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ADDIS ABABA UNIVERSTY

FACULTY OF VETERYNARY MEDICINE

MICROBIOLOGICAL STUDY OF BACTERIAL CAUSE BOVINE MASTITIS AND ITS ANTIBIOTICS SUSCEBTIBILITY PATTERNS IN EAST SHOA ZONE OROMIA REGIONAL STATE

By

REDEAT BELAYNEH

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DEBRE ZIET ETHIOPIA

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A thesis submitted to the school of graduate studies of Addis Ababa University in partial

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BY
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LIST OF ABBREVIATIONS

A.A.U	Addis Ababa University
CAMP	
CI	Cofidence interval
CMT	California Mastitis Test
CSA	Central Statistics Authority
CNS	Coagulase negative staphylococcus
DNA	Deoxyribo Nucleic Acid
FAO	Food and Agricultural Organization
FL	Front left
FR	Front right
FVM	Faculty of veterinary medicine
HL	Hide left
HR	Hide right
IgA	Immuno globulin A
IgG	Immuno globulin G
IMI	Intra-mammary Infection
IMVIC	Indol, Methyl Red, Voges-Prouskaue,Citrate
SCC	Somatic Cell Count
SPSS	Statistical Package for social science
TSI	Triple Sugar Iron
MR	Methyl Red
MSA	Manitol Salt Agar
NCCLS	National comitte for clinical standard
OF	Oxidation fermentation
OR	Odds ratio
PAB	Purple Agar Base
PCR	Polmeras Chain reaction
VP	Voges- Prouskauer

ABSTRACT

Mastitis is inflammation of mammary gland and primarily results from invasion of pathogenic microorganisms through teat canal. Mastitis has great effect on milk quality, production, composition and economy, and public health significance. Mastitic milk losses its appearance, flavor, maintenance of original nutritional qualities and has no long shelf life. Factors that influence infection rate with mastitic organisms are host factor, environmental factor and pathogenic factor. Mastitis can be classified as contagious and environmental depending on the source of infection. A variety of diagnostic tests can be employed to screen and detect mastitis, as well as to isolate and identify the causative agents.

The study was conducted in east shoa zone Adama and Akaki woredas with the objectives of determining the prevalence of clinical and subclinical mastitis to identify risk factor of bovine mastitis, to isolate and identify the bacterial causes of bovine mastitis and to conduct in-vitro antimicrobial susceptibility test of isolated pathogens from September 2008 to 2009. For this study, 102 and 46 small holder dairy farms and 303 and 200 dairy cows were selected .by one stage cluster sampling in Adama and Akaki woredas respectively. Questionnaire survey was administered to the small holder dairy farm owners to collect data on clinical examination of animal and farm attribute. Farms inspection and clinical examination of cow was performed to associated mastitis with farm based factors and animal factors. California mastitis test (CMT) was used as screening for selection of culture samples for the cow under study. Strict aseptic producers were used when collected milk samples in order to prevent contamination with microorganism. The bacteriological culture was performed following the standard microbiological technique to diagnose the occurrence of clinical and sub clinical mastitis. All collected data analyzed by using descriptive statistics SPSS and Stata. In both the study farms the prevalence of clinical and sub-clinical mastitis were made at herd, caw and quarter level. Based on clinical manifestation for clinical mastitis prevalence and on California mastitis test for sub-clinical mastitis prevalence infections. In the result revealed that the prevalence of clinical mastitis at herd, cow and quarter level (15.6%, 5, 9% and 2.9%) and (17.3%, 3.0% and 1.5%) in Adama and Akaki respectively.

The prevalence of subclinical mastitis at herd, cow and quarter level (57.8%, 33.6%and 20.8%) and (60.8%, 25.0 % and12.7%) in Adama and Akaki respectively. The major isolates of pathogen in both areas from subclinical mastitis cases were *S.aureus* (26.8% and 34.8%), *S.intermidiua* (2.48% and 0.49%) ,*CNS* (18.7% and 20.9%) , *St. agalactiae* (5.77%and5.8%), *St dysgalactaie (*1.99% and1.16%), *St uberis* (4.47% and 1.16%) ,*Bacillus Species* (7.96% and9.3%), *E.coli* (6.46% and 6.98%).and from clinical mastitis *S.aureus* (2.48% and 2.38%), *St agalactiae* (_3.84% and 3.48%) in Adama and Akaki woredas respectivly . Regarding to association risk factor with prevalence of mastitis, the univariate logistic regression in cow level showed that among the risk factor considered, stage of lactation ($p< 0.05$), parity number ($p< 0.05$) and presence of teat lesion ($p<0.05$) significant effect of sub clinical mastitis in both woredas, milking mastitis cow last ($p<0.05$) in both woredas in herd level was significant effect .In considering multivariate logistic regression only stage of lactation had significant on sub clinical mastitis in both woredas in cow level. Regarding the antimicrobial tests comparing the over all efficiency (on all isolates) Gentamycine, Kanamycine, Chloramphenicol and Vancomycine were the most effective antibiotics were found susceptible in both woredas. Fallowing that Streptomycin and penicilline were effecective. The least effective drug was Bacitracine. Amoxaciline was also with relatively weak efficacy.

Keywords: Antibiotics sensitivity test, control, Isolation, Identification, mastitis, Prevalenence, risk factor, study design, study area, bacterial pathogen.

1. INTRODUCTION

Dairy production is a biologically efficient system that converts large quantities of roughage, the most abundant feed in the tropics (Bradley, 2002). Milk is a very nutritional food that is rich in carbohydrate, proteins, fats, vitamins and minerals. However, milk can be associated with health risk to consumers, which is linked to presence of zoonotic pathogen and anti-microbial drug residues. The quality of milk may be lowered by a number of factors such as milk adulteration, contamination during and after milking and presence of udder infection Esron *et al*., 2005).

Milk serves as an excellent medium for certain microorganism. Particularly bacterial pathogen whose multiplication depends mainly on temperature and competing microorganisms and their metabolic products .with regards to their disease producing capacity. These pathogens depends upon the initial load of infection in the milk and on the subsequent dilution, processing, time lapse before the milk is consumed and other factors (Wallenberg *et al*., 2002) pathogenic organisms in milk are derived from the cow it self, from the human hand or the environment. These organisms can be excreted from the udder through the milk or may originate from the skin and mucous membranes of the animal or milker and contaminate the milk and utensils (Bradley, 2002).

Mastitis is the inflammation of mammary gland, is a highly prevalent problem in dairy cattle and is one of the most important threats affecting the world's dairy industry. Although it may be caused by chemical or physical agents, the causes are almost entirely infectious and mostly bacterial. At least 137infectious causes of bovine mastitis are known to date and in large animals the commonest pathogens are *Staphylococcus aureus*, *Streptococcus agalactiae*, other *Streptococcus species* including Coliforms . It may be associated with many other micro organisms including *Actinomysis pyogens, Pseudomonas aeruginosa,Nocardia asteroides Clostridium Perfringens* and other like Mycobacterium, *Mycoplasma*, *Pastuerella* and *Prototheca* species and *Yeasts.(*Radostits *et al* 2000)

Bovine mastitis can be clinical ,and the clinical signs vary with the severity of the disease wich includes pain, heat and swelling of the affected quarter half of the gland show abnormality of milk either as clots or flakes and wateriness of the liquid phase. Sub-clinical mastitis manifests with production losses and lowered milk quality. Both forms of mastitis produce significant economic losses due to rejection of milk), degraded milk quality, early culling of cows. (Mifflin, 2004).

Staphylococcus aureus present in the udders of chronically infected cows and also in cuts and chaps on the teat skin *Streptococcus agalactiae* found only in the udder, and can survive for 2-3 weeks with out multiplication whereas *Steptococcus dysagalactiae found* in the udder and on the teat skin are the main pathogenic bacteria that are involved in contagious mastitis (Wallenberg *et al*., 2002). *Streptococcus uberis* is found in the mouth, vulva, teats and faeces of the cows as well as in the environment. It is probably the common cause of environmental mastitis with less severe clinical signs than *Escherchia coli.*

Escherchia coli can cause severe even fatal mastitis moreover it is enormously present in the faeces and contaminates the environment and can multiply to greater concentration. Some species like Pseudomonas, Klebsialla, and Yaests are pathogens that are considered as causal agents for environmental mastitis. Actinomyces Pyogens, Streptococcus dysagalactiae and Peptococcus indolicus are bacterial agents that are involved in summer mastitis. (Gruent, 2001).

Infections due to those organisms are not particularly common and if one has a mastitis problem in this herd it is more likely that environmental organisms or cow-to-cow transition is involved. *Mycoplasma* species, *Corynobacterium bovis*, *Staphylococcus epidermidis* and *Micrococcu*s are some of uncommon causes of mastitis. (Deluyker, 2005)

The economic impacts of bovine mastitis and IMI have lead to the development of various therapeutic strategies to control them. Many drugs belonging to various therapeutic classes have been assessed (Koivula *et al*., 2005). The production of high quality milk is realistic and

meaningful goal for all aspects of dairy industry and the primary motivator for establishing a mastitis control program in dairy herd. Herds that have successful comprehensive mastitis control program also need to develop strategies to control infection with environmental organism and need to use an effective monitoring system for new infection (Smith *et al.*, 1985).

The diagnosis of mastitis is based on clinical signs such as swelling of the udder, tender to the touch, fever and depression. In many cases reduced milk production can be observed, because of the large number of sub-clinical mastitis cases. The diagnosis of mastitis can also depend on indirect test, which in turn depends on somatic cell count (Radostits *et al.,* 2000).

Treatment of clinical bovine mastitis has been antimicrobial therapy and currently there are a number of conventional antibiotics with different degree of spectrum that are used for the treatment of the disease. An important aspect of mastitis therapy is the alleviation of inflammation that can result swelling and subsequent pain associated with clinical mastitis that can cause considerable discomfort to the cow in the udder. Then the purpose of mastitis therapy is to assist the affected quarter to clear as rapidly as possible and to enable a quick return of the cow to normal milk production (Radostits *et al* 2000). Antibiotics have a potential high cure rate when the treatment is well targeted.

In Ethiopia the disease is insufficiently investigated and information related to its cause, magnitude of distribution and the risk factor is scanty. Such information is important to envisage when designing appropriate strategies that would help to reduce its prevalence and effect. The conventional drugs used for treatment of mastitis are of limited values in most of the woredas, and due to this and other factors causative agents have showed variable degree of resistance. Some of the bacteria like *Staphylococcus aureus*, *Streptococcus species* and some other pathogens have already developed resistance to many antibiotics (Kerro. 1997).

The present study attempt to envisage the following objectives:-

-To determine the prevalence of bovine mastitis in selected woredas

-To identify risk factor of bovine mastitis in the study areas

-To Isolate and Identify the Bacterial causes of Bovine mastitis

- To conduct in-vitro antimicrobial susceptibility test on those isolated pathogens

2. LITERATURE REVIEW

2.1. The bovine udder and natural resistance mechanism

The mammary glands are transformed dermal glands, in which the secreting tissue is located between the skin and abdominal wall in a capsule formed by connective tissue. The udder becomes infected when the bacteria penetrates the teat canal and multiply in there. Infection of mammary gland is almost always via the teat canal. In the cows this often occurs when the teat sphincter in slack, for a period of 20 minutes to 2 hours after milking (Quinn *et al.*, 2002). Up on introduction of any pathogen in to the mammary gland the udder will respond or react to the particular agent in order to defend against the incoming invader and this is manifested as information (Shook,1989).

2.1.1. Physical defenses mechanisms

The teat streak canal having a keratin lining provides the most important physical deterrent to the entry of pathogens. Keratin also inhabits pathogens through chemical defence system composed of antimicrobial lipids and proteins. Bacteria attached to the keratin in the teat canal may be sealed in this location by tight closure of the sphincter muscle or extruded during milking as keratin desquamates. The udder is very susceptible to new infections during the early dry periods before the teat canal has forms a thick keratin plug (Rebhun, 1995).

2.1.2.. Cellular defenses mechanisms

Macrophages neutrophils, sloughed alveolar epithelial cells and small fractions of leucocytes compose the majority of somatic cells in milk. Macrophages may be the most populated in non inflamed glands but neutrophils predominate (90% or more) in inflamed glands. Neutrophils have a relative impairment in milk as compared to blood and a large number of

neutrophils are necessary during infection. This is thought to be due to lack of opsonin, energy source and interference by casein and fat. (Howard, 1993).

2.1.3. Secretary antibodies

Immunoglobulin G transferred to milk from serum is the major antibody fraction in milk, where as IgA and IgM may be locally synthesized and transferred through the mammary epithelium.

2.1.4. Lactoferrin

Lactoferrin is a whey protein, which binds iron in the presence of bicarbonate and there for makes iron less available for the bacteria requiring iron for their growth. Coliforms and most Staphylococci require iron, where as Streptococci needs very little. Lactoferrin increase greatly in the well involutes dry cow mammary gland along with increase in bicarbonate. As parturition approaches and colostrums is secreted in the udder, lactoferrin is reduced and citrates which compete for iron (Rebhun, 1995).

2.1.5. Lysozome and lactoperoxidase

They are other soluble components of the defence mechanism of the mammary gland. Lactoperoxidase produced by mammary epithelial cells may oxidize thiocyanate to hypothiocyanate, which is lethal to some bacteria through cell membrane damage (Rebhun,. 1995)

2.2. Definition of bovine mastitis

Inflammation of the mammary gland, although it may be caused by chemical or physical agents, the causes are almost entirely infectious and mostly bacteria, that invade the udder, multiply and produce toxin that are harmful to the mammary gland (Mifflin, 2004).
Clinical mastitis: is expressed by the production of abnormal milk (flakes, clots or watery secretions) from the infected quarter showing clinical signs such as, swelling, heat and pain on palpation. In case of acute clinical mastitis, these signs maybe combined with general signs (hyperthermia, anorexia, depressed general condition) (Gruent, 2001).

Sub-clinical mastitis: causes decreased milk production and lower milk quality and contagious bacteria may spread to other quarters. Nevertheless, is often go untreated during the lactation (Deluyker , 2005), sub clinical mastitis does not pose threat to the cow's life or its ability to produce the main symptoms, but is an increased somatic cell count (SCC) which leads to financial losses to the farmer (Gruent, 2001).

2.3. Etiology of bovine mastitis

There are many types of micro organisms found inside and outside the udder. According to Watt (1988) 137 species and sub species of microbes that can be associated with mammary gland of cow. Several of them are part of the normal flora. The microbial cause of mastitis include a wide varitey of bacteria (aerobic ,facultativly anaerobic ,microaerobic and anaerobic), Mycoplasmas ,Yeasts ,Fungi, Moulds ,Algae, Virus , and Rikettsias (Savanan, 2002) Among the micro organisms that cause infection in mammary gland, the most common causes are divided in to two categories. The first one contagious pathogens that colonize the mammary gland and can be spread by milking machines and hands of milkers and the second one is environmental pathogens that do not normally infect the mammary gland but can do so when the cows' environment the teats and udder, or the milking machine is contaminated with these organisms and they gain access to the teat cistern (Quinn *et al*., 2002).

2.3.1. Major pathogens of bovine mastitis

2.3.1.1. Major contagious mastitis:-

The major pathogens causing contagious mastitis are *Staphylococcus aureus, Streptococcus agalactiae*, and *Mycoplasma bovis* There are pathogenic micro organism multiply in cow mammary gland and spread from cow to cow primarily through milker hands and udder wash clothes during milking (Quinn *et al*., 2002).

Staphylococcus aureus: – One of the most common types of mastitis is caused by gram positive bacteria and a major cause of economic losses to dairy industry. Often, it is subclinical, where there is neither abnormal milk nor detectable change in the udder, but somatic cell count has increased. Some cows may flare-up with clinical mastitis, especially after calving. The bacteria persist in mammary glands, teat canals, and teat lesions of infected cows and are considered contagious (Fox and Gay, 1993). The infection is spread at milking time, when *S. aureus* contaminated milk from infected cows comes into contact with teats of uninfected cows, and the bacteria penetrate the teat canal. Once established, *S. aureus* usually does not respond to antibiotic treatment, and infected cows eventually must be segregated or culled from the herd. In some herds with somatic cell counts (SCC) below 200,000, dairy managers have not been able to eradicate S. aureus, even when they practiced standard milking time hygiene techniques (Quinn *et al*., 2002).

It causes per acute, acute, and chronic mastitis. The chronic subclinical form is the predominate form. It produces many enzymes/toxins (catalase, coagulase), it is highly invasive (produces hyaluronidase which allows it to invade tissues), is often can resist phagocytosis (it has Protein A on its surface), it resists the immune system (produces teichuronic acid), it is a facultative intracellular pathogen (a live inside phagocytic cells). *Staph. Aureus* can survive to a limited degree in the environment.(Ahmed *et al*.,, 2005).

Clinically infected quarters often show moderate swelling and visible signs of chunks of milk, especially in fore stripping. Acute *S. aureus* infections generally develop late in the lactation or just prior to calving. However, the clinical symptoms (udder swelling or hardness, changes in appearance of milk) do not show up until calving or early in the next lactation. It becomes difficult to successfully treat an infection because drugs are not able to penetrate to all infection sites and because the bacteria live inside the white blood cells. *S. aureus* produces an enzyme that inactivates most penicillin-based treatments, resulting in ineffective antibiotics (Ahmed *et al*,, 2005).

Streptococcus agalactiae. - is the classical example of contagious mastitis, because it is highly contagious and an obligate inhabitant of the mammary gland. *St agalactiae* is a gram positive bacterium which inhabits ducts and cisterns of the gland. It causes an inflammation which blocks the ducts, leading to decreased milk production, increased somatic cell count, and eventually to involution (Quinn *et al.*, 2002). It has few enzymes/toxins and is very sensitive to antibiotic treatment. The agent can survive for 2-3 weeks away from the cow but multiplication occurs only in the udder. The bacterium does not invade the glandular tissue and hence does not cause fibrosis and abscess. The Streptococcal mastitis is largely subclinical with occasional acute flare-ups. It will permanently decrease productivity in the affected gland in chronic infections (Bradley, 2002). Mastitis caused by *Strep. agalactiae* should be suspected in a herd if cow or bulk tank SCC's begin to rise and remain high, especially when bulk milk SCC is 1,000,000 cells/ml or higher. Occasionally high bacteria counts in bulk tank milk will occur when infected udders shed high numbers of *Strep. agalactiae* in the milk.(Coin, 2006)).

Mycoplasma species: - *Mycoplasma* is a pleomorphic organism (has no cell wall). It can be isolated from the respiratory and urogenital tracts. It is mastitis epizootic. There is no treatment; must segregate or cull the cow. *Mycoplasma* infection is associated with contagious mastitis. Cows of all ages and all stages of lactation can be affected by mycoplasmal mastitis,however, those that have recently calved show the most severe signs, These can be due to the long term persistence of the organisms in the udders(up to 13 months

and some cow may become shedders of mycoplasm (Rebhun, 1995).as with out severe clinical sign.(Quinn *et al.,* 2002). *Mycoplasma bovis* is the most common cause and *Mycoplasm californicum* and other species have been isolated from the milk, it cause herd endemic of acute mastitis that subsequently envolve into chronic mastitis. Following acute attack cows may show chronic mastitis, intermittent acute flare-ups or have subclinical infection requiring culture confirmation (Andrew, 2002)

2.3.1.2. Major environmental mastitis:-

Major pathogens causing environmental mastitis pathogens include gram negative bacteria (such as *E.coli* , klebseila species and *Enterobacter species*) and *streptococcal species*(such as *streptococcus uberis* and *St. dysgalactaie*).Environmental mastitis pathogens may occur continuously because the primary route of exposure is contact with moisture, mud and manure. Unlike mastitis caused by contagious pathogens mastitis caused by environmental pathogens cannot be eradicated from a dairy herd. Maceovek and Ruegg (2003).

Coliforms: - Lactose fermenting gram-negative rods, such as *E.coli, Klabsiella* species and *Enterobacter species* are the causative agents of mastitis. Which are the classic examples of environmental mastitis. Summer heat and humidity contribute to multiplication and persistence of Coliforms in the environment. Cows in herds with low somatic cell counts had the highest incidence of clinical mastitis with in 30 days of lactation (Marion 2007).Inflammatory reactions destroy a large proportion of gram negative bacterial populations and there lyses results in release of endotoxine that cause a severe, life treating toxemia. A unique feature of coliform infections is that, in the cows that recover, the udder tissue gradually returns to normal without fibrosis and in its subsequent lactations the gland produces to its optimal capacity (Quinn *et al*.,, 2002). Dry cows are at greater risk of infection just after drying off and just before calving. Organisms in the gland release lipolysacharide endotoxin through destruction or rapid multiplication of the organisms which creates local and systemic signs associated with coliform mastitis (Katholm, 2003).

Streptococcus uberis: - It is gram positive bacteria, may be associated with an acute mastitis, which later becomes chronic. Its course is more transient with fewer tendencies to permanent infections. The organism also has been found in the udder of the cows not showing any obvious evidence of infection and less than 10% of the causes of Streptococci mastitis are found to be caused by this organism (Quinn *et al*., 2002). *Streptococcus uberis* is ubiquitous through out the farm environment because of fecal contamination by cows harbouring the organism in the rumen. Most infection occurs in early lactation or late dry period (Bradley, 2002).

Streptococcus dysgalactiae: - Is gram negative microorganism found in the udder and teat lesion and tends to have lower prevalence than *streptococcus agalactiae*. And become overtly clinical (Rebhun, 1995).

Table 1. Major Pathogens involved in mammary infection

Species	Main source	Living condition	Propagation factor
Sterptococcus agalactiae	Infected cows	Infected quarter and udder only	Using same rag for cleaning udder
Staphylococcus aureus	Infected cows	An abnormal udder and teat, milkers, vagina, tonsillitis	Transmitted by milkers' hands and a milking equipment Entry during milking
Staphylococcus dysgalactiae	Infected cows	Infected cows, injuries	Transmitted during milking time
Streptococcus uberis	Contaminated environment, Teat ends	On cow's skin mouth ground and soil	Neglectedudder washing, insufficient drying, lack of bedding, muddy yard
Eschericha coli	Contaminated environment, Teat ends	Ground, bedding,(saw dust and shavings), manure and water	Environmental source (dirty calving stall, lack of bedding, inadequate udder washing)
Corynebacterium pyogens	Certain insect	Humid valley, wooded areas	

Source: (Duval, 1997)

2.3.2. Minor pathogens of bovine mastitis:-

The minor pathogens include coagualase negative Staphylococcal species, Actinomycoses bovis, Bacillus cereus and *Serratia marcecness*. *Streptococcus dysgalactiae* appears to occupy an intermediate position between the contagious and environmental groups of mastitis {Brooks et al., 1983). Nocardia asteriodes, Serratia marcecness, Prototheca zapfii, Leptospira interrogans and Pseudomonas aerugenosa are the environmental minor pathogens

of bovine mastitis (Quinn *et al*., 2002). Bovine mastitis caused by Prototheca species unicellular algae related to green algae but, without chlorophyll it can assume high significance because of economic losses and the potential risk to public health. (Marques *et al.,* 2006).

Coagulase negative staphylococcus (CNS): - Is gram positive non pathogenic bacteria causing bovine mastitis. In mastitis diagnostics they are not identified at species level but are treated as a uniform group. CNS mastitis is most common on the first lactation, it is assumed to cure spontaneously and antimicrobial treatment is not recommended. Although usually subclinical or mildy clinical mastitis, and are considered to be harmless bacteria of the normal skin flora. CNS mastitis causes increase of milk leucocytes and may decrease the milk quality. It seems to be a problem in well managed dairy herds with good udder health. In contrast to the usual assumption that CNS mastitis cures spontaneously, it may persist in the udder throughout the entire lactation. In addition, the CNS species are not necessarily a uniform group but differences between the species in virulence and clinical characteristics may exist (Marques *et al.,* 2006) . .

Actinomyces pyogens:-Is caused by cow or summer mastitis and infection is extremely purulent. The incidence of infection for the dry cow is increased by fifty, wet or muddy environment. Most of the infection begins after the udder being dry for two weeks or more and muddy or wet, dirty environment usually are present. Epidemics are also possible with up to 25% of the dry cows being affected. (Rebhun, 1995).

Pseudomonas species: - These are gram negative, causes clinical or sub clinical mastitis and are ubiquitous in the environment, occasionally agents of mastitis and infection may be epidemic, sporadic, and endemic in the herd. They often are found in contaminated water/pipelines/wash hoses. The only way to get rid of them may be to throw away the pipeline or heater, etc. They may also come from contaminated teat dips or antibiotic infusion they are resistant to antibiotics (Rebhun, 1995). Pseudomonas infections would be associated with environmental mastitis, infections by this organism can have a pathogenesis

similar to coliform mastitis and a severe endotoxemia can occur. The infection may result in a sub clinical mastitis the pathogen persisting in the mammary gland (Quinn *et al*, 2002).

Yeasts: - Mastitis usually caused by candidia species is almost always secondary to acute enter the gland by way of needles and syringes that are reused. This happens when there is excessive antibiotic therapy occurring. Yeast infections would be associated with environmental mastitis (Quinn *et al*, 2002).

Fungus: - Aspergillus species are opportunistic fungi and rarely gain access to the udder through the same mechanism as yeast Crytococcus neoformans has been reported to cause mastitis and present a public health hazard if contaminated raw milk is consumed (Quinn.,*et al* 2002)

2.4. Risk factor associated with bovine mastitis

Mastitis is a difficult problem to handle because it is caused by many factors How ever many research workers have restricted the disease causation to microbial infection, ingoing without group in to epidemiological factors like environmental and management (Leslie, 2004).

2.4.1. Environmental factor

External Environment: - Encompass all aspects of the environment out side the building. Accordingly, out breaks of mastitis have been ascribed to climatic extremes various geographic factors and traditional practices. It is most likely that these factors are indirectly associated with management and housing in causing mastitis. Climatic conditions have a direct or indirect influence on the onset of mastitis; exposure to cold, draught, conditions high humidity or heat predisposes cattle to mastitis (Peeler *et al*., 2000).

Internal environment: - This includes housing, feeding, management, hygiene and milking techniques. With in these areas that most researches on environmental influences on bovine mastitis has been conducted and the clearest evidence of significant influence exists.

Good management of housing for dairy cattle has a major influence on the type mastitis pathogens. General Management of animal, for example coliform mastitis is much more frequent in housed cattle (Radostits *et al*., 2000). There are several other risks factors in addition to the suppression of the immune system during calving. Many of these factors are related to the cow's surrounding such as hygiene and milking technique. Unfavorable levels of environmental factors maybe more uniformly distributed over the lactation, resulting in fairly constant risk of infection and disease (Saebo *et al.,* 2005)

2.4.2. Host factor

Host characteristics are important risk factor in pathogenesis of mastitis, which are associated with the development of specific immunity and with non-specific host defense mechanism (Erskine, 2001). Regarding the genetic factor, recently there have been a lot of researches on how hereditary factor influence susceptibility to mastitis. Genetic association has been demonstrated, studies have indicated that animal with genetically high productive are more susceptible to mastitis (Koivula *et al.,* 2005). Stage of lactation also influence mastitis, cows are generally more susceptible just after calving and for the following two months Stage of lactation is a further determinant of both subclinical and clinical mastitis. Mastitis due to environmental organism is most common in the first few days after calving and regresses as lactation progresses. This is due to a reduction in the minimum function soon after calving. (Quinn *et al*., 2002). Genetic variation in conformation of the udder, teat, sphincter tone and anatomy of the teat canal is determined in part by hereditary and may be considered one component genetic resistance (Radostits *et al.,* 2000). Once inside teat cistern, pathogenic encounters a group of non specific bacteriostatic and bacteriological factors. Parity has been shown to be a further risk factor for both subclinical and clinical mastitis. It is postulated that younger animals have a decreased susceptibility through a more effective host defense mechanism. Aged cow especially after four lactation are more prone to mastitis. (Quinn *et a.,l* 2002). Prepaturent diseases such as dystocia, parturient paresis, retend plasenta and ketoses also have been identified as risk factor for subsequent development of mastitis (Erskine,.2001). The other host factors which affect mastitis occurrence are immunological factor such as the level of IgA, IgG, lactoferon and phagocytes in mammary gland. Presence

of lesions on the teats that may predispose to inadequate milking or may harbor mastitis producing bacteria (Quinn *et al* 2002).

2.4.3. Agent factor

It is estimated 137 deferent microorganisms have been attributed to causes of mastitis. The microbial factor which affect mastitis are;-

Bacterial viability – The ability of organism to survive in cow's immediate environment – that is its resistance to environmental influences including cleaning and disinfection procedures is a characteristic of each species of bacteria.

Colonizing ability – The ability of the organism to colonize the teat duct, then to adhere the mammary epithelium and set up a mastitis reaction, is major characteristics of the principal bacterial causes of mastitis

Susceptibility to antibiotics- Inherent or acquired resistance to antibiotics the raspy usually due to excessive exposure to the agent is potent reason why some mastitis control program is unsuccessful. The degree of invasiveness for example, streptococci cause little pathogenically change to secretory cells but staphylococci initiate degenerative changes (Quinn *et al.*, 2002).

Seasonal prevalence of individual bacterial includes *Actinomyces pyogens*, commonest in dry cows. *E.coli* most common immediately after calving. *Streptococcus species* common in all seasons except winter, *S. aureus* common in all years (Radostits *et al*., 2000).

2.5. Significance of bovine mastitis

2.5.1. Economic importance:

Mastitis is an endemic disease that is considered to be one of the most frequently occurring and costly disease in dairy industry. The economic consequences of mastitis (clinical or sub-clinical) are due to diagnose and treatment, production loses, culling, changes in product quality, condemning of milk (Haliaset *et al*., 2007)

Economic loss to mastitis in the United States is estimated to be approximately 200 dollars per cow annually. If we assume the same milk price and this value is multiplied by the total number of milking cows (9.5 million head), the total annual cost of mastitis is about $1.8 billion. This is approximately 10% of the total value of farm milk sales, and about two-thirds of this loss is due to reduced milk production in sub clinically infected cows (Schroeder,, 1997)

The average production loss per lactation for one infected quarter is about 1,600 pounds. Other losses are due to discarded abnormal milk and milk withheld from cows treated with antibiotic, costs of early replacement of affected cows, reduced sale value of culled cows, costs of drugs and veterinary services, and increased labor costs (Schroeder, 1997)

2.5.2. Public health importance:-

Milk from mastitic cow may contain harmful pathogenic microorganisms to human beings. Bad milk would be responsible for more sickness and death (Howard, 1993). Although pasteurization has eliminated the public health significance of milk, there are still enough consumers of row milk to mention the various mastitis or milk related factors affecting human health. In recent years a human group B streptococcus, not dissimilar to *streptococcus agalactiae* has been reported as a cause of meningitis and death in new born infant and also urinogenital tract infection in adults. There has also been reported of individuals taken ill after consuming milk products high in toxins produced by *staphylococcus aureus* that pasteurization did not eliminate. Besides *Escherdichia coli* can cause enteritis, diarrhea and vomition ((Schroeder, 1997). Disease like Tuberculoosis, Brucellosis, Listeriosis may be transmitted through milk to human being. *Cryptococcus neoformans* and Protheca species also have zoonotic importance (Hugh-Jones *et al*, 1995).

Public health hazards associated with the consumption of antibiotic contaminated milk and milk products causes allergic responses, changes in intestinal flora and development antibiotic resistance pathogenic bacteria (Bradley, 2002).

2.6. Pathogenesis of bovine mastitis

Infection of intramammary gland always occurs via teat canal. Nevertheless, 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 teat canal, which multiples rapidly and extended into mammary gland tissue where infection occurred frequently or occasionally depending on its susceptibility (Radostits *et al.,* 2000). The potentiality for invasion is greatly increased by bacteria that reside in or colonize the teat duct. Such colonization occurs in both lactating and dry cows, and they may survive for months, serving as source of bacteria for infecting the gland (Schroeder, 1997).

2.7. Diagnosis of mastitis

2.7.1. Screening test

Clinical examination: Cows with clinical mastitis can be readily identified by visible change in milk composition and physical examination of the udder (Bansan *et al.,* 2005). Clinical examination of mastitis based on clinical signs, e.g. swelling of udder tender to touch, fever and depression. Proper examination requires the use of strip cup. Preferably one that a shinny, black plate permitting the detection of discoloration as well as clots, flakes and pus. In this method, milk is drawn on the plate in pools and comparison made between the milk of the different quarters (Radostits *et al.,* 2000).

Direct microscopic count: This method involves counting leucocytes from the milk directly. A volume of 0.01 ml of milk is spread over a microscope slide, defatted and then stained by methylene blue stain. The leukocytes are counted in 50 fields in the collaborated microscope and the leukocyte number is expressed per ml of milk (Minna, 2004).

California Mastitis Test (CMT): California mastitis estimates the somatic cell content of milk. The scores are related broadly to the number of somatic cells in milk. Somatic cell in milk tends to increase during milking and remain for several hours afterward even in uninfected quarters (Table 2). For reliable results, teats should be conducted just before

milking after stimulating the cow and discarding the foremilk. (Bansan *et al.*, 2005). The CMT reagent reacts with genetic material of somatic cells present in milk to form a jell. A squirt of milk, about 2ml from each quarter will be placed in each of four shallow wells (cups) in the CMT paddle an equal amount of the commercial reagent will be added to each cup. A gentle circular motion is applied to the mixture on a horizontal plane and gelling reaction occurs in a few seconds with the positive samples. This reaction must be scored with in 15 seconds of mixing because the reaction will be disappearing after that time. The test results are interpreted subjectively as a negative, trace, weak +, distinct and strong +. (Quinn *et al*., 2002).

Table 2 .The correlations between CMT and SCC

CMT	Interpretation	Visible reaction	Total cell count/ml
0	Negative	Milk fluid & normal	0-200,000 0.25% neutrophils
T	Trace	Slight precipitation	150,000-500,000 30-40% neutrophils
1	Weak +	Distinct precipitation but no gel formation	400,000-1,500,000 40-60% neutrophils
2	Distinct	Mixture thickness with a gel formation	800,000-5,000,000 60-70% neutrophils
3	Strong +	Viscosity strong gel that is cohesive with a convex surface	$\geq$5000,000 70-80%neutrophils

Source: (Quinn *et al*., 2002)

Somatic cell count (SCC): The large number of sub-clinical mastitis causes diagnoses of SCC Elevation of SCC is a clear indication of infection in the udder. However somatic cells are (leukocytes predominantly neutrophils) along with sloughed epithelial or milk secreting cells. Count is a normal cellular defense against udder infection (Wallenberg *et al*., 2002).

pH Determination

The pH of normal milk may vary between 6.5 and 6.8. In mastitis as lactose production decreases and alkaline salts from the blood enter the milk, the milk becomes more alkaline. Thus increasing alkalinity of the milk is characteristics of a progressive mastitis condition. Mastitic milk when drawn from the teat may on rare occasion be acidic and is yellow with Bromocresol purple. The pH should be determined on freshly drawn milk, although milk held at refrigerator temperatures for 24 to 28 hours may be used (Bradley, 2002).

2.7.2. Microbiological test

Detection of sub clinical mastitis is based on bacteriological examination of milk and assessment of udder inflammation. For the latter one possible indication is SCC, which reflects the disease combating response of the animal to the pathogen (Bansan *et al.*, 2005). Microbiological diagnosis is sought in case of elevated somatic cell count as given by CMT, direct or electronic somatic cell count to differentiate the micro organisms involved and also to act as a confirmatory test (Erskine, 2001).

Direct microscopy: The milk collected can be centrifuged and stained. A gram stain is used routinely to detect organisms such as *Staphylococci, Streptococci, and Bacillus* and also yeast such as *Candida albcans* that stains deeply by cryctalviolet. A modified Ziehl-Nielsen stain can be used to detect *Mycobacterium fortuitum or Mycobacterium bovis* (Quinn *et al.*, 2002).

Culture: In working with mastitis problem herd, it is desirable to know the types of microorganism causing infection. Combining data from milk cultures and SCC information provides with a herd inventory of mastitis pathogens, a picture of their distribution and an indication of the relative importance of each pathogen within the herd. This information can be used to identify risk factors and critical control points for control program development. It will also serve as a guide for developing realistic short and long milk quality goals and

provide the means for monitoring progress towards those goals. Most mastitis pathogens can be isolated easily using routine culture method. This performed by microbiological culturing of milk sample from individual quarters of a cow (Radostits *et al*., 2000).Sub-culture of the bacterial colony type (s) considered most significant should be made to obtain pure cultures for use in identification tests. Once a pure culture is obtained, the results from a few comparatively simple tests can often identify the bacterium to genus level. A gram-stained smear from the culture will establish the gram reaction (gram positive or gram negative) along with the cellular morphology (Dwight and Yuan, 2002) The culture results are important for an adequate understanding of specific herd problems, for making recommendation for therapy, and for making culling decision on individual cow (Radostits *et al* 2000

Most of fastidious and non fastidious bacterial pathogens causing mastitis grown on ox or sheep blood agar which supports the growth of mastitis pathogens. McConkey agar plate is streaked in parallel to detect Coliform and other gram negative bacteria that are able to grow on the medium. Edwards' medium is highly selective for streptococcus and also acts an indicator medium for hydrolysis of aesculin (Radostits *et al*., 2000). Interpretation was made as provided by national mastitis counsel (1990).

2.7.3. Biochemical tests

Once the bacteria have been identified to a genus level, further tests can be carried out to identify the species. Pure culture of a single colony type from blood agar will be transferred to nutrient agar slants from which a series of biochemical tests which aid final identification of various pathogens can be done following standard methods (Quinn *et al.,* 2002).

2.7.4. Polymerase Chain Reaction (PCR)

The multiplex PCR assay could be also used as an alternative method in routine diagnosis for rapid, sensitive, and specific simultaneous detection of *Staphylococcus aureus*, *Streptococcus agalactie*, *Streptococcus dysgalactiae*, and *Streptococcus uberis* in milk samples (Phuekets *et al.*, 2002).

2.8. Treatment of bovine mastitis

Antibiotic treatment of bovine mastitis

Antimicrobial agents are used extensively for the treatment and control of bovine mastitis. Antibiotics used in the treatment of mastitis can be administered by Parenteral or intramammary routes (Quinn *et al.*, 2002). The treatment of intramammary concern anti-microbial substance, which delivered intracisternally into the udder through the teat canal, they are essentially targeted at the treatment of mastitis (Gruent, 2001). Intramammary antibiotic preparations are readily available to farmers in many countries and this easy access is likely to result in excessive antimicrobial chemotherapy (Quinn *et al*., 2002). Chemotherapy agents given by the parental route for the treatment of mastitis should ideally, have certain characteristics such as low minimal inhibitory concentration for pathogen causing mastitis (Edward *et al*., 2002). High availability and distribution in mammary tissue after intramuscular or intravenous administration, chemical structure favoring accumulation in milk is low serum protein-binding activity and long half life (Hillerton and Semmens, 2000).

Antibiotic therapy in lactating cows: - During lactation antimicrobial therapy is generally used for treatment of clinical mastitis. Mastitis caused by *Streptococcus agalactiae* is exceptional as both clinical and sub clinical mastitis successfully treated during lactation. *Streptococcus agalactiae* is usually treated by the intramammary route with success rates approaching 100% (Quinn *et al.*, 2002).. Treatment of *Staphylococcus arueus* infection during lactation results in clinical recovery rates of 30-60%. There is uncertainty about the value of antibiotics therapy for treatment of *E. coli* mastitis (Suzkiw.J, 2006).

Antibiotics therapy in Dry cows: - It is employed for controlling sub clinical mastitis. Administration of intramammary antibiotics at the beginning of the dry period is used for the treatment of mastitis caused by contagious pathogens, particularly *Staphylococcus aureus* (Katholm, 2003). Treatment of subclinical cases of mastitis due to environmental organisms such as *St. uberis* which are detected late in lactation may defer until the dry period. (Quinn *et al.,* 2002).

Antibiotic therapy of drying off is an important means of controlling bovine mastitis because antibiotics may be administered by parentral route as there in no concern about withdrawal period and residue in milk (Andrew Biggs, 2002).

2.9. Antibiotic resistance

Indiscriminate use of antibiotics for the treatment of mastitis has lead to the emergence of strain of micro organism resistant to antibiotics and formation of highly complex form of micro organisms in bovine mammary gland (Reena and Snehal, 2007). In many instances, there are improper uses of antimicrobial agents in treatment of mastitis. A single strain may predominate because of the antimicrobial resistance, host adaptation or other factor (Leptolainen *et al.,* 2003*).* During the past decade, bacteria that cause a human disease have developed resistance to many of the antibiotics, commonly used for treatment (Sanmartin *et al.,* 2007). *Staphylococcus aureus* (major pathogen) and coagulase negative *Staphylococcus species* (minor pathogen) of mastitis are resistant to penicillin or ampicillin because of the long term use of B-lactum antibiotics in agricultural and health care settings (Kang *et al.,* 2007).

2.10. Control of bovine mastitis

Mastitis remains one of the major problems in dairy herds causing profound economic losses to the entire milk production. Therefore, strategies to reduce mastitis are important in decreasing cost and improving the quality of production (Koivula *et al.,* 2005). Mastitis

remains a complex disease and its management is an increasing challenge. The novel approach to control bovine mastitis is though a greater understanding the ecology of mammary gland. There are undoubtedly complex interaction between different strains and species of bacteria with in mammary gland (Kitchen *et al*., 2005)

2.10.1. Controlling contagious mastitis

Staphylococcus aureus infection is the major problem. Curing rate with antibiotics therapy during lactation is very low; cows becoming chronic and have to be culled. *Streptococcus agalactiae* responds well to antibiotic therapy and can be eradicated from dairy herds with good control practices including teat dipping and dry cow treatment (Schroeder, 1997). *Streptococcus dysagalaotiae* may live almost anywhere in udder, rumen and faces in the barn. They can be controlled with proper sanitation (Leslie, 2004). According to Erskine (2001) to reduce the prevalence of new infection in a herd one has to achieve two goals:

1- Elimination of contagious intramammary infection
2- Preventing new infection

Elimination of contagious intramammary infection:-

Elimination of IMI during lactation: The basic options for clinical mastitis therapy protocol include treating all cows with antibiotics. Different organisms require different treatment regimens and control strategies (Leslie, 2004). Sub clinical mastitis doesn't present an urgent potential loss of gland function or threat to the life of cow. There fore therapy is administered on the premise that treatment cost will be out weighted by compensatory production gain after elimination of infection. It is advisable to avoid treatment during lactation because it is inefficient and much loss due to prolonged withdrawal time. Only critical cases should be treated during lactation (Radostits *et al.,* 2000).

Elimination of IMI dry period: Udder health management during the dry period is an integral period for elimination of existing and prevention of new IMI. Intramammary infection is a highly used and widely recommended procedure for mastitis therapy. However, there are potentials for identification of organisms during the infusion process (Erskine, 2001). Quarter that closed during dry period was 1.8 times less likely to develop IMI. These recent field studies provide strong evidence for the importance of management strategies aimed at enhancing teat canal closure (Leslie, 2004).

Elimination of infection by culling: Culling is a corner stone for the control efforts for some recent studies have elucidated significant factor that affect the cure *S. aureus* (Leslie, 2004). Culling may be the most effective method of decreasing the prevalence of chronically infected cows. Culling must be done judiciously with respect to production, genetic potential, breeding performance and other health problems (Erskine, 2001). Cows that maintain a high cell count during all lactation should also be culled (Schem *et al*., 2002)..

Preventing new infection:-

Pre-milking hygiene and udder preparation: - Pre-milking udder preparation is important for minimizing bacterial contamination avoiding disinfectant residues and properly stimulated milk let down (Leslie, 2004). Washing the udder is hygienic and has a stimulating effective on milk flow, using individual moist paper towels, wet and wash the teats only. Wetting the udder and the teats results in more bacteria getting into milk that if and only if the teats are wet (Duval, 1997).

Rest-milking teat dipping: - Implementation of post-milking teat disinfection is almost unanimous through out the North America dairy industry for the control of contagious pathogens. It is recommended that all teats of all cows be dipped in an effective rest-milking teat disinfectant after every milking (Leslie, 2004). Dipping the teat of all cows immediately after each milking is the backbone of modern mastitis control program. Teat dipping with germicidal solution management practices immediately to reduce the rate of a new intra mammary infection (Radostits *et al.,* 2000).

Milking sequence: In herds with significant prevalence of contagious cows pathogens, establishing a specific milking order maybe helpful to limit the rate of new infection (Erskine 2001). If possible milking sequence should be as follows: - first lactation cows (heifers), fresh (uninfected cows), second and current clinical cases, cows with high SCC and chronic mastitis should be milked last (Duval, 1997). Monitoring of udder health status: -An excellent review of milk quality and mastitis has recently been published. There have been considerable developments in the use of SCC data for monitoring udder health and milk quality. Implementation of these methods must lead to wide spread improvement in mastitis control. However, even with advent and implementation of comprehensive computerized health management program, it continue to be a challenge to collect analyze and use clinical mastitis records. The development of an automated link where the milk bacteriological culture information is automatically recorded into individual and herd level dairy herd computerized records yield great promise (Leslie, 2004).

2.10.2. Control of environmental mastitis

Prevention (reduce exposure): The goal of every farm should be to reduce the exposure of the teat of the cow to bacteria as much as possible. Trends towards increased size of herds, more confinement of housing greater milk production and genetic selection toward production and away from disease resistance are all modern dairy management realities that work against practitioners in the control of environmental mastitis (Erskine, 2001). Environment around cow should be as clean and dry as possible and should not have access to manure, mud, or pools or stagnant water. Organic bedding such as straw supports the growth of environmental bacteria more than inorganic bedding such as sand or limestone (Schroeder, 1997). Dry cow housing and particularly maternity areas should receive strict attention. Particularly, for lactating cows, adequate free stall space should be provided. Sand bedding used in well designed free stalls should be considered as the standard for housing
Enhanced resistance: Natural defense mechanism of the udder can be used to our advantage in mastitis control the teat sphincter is primary barrier to infection (Erskine, 2001). Proper nutrition will reduce the risk of environmental mastitis adequate level of vitamin E and

selenium reduce the incidence of environmental mastitis. On going research at the University of Kentucky indicates that copper supplement may play a role in maintaining the immune system in dairy cattle (Leslie, 2004).

2.10.3. Proper milking procedures

Proper milking procedures are important for the prevention of mastitis and ensuring complete milk removal from the udder. A good milking reduces the risk of environmental as well as contagious infections. In general use of less water in preparing teats before milking is better. The aim is to put the milking unit on clean and dry teats, so that the risk of environmental organism entering the udder is minimized. Generally if the cows come into the parlor with clean teats dry wiping each teat with paper towel will suffice. In the event of contaminated teat, these must be washed and dried. Badly functioning milking machines results in frequent linear slips and teat and impact will increase environmental mastitis maintain and operates properly milking machine (Schroeder, 1997). Teat should be dipped after milking and cows should be remain standing for about 20-30 minutes to allow the teat canal to seal over before having access to lie down in the cubicles. This is an essential way of reducing infections (Erskine, 2001). Milking May began with a check of or quarters it is acceptable to stripe milk on to the floor in a milking par lour or flat barn. Any cows that show clinical mastitis should be examined and appropriate action taken if fore milking is not done. Visual checking for inflamed quarters is done by milkers and herds health people (Schroeder, 1997).

2.10.4. Vaccination

There is no clear-cut evidence of the effectiveness in diary farms more over development of vaccine for streptococcal mastitis. It is also worthwhile mention that vaccination will not cure existing infection and will not eliminate the need for employing sound milking management However there has been considerable effort to develop vaccine against *S. aureus.* Further

research focusing on the development of sub unit *E.coli* vaccine it may hold promise in the future (Bradley, 2002).

2.11. Bovine mastitis in Ethiopia and its economic impact

2.11.1. Prevalence of bovine mastitis

Ethiopia has the largest domestic animal population in Africa and the latest animal population census (CSA, 2004) shows that Ethiopia has 44.32 million cattle, 23.62 million sheep, 23.33 million goats, 2.31 million camels and over 42 million poultry (excluding agro–pastoral and pastoral areas).

According to the report of FAO (2003), the total annual national milk production in Ethiopia ranges from 797.900 to 1,197,500 metric ton raw milk equivalents out of total national milk production between 85%-89% is from cattle. However this amount is by far below the national demand for milk and milk products in the country, the number of reason could be described for law annual national milk yield among which mastitis is one of the most important factors.

A number of epidemiological studies of bovine mastitis were carried out in Ethiopia (Nesru, 1999) reported the prevalence of clinical and sub clinical mastitis to be 1.9% and 5.3% on cow basis respectively and 1.9% and 7.4% on quarter basis respectively, in central Ethiopia, Tesfu *et al*., 1999) Reported on the overall prevalence of mastitis to be 30.2% and 5.5% for subclinical and clinical mastitis respectively, in study conducted in urban and peri-urban dairy production system in and around Addis Ababa. According to (Nesru, 1999) a survey carried out on mastitis in dairy herds of the Ethiopian central highland, the prevalence of clinical and subclinical mastitis on cow basis to be 1.2% and 38.9% respectively. (Mungube, 2001) reported an overall 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 the study conducted at

Rapi and Debreziet dairy farms, out of 186 lactating cows, 21.5% and 38.0% clinical and sub clinical mastitis (Workeneh et al (2002). In another study in the same area, (Mekonnen, *et al*., 2001) reported the clinical mastitis was the second most frequent disease next to reproductive disease. Variation of the prevalence of mastitis due to differences in environments and management were reported (Odessa, 1997). Recently, Getahun, (2006) in small holder dairy farms of central highlands of Ethiopia identified a prevalence of clinical and sub clinical mastitis of 2.6% and 22.3% respectively. Similarly, Tamrat (2006) reported a prevalence of 23.4% and 54.3% clinical and sub clinical mastitis, respectively, in Sodo area. Demelash *et al.,* (2005) confirmed 34.9% prevalence with 16.11% clinical and 36.6% sub clinical mastitis in lactating dairy cows in southern Ethiopia.

2.11.2. Economic impact of mastitis

Economic losses due to mastitis in general in Ethiopia are not known. However, some research works indicate that the losses in significant (Bishi, 1998) reported that the economic losses from clinical and subclinical mastitis in the urban and pri-urban area of 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 115,526.34 Birr in a single lactation for 363cows. Annual loss caused by mastitis in central highland of Ethiopia is summarized in table 3.

Table 3. Estimated annual losses due to mastitis in central highland of Ethiopia

Source of loss	Loss in birr	%
Milk production loss	29,356.17	25.4
Treatment cost	7122.60	6.2
Withdrawal loss	2534.40	2.2
Loss due to culling	76513.17	66.2
Total	115,526.34	100

Source: (Mungube, 2001

3. MATERIALS AND METHOD

3.1 Study area

The study is conduct in east shoa zone selected wordas of oromia regional state, which located south east of Addis Ababa 35-198Km. The total coverage of the zone is 11,607 KM^2 while the altitude of zone is ranging from 1000-3100 above sea levels (CSA, 2003). The mean annual rain fall of the zone is 500-1200 mm and the daily mean maximum and minimum temperature30^{oc}-42^{oc}and10^{oc}-28^{oc} respectively.The livestock population is estimated to be 865,106 bovine, 447050 ovine, 549,993caprine,216,174 equine,68331 camel. The specific study area from zone is Adama and Akaki woredas.

Adama woreda- Located in the rift valley about 99km southeast of Addis Ababa, at 10^0 - $12^0$25'North latitude and $37^0$57'-$40^0$36' East latitude with an altitude range of 1450-2300 meter above sea level, the range maximum and minimum temperature vary between 15^0c to 33^0c.The mean annual rain fall of the woreda is 650-850mm and the short rain occurs during the month march and April, the long rain extends from June up to August. Live stock population is estimated to be 70,622 bovine, 36.142 ovine, 42.968 caprine and 31.193 equine.

Akaki woreda: - Located 35km from Addis Ababa at 9^0-$10^0$24 North Longitude and $37^0$56'-$40^0$35' East Latitude with an altitude range of 1500-3100 meter above sea level. Its annual temperature ranges from 15^0c-27^0c. The mean annual rain fall of the woreda is 800-900 mm and the short rain occurs during February, March and April and the long rain extends from June up to August. Life stock population is estimated to be 62520 bovine, 15806 ovine, 21911 caprine, 23004 equine.

3.2. Study population and sample determination

The total cattle population of east shoa zone is 3,061,982.The majority of cattle population is ingenious breed 99.1% and 0.9% cross breed. The study will be conducted from September 2008- May 2009 on smallholder dairy farms in Adama and Akaki woredas. Therefore the

target population for the study will be all (market oriented) smallholder dairy farms and their lactating cows in Adama and Akaki.

The sample size is determined at the existing of the actual cluster of small holder dairy farms in the study areas are 102 small holder dairy farms in Adama which involved in the study site of awash melkasa, wonji town, kurftu town, Adama town (22 kebeles). In Akaki 46 small holder dairy farms in the study site of Gelan town, Akaki around Addis Ababa and around Dukem. The sampling frame from both study sites indicated that those are smallholder dairy farms having in average two-eight animals in the sampling frame will be sampled as a cluster In this case sample size were all 303 lactating cows from109 herd were sampled in Adama and in Akaki all 200 cows from 46 herds were sampled.

3.3. Study design

3.3.1. Prevalence study

Study will be conducted cross sectionally in the smallholder dairy farms found in Adama and Akaki woredas from October 2008 to March 2009. Disease identification was made based on clinical examination, nature and appearance of milk secretion and reaction to CMT. Accordingly, milk with pus flaks, clots or blood-tinged watery secretion,. Subclinical mastitis yet no visible or palpable changes in mammary quarter determine based of CMT. Determination of prevalence of clinical and sub clinical mastitis at herd, cow and quarter level in the following formula:-.

$$\text{Prevalence} = \frac{\text{number of cows with mastitis} \times 100}{\text{Total number of investigating (cow, herd, quarter)}}$$

3.3.2. Data collection

Questionnaire survey- Questioners survey will be made to evaluate risk factor on the occurrences of mastitis. The factor considered will be categorized in to two types:-Animal factor and farm factor. Parity numbers, herd size, stage of lactation, presence of teat lesion,

previous study of mastitis are included in animal based factor. The farm based factor will included housing ,farm cleanness, barn floor status, milking hygiene, use separate cloth for teat drying, milking mastitic cow at last, wash hand and udder before milking, a person treating mastitic cow and problem of treating mastiting cow. .Questionnaire survey was developed; pre testing and administered to owners of the animal. Data on each cow was collected in format designed for this purpose (Annex-1).

Clinical examination of cows:-The clinical inspection of the udder was done in the fallowing way; the udder was first examined visually and then by palpation to detect fibrosis, inflammatory swelling, visible injury, tick infestation, atrophy of tissue and swelling of supra mammary lymph nodes. The size and consistency of mammary quarter were inspected for presence of any abnormalities such as disproportional symmetry, swelling, firmness, and blindness (photo 2). Mammary quarters often blocked when exposed to repeat infectionious and little or no treatment were provided. Information related to the previous health history of the mammary quarters and cause of blockage was obtained from intervenes with the owner of the farm. The udder was also inspected for the presence of any grossly visible injury and ticks. Injuries caused by ticks and vigorous calf sucking were described based on location, size and nature injuries and identified as circumscribed lesions around teats.

3.3.3. California mastitis test

The California Mastitis Test (CMT): was carried out as screening test for sub-clinical 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 commercial reagent was added to each cup. A gentle circular motion was applied to the mixtures, in 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 result were scored as negative (0), 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, 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.

3.3.4. Milk sample collection

Producer for collected milk sample will conducted according to Quinn *et al* (1994) strict aseptic procedures will used when collecting of milk samples in order to prevent contamination with microorganisms present on the skin of cows flasks, udder and teats, on the hands of the samplers and in the barn environment udders and especially teats are have to be clean and dry before sample collection.

The following procedures were used to reduce contamination during sample collection:-

Equipment: - Sterile universal bottle with tighten fitting caps were used. The universal bottle was marked with permanent marker before sampling. So that the markings were easy to read when the bottle were placed in a rack. Preparing udders and teats were cleaned and dried before sample collection is attempted. First few streams of milk were removed and discarded to reduce the number of contamination bacteria in the teat canal. The teats were washed with soap and water and dried with towel and disinfect with 70% ethyl alcohol.

Collecting samples: - To reduce contamination of the teat ends during sample collection, the near teats first were sampled then the far one .Remove the cap from the sample vial and with out touching its inner surface, The collecting universal bottle was near horizontal as possible and by turning the teat to a near horizontal position, 15 ml of milk sample was collected in to the universal bottle.

Handling and storing samples: - After samples have been collected they were held in icebox, properly packed and kept cold. In the laboratory, the samples were cultured immediately as soon as possible.

3.3.5. Bacterial isolation and identification

Bacterial isolation and identification was conducted at A.A.U. FVM in microbiology laboratory Debrezeit. Bacteriological examination of the milk was carried out used the following standard procedure (Quinn *et al*., 1994) one standard loop (0.01ml) of milk sample

streak on 5% blood agar. The inoculated plate then incubate aerobically at 37^{0c} for 24-48 hour to rule out show growing bacteria and morphologic features that is colony, size, shape, color and hemolytic characteristics .The first step was staining of randomly selected representative colonies with gram stain used to classify bacteria as positive or negative and as cocci, rods and pleomorphic rods and examined under the microscope and in suspected organism acid fast staining was conducted in addition to gram staining. The finding was correlated with colony characteristics. Based on the above results suspect colonies were subcultured to nutrient and blood agar plates for further investigation, then isolated pure colony organisms were subjected to different biochemical tests, *Staphylococcus* were identified and differentiate by catalase test, coagulase test by rabbit plasma, oxydase test, OF test, purple agar (maltose fermentation) and mannitol salt fermentation. *Streptococcus* species were isolated and identified using catalase test, CAMP test, esculin hydrolysis test. Coliform(*E.coli*, *klebsiela* species, *Enterobacter* species) identified and differentiate by Maconcey agar midia and identified by biochemical test (TSI, Indol ,MR-VP Citrate, ureas). Bacillus species was identified using hemolytic (wide range hemolysis), urease, and character of colonies. Other micro organisms such as C.bovis, A.pyogens, and micrococcus are identified by their rule of identification.(Annexes 3,7,8,9,10,11,12,13)

3.3.6. Antimicrobial sensitivity test

The antimicrobial resistance pattern of the bacterial isolates was determined using the Kirby-Bauer-disk diffusion method (Quinn *et al* 1994). The disks were implements with the fallowing antibiotics: Kanamycine (K 30), Streptomycin (S 10), Penicillin 10units (P 10), Amoxaciline (Aml 2), Gentamycine (CN10) , Chloramphenicol (C 30), Polymyxine (PB 300) , Bacitracine (B 10) and Vancomycine (VA 30).

Disks were stored under refrigeration to ensure maintenance of their potency. Well isolated bacterial colonies of the same morphologic type were inoculated in to 5ml of a tryptophan Soya broth and incubated at 37^0c for 8 hours until a visible turbidity was compared to the 0.5 Mc farland standards. Muller Hinton agar for less fastidious bacterial isolates and 5% sheep blood agar for streptococcus species isolates were used as planting medium.15 minutes after the plates were inoculated antibiotic impregnated disks were applied to the surface of the

inoculated plates with sterile forceps. All disks were gently pressed down on to the agar with forceps to ensure complete contact with the agar surface; the plates were inverted and then aerobically incubated for 18 hour at 37^0c. The diameters of the zone inhibition were measured to the nearest whole millimeter using the transparent ruler. Zones of inhibition for individual antimicrobial agents were translated in to susceptible, intermediate and resistant categories by referring the recommended NCCLS (1997) interpretative standards (Annex 15)

3.4. Data storage and analysis

Data collected through questionnaire survey, farm inspection, animal examination, bacterial isolation and identification and antibiotic susceptibility test will be entered in to the data base management soft ware Microsoft- Excel computer program (Version 6.0. 2000). Descriptive statistics were estimated using SPSS for windows (release 14.0, 2007). 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 intercooler Stata 7.0 (2000).

Statistical analysis in this study, the occurrence or non occurrence of clinical and sub clinical mastitis at herd, cow, and quarter level as defined by CMT score and bacteriological result was dependent variables. Independent variable at cow levels included parity number, stage of lactation, and presence of teat lesion and. The independent variable at herd level included barn floor status, milking hygiene, milking mastitic cow at last. Stage of lactation was classified in to three in such a way that beginning of lactation referred to the 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 udder before milking, use of separate towel for each lactating cow and milking mastitic cow last, wash milker’s hand before and between milking.

RESULT

4.1. Prevalence study

A total 503 lactating cows in 148 herds in east shoa zone were investigated. From these (303and 200) cows in (102 and 46) herds in Adama and Akaki woredas respectively, all are cross breed lactating cows, investigated from October (2008) to March (2009) cross sectional to determined the magnitude of mastitis and determine prevalence of mastitis at herd, cow and quarter level. The results of this study showed that out of 2012 quarters 47 (2.34%) were blind and 1965 (97.66%) teats were open. The overall quarter level prevalence of clinical and subclinical mastitis was 2.39% (n=47) and 88.01% (n=345), respectively. The quarter level prevalence of subclinical mastitis was 20.48% (n=103), 22.00% (n=110), 16.39% (n=78) and 20.78% (n=101) for the front right, front left, hind right and hind left quarters, respectively. The results of univariate logistic regression revealed that quarter level prevalence of subclinical mastitis was not significantly different between the hind (21.44%) and front (26.03%) quarters and also the right (25.63%) and left quarters (21.96%).

4.1.1. Prevalence of clinical mastitis

On the bases of California mastitis and clinical observation 16(15.6%) and 8(17.3%) herds, 18(5.9%) and 6(3.0%) cows and 35(3.0%) and 12 (1.5%)) quarters had clinical mastitis Adama and Akaki woredas respectively. Out of clinical positive quarters most of them were acute mastitis (swelling of udder, change of milk content fig 1and 2). Distribution of bacterial mastitis based on culture is 15(14.7%) and 8(17.3%) herds, 16(5.2%) and 6(3.0%) cows, and 32(2.7%) and 10(1.2%) quarters (Table 4)

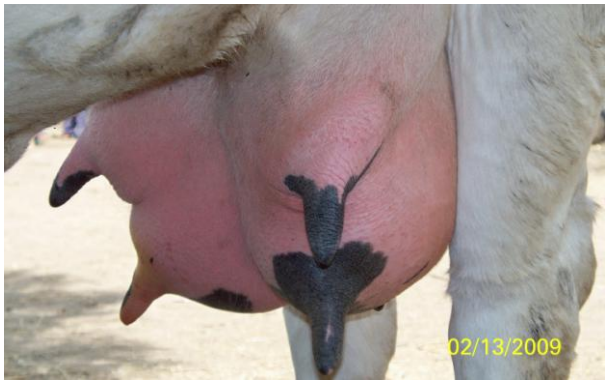
Table 4. Prevalence of clinical mastitis at herd, cow quarter levels based on CMT and culture.

Observation level	Adama			Akaki			Over all		
	N	Prevalence		N	prevalence		N	prevalence	
		CMT(n)	Culture(n)		CMT(n)	Culture(n)		CMT(n)	Culture(n)
Herd level	102	15.6 (16)	15.6 (16)	46	17.3 (8)	17.3(8)	148	16.2 (24)	16.2 (24)
Cow level	303	5.9 (18)	5.2(16)	200	3.0 (6)	3.0(6)	503	4.7 (24)	4.3 (22)
Quarter level	1172	2.9(35)	2.7(32)	793	1.5 (12)	1.2(10)	1965	2.39 (47)	2,1 (42)

N= number of observation n= number of positive

Figure 1.change of milk and swelling of udder in clinical mastitic mastitic cow

Source- Adama cow ID 22 herd 11



Source- Adama cow ID 204, herd ID 72

4.1.2. Prevalence of sub clinical mastitis

The prevalence of sub clinical mastitis was determined by CMT and microbiological cultures are presented in Table 5. From a total of 59(57.8%) and 28(60.8%) from herds, 102(33.6%) and 50(25%) from cows and 244(20.8%) and 101(12.7) from quarters, which were CMT positive bacterial cultures were isolated from 58(56.8%) and 23(50%) herd, 92(32.2%) and

48(24%) cow and 229(19.5%) and 91(9.1%) quarters in Adama and Akaki woredas respectively.

Table 5. Prevalence of Sub clinical mastitis at herd, cow quarter levels based on CMT and culture.

Observation level	Adama			Akaki			Over all		
	N	Prevalence		N	prevalence		N	prevalence	
		CMT(n)	Culture(n)		CMT(n)	Culture(n)		CMT(n)	Culture(n)
Herd level	102	57.8(59)	56.8(58)	46	60.8(28)	50(23)	148	58.7 (87)	54.7 (81)
Cow level	303	33.6(102)	32.2(92)	200	25 (50)	24(48)	503	30.2(152)	27.8(140)
Quarter level	1172	20.8(244)	19.5(229)	793	12.7 (101)	9.1(91)	1965	17.5 (345)	16.2 (320)

N= number of observation n= number of positive

4.1.3. Risk factors affecting the overall prevalence of subclinical mastitis

The results of a univariate logistic regression revealed that the overall cow level prevalence of sub-clinical mastitis was significantly affected by stage of lactation, parity number and presence of teat lesion ($p<0.05$). The prevalence of subclinical mastitis was significantly higher in cows at the end of lactation (81.25%) and in those with high parity number (61.33%) and with teat lesion (92.31%) (Table 6).

Table 6. Risk factors affecting the prevalence of subclinical mastitis at cow level

Factor	Categories	N	Number positive (%)	P value	OR	95% CI of OR
Herd size	1-5	386	138 (35.75)			
	> 5	117	34 (29.06)	0.179	0.74	0.47-1.15
Stage of	Beginning	72	9 (12.50)			
lactation	Middle	303	59 (19.47)	0.201	1.69	0.75-3.80
	End	128	104 (81.25)	0.000	30.33	12.24-75.14
Parity	1-3	322	61 (18.94)			
	> 3	181	111 (61.33)	0.000	6.78	4.40-10.46
Presence of	Yes	13	12 (92.31)			
teat lesion	No	490	160 (32.65)	0.002	24.75	3.23-189-55

When those factors with p-value less than 0.25 were fitted in the multivariate model, only stage of lactation had significant effect on cow level seroprevalence ($p<0.05$). Cows at the end of lactation were more affected by subclinical mastitis than others (OR=30.33).

According to the results of univariate logistic regression, from the farm attributes considered as potential risk factors, only the practice of milking mastitic cow last had significant effect on herd level prevalence of subclinical mastitis. Farms which milk mastitic cows last were less affected by subclinical mastitis (25.71%) than which does not do such a practice (87.61%) (Table 7).

Table 7. Risk factors affecting the prevalence of subclinical mastitis at herd level

Factor	Categories	N	Number positive (%)	P value	OR	95% CI of OR
Herd size	1-5	131	94 (71.76)			
	>5	17	14 (82.35)	0.361	1.84	0.50-6.76
Udder washing	Yes	72	49 (68.06)			
before milking	No	76	59 (77.63)	0.192	0.61	0.30-1.28
Milking mastitis	Yes	35	9 (25.71)			
cow last	No	113	99 (87.61)	0.000	0.05	0.02-0.13
Hand washing	Yes	7	5 (71.43)			
before milking	No	141	103 (73.05)	0.925	0.92	0.27-4.97
Drainage	Good	84	60 (71.43)			
structure	Bad	64	48 (70.59)	0.628	1.2	0.57-2.51
Barn floor status	Good	90	61 (67.78)			
	Bad	58	47 (81.03)	0.079	2.03	0.92-4.48

Risk factors with p-value less than 0.25 were fitted in a multivariate model and the practices of milking mastitic cow last (OR=0.04, p=0.000) and washing udder before milking (OR=0.34, p=0.031) had significant effect on herd level prevalence of subclinical mastitis. Subclinical mastitis was significantly higher in farms, which do not milk mastitic cows last (87.61%) (OR=0.33) and in those, which do not wash udder of cows before milking (77.63%) (OR=0.04).

4.1.4. Risk factors affecting the prevalence of subclinical mastitis in Adama area

In Adama area, similar results were found as it was in the overall study area. The results of a univariate logistic regression revealed that the cow level prevalence of sub-clinical mastitis in Adama area was significantly affected by stage of lactation and parity ($p<0.05$). All the cows (n=8) with teat lesion in Adama area had subclinical mastitis. The prevalence of subclinical mastitis was significantly higher in cows at the end of lactation (78.82%) and in those with high parity number (65.69%) (Table 8).

Table 8. Risk factors affecting the prevalence of subclinical mastitis at cow level (Adama)

Factor	Categories	N	Number positive (%)	P value	OR	95% CI of OR
Herd size	1-5	252	99 (39.29)			
	> 5	51	17 (33.33)	0.448	0.77	0.40-1.50
Stage of	Beginning	40	6 (15.00)			
lactation	Middle	178	43 (24.16)	0.237	1.80	0.68-4.81
	End	85	67 (78.82)	0.000	21.09	7.00-63.66
	1-3	Parity	49 (24.38)			
	> 3	102	67 (65.69)	0.000	5.93	3.41-10.32

When those factors with p-value less than 0.25 were fitted in the multivariate model, only stage of lactation had significant effect on cow level seroprevalence ($p<0.05$).

In the case of farm level prevalence of subclinical mastitis, only the practice of milking mastitic cow last had significant effect on the prevalence of subclinical mastitis ($p<0.05$). The prevalence was significantly higher (86.42%) in those which were not milking mastitic cows last than otherwise (Table 9).

Table 9. Risk factors affecting the prevalence of subclinical mastitis at herd level (Adama)

Factor	Categories	N	Number positive (%)	P value	OR	95% CI of OR
Herd size	1-5	94	66(70.21)			
	>10	8	6 (75.00)	0.776	1.27	0.24-6.70
Udder washing	Yes	62	39 (62.90)			
before milking	No	40	33 (82.50)	0.38	0.36	0.14-0.94
Milking mastitic	Yes	21	2 (9.52)			
cow last	No	81	70 (86.42)	0.000	0.02	0.00-0.08
Hand washing	Yes	6	4 (66.67)			
before milking	No	96	68 (70.83)	0.828	0.82	0.14-4.76
Drainage	Good	60	41 (68.33)			
structure	Bad	42	31 (73.81)	0.551	1.30	0.54-3.14
Barn floor status	Good	63	41 (65.08)			
	Bad	39	31 (79.48)	0.125	2.08	0.82-5.29

Risk factors with p-value less than 0.25 were fitted in a multivariate model and only the practices of milking mastitic cow last had significant effect on the prevalence of subclinical mastitis ($p<0.05$).

4.1.5. Risk factors affecting the prevalence of subclinical mastitis in Akaki area

In Akaki area, cow level prevalence of subclinical mastitis was significantly affected by stage of lactation, parity number and presence of teat lesion ($p<0.05$). The prevalence was significantly higher in cows at the end of lactation (86.05%) and with high parity number (55.70%) and with teat lesion (80%). When factors with p-values 0.25 or less were fitted in a multivariate logistic regression model, only stage of lactation had significant effect ($p<0.05$) (Table 10)

Table 10. Risk factors affecting the prevalence of subclinical mastitis at cow level (Akaki)

Factor	Categories	N	Number positive (%)	P value	OR	95% CI of OR
Herd size	1-5	134	39 (29.10)			
	> 5	66	17 (25.76)	0.568	0.84	0.47-1.51
Stage of	Beginning	32	3 (9.37)			
lactation	Middle	125	16 (12.80)	0.643	1.41	0.32-6.23
	End	43	37(86.05)	0.000	59.61	11.58-306.80
Parity	1-3	121	12 (9.92)			
	> 3	79	44 (55.70)	0.000	11.42	4.89-26.65
Presence of	Yes	5	4 (80.00)			
teat lesion	No	195	52 (26.66)	0.032	11.00	1.23-98.69

Regarding herd level prevalence, only the practice of milking mastitic cows last had significant effect ($p<0.05$). All farms which were not practicing washing udder before milking were infected (100%) while only 64.29% of the farmers which practices udder washing were infected. In case of hand washing between milking only one farm was doing the practice which was infected.(Table 11)

Table 11. Risk factors affecting the prevalence of subclinical mastitis at herd level (Akaki)

Factor	Categories	N	Number positive (%)	P value	OR	95% CI of OR
Herd size	1-5	37	28 (75.68)			
	>10	9	8 (88.88)	0.402	2.57	0.28-23.45
Milking mastitic	Yes	14	7 (50.00)			
cow last	No	32	29 (90.63)	0.005	0.10	0.02-0.50
Drainage	Good	23	18 (78.26)			
structure	Bad	23	18 (78.26)	1.000	1	0.24-4.06
Barn floor status	Good	28	20 (71.43)			
	Bad	18	16 (88.88)	0.176	3.20	0.59-17.22

Risk factors with p-value less than 0.25 were fitted in a multivariate model and only the practices of milking mastitic cow last had significant effect on herd level prevalence of subclinical mastitis.

4.2. Questionnaire survey result

Around 50% of housing material used were local material .Most of small holder had semi-closed housing system, floor types were concrete, arranged stone and earth. Almost all the houses provide adequate space for the animal and overcrowding is not a problem. In some structure had poor drainage system resulting in the accumulation urine and fecal matter. This was particularly observed in houses with the stone floors. An assessment of the cows cleanness was also done most of the cows especially in Adama had soiled udder and this was correlated with dirty, houses and more having drainage problem. (Photo 1). All the dairy holders milked their cows by hand twice a day, they have problem washing their hands before and between milking. The majority of the small holder said they did not cull a cow because of mastitis and those that had done so would only cull after the lost of two or more teats. Some of the milkers washed the udder using water from a common bucket for several cows until the water become dirty, and they have no separate cloth for drying teats. Among those who had experienced their cows for mastitis about 80% of consulted animal health professionals and others followed their cows themselves in both woredas.

Figure 2. poor barn floor status

Source-Adama, herd ID 60cow ID 172

4.3 .Bacterial isolates

In order to get pure culture colonies of the organisms, we used deferent media such as blood agar, nutrient medium, Macconkey agar, Edward's medium and Eosin methyl blue medium (Table). On blood agar some colonies of bacteria showed hemolysis (Alpha, Beta and Gama hemolysis), while others were found to be nonhemolytic. Physical properties of bacterial colonies were also characterized based on size, color and consistency on the agar media. Macconkey agar was used to grow Gram negative lactose fermenter microorganisms, which grew in pink color. Colonies had grown on Edward's medium with dark brown color which indicated Aesculin hydrolysis. On the other hand, we inoculated coliforms on Eosin methyl blue medium, where all were grown. However, *E. coli* grew with greenish shiny appearance which is referred to as "metallic sheen".

Table 12. Result of cultural characteristics of pathogenic organism

Isolates	Blood agar midia	Nutrient agar midia	MacCon key agar	Eosin methyln blue agar midia	Edward midium
S.aureus	Hemolysed	+	-	-	-
S.intermidius	Hemolysed	+	-	-	-
CNS	Hemolysed	+	-	-	-
St.agalactaie	Hemolysed	+	-	-	+
St.dysgalactaie	Hemolysed	+	-	-	+
St.uberis	Hemolysed	+	-	-	+(aisculin hydrolysis)
E.fesalis	Hemolysed	+	+	-	+(aisculin hydrolysis
Bacillusspecies	Hemolysed	+	-	-	-
Micrococcus	Non Hemolysed	+	-	-	-
C.bovis	Non Hemolysed	+	-	-	-
A.pyogens	Non Hemolysed	+	-	-	-
E.coli	Non Hemolysed	+	+	+(metalicsheen)	-
Enterobacter	Non Hemolysed	+	+	+	-
Klebsella	Non Hemolysed	+	+	+	-

After pure culture was obtained the results from a few comparatively primery testes have done in order to identify the organism to a generic level (Table). Gram staind smear had been made from pure culture which have established the gram reaction of gram positive and gram negative organism and also a cellular morphology of bacteria was observed. In this case all lactose fermentative coli forms (in macconkey medium) were observed as gram negative (rod) and some others were gram positive (cocci). Catalase test was done by 3% of hydrogen per oxide; in the result all isolates were positive for catalase test except *Streptococcus* and *Micrococcus* unlike *staphylococcus* which was catalase positive. Motility test demonstrated that all isolates of observed organisms were non motile except lactose fermentative coliforms and *bacillus species*. Primary identification of the colonies is presented in Table 13.

Table 13. Result of primary identification of pathogenic organism

Isolates	Gram stain	Catalase test	Oxydase test	OF test	Motility test
S.aureus	Gram^{+ve} Cocci(cluster)	+	-	Fermentative	Non motile
S,intermidius	Gram^{+ve} Cocci(cluster)	+	-	Fermentative	Non motile
CNS	Gram^{+ve} Cocci(cluster)	+	-	Fermentative	Non motile
St.agalactaie	Gram^{+ve} Cocci(Chain)	-	-	Fermentative	Non motile
St.dysgalactaie	Gram^{+ve} Cocci(Chain)	-	-	Fermentative	Non motile
St. uberis	Gram^{+ve} Cocci(Chain)	-	-	Fermentative	Non motile
E.fesalis	Gram^{+ve} Cocci(Chain)	-	-	Fermentative	Non motile
Bacillus species	Gram^{+ve} (rod)	+	-	Fermentative	Motile
C.bovis	Gram^{+ve} (mixed)	+	-	Fermentative	Non motile
A.pyogens	Gram^{+ve} rod	+	-	Fermentative	Non motile
Micrococcus	Gram^{+ve} pair	-	+	Oxydative	Non motile
E.coli	Gram^{-ve} rod	+	-	Fermentative	Motile
Enterobacter	Gram^{-ve} rod	+	-	Fermentative	Motile
Klebseila	Gram^{-ve} rod	+	-	Fermentative	Motile

OF= Oxydation - Fermentation

Biochemical tests were carried out for identification of the bacteria to the species level (Table). *Staphylococcus species* were differentiated and identified by using MSA, PAB and coaculase test. Those organisms which fermented the mannitol salt agar within 24 hours were *St. aureus* while other organisms that delayed up to 48 hours were *St. intermidius*. Purple agar base was applied in the same way as MSA. Coagulase test, very important for identification of pathogenic *staphylococcus*, 1ml rabbit plasma was mixed with equal amount of suspension of bacteria in test tube. In this case, coagulation of the mixture within 4 hours indicated that the organism was *S. aureus*. Others *Staphylococcus species* which coagulated after 24 hours were *S. intermidius*. Those which did not show coagulation were considered as CNS. In order to identify and differentiate between *St agalactaie* and *St.dysgalactaie*, CAMP test was used. All isolated gram negative bacteria were identified and defferenciated by IMVIC test. For MR test *E.coli* showed positive reaction while *Enterobacter* and *Klebseilla* species had negative reaction .VP test showed the reverse reaction to MR test. .In case of Indol and citrate tests, *klebseila* was positive and *E. coli* was negative in both tests. However *Enterobacter* was positive for Indole and negative for citrate. In TSI test all coliforms

showed A/A with gas production, which means acid slant yellow and acid butt yellow with gas production. Results of biochemical tests are presented in Table 14. .

Table 14. Result of seconary biochemical tests of pathogenic micro organism

Isolates	Coagulase test	MSA ferm	PAB ferm	CAMP test	MR_VP test		TSI test	Citrate test	Indol test	ureas test
S.aurues	Coagulat (in 4hrs)	++	++	-	-	-	-	-	-	-
S.intermidius	Coagulat (in24h)	+	+	-	-	-	-	-	-	
CNS	-	-	-	-	-	-	-	-	-	-
St.agalactaie	-	-	-	+	-	-	-	-	-	-
St.dysgalactaie	-	-	-	-	-	-	-	-	-	-
St. uberis	-	-	-	-	-	-	-	-	-	-
E.fesalis	-	-	-	-	-	-	-	-	-	-
Bacillus species	-	-	-	-	-	-	-	-	-	-
C.bovis	-	-	-	+	-	-	-	-	-	-
A.pyogens	-	-	-	+	-	-	-	-	-	-
Micrococcus	-	+	+	-	-	-	-	-		-
E.coli	-	-	-	-	+	-	(A/A) ,and Gas prod.	-	+	-
Enterobacter	-	-	-	-	-	+	(A/A),and Gas prod.	+	-	-
Klebsella	-	-	-	-	-	+	(A/A, and Gas prod.	+	-	+

A/A= Acid slant yellow, Acid butt yellow and gas production

From the total 118 and 56 lactating cows and 279 and 113 quarters that were positive either clinically or under screening tested using CMT .Over all 261and101 quarters milk sample were cultured from which a total of 201 and 86 isolates were found presented in Adama and Akaki woredas respectively presented in (Table15)

From a total isolated agents 24 and 9 (11.9% and 10.4%) the most isolates from the clinically mastitic quarters were *S.aureus*- 5and2 (20.8% and 22.2%), *CNS*- 3 and 2 (12.5% and 22.2%), *St. agalactaie*– 7 and 3 (29.1% and33.3%), *St. dysgalactaie*- 2 and 1 (8.3% and11.1%) in Adama and Akaki woredas respectively

About 88%and 89% (n=177 and n=77) of the isolates were found from milk samples originating from cow with out any apparent clinical signs of mastitis in both woredas.The most commonly found isolates of sub clinical mastites in a descending order of frequency are *S.aureus* 54 and30 (26.8%and 34.8%), *CNS* 39 and18 (19.4% and20.9), *Bacillus species* 16 and 8 (7.96% and 9.3%), *E.coli* 13 and 6 (6.46% and 6.9%), *St agalactaie* 12and5 (5.97% and 9.3%) in Adama and Akaki woredas respectively. Other isolates including *E.fesalis*, *Micrococcus*, *C.bovis*, *A.pyogens*, *Klebseila*, and *Entrobacter*.were isolated. Of the total isolates from sub clinical mastitis cases, 169(95.4%) and 72(93.5%) showed single growth while 8(4, 6%) and 5 (6.5%) had mixed growth.

Table 15. Bacterial isolates from quarters affected by clinical and subclinical mastitis

Name of bacteria	Frequency of isolation					
	Adama		Akaki		Overall	
	Clinical (%)	Subclinical (%)	Clinical (%)	Subclinical (%)	Clinical (%)	Subclinical (%)
S aureus	5 (2.48%)	54 (2.68)	2 (2.38%	30(34.8%)	7(2.4%)	84 (32.6%0
CNS	3 (1.49%)	39(11.4%)	2(2.38%)	18 (20.9%)	5(1.7%)	61(21.2%)
S.intermidius	1 (0.49%)	5 (4.6%)	-	4(4.46%)	1(0.3%)	9(3.1%)
St. agalactaie	7 (3.48%)	12(5.77%)	3 (3.48%)	5(5.8%)	10(3.48%)	17(5.9%)
St.dysgalactