm. Tell. 251- 1- 3200704,(Mobile) 0911340293 A.A. Ethiopia

2. Educational background

1971 – 1977	Shimelis Habte, Beyene Merid Primary and Junior High School
1978 – 1981	Abyot Kirs Secondary High School
1982 – 1983	Addis – Ababa Technical School
1984 – 1989	Faculty of Veterinary Medicine, Kishinev Agriculture Institute, Moldavia. (USSR)

3. Professional experiences

1990 – 1991	Head of veterinary service at Dida – Tuyura boran Cattle Breeding Center
1992 – 1995	Southern Rangeland Development project, Mega Animal Health Service unit head
1996 – 2003	Team leader of animal health service at Adola, and Girar Jarso woreda in Borena, and North Shoa Zone

4. Membership

Member of the Ethiopian veterinary associations

5. Publication:

1. DVM thesis, the effect of histotherapy of an emulsion of denatured placenta on the clinical status, haemopoetic, and antioxidation system of newborn calves, Faculty of veterinary medicine, Kishinev agricultural institute, 1989, Moldavia (USSR).
2. Bovine Mastitis and Somatic Cell Count. A review, May 2005, Faculty of Veterinary Medicine, Addis Ababa University, Debre Zeit, Ethiopia.
3. MSc. Thesis, Bovine Mastitis and Antibiotic Resistance Patterns of Major Pathogens in Smallholder Dairy Farms in the Central Highlands of Ethiopia. Jun 2006, Faculty of Veterinary Medicine, Addis Ababa University, Debre Zeit, Ethiopia.

Signed Declaration Sheet

I the under signed, declare that the thesis is my original work and has not been presented for a degree in any University

Name _____

Signature _____

Date of Submission _____

This thesis has been submitted for examination with our approval as the University advisors

Dr. Kelay Belihu _____

Dr. Merga Bekana _____

Bovine Mastitis & Antibiotic Resistance Patterns Of Major Pathogens In Small Holder Dairy Farms In The Center Highlands Of Ethiopia

Getahun Kassaye