ADDIS ABABA UNIVERSITY

FACULTY OF VETERINARY MEDICINE

COMPARISON OF CLINICAL TRIALS OF BOVINE MASTITIS WITH THE USE OF ANTIBIOTICS AND HONEY

A thesis submitted to the School of Graduate Studies of Addis Ababa University in partial fulfillment of the requirement for the Degree of Master of Veterinary Science in Tropical Veterinary Microbiology

BY

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JUNE 2007

DEBRE ZEIT, ETHIOPIA

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ACKNOWLEDGEMENTS

I would like to acknowledge Professor A.R.S. Moorthy, who was willing to assist and advice me from the beginning. I also express my heartfelt gratitude to Soddo Veterinary Laboratory personnel; especially to Dr. Terzu Daya, Ato Endashaw Fikru, Wro. Mulualem Almazu Wro. Zemzem Arebo and Ato Mohamedcha Mana; Soddo dairy farm; Soddo Woreda Veterinary Clinic; Soddo Christian Hospital; Soddo Public Health Center; Soddo A Y M farm and all the colleagues and my family who forwarded valuable suggestions and assisted me in one or the other way, during my research work.

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ABBREVIATIONS

BHI	Brain heart infusion
CMT	California mastitis test
FMD	Foot and mouth disease
ELISA	Enzyme linked immuno-sorbent assay
IM	Intramuscular

IMI	Intramammary infections
IMM	intramammary infusion
KOH	Potassium hydroxide
LSD	Lumpy skin disease
MZN	Modified Ziehl-Neelsen stain
NMC	National Mastitis Council
O-F	Oxidation-fermentation
SCC	Somatic cell count
SDA	Sabouraud's dextrose agar
SNNPR	Southern Nation Nationalities and Peoples Region
TSI	Triple sugar iron agar
URT	Upper respiratory tract
ZN	Ziehl-Neelsen stain

ABSTRACT

A total of 423 representative randomly selected Holstein, Jersey and local zebu lactating cows located in Wolaita zone at Soddo Zuria Woreda of Southern Nation Nationalities and Peoples Regional State were examined. All the 46 lactating cows of Soddo dairy farm, 11 lactating cows from Soddo Veterinary Clinic, 103 lactating cows from urban dairy holders and the rest 263 were

randomly selected lactating dairy cows from 18 rural Kebeles were included and tested to determine the prevalence of mastitis by using mastitis detecting BOVI-VET (RUUSE, DENMARK), indicator paper and white side test. Milk samples were cultured from 329 cows infected with different grades of mastitis. Examination for mastitis infection in Soddo Zuria Woreda revealed that there were 99 (23.4 %) clinical and 230 (54.3 %) sub clinical cases. Out of 423 cows 99 clinical cases and 230 sub-clinical cases were recorded and grouped into grade I, II, III, IV level of infection and only clinical cases were subjected to antibiotics and honey intramammary infusion treatment. The prime objective of the study is to examine the safety and efficacy of honey compared to a known antibiotic infusion in lactating mastitic cows. Minimum Inhibitory Concentration (MIC) of honey was determined by using different dilutions and different species of bacteria available at the microbiology laboratory of FVM. And it was revealed that minimum concentration of honey that inhibited *E. coli* was 10 %; *L. monocytogenes* and *K. pneumoniae* inhibited at 20 %; *Staphylococcus and Streptococcus spp,* were inhibited at 30% concentration, where as *Micrococcus spp*. at 40 % concentration. *Salmonella enteritidis* was resistant (shown growth in all concentrations). Out of ninety-nine clinical cases, forty-eight cases were treated with antibiotic (Multiject IMM.) infusion and fifty cases were treated with Beza honey. Milk samples from the cows identified and recorded for treatment trial groups were re-cultured within one month post-treatment. Cases included in the analysis had at least one mastitis pathogen isolated from the initial milk samples. Overall bacteriological cure rate of treated cases was 54 % (53 of 98). The effectiveness of honey in clearing bacterial infection in the intramammary infusion for mastitis treatment revealed similar to antibiotic (54.2% (Multiject IMM.) treated cases. Antibiotic and non-antibiotic treatment responses were associated with grade of affection. Honey treatment was more effective 78.1% in grade III type of mastitis when compared to treatment of grade II type of mastitis (11.1%); where as multiject was more effective in grade II type of mastitis (41.9%) and slightly less curative rate (76.4%) for grade III type of mastitis when compared to honey. The present study indicated that honey treatment could be an alternative treatment to antibiotics so that indiscriminate use of antibiotics and emergence of antibiotic resistance strains of microorganisms could be avoided and at the same time financial benefits can be obtained. Treatment with honey is more economical i.e.7-8 times cheaper than the available mastitis treatment drugs in the country. As an alternative to antibiotic treatment for

mastitis, 10 ml of 40% honey intramammary infusion for each quarter for 3 consecutive days can be tried.

INTRODUCTION

Even though great technological advances have been made in dairy industry, mastitis continues to be a major economic issue for dairy producers (Fetrow *et al*., 2000). Thus, researchers and dairy advisors continue to refine the National Mastitis Council (NMC, 2000) recommended mastitis control program.

Mastitis accounts for the majority of financial loss in dairy cattle and despite the numerous endeavors invested in prevention; hitherto most treatments resulted in partial success. Recently the awareness of healthy foods has grown and became evident in the aim to minimize the usage of antibiotics among farms including mastitis in dairy cattle (Pinchasov *et al*., 2004).

The indiscriminate use of antibiotics has made many microorganisms to develop resistance to them. This has created immense clinical problems in the treatment of infectious diseases. Therefore, there is a need to develop alternative anti-microbial agents for the treatment of infectious diseases (Al-Jabri, 2005).

Studies on mastitis in Ethiopia indicated a high prevalence of 52-67.4% (Biru, 1989). In another Study by Simuka, (1998) isolated *Staph. aureus, Staph. epidermidis, Str. agalactiae, Str. uberis, Actinomyces bovis* and *Actinomyces pyogenes* from mastitis cases in Ethiopia.

Only a few clinical trial researces were conducted with non-antibiotic treatment in this country. Hence, in the present study, comparative clinical trials were conducted in bovine mastitis cases by using antibiotics and honey (non-antibiotics) to compare their curative effect in dairy herds at Soddo Zuria Woreda of Southern Nation Nationalities and Peoples Regional State from September 2006 to April, 2007.

The objectives of the study are to:

Estimate the prevalence of mastitis in dairy herds at Soddo Zuria Woreda.

Identify the bacterial isolates from mastitis cases.

Determine the inhibitory concentration of honey to some known bacteria *in vitro.*

Conduct clinical trials of mastitis cases with antibiotics and honey to compare the curative effect.

2. LITERATURE REVIEW

2.1. Definition of bovine mastitis

According to Quinn *et al*. (1994), mastitis is an inflammation of the mammary gland that can be caused by physical or chemical agents but the majority of the cases are infectious and usually caused by bacteria.

2.2. Clinical forms of mastitis

The main clinical forms of mastitis are: 1) per acute form, in which swelling, heat, pain and abnormal secretion in the mammary gland are accompanied by fever and other signs of systemic disturbance such as marked depression, rapid weak pulse, sunken eyes, weakness and anorexia; 2) Acute form, in which similar changes in the mammary gland occur with only slight to moderate fever and depression; 3) Sub-acute form, in which there is no systemic change and changes in the gland and the secretions are less marked; and 4) Sub-clinical form, where the inflammatory reaction within the gland is only detectable by tests, such as the California Mastitis Test (CMT), the Wisconsin Mastitis Test, the White side test and the cell count methods (Britt, 1998).

Changes in the secretion can vary from a slight wateriness with a few flakes (e.g. sub acute staphylococcal mastitis), wateriness with large yellow clots (e.g. acute and per acute streptococcal and staphylococcal mastitis) watery to brownish secretion with fine flakes (e.g. coliform mastitis). Without treatment, the affected quarter gradually loses its productive capacity and may become either atrophied or slowly develops firm nodular granuloma like masses within the parenchyma of the udder (fibrosis). The bacterial pathogens most commonly responsible for bovine mastitis (in order of frequency) are: *Staphylococcus aureus*, *Streptococcus agalactiae*, other Streptococci, Coliform organisms, *Actinomyces pyogenes* and *Pseudomonas aeruginosa*. Less commonly, mastitis may be associated with infection of the udder by *Nocardia asteroides*, *Clostridium perfringens*, *Mycobacterium spp.* and yeasts (Britt, 1998).

2.3. Prevalence and economic importance

In Ethiopia, even though the disease has been studied, it was not systematic. Hundera *et al*., (2005) studied the prevalence of mastitis and associated economic loss and they reported a prevalence of clinical and sub clinical mastitis as 16.11% and 36.67%, respectively, Demelash *et al*., (2005) reported 34.9% prevalence of bovine mastitis. In general, the incidence of clinical mastitis is reported to be between 30% and 55% annually (Britt, 1998). In contrast to this, the incidence of clinical mastitis has been very low in smallholder dairies in Eastern Africa .The incidence was reported to be as low as 13.2% per annum in Kiambo district of Kenya but in other parts of Kenya it ranged from 4-10%. The prevalence of sub-clinical mastitis however was very high (71%) when threshold of 300,000 cells / ml of milk was used. The most common bacterial isolates were *Staphylococcus aureus* and *Streptococcus species* (Simuka, 1998).

Major economic loss can occur in a dairy herd with a mastitis problem. The losses due to mastitis are some or all of death due to per acute forms of mastitis, loss of cows through premature culling, loss of milk production, cost of treatment and veterinary fees etc (Quinn *et al*., 1994).

2.4. Aetiology

Many infectious agents have been implicated as cause of mastitis in cattle. The causes of contagious mastitis particularly are *Streptococcus agalactiae* and *Staphylococcus aureus*. *Escherichia coli*, *Streptococcus dysgalactiae*, and *Streptococcus uberis* are environmental opportunistic pathogens of udder. *Staphylococcus hyicus*, *Staphylococcus epidermidis*, and coagulase negative Staphylococcus and *Actinomyces bovis* are normal teat flora often isolated from bovine intramammary infections (Radostits *et al*., 1994).

Mastitis causing pathogens from etiological point of view, a total of 133 microbial species, sub-species and serovars has been isolated from bovine udder. However, epidemiology has allowed their classification into:

Major pathogens are classified as causes of either contagious or environmental mastitis. The major pathogens causing contagious mastitis are *Streptococcus agalactiae* and *Staphylococcus aureus*. These are mainly spread from cow to cow through milker's hands, udder wash-cloths,

residual milk in teat cups etc. Major pathogens causing environmental mastitis are coliform and the other Streptococci with the most prevalent species being *Escherichia coli*, *Streptococcus uberis, Streptococcus dysgalactiae*. The other environmental infection is of opportunistic nature, namely those caused by *Pseudomonas spp*., Yeasts and Nocardia.

Minor pathogens, fall in to the group of environmental pathogens include, coagulase negative Staphylococcus species, *Actinomyces bovis*, *Bacillus cereus*, and *Serratia marscescens*. Minor pathogens are rarely associated with the clinical changes and often induce only moderate somatic cell response. The minor pathogens have been credited for maintenance of the high somatic cell count and increased resistance of the udder to invade by major pathogens (Radostits *et al*., 1994 and Simuka, 1998).

2.4.1. Streptococcal mastitis

The *Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis* are the principal pathogens involved in streptococcal mastitis. *Streptococcus pyogenes Streptococcus zooepidermicus* are less common isolates from cases of mastitis (Quinn *et al*., 2002)

2.4.2. Staphylococcal mastitis

The most important type of mastitis in most dairying areas to-day is staphylococcal mastitis because the organism is ubiquitous and can colonize teat sores, as well as the udder. A high proportion of isolates in many herds are now penicillin resistant. In herds where staphylococcal mastitis is a problem, 50% or more of the cases may have sub-clinical infection. *Staphylococcus aureus* may cause per acute mastitis, per acute gangrenous mastitis (in which the skin of the quarter and the teat become cold and bluish in color and eventually slough), as well as acute, sub-acute and sub-clinical types. Infections of a year or more in duration are often refractory to treatment because of the development of tissue barrier between the antibiotic and the organism (Britt, 1998).

Staphylococcus aureus causes both acute and chronic mastitis that responds poorly to treatment. It is easily transmitted at milking time and colonizes the teat canal. In herds, in which

staphylococcal mastitis is a problem, >50% of the cows may have chronic or sub-clinical infections (Britt, 1998 and NERA, 2002).

2.4.3. Coliform and Klebsiella mastitis

The species *of Klebsiella pneumoniae* and *Enterobacter aerogenes* are two opportunistic pathogens commonly encountered in coliform mastitis of dairy cattle and also include *Escherichia coli* whose source of infection is fecal contamination of the skin of the mammary gland and relaxation of the teat sphincter following milking, increases vulnerability to infection. Cows with low somatic cell count are particularly susceptible to infection. The acute form of the disease is characterized by endotoxemia and can be life threatening. Per acute disease may be fatal in 24-48 hours. Affected animals are severely depressed with drooping ears and sunken eyes. Mammary secretions are watery and contain white flakes (Quinn *et al*., 2002).

2.4.4. *Pseudomonas aeruginosa* mastitis

Bovine mastitis associated with *Pseudomonas aeruginosa* is often linked to contaminated water used for udder washing or to the insertion of contaminated intramammary antibiotic tubes (Crossman and Hutchinson, 1995). *Pseudomonas aeruginosa* is extremely resistant to many antibiotics and antibiotic susceptibility testing should be carried out on isolates before starting treatment. A combination of either gentamicin or tobromycin with either carbencilin or ticaricillin may be effective (Quinn *et al*., 2002)

2.4.5. *Arcanobacterium pyogenes* mastitis

Bovine mastitis caused by *Arcanobacterium pyogenes* occurs most frequently in heifers and dry cow during the summer months. It has been suggested that this may be responsible for the transmission of infection to other cows (Buxton, 1977).

2.4.6. Mycoplasmal mastitis

Mycoplasmal mastitis is an unusual form of mastitis; the infection may spread rapidly through herd with serious consequences. *Mycoplasma bovis* is the most common cause (Britt, 1998). Typically all quarters become involved following a rapid onset. Loss of production is often

dramatic, secretion soon being replaced by serous or purulent exudates. Initially fine granular or flaky sediment is characteristic of the fluid removed from infected glands. Despite severe local effects on udder tissue, the cow usually does not manifest signs of systemic involvement. The infection will persist through the dry period since there is no satisfactory treatment (Britt, 1998).

2.4.7. *Nocardia asteroides* mastitis

Bovine Nocardial mastitis is caused by *Nocardia asteroides* and is often introduced with udder infusions. Onset is sudden with fever, anorexia and abnormal milk secretion. The affected gland is swollen, hot and painful. Discharging fistulous tracts may develop and lymphadenopathy is common (Hirsh and Yuan, 1999)

2.4.8. Serratial and other organisms causing mastitis

Serratial mastitis may arise from contamination of milk hoses, teat dips, water supply, or other equipment used in the milking process. The *Serratia marscescens* is resistant to disinfectants. Cows with this form of mastitis should be culled.

Mastitis due to a variety of yeasts has appeared in a number of dairy herds following the use of penicillin in an attempt to eradicate *Streptococcus agalactiae* or in association with prolonged repetitive use of antibiotic infusions in individual cows (NERA, 2002). A chronic indurative mastitis similar to that caused by the tubercle bacillus has been reported to be caused by acid-fast bacilli derived from the soil. *Mycobacterium fortuitum*, *Mycobacterium smegmatis*, *Mycobacterium vaccae*, and *Mycobacterium phlei*, organisms are introduced into the gland along with antibiotics, in-oil or ointment vehicles. The oil is required for the organism to become invasive for the mammary tissue (Britt, 1998 and NERA, 2002).

2.5. Transmission

Infection of the mammary gland is almost always via the teat canal. In cows this often occurs when the teat sphincter is slack for a period of 20 minutes to 2 hours after milking. The pathogenic microorganisms generally come from one or two sources, the environment or from inside the udder of the animal and are transmitted via milking machine, or milker's hands. Once

the microorganisms have passed into teat canal they establish themselves there and multiply (Quinn *et al*., 1994).

2.6. Diagnosis

Clinical findings: Clinical findings in mastitis include abnormalities of secretion, abnormalities of the size, consistency and temperature of the mammary gland and frequently a systemic reaction (Radostits *et al*., 1994). The udder to be first examined visually and then through palpation to detect possible fibrosis, inflammatory swellings, visible injury, tick infestation, atrophy of the tissue and swelling of supra-mammary lymph nodes etc. Mammary quarters often become blind when there had been repeated infections and little or no treatment is provided. Information related to the previous health history of the mammary quarters and causes of blindness to be obtained from interviews with owners of the farm (Demelash *et al*., 2005).

Abnormalities of milk: Detection of clots, flakes and pus. Discoloration may be in the form of blood stained or wateriness. The latter usually indicating chronic mastitis (Radostits *et al*., 1994).

Screening tests: Indirect tests that detect only the presence of inflammatory changes. They are of value as screening tests and need to be supplemented by bacterial examination for the detection of the causative organisms. These are California Mastitis Test (CMT), individual cow milk cell count or bulk cell count, a direct capture ELISA test, (Radostits *et al*., 1994). Bromo-thymol blue blotting mastitis-detecting paper, the white side test that is depended on the cell count, plus chloride content. White side test produces a slime layer and flakes if many nucleated cells are present (Hall, 1985).

Cell counts on milk: Many cell-counting methods have been developed.

- Electronic somatic cell counts using equipment such as Coulter counter. It gives a total cell count of both exfoliated epithelial cells and leukocytes.

-Direct microscopic count: In this case, leukocytes can be counted directly. A known volume of (0.01ml) spread over a microscope slide defatted and stained by methylene blue based stain. The

microscope is calibrated and from an average number of leukocytes per field, the number of leukocytes per ml. of milk can be calculated.

-Normal variations in cell count of milk: Cows in early and late lactation have higher counts than those expected in mid-lactation. However, the milk from all four quarters will be equally affected. Individual variation exists between cows and normally a cow will maintain a certain cell count level throughout the life. The presence of *Actinomyces bovis*, regarded as non–pathogenic, in the teat duct will cause a rise in the cell count (Radostits *et al*., 1994).

Direct microscopy: Milk sample can be centrifuged and a stained smear made from the deposit. Gram's stain is used routinely to detect Gram-positive pathogens such as Staphylococci, Streptococci and this will also reveal yeasts, such as *Candida albicans*, that stain deeply by crystal violet. A modified Ziehl Neelsen stained smear can be made, if *Nocardia asteroides* is suspected and Ziehl Neelsen stained smear for the rare cases when bacteria such as *Mycobacterium fortuitum* or *Mycobacterium bovis* are present (Quinn *et al*., 1994).

Culture: Most of the bacterial pathogens causing mastitis grow on ox or sheep blood agar. A MacConkey agar plate is streaked in parallel to detect *Enterococcus fecalis* and any Gram-negative bacteria that are able to grow on the medium. Edward's medium is highly selective for Streptococci and also acts as an indicator medium for hemolysis and for hydrolysis of aesculin. A Sabouraud's dextrose agar plate is inoculated if a fungal pathogen is suspected. However, pathogens such as *Candida albicans* and *Aspergillus fumigatus* form colonies on blood agar at 37°C in 2-3 days, if there is little or no competition from faster growing bacteria (Quinn *et al*., 1994).

The cultural, sugar fermentation, biochemical and other tests to differentiate different species of Streptococci and Staphylococci are described by Wolfgang and Gunter, (1988).

2.7. Prevention and control

The occurrence of mastitis depends on the complex interaction of the three epidemiological components: host, agent and the environment. Therefore, any control program to be successful it should be geared towards correcting mastitis problem associated with these three factors

(Radostits *et al*., 1994). They have extensively reviewed the following essential components of a comprehensive bovine udder health program.

- Employ proper milking management methods.
- Proper maintenance and use of milking equipments.
- Dry cow management.
- Appropriate therapy during lactation.
- Cull chronically infected cows.
- Maintenance of clean environment.
- Good record keeping.
- Monitoring of udder health status.
- Periodic review of the udder health program.
- Setting goals for udder health status, with awareness created and a system of monitoring udder health in place.

A potentially successful mastitis control program should be:

1 Cost effective
2 Within the scope of the average dairy producer to understand
3 Currently used dairy management systems
4 Should lead to visible success by a rapid reduction in numbers of clinical cases and steady improvement in the parameters used for monitoring udder health status (Simuka, 1998).

Prevention of new cases of mastitis depends on reducing exposure of uninfected cows to infected cows during milking. First-calf heifers should be milked before older or infected cows. This reduces contamination with contagious mastitis bacteria and limits exposure to the first-lactation cows. A clean environment that limits bacterial growth is desirable. Inorganic bedding in the form of sand reduces the food supply for bacteria. Stall and barn management should assure cow comfort and cleanliness. Limited water should be used during udder preparation (Britt, 1998)

Environmental mastitis and contagious mastitis can be reduced by 50 % with effective pre- and post milking teat dips respectively. Dry cow therapy will cure some infections that exist at the

time of drying off (Jones, 1965). A clean environment for dry cows will reduce new infections during the dry period, especially the last 2 weeks before calving. Culling cows with chronic infections will also reduce herd exposure. Recent development of improved mastitis vaccines that reduce the clinical severity of the infections may help in reducing herd incidence of mastitis caused by Gram-negative organisms as well as by *Staphylococcus aureus* (Britt, 1998).

A much smaller percentage of cows infected with *Staphylococcus aureus* are cured by either lactation or dry cow therapy. Therefore, all infected cows should be maintained in a segregated group until culled from the herd. This group is always milked last (unless there is also a group infected with Mycoplasma) to stop transfer of infection at milking. Dry-cow therapy is administered to this group and clinical cases are treated, although never regarded as cured, even if a negative culture is obtained. Any other cow with new *Staphylococcus aureus* infection should be transferred to this group. The herd should be monitored by culture of milk samples from cases of clinical mastitis and from the bulk tank to detect reintroduction of infection (Radostits *et al.*, 1994).
Any cow in a herd having mastitis twice in a single lactation period should be culled promptly.

2.8. Mastitis therapy

Parentral treatment is advisable in all cases of mastitis in which there is a marked systemic reaction to control or prevent the development of septicemia and to assist in treatment of the infection of the gland and it is also advisable when the gland is badly swollen.

Intramammary antibiotic infusion like penicillin 16,500 units/kg of body weight for 5days, oxytetracycline 10 mg/kg, tylosin or erythromycin 12.5 mg/kg body weight, sulfadimidine 200-mg/kg body weight is valuable to diffuse to all parts of the glandular tissue (Radostits *et al*., 1994).

Udder infusion is the preferred method of treatment. Disposable tubes containing suitable drugs in a water-soluble ointment base are best suited for dispensing and the treatment of individual cows with cephalosporin, penicillin G and Neomycin combination or other approved broad-spectrum mammary infusions (Radostits *et al*., 1994).

Cows or even young heifers with multiple udder abscesses due to *Actinomyces pyogenes* or other infections should be slaughtered. Per acute and acute cases should be treated with systemic and intramammary penicillin. Long acting penicillin infusions at drying off and half way through the dry period may prevent *Actinomyces pyogenes* mastitis in a dry cow (NERA, 2002).

Sulfonamide: The use of 35% sulfonamide in mineral oil resulted much effect to over come streptococcal mastitis (Jones, 1965 and Goodman *et al*., 1996).

Penicillin: Penicillin produces little irritation in the udder and is more efficient against streptococcal mastitis, but less effective against staphylococcal and ineffective against coliform mastitis. Average curing rate of 89% by intramammary infusion for *Streptococcus agalactiae*, while mastitis with *Staphylococcus sp*. intramammary infusion of penicillin 300,000 -500,000 units resulted a cure rate of 41% of cases (Jones, 1965 and Goodman *et al*., 1996).

Tetracycline: Tetracycline, 440 mg in pomade every 24 hours by intramammary infusion, 70-90% effective results on staphylococcal and streptococcal but of little value for pseudomonas and coliform infections (Jones, 1965 and Goodman *et al*.,1996).

Streptomycin: Streptomycin, 0.25 - 0.5 gr. per infusion for each quarter daily for 3-4 days acute coliform mastitis and can be treated to get satisfactory clinical results by infusion of 1 gr. Streptomycin dissolved in 50 ml of distilled water for 4 days; in case of generalized infection, intra-muscular injection is required i.e. 10 gr. of streptomycin in divided doses (Jones, 1965).

Neomycin: Infusion of 0.5 gr. Neomycin dissolved in 10-20 ml of water or saline solution or incorporated with hydro soluble pomade for 3-4 days every 12 hours; 80-90% cure rate result for streptococcus and staphylococcal mastitis, for *Escherichia coli* mastitis 90-95% for pseudomonas 60% was obtained. Variable results have been reported when neomycin and carbencillin have been infused into the udder and carbencillin appears to be the drug of choice (Jones, 1965 and Goodman *et al*., 1996).

Cloxacillin: Infections of a year or more in duration are often refractory to treatment because of the development of a tissue barrier between the antibiotic and the organism. A high proportion of penicillin resistant Staphylococci are sensitive to drug cloxacillin. Per acute and acute

staphylococcal mastitis may be treated systemically with an appropriate antibiotic. For intramammary therapy, cloxacillin is recommended but sensitivity tests may reveal that others such as erythromycin, lincomycin, penicillin-streptomycin, chlortetracycline and neomycin infusion may be more effective in some instances (Britt, 1998).

Antibiotics in milk pose a potential human health hazard to allergic individuals, and research areas that emphasize prevention rather than treatment, can help reduce this risk of antibiotics to human health (Simuka, 1998).

The indiscriminate use of antibiotics has made many microorganisms develop resistance. This has created immense clinical problems in the treatment of infectious diseases. Therefore, there is a need to develop alternative anti-microbial agents for the treatment of infectious diseases. Non-antibiotic treatment and prevention of infection includes the application of honey and milk for mastitis and wound treatment (Al-Jabri, 2005).

2.9. Antibacterial activity of honey

There are many reports of the effectiveness of honey in clearing bacterial infections in ulcers and abscesses, which suggest that it may be suitable for the intramammary treatment of mastitis (Al-Jabri, 2005).

Honey could possibly be suitable for the treatment of mastitis if inserted into the infected udder via the teat canal, as it is harmless to tissues and would leave no undesirable residues in milk (Molan and Allen, 1996).

In many cases, honey is used with success in infections not responding to standard antibiotic and antiseptic therapy. Its effectiveness as an antibacterial agent is reported by Allen and Molan, (1997) and Al-Jabri, (2005).

It has been observed clinically that when honey is applied to a wound, it visibly reduces inflammation (Subrahmanyam, 1998). Honey is used at a concentration of 30 – 50% found to be superior to cephaloridine, ampicillin, gentamycin, nitrofurantoin, nalidixic acid and co-trimoxazole in inhibiting the growth of nine types of pathogenic organisms isolated from urine samples of 149 patients with confirmed urinary tract infection (Karayil *et al*., 1998)

3. MATERIALS AND METHODS

3.1. Study area

3.1.1. The study area-location and topography

The present study was conducted in Southern Nation Nationalities and Peoples Regional State, Wolaita zone, at Soddo Zuria Woreda, about 390 km away from Addis Ababa. The town Soddo is located at latitude 8º 50’ N and longitude 37º 45’E.

The area covers about 63,282 ha of land with the total population 285,598 (population density 171.1 ha/sq km). Topographically the area is marked by plain, hilly, steep slopes and gorges and a number of streams and mountains, the highest is mount Damota, 2,500 meters above the sea level, which is located near Soddo town (Demelash, *et al.* 2005).

3.1.2. Climate

Altitude range varies from 1100-2500 m.a.s.l. The area experiences a mean annual temperature of about 19°C. The highest average monthly temperature occurs in January when the mean maximum temperature is 26.2°C and the coolest month is August when the average monthly minimum temperature is about 11.4º C.

The rainfall over much of the area is typically bimodal with the big rainy season extending from June to September and a small rainy season occurring from February to April. The mean annual rainfall of the area ranges from 450- 1446 mm with the lowest being in the low lands and the highest in the highlands (Soddo meteorological station record) (Demelash *et al.*, 2005)

3.1.3. Agricultural system, livestock population and major constraints in the region.

The prevailing agricultural system in the region is mixed farming with crop and livestock production. The subsistence needs of rising human population are resulting in the progressive extension of the area under cultivation and subsequent limitation of grazing lands. The livestock population in Soddo Zuria is estimated to be 94,006 cattle, 11,384 sheep, 3,164 goats, 429 horses, 500 mules, 4,020 donkeys, and 55,191chicken (Wolaita zone finance and economic development

main department, 2004). The total livestock population of the woreda is declining through time. The region features overgrazing due to continuous pressure, competition among animals and inappropriate stocking rate practices which lead to scarcity of food supply.

The major livestock diseases in the region are: Trypanosomosis, Foot and Mouth Disease, Lumpy Skin Disease, Anthrax, Contagious Bovine Pleuro-Pneumonia, Mastitis, Blackleg, Pasteurellosis, gastrointestinal and external parasitism.

3.1.4 Management system of the study animals in Soddo Zuria Woreda

Selected indigenous Zebu, Holstein and Jersey crossbred lactating cows with clinical mastitis around Soddo under mixed and peri-urban management system and lactating cows of Soddo pure Jersey dairy farm under the Ministry of Agriculture were studied.

The indigenous zebu found in the region is managed under traditional extensive system as a source of milk, meat, and draught power production and as a means of generating income for household. Livestock under peasant holdings are usually grazed on communal grazing lands, are not provided with nutritional supplement although occasions exist when they are offered pasture grasses and false banana in a cut and carry system and are provided with straws of various crops and hay especially during dry seasons. Before milking, calves are allowed to suckle dams in order to obtain let down of milk. Teat injuries induced by calves in an attempt to suckle and force to remove from their dams are common occurrence and precautions for hygienic milking of the cows such as udder washing and drying, routine screening of clinical mastitis using strip cup are not practiced. Cows are milked twice daily, manually.

The dairy herd of Jersey at Soddo farm is under the management of semi-trained personnel. Cows are kept in exclusive stalls built with concrete flooring and are allowed a grazing time of five hours a day on pastures. In addition to grazing, cows are supplemented with green feed, fodder and concentrate. Manure is removed regularly once daily and more frequently whenever required. But the floor was not maintained for a long period of time so there are pot holes on the surfaces that lodge moisture. Pre-milking preparation includes washing of udder by flushing with warm water and drying the udder by wiping with towels is routine activity but the towels were not provided for each and individual cow. Satisfactory tick control program was not established.

Calves are reared artificially in individual pens away from their dams. At Soddo Veterinary Clinic, more than 40 clinical cases are being attended daily but some individuals discontinue the treatment program as advised.

3.2. Study population.

Representative Kebeles to identify 423 lactating cows were selected. From these, all the 46 lactating cows of Soddo dairy farm, 11 lactating cows from Soddo Veterinary Clinic, 103 lactating cows from urban dairy holders and the rest 263 were randomly selected lactating dairy cows from 18 rural Kebeles were included.

3.3. Study design

3.3.1. Prevalence study

Prevalence of mastitis has been determined by systematic cluster sampling method from September 2006 to April 2007 in Soddo Zuria Woreda at cow and quarter level based on clinical examination for clinical prevalence and indirect BOVI-VET indicator paper test, White Side Test for sub-clinical mastitis prevalence and bacterial culture method for clinical and sub- clinical mastitis cases.. The prevalence of blind/blocked quarters was also been included, in the study.

$$\text{Prevalence of sub clinical/clinical mastitis} = \frac{\text{Number of cows with mastitis} \times 100\%}{\text{Total number of lactating cows examined}}$$

$$\text{Quarter prevalence} = \frac{\text{Number of positive quarters} \times 100\%}{\text{Total number of quarter screened}}$$

$$\text{Prevalence of blind/blocked quarters} = \frac{\text{Number of quarters blind/blocked} \times 100\ \%}{\text{Total number of quarters examined in cows}}$$

3.3.2. Sample size determination and sampling strategy

Sample size was determined at 95% confidence interval, 5% precision and from previous studies in similar study area (Demelash *et al*., 2005), with an expected prevalence of 34.9%. The sample size was determined using the formula for prevalence of cluster sampling (Thrusfield, 2005).

$$g = 1.96^2 \frac{[nVc + Pexp\,(1-Pexp)]}{nd^2}$$

Where:

g = number of clusters to be sampled,

n = predicted average number of animals per cluster,

Pexp = expected prevalence,

d = desired absolute precision,

Vc = between cluster variance,

G =Total number of cluster in the population.

The sample size value was determined by substituting the given data required and found to be 46 clusters. The population of clusters from which the sample to be drawn was small. So the estimated number was adjusted by: Gadj = G g / (G+g).

Therefore 315 samples from 21 clusters have been selected. Considering the existence of different management system in different sites, 263 lactating cows from 18 clusters of Soddo Zuria (rural) were selected by simple random sampling method and all 46 lactating cows of Soddo dairy farm 103 lactating cows from Soddo town and 11 cows from Soddo Veterinary Clinic were included, and made to increase the sample size to 423.

3.4. Clinical examination of cows for mastitis infection

In the present study, the lactating cows were clinically examined the udder and teats by palpation to know the abnormalities, injuries, warts, tick infestation etc before the collection of milk samples for examination. Based on the clinical examination, physical appearance of milk and BOVI-VET indicator paper or white side test results, the mastitis cases were grouped into grade I, II, III, and IV. Grading of mastitis was done according to Faull and Huges, (1985) and Ouinn, (1994) with some modification in numbering. In this study mastitis infection was graded in into four levels by its form and severity.

Grade I, a quarter with visible and palpable changes, and the cow with systemic involvement.

Grade II, if a quarter with visible and palpable changes, the cow is not ill but a quarter may be swollen, hot, painful and some times discolored (acute) or a quarter is hard and lumpy and not painful (chronic).

Grade III, a quarter with visible changes in the milk (usually a few clots in the fore-milk) plus presence of pathogens. However the quarter feels normal and the cow is not ill.

Grade IV (sub-clinical) mastitis; a quarter with pathogens in milk, but the milk looks normal and the quarter feels normal and the infection is detected by screening tests only.

3.5. Milk Sample collection

Procedure for collection of milk sample was according to Schalm *et al*., (1971), and Quinn *et al*., (1994). Strict aseptic procedure was adopted when collecting milk samples in order to prevent contamination with microorganisms present on the body of the animal, hindquarters etc and from the barn environment. The time chosen for sample collection was before milking.

3.5.1. Preparing udders and teats

Udders and especially teats were cleaned and dried before sample collection. Each teat end is scrubbed vigorously with cotton or gauze sponge moistened with 70% ethyl alcohol till all the

dirt comes out. Recontamination of the teats during scrubbing, has been avoided by scrubbing, the teats on the far side of the udder first, then those on the near side. A separate swab was used for each teat.

3.5.2. Collection of milk samples

A teat towards sample collector was sampled first, and then the far ones. The first 3-4 streams of milk was discarded. The sterile collecting vial was held in a slanting position to avoid dirt / dust falling into the sample and 15 ml of milk was collected into the vial, from each teat of the cow separately.

3.5.3. Handling and Storing of Samples

After collection, each vial was marked with identification number and the milk were kept in racks for easy handling and the racks were kept in an ice box with ice packs and brought to the laboratory immediately after collection for processing. In case of delay in processing, the milk samples were stored at 4 °C until further processing.

3.6. Diagnosis of mastitis

3.6.1. BOVI-VET indicator paper (KRUUSE, DENMARK) and White Side Tests

According to Hall, (1985) the bromo-thymol-blue blotting mastitis detecting paper (BOVI-VET indicator paper of KRUUSE, DENMARK), about 10 cm^2 with 4 circular areas each about 2 cm in diameter which have been saturated with bromo-thymol-blue and white side tests were used as screening tests for detecting sub-clinical mastitis. The milk from each quarter was stripped on to each circular area from infected quarters, which turned the yellow area of BOVI-VET indicator paper card to a light or dark greenish-blue colour depending on the degree of alkalinity. Also for white side test, 4% sodium hydroxide was mixed with milk in the ratio of 1:5 on a slide kept on dark background. The normal milk showed no change in the consistency but mastitis milk showed a few white flakes and becomes viscid and thick, depending on the number of cells present in the milk sample.

3.6.2. Bacterial Isolation

Only those clinical mastitis milk samples and sub-clinical mastitis samples were processed for the isolation of bacteria using standard bacteriological procedures and processed according to Quinn *et al., (*2002). One standard loop (0.01ml) of thoroughly mixed milk sample was streaked on 5% sheep blood agar plate. The inoculated plates were incubated aerobically at 37 ^{0}C for about 24 to 48 hours. The plates were examined for growth i.e., colony and haemolytic characteristics.

Individual well-separated different colonies from each sample were sub-cultured on to nutrient agar/ brain Heart Infusion / blood agar slants and incubated at 37° C for 24-48 hours.

These slant cultures were used for characterization of the isolates i. e. to determine the morphological features by Gram's method of staining, catalase and oxidase tests, growth on MacConkey and O-F test as primary identification tests.

Depending on presumptive identification of the isolates, secondary identification / confirmatory tests like sugar fermentation, growth on mannitol salt agar, coagulase tests and biochemical tests like Methyl red, Voges Prosekauer, citrate utilization, urease production, hydrogen sulphide production, Indole test, growth on triple sugar iron agar etc. were conducted and based on these results, bacterial isolates were identified as per Quinn *et al.*, (2002). Different isolates of Staphylococci and Streptococci were identified based on characteristics presented in table1, 2 and 3. The tests other than these were used for the identification of Gram-negative bacteria, as per Quinn *et al.*, (2002).

Interpretation

The milk sample was considered negative if no growth occurred after 72 hours of incubation. Isolation of two or more different organisms from a quarter milk samples were also considered as originated from the sample. However, isolation of *Staph. aureus* and *Str. agalactiae* from the sample was considered as causal.

Table 1. The main differentiating characteristics used for Gram-positive cocci

	Coagulase	Catalase	Oxidase	O-F glucose	Hemolysis
Pathogenic Staph.	+	+	-	F	+ (-)
Non-pathogenic Staph.	-	+	-	F	- (+)
Enterococci	-	-	-	F	(+)
Streptococci	-	-	-	F	(+)
Micrococci	-	+	+	O	- (+)

+ = positive, - = negative, F = fermentative, O = oxidative, (+) = some strains positive, (-) = some strains negative

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

Table 2. The characteristics used in differentiating important Staphylococcus isolates from the infected bovine udder.

	Staph aureus	*Staph. intermedius*	*Staph. epidermidis*
Coagulase test	+	+	-
Catalase test	+	+	+
Glucose fermentation	+	+	+
Hemolysis	+	V	-
Sucrose fermentation	+	+	+
Trehalose fermentation	+	+	-
Mannitol fermentation	+	(+)	-

+ = positive, - = negative, V = variable, (+) = slight

Source: Wolfgang and Gunter, (1988)

Table 3. The characteristics used in differentiating important Streptococcal isolates.

Properties	*Str. agalactiae*	*Str. dysgalactiae*	*Str. uberis*
Morphology in infected material	Long chains with cocci or in pairs,	Chains of medium length ovoid cocci	Ovoid cells in diploid forms, chains upto medium length
Broth	Sediment, long chains	Uniform turbidity, chains of medium length	Uniform turbidity, small ovoid cocci, short to medium chains, diploid form.
Hemolysis on blood agar	∝, β, ∂	∂	∝, ∂, vir
CAMP test	+	-	V
Aesculin hydrolysis	-	-	+
Mannitol fermentation	-	-	+

+ = positive, - = negative, V = variable,

Source: Wolfgang and Gunter, (1988)

3.7. Determination of inhibitory concentration of honey

Sensitivity test was carried out according to Tawetz *et al.,* (1984) and Carter, (1984) as *in vitro* antibiotic sensitivity test.

This test was performed to know whether honey can inhibit growth of Gram-positive and Gram-negative bacteria and at what concentration it can inhibit the growth *in vitro.* The cultures used in this test were available at Microbiology laboratory of Faculty of Veterinary Medicine, Debre Zeit.

For this test, Honey (Beza of Ethiopian agro-industry) was diluted i.e. 0% (no dilution), 10%, 20%, 30%, 40% and 50% in BHI broth and 0.1 ml young broth culture of *Micrococcus spp., Staphylococcu aureus, Staphylococcus epidermidis Streptococcus agalactiae, Listeria monocytogens, Enterococcus spp, Escherichia coli, Salmonella enteritidis* and *Klebsiella*

pneumoniae were inoculated into 10 ml of each dilution of honey in duplicates and incubated at 37°C for 24 hours. Control for each culture was BHI broth, which was also incubated. After incubation, from each dilution, a loop-ful of the inoculum was plated on to either BHI / Blood agar plates and incubated at 37°C for 24 hours and the growth recorded. The highest concentration of the honey, which inhibited the growth of each bacteria, was recorded.

3.8. Mastitis Treatment trials

For clinical trials of mastitis cases, 99 cows with mastitis were selected and grouped randomly into two groups. These 99 lactating cows were comprised of 15 clinical mastitis cases from Soddo dairy farm, 10 cows from Soddo Veterinary Clinic, 52 cows from Soddo Zuria (rural part) and 22 cows from Soddo urban area.

In the present study, there was only one case of grade I mastitis in Soddo town and it was not included in the clinical trial. Only grade II and III mastitis cases were included and divided into two groups.

Group A was treated with commercially available honey (Beza Honey), 10 ml of 40% concentration of intramammary infusion once daily for three consecutive days. That means 10 ml of 40 % honey was diluted in sterile distilled water and was infused into teat canal of affected quarter for three consecutive days at 24 hours interval. Sterile stainless steel syringe with 16 G, 11/2 obtuse hypodermic needle were used for the application of honey infusions.

Group B received Multiject IMM intramammary infusion (milking cow therapy) (Norbrook, UK.) each of 5 gram tube that contained Procaine Penicillin G 100,000 IU; Streptomycin sulphate 100 mg; Neomycin sulphate 100 mg and Predinsolone 10 mg in milk dispersible mineral oil base for three consecutive days at 24 hours interval.

Before the administration of the drug into the quarters, they have been stripped and the drugs were infused into the affected quarter and massaged gently upwards on the teat and quarter in order to distribute to the upper parts of the quarter.

Following the treatment, cows were examined daily for the progress for 5 days. All the treated cows have been re-sampled after 30 days for bacteriological analysis. Clinical mastitis case with systemic involvement was treated differently according to the grade and severity, by antibiotics infusion and parentral injections.

Cure from infection

Milk sample from each cow with mastitis was examined and bacterial isolation was carried out before the treatment starts. After the treatment completed, a second sample of milk was collected 30 days after the treatment and examined for bacterial isolation. If no bacteria were isolated, then that cow was considered as cured. If not (if the original organism was isolated again) then that animal was not considered as cured.

3.8.1 Economics of Honey Treatment

Economical advantage by using antibiotic and non-antibiotic for treatment of mastitis was analyzed by actual market price of intramammary infusions and honey available in the country.
An average cost of honey is 0.54 Birr / syringe of 5gram for three days application and the cost of syringe equals to 2.00 Birr and if the other expenses like sterilization cost is 0.46 birr. Total cost will be 7.00 birr / quarter. And if the cost of Multiject is 17.00 birr per tube, then an average expense needed for treatment of mastitis with antibiotic infusion will be 51.00 birr per quarter for three consecutive day's administration. So, greater than seven fold extra treatment cost per-quarter is needed to treat a cow affected by mastitis with Multiject IMM infusion than honey. Actual Market price of intramammary infusions and honey available in Ethiopia are listed in table 16.

3.8.2. Statistical analysis

Chi-square was employed to see whether there is a significantly different response in grade of mastitis between antibiotic and honey (non-antibiotic) treatments and also if relationship exists between age and breed variation in treatment response.

4. RESULTS

In this study, 423 lactating cows were examined, of which, 11 from Soddo clinic; 46 from Soddo Dairy Farm; 103 from Soddo town and 263 from Soddo Zuria Woreda (rural). These cows were belonged to Holstein (108) Jersey (93) and local Zebu (222) breed. These cows also were grouped in to three based on their age i.e. young ones (< 5 years) adults (6-8 years) and aged (> 9 years), (Table 4 and 5).

Table 4. Breeds of lactating cows screened for mastitis infection.

		Breed		
Place	Screened cows	Holstein	Jersey	Zebu
Soddo clinic	11	3	3	5
Soddo dairy farm	46	0	46	0
Soddo town	103	75	20	8
Soddo Zuria Woreda	263	30	24	209
Total	423	108	93	222

Table 5. Age of lactating cows screened for mastitis infection

		Age		
Place	Screened cows	Young	Adult	Aged
Soddo clinic	11	5	2	4
Soddo dairy farm	46	26	15	5
Soddo town	103	48	45	10
Soddo Zuria Woreda	263	95	133	35
Total	423	174	195	54

4.1. Clinical Examination Results

In the present study, a total of 1621 quarters from 423 lactating cows were clinically examined and observed that 71 cows (4.4%) had fibrosis, atrophy and blind teat canals (Table 6).

Table 6. Prevalence of blind quarters in the cows examined**.**

Place	No. of cows screened	Quarters screened	No. of blind quarters
Soddo clinic	11	38	6
Soddo dairy farm	46	158	26
Soddo town	103	391	21
Soddo Zuria Woreda	263	1034	18
Total	423	1621	71

Clinical examination of the udders of 423 cows and examination of milk samples collected for physical examination and indirect screening tests by BOVI- VET (KRUUSE, DENMARK) indicator paper and White Side Tests for sub-clinical mastitis, revealed that only one cow (0.24%) had grade I type of mastitis accompanied with systemic involvement; 49 (11.6%) cows showed grade II; 49 (11.6%) cows with grade III type of mastitis and 230 (54.4%) cows showed sub-clinical mastitis by screening tests and the remaining 94 (22.2%) cows showed no abnormalities of the udder by clinical examination or by screening tests of milk.

Figure 1. BOVI-VET indicator card test (Strong positive spots at top-right; slight positive at the middle and negatives at right-bottom and left side).

Among the 1621 quarters from 423 cows examined, 106 (6.5%) quarters showed clinical mastitis and 342 (21.1%) quarters had sub-clinical mastitis (Table 7).

Table 7. Prevalence of clinical and sub-clinical mastitis in cows and their quarters screened**.**

Place	No. Of Cows			No. Of Quarters		
	Scr-eened	+ve for clinical mastitis	+ve for sub-clinical mastitis	Scree ned	+ve for clinical mastitis	+ve for sub-clinical mastitis
Soddo clinic	11	10	-	38	12	2
Soddo dairy farm	46	15	10	158	18	33
Soddo town	103	22	38	391	23	71
S. Zuria Woreda	263	52	182	1034	53	236
Total	423	99	230	1621	106 (6.5%)	342 (21.1%)

Number in parenthesis indicates percentage positive.

Physical examination of the udder secretions from the clinical mastitis cases revealed that the discharge was watery to thick, purulent discharge, secretion with putrified smell and one case had blood tinged watery secretion.

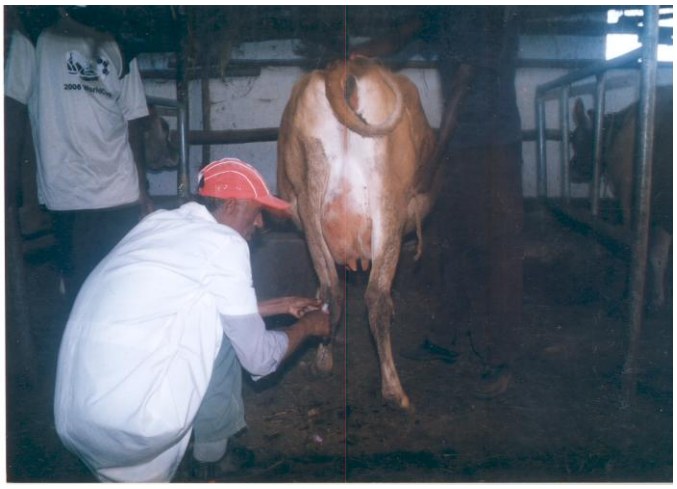

Figure 2. A quarter of a cow affected by grade II mastitis (Left hind quarter).

Prevalence of clinical mastitis

The prevalence of clinical mastitis at Soddo dairy farm, at Soddo town and Soddo Zuria (rural area) was 32.6%, 21.3% and 19.7%, respectively. Overall clinical prevalence of mastitis of the Woreda was 23.4% (Table 8).

Table 8. Distribution of clinical mastitis and its grade of infection in cows.

Place	No. of cows screened	No of clinical cases	Mastitis Grade		
			I	II	III
Soddo clinic	11	10 (99 %)	0	5	5
Soddo dairy farm	46	15 (32.6 %)	0	6	9
Soddo town	103	22 (21.3 %)	1	14	7
Soddo Zuria Woreda	263	52 (19.7 %)	0	24	28
Total	423	99 (23.4%)	1 (0.24 %)	49(11.6 %)	49(11.6 %)

Figure 3. Milk originated from a quarter affected by grade II mastitis.

4 **2. Isolation of bacteria from clinical and sub- clinical mastitis cases.**

In the present study, a total of 425 bacteria and four fungi were isolated from clinical and sub-clinical mastitis cases. All the isolates were belonged to the Genera Staphylococcus, Streptococcus, Micrococcus, Corynebacterium, Klebsiella and Candida. Among these, Staphylococcus Spp. dominated (57. 8 %) followed by Streptococcus Spp. (26.3 %); Micrococcus Spp (11.2 %), Candida (0.93 %) and Corynebacterium (0. 47 %).

In grade I type of mastitis, there was only one case and in that Staphylococcus. Spp. was isolated, where as in grade II type of mastitis, Streptococci Spp. dominated followed by Staphylococcus Spp. In case of grade III type, Staphylococcus spp. followed by Streptococcus and Micrococcus. In sub-clinical mastitis milk samples, prevalence of Staphylococci were more when compared to Streptococci and Micrococcus Spp. etc. Candida Spp. was isolated only from grade II type of mastitis cases, (Table 9).

Figure 4. Mastitis milk from a quarter affected by grade III mastitis (Left) and milk from normal quarter (Right).

Table 9. Distribution of bacterial genera isolated from clinical and sub-clinical cases of mastitis.

Grades of mastitis	Organisms isolated						
	Staphylo-coccus	Strepto-coccus	Klebsiella	Micro-coccus	Coryne-bacterium	Fungi	Over all
I	1	-	-	-	-	-	1
II	40	59	9	7	-	4	119
III	109	20	15	16	-	-	160
Sub-clinical	98	34	-	15	2	-	149
Over all	248	113	24	48	2	4	429

Among the bacterial species *Staphylococcus epidermidis* (113) dominated; followed by *Staphylococcus intermedius* (81); *Staphylococcus aureus* (54); *Streptococcus agalactiae* (42) *Streptococcus dysgalactiae* (39); Micrococcus Spp. (38); and other species (30). Only one species of fungi i.e. *Candida albicans* (4) was isolated from clinical mastitis cases, (Figure 5)

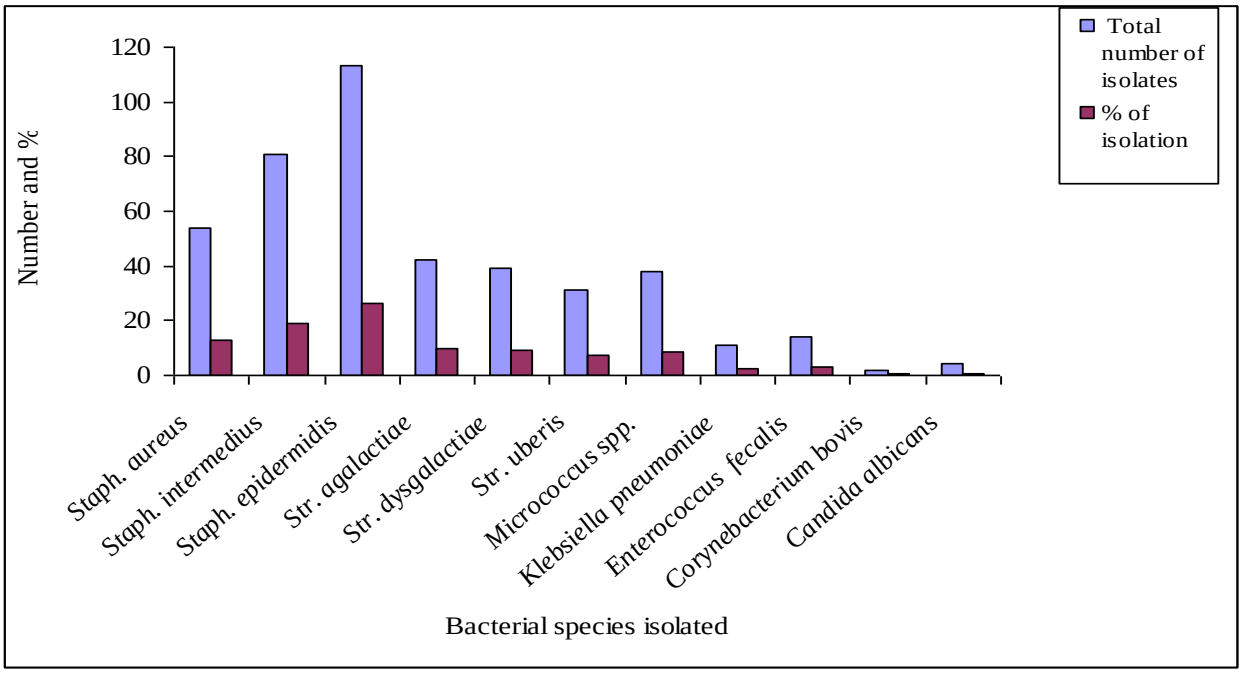

Figure 5. Distribution of different species of bacteria isolated from clinical and sub-clinical mastitis cases.

Determination of susceptibility of different known species of bacteria to honey.

In the present study, susceptibility of various Gram-positive and Gram-negative organisms to honey was determined by using different dilutions of honey in the laboratory. It was revealed that none of the organisms other than *S. enteritidis* tested showed growth in whole honey (without dilution) minimum concentration of honey that inhibited *Escherichia coli* was 10%; *Listeria monocytogenes* and *Klebsiella pneumoniae* inhibited at 20%; *Staphylococcus and Streptococcus spp,* were inhibited at 30% concentration, whereas *Micrococcal spp* required a 40% concectration. *Salmonella enteritidis* was tested resistant (shown growth in all concentrations i.e. 0- 50% in BHI broth).

The determination of minimum inhibitory concentration of honey was necessary for use in clininical trials with honey in the treatment of mastitis when compared to antibiotic (Multiject IMM.) infusion treatment.

Table 10. *In vitro* determination of minimum inhibitory concentration of honey for some of the known species of bacteria.

Microorganisms	Concentration of honey						
	Control *	10%	20%	30%	40%	50%	100%
Staph. aureus	-	-	-	+	+	+	+
Staph. epidermidis	-	-	-	+	+	+	+
Micrococcus spp	-	-	-	-	+	+	+
Streptococcus agalactiae	-	-	-	+	+	+	+
Listeria monocytogenes	-	-	+	+	+	+	+
Escherichia coli	-	+	+	+	+	+	+
Salmonella enteritidis	-	-	-	-	-	-	-
Klebsiella pneumoniae	-	-	+	+	+	+	+
Enterococcus spp	-	-	-	+	+	+	+

- = growth; + = growth inhibition; * = Brain Heart Infusion broth only.

4. 3 Clinical trials with honey and Multiiject IMM intramammary infusion.

In this study, all the clinical cases except one case were divided into two groups randomly i.e. group A and group B. Group A had 50 cases which received honey treatment and group B had 48 cases, which received Multiject IMM. The dose and division of treatment for both the groups were same i.e. 40 % concentration of Beza honey in sterile distilled water given intramammary infusion in a dose of 10 ml per quarter for three days after milking the infected quarter. The Multiject IMM. infusion was also given in a dose of 5-gram infusion per quarter for three consecutive days after milking. This is as per the manufacturer's instruction.

Table 11. Comparative treatment response of mastitis with honey and Mulltiject IMM infusion at different places.

Place	Group A (honey)		Group B (Multiject IMM.)	
	No of cows treated	Number cured	No of cows treated	Number cured
Soddo clinic	5	3 (60%)	5	2(40%)
Soddo dairy farm	8	4(50%)	7	5(71.4%)
Soddo town	9	5(55.5%)	12	8(66.7%)
Soddo Zuria (rural)	28	15(53.6%)	24	11(45.8%)
Total	50	27 (54%)	48	26 (54.2%)

Number in parenthesis indicates percentage.

One of the 99 clinical mastitis was not included in the clinical trial and this case was of grade I, and was treated separately with different line of treatment. In the group A, 18 cows were of grade II and 32 cows belonged to grade III mastitis cases, where as in group B which received Multiject IMM. infusion had 31 grade II and 17 grade III mastitis cases.

In both the groups, cows were declared cured or recovered from infection only when there was physical appearance of the milk was normal and the causative organisms could not be isolated 30

days after the treatment from milk sample and if the causative organism recovered even 30 days after the treatment from milk sample, that animal was declared as not cured /not recovered.

Based on the above criterion, the over-all curative rate for group A (honey treated) was 54% and for group B (Multiject IMM.) 54.2 %. Two out of 18 (11.1 %) grade II and 25 out of 32 (78.1%) grade III responded to honey treatment; whereas 13 out of 31 (41.9%) grade II cases and 13 out of 17 (76.4%) grade III cases responded to Multiject IMM. treatment (Table 12).

Honey treatment was safe for intramamary administration. No adverse events were observed except temporary aggravation of inflammation process. A reduction in milk yield in the first three days of application was reported from some owners of the herd.

Table 12. Mastitis treatment response to honey and Multiject IMM by grade of infection.

Grade of mastitis	Group A (Honey)			Group B (Multiject IMM)		
	Total no	No of cows cured	% Cured	Total no	No of cows cured	% Cured
II	18	2	11.1	31	13	41.9
III	32	25	78.1	17	13	76.4

Breed wise response to the treatment with honey group, 6 of 12(50%) Holstein cows; 5 of 12 (47.7%) Jersey cows and 16 of the 26 (61.5%) Zebu cows responded to honey treatment, where as in group B i.e. Multiject IMM infusion treated group, 9 of the 12 (75%) Holstein cows; 8 of 15 (53.3%) Jersey cows; 9 of the 21 (42 9%) Zebu cows responded to the treatment (Table 13)

Table 13. Mastitis treatment response to honey and Multiject IMM by breed of animals.

Breed	Group A (Honey)			Group B (Multiject IMM)		
	Total number	No of cows cured	% Cured	Total number	No of cows cured	% Cured
Holstein	12	6	50	12	9	75
Jersey	12	5	41.7	15	8	53.3
Local Zebu	26	16	61.5	21	9	42.9

Honey treatment responded well in young cows i.e. 15 of the 25 (60%) cows; cured 50% of adults and two out of 5 cows (40%) in aged group. In group B which received Multiject IMM. 12 of 22 (54.5%) young cows 11 of the 21 (53.4%) adult cows and 3 of the 5 (60%) cows got cured (Table 14).

Table 14. Mastitis treatment response to honey and Multiject IMM. by age of animals.

Age	Group A (Honey)			Group B (Multiject IMM.)		
	Total number	No of cows cured	% Cured	Total number	No of cows cured	% Cured
Young cows	25	15	60	22	12	54.5
Adult cows	20	10	50	21	11	52.4
Aged cows	5	2	40	5	3	60.0

4.4. Economics of Honey Treatment

Economic advantage by using antibiotic and non-antibiotic for treatment of mastitis was analyzed by obtaining actual market price of intramammary infusions and honey available in the country. An average cost of honey is 0.54 Birr / syringe of 5gram for three days application and the cost of syringe equals to 2.00 Birr. And if the other expenses like sterilization cost is 0.46 birr, and the

total cost will be 7.00 birr / quarter. The cost of Multiject IMM is 17.00 birr / tube, then the cost of treatment of mastitis will be 51.00 birr / quarter for three consecutive days of administration. So the cost would be 7-8 times more than the cost of honey treatment. Market price of intramammary infusions and honey available in Ethiopia are listed in Table 15.

Table 15.Market price of some antibiotics and honey used for mastitis treatment.

Item	Unit	Unit price	Amount needed	Total cost (Birr)
Multiject	Tube of 5 gm	17.00	3 tubes	51
Mastiject	Tube of 5 gm	15,00	3 tubes	45
Beza honey	Bottle of 500 gm	18.00	15 gm	0.54
Yeshi honey	Bottle of 500 gm	16.00	15 gm	0.48
Syringe	Piece	2.00	3 syringes	6.00

5. DISCUSSION

The study was carried out to determine the prevalence of bovine mastitis and the efficacy of honey in mastitis treatment in Soddo Zuria Woreda revealed that 23.4% of animals examined had infections in their udders as evidence of clinical mastitis and 54.3% sub clinical mastitis. This finding was lower than those of Abaineh, (1997) who reported 65% in Fiche and was higher than the finding of Demelash *et al*., (2005) who reported the incidence of 11.9% and 23.0% clinical and sub-clinical mastitis respectively in Soddo. The prevalence was higher than the findings of Hundera *et al*., (2005) who reported that clinical mastitis was present in 16.11%, and sub-clinical mastitis in 36.67% of the population.

The variability in the prevalence of bovine mastitis between reports could be attributed to the differences in management of the farms, breeds considered, or diagnostic tests employed. In this study the prevalence of mastitis as sub-clinical disease entity was higher (54.3%) than clinical forms.

Robertson, (1985) concluded that sub clinical mastitis was usually far higher than clinical mastitis. However, the relatively high proportion of clinical cases of mastitis in this study (23.4%) of the total mastitis cases may be attributed to environmental and management problems of the dairy herds in the region, but mainly due to 10% of the clinical cases were from the Soddo clinic and this has boosted the percentage of clinical cases in the present study.

The results of previous studies and the present field study indicated that the non-antibiotic treatment is non-harmful for both animal and consumer. In the long run, the product poses two major advantages over antibiotics i.e. minimize the chance for selecting for resistant and more virulent strains of microorganisms as a result of repeated use, and neutralizing the risk for consumers due to drug residues in the milk. Further to these advantages, there is an economical benefit to the farmer in the short run by continuing to market the milk from untreated quarters. The basic calculation of milk loss from systemic antibiotic treatment till complete clearance of medication results in 5 days of milking cost. According to milk records in the study of Pinchasov

et al., (2004) each cow has lost on average 125 liters (25 liters per day for five days) as a result of milk discarding.

The antibacterial property of honey has been known for more than a century (Dustmann, 1979). Although it has been used as a medicine since ancient times in many cultures, in its ancient usage there was no recognition of its antibacterial properties (Majno, 1975 and Ransome, 1937). It was just known to be an effective remedy. This is not surprising considering that it is only since the latter part of the last century that it has become known that many ailments are the result of infection by microorganisms. Now it can be seen that the effectiveness of honey in many of its medical uses is possibly due to its antibacterial activity. It is well established that honey inhibits a broad spectrum of bacterial species. There are many reports of bactericidal as well as bacteriostatic activity. There have also been reports of honey having antifungal activity. These reports of the antimicrobial activity of honey have been comprehensively reviewed by Molan, (1992). Also, there has not been much distinction made in the different types of antimicrobial activity in honey to which the various microbial species are sensitive. For serious consideration to be given to the use of honey as a therapeutic agent it is necessary that these aspects be further investigated.

Allen *et al*., (1991) reported the minimum inhibitory concentration of 345 samples of New Zealand honey tested on various bacteria ranged from 0.25% to 25% concentration.

In recent times it has been re-discovered, and honey is in fairly widespread use as a topical antibacterial agent for the treatment of wounds, burns and skin ulcers reported by Bulman, (1955) and McInerney, (1990).

Molan, (1992) in his comprehensive review, states that the antimicrobial effect of honey is mainly due to the osmotic effect of honey in which 84% sugars made up of fructose and glucose; its acidity i.e. pH ranged between 3.2 - 4.5 and at this pH most of the bacteria will not grow, hence it is effective inhibitor of many species of bacteria. The other effect of honey is by enzymatic production of hydrogen peroxide in the honey. The hydrogen peroxide and acidity serve to preserve the honey. Hence honey serves in many respects as an antibacterial. In the present study, minimum inhibitory concentrations of honey tested with known bacteria were

found to be between 10 – 40% except *Salmonella enteritidis* was resistant in all concentrations including whole honey. The present results of minimum inhibitory concentration of honey when compared to Allen *et al*. (1991) was bit higher and this may be due to many samples of honey was used by them for testing and in this study only one brand i.e. Beza Honey was tested.

By reviewing the antibacterial activity of honey for the treatment of various ailments in humans, it was decided to test the efficacy of honey for the treatment of bovine mastitis in the present study. It appears to be first of its kind to undertake clinical trials in the treatment of bovine mastitis, but there are numerous reports of using antibiotics in its treatment all over the world including Ethiopia (Pilpot and Nickerson, 1992; Wilson *et al.,* 1999; Leslie 2004).

There is a strong suggestion that non-antibiotic treatment is superior to antibiotic treatment. It is assumed that honey treatment may not induce resistance as seen in antibiotic treatment residues in milk as observed in antibiotic treatment and may be safer to utilize the milk soon after recovery for human consumption unlike in antibiotic treatment. When antibiotics are used there is a variable withdrawal period (Pilpot and Nickerson, 1992; Wilson, *et al*., 1999).

In this study, the honey treatment response to the cows affected by grade III type of mastitis was significantly high (78.1%) $p < 0.001$ when compared to cows affected by grade II type mastitis which accounted for 11.1%. This may be due to local curative effect of honey that can be affected by low concentrations. In grade II type of mastitis infection, Multiject IMM intramammary infusion was more effective (41.9%) than with honey treatment. This may be due to the fact that grade II infection is more severe than grade III type of mastitis and Multiject IMM contains three antibiotics and corticosteroid prednisolone and the combined effect of these may be the reason for it being more effective than honey.

There was no significant treatment response differences were observed between age and breed variation, with respect to either Multiject IMM or Honey treatment in the present study.

Also there was no difference in the type of bacterial isolated from the present study with that of others in Ethiopia as well as in other countries (Crossman and Hutchinson, 1995; Demelash, 2005).

Honey treatment was found to be safe for intramammary administration. No adverse events were observed except temporary aggravation of inflammation process. Some farmers reported some reduction of milk yield in the first three days of application in a few cases, in this study. Since there were no reports on honey treatment hence, it is difficult to compare and contrast the present findings.

One of the problem each farmer encounters is the lack of identification of the causative microorganism at the time of diagnosing the clinical symptoms of infection, establishment of sensitivity to specific antibiotics, availability and the cost of antibiotic infusions. This is the major drawback in making a decision or to what to treat an infected quarter based on prevalence of the pathogen on the farm.

In the present study, no significant difference was observed in the recovery of bovine mastitis with the antibiotic and honey treatment in grade III mastitis infection. Hence, honey can be used as an alternative to antibiotic treatment on grade III type of infection, since honey is available all over the country at a very cheap rate and it is affordable by the livestock owners in the treatment of bovine mastitis. Many times, antibiotics for the mastitis treatment may not be available in the rural and remote areas except in large towns and cities of Ethiopia. Hence, honey can provide a substitute to antibiotic treatment. How ever, more clinical trials are needed in different areas of the country on the use of honey as an alternative to antibiotic treatment.

6. CONCLUSIONS AND RECOMMENDATIONS

Based on the findings in the present study on bovine mastitis, following conclusions are drawn:

- A moderate clinical and high sub-clinical mastitis prevalence in dairy herds was observed in Soddo Zuria Woreda.

- Regarding the aetiology of mastitis, among the infectious agents, *Staph. epidermidis, Staph. intermedius* and *Staph. aureus* are the most frequently isolated infectious agents, followed by *Streptococcus agalactiae, Streptococcus dysgalactiae,* Micrococcus Spp. Only one species of fungi i.e. *Candida albicans* was isolated from clinical mastitis cases.

- *In vitro* sensitivity test to honey revealed that all the bacteria tested were inhibited between 10- 40% concentration of honey except *Salmonella enteritidis.*

- The overall curative efficacy in treating mastitis cases with honey was similar to that of antibiotic (Multiject IMM). However honey intramammary infusion was less effective in grade II type of mastitis compared to the antibiotic used.

- Treatment with honey was found to be seven to eight times cheaper than that of antibiotic treatment.

Based on the above conclusions, following recommendations are forwarded:

- Milking hygiene has to be practiced regularly.

- Prompt treatment of injuries and wounds on udder and teats to be done and their prevention to avoid occurrence of mastitis.

- When a cow has mastitis twice in a single lactation period it should be culled.

- Regular monitoring of sub-clinical mastitis and its treatment during drying off. This will prevent clinical mastitis after calving and cure summer mastitis.

- As an alternative to antibiotic treatment for mild (grade III) type of bovine mastitis cases 10 ml intramammary infusion of 40% of honey in sterile water in a dose of 10 ml per day for three consecutive days after milking.

- Education and demonstration of good dairy management, milking hygiene and correct method of hand milking to the dairy farmers through mass media like radio and TV programs.

- Dairy Enterprise of Ethiopia /Ministry of Agriculture /should grade dairy farms based on milking hygiene practices and milk rate to be fixed on the grade of the dairy like in developed countries to encourage dairy farmers to practice good milking hygiene.

- Further research on traditional herbal medicines and the use of honey as an alternative to antibiotics in the treatment of bovine mastitis to be explored and further clinical trials to be conducted with honey in the country.

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8 ANNEXES

Annex 1. Biochemical tests performed for identification of bacteria.

Tube coagulase test: 0.5 ml of human plasma is placed in a small (7mm) tube .Two drops of an overnight broth culture of the Staphylococcus is added. The tube is rotated gently to mix the contents and then incubated at 37˚ C. A positive, with clothing of plasma can occur in 24 hours. Many coagulase-positive strains will coagulate the plasma only after overnight incubation.

Oxidase test: Apiece of filter paper is moistened in Petri dish with1% aqueous solution of tertramethyl-p-phenylenediamine dihydrochloride. 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.

Catalase test: 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% hydrogen peroxide is added. An effervescence of oxygen gas, with in a few seconds, indicates positive reaction.

Annex 2. Composition of bacteriological media.

Nutrient Agar	
Agar	15.0 g
NaCl	5.0 g
Peptone	5.0 g
Yeast extract	2.0 g
Beef extract	1.0 g
Distilled water	1.0 L

pH 7.4 ± 0.2 at 25˚C

MacConkey Agar

Pancreatic digest of gelatin	17.0 g
Agar	13.5 g
Lactose	10.0 g
NaCl	5.0 g
Bile salts	1.5 g
Pancreatic digest of casein	1.5 g
Peptic digest of animal tissue	1.5 g
Neutral Red	0.03 g
Crystal Violet	1.0 mg
Distilled water	1.0 L

pH 7.1 ± 0.2 at 25˚C

Sabouraud's Agar

Glucose	40.0 g
Neopeptone	10.0 g
Agar	20.0 g
Distilled water	1.0 L

pH 5.3 – 5.6 at 25˚C

Oxidation-Fermentation Medium

NaCl	5.0 g
Agar	3.0 g
Peptone	2.0 g
K2HPO4	0.3 g
Carbohydrate solution (1%)	100.0 ml
Bromo-thymol-blue solution (0.2%)	15.0 ml
Distilled water	1.0 L

pH 7.1 ± 0.2 at 25˚C

Simmon’s Citrate Agar

Agar	15.0 g
NaC	l5.0 g
Sodium citrate	2.0 g
Dipotassium phosphate (K2HPO4)	1.0 g
Ammonium dihydrogen phosphate (NH4) H2PO4)	1.0 g
Magnesium Sulfate (MgSO4.7H2O)	0.2 g
Bromo-thymol-blue	0.08 g
Distilled water	1.0 L

pH 6.9 ± 0.2 at 25˚C

Christensen’s Urea Agar

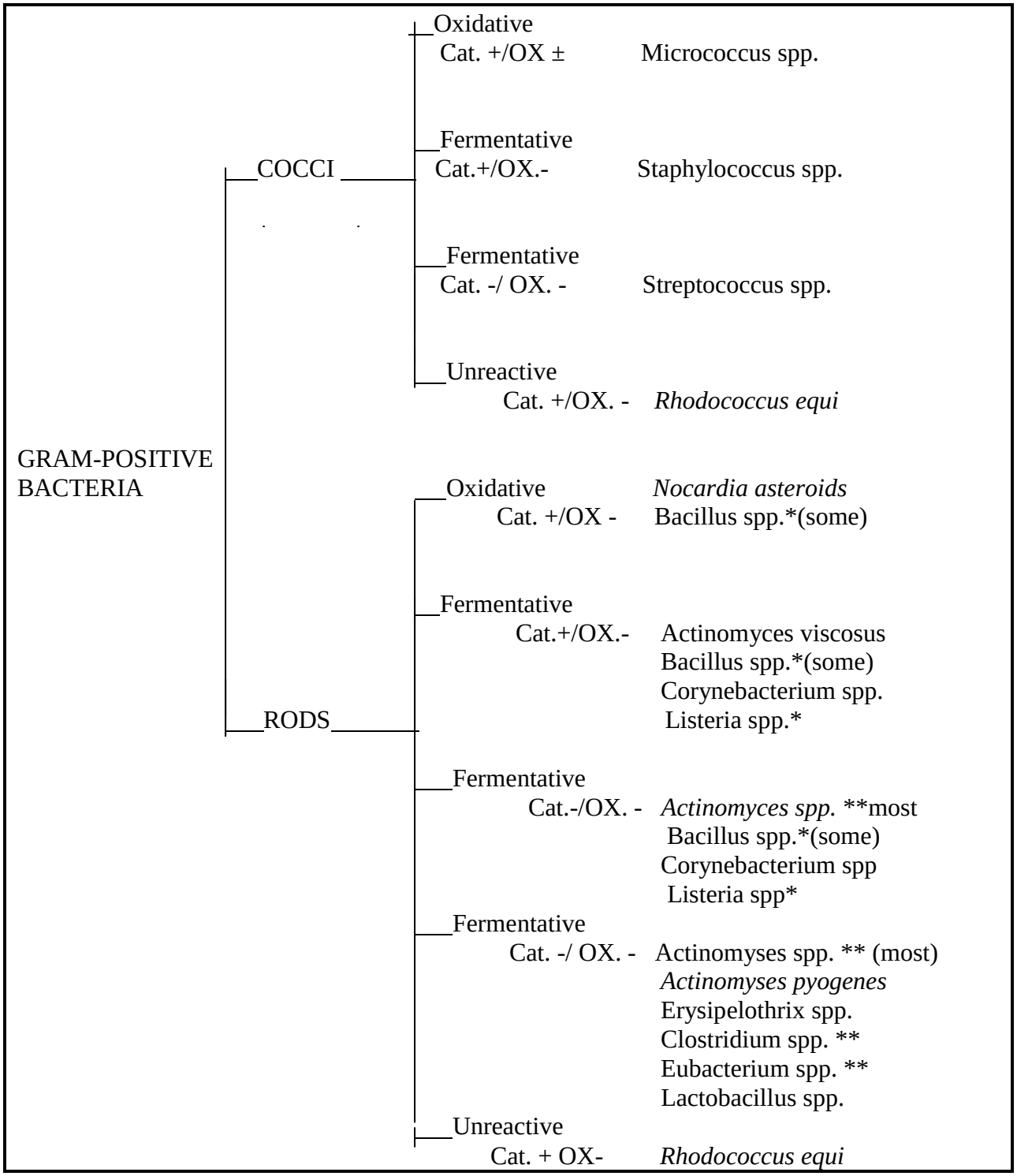
Urea	20.0 g
Agar	15.0 g
NaCl	5.0 g
K2HPO4	2.0 g
Peptone	1.0 g
Glucose	1.0 g
Phenol Red	0.012 g
Distilled water	1.0 L

pH 6.8 ± 0.2 at 25˚C

Annex 3. Gram's method of staining

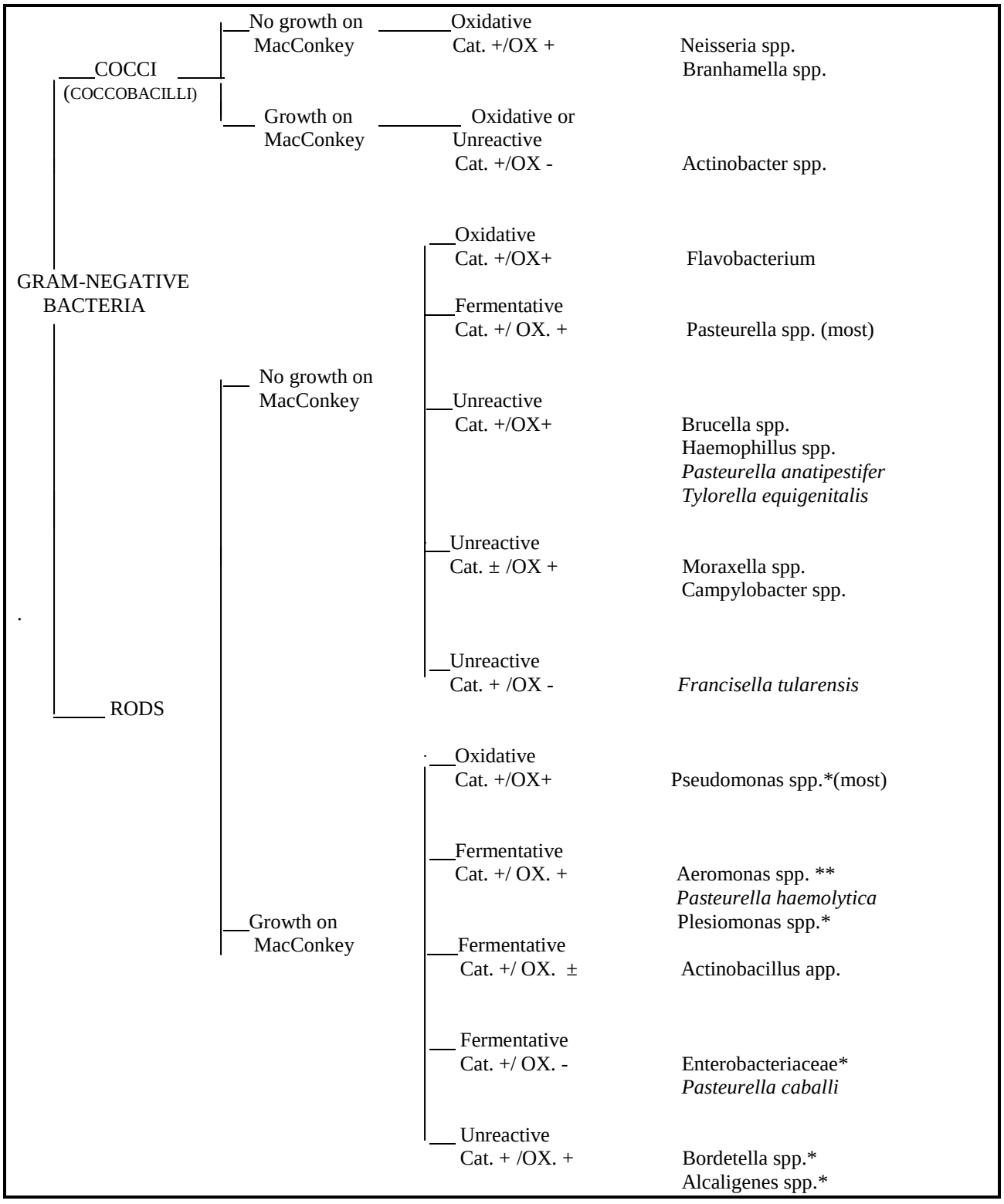
1-Make a thin smears of the material for study and allow to air dry.
2-Fix the material to slide by passing the slide three or four times through the flame of a bunsen burner so that the material does not wash off during the staining procedure.
3-Place the smear on staining rack and overlay the surface with crystal violet solution.
4-After one to two minutes of exposure to the crystal violet stain and wash the remaining stain off with iodine solution. Leaving the slide covered with for 1-2 minutes.
5-Drop of the iodine solution and wash in alcohol unless no crystal violet dye is washed off.
6-Counter stain with safranine for 2 minutes.
7-Wash with water, place the smear in an upright position in a staining rack and allow the excess water to drain off the smear to dry.
8-Examine the stained slide under oil immersion objective of the microscope.

Annex 4. Diagram showing primary identification of Gram-positive bacteria.



Cat = catalase; ox = oxidase; + = Positive reaction; ± = variable; * = motile; ** anaerobic.
Source: (Quinn, 1994).

Annex 5. Diagram showing primary identification of Gram- negative bacteria.



Cat = catalase; ox= oxidase; + = Positive reaction; ± = variable;* =motile; ** = anaerobic.
Source: (Quinn, 1994).

9. SIGNED STATEMENT OF DECLARATION

This thesis is my original work, has not been presented for a degree in any other university and that all sources of material used for the thesis have been duly acknowledged.

Name Tamrat Tomas Abraham

Signature _____

Date of submission 25- 06- 2007

This thesis has been submitted for examination with our approval as university advisors.

1. Prof. A.R.S. Moorthy _____

2. _____ _____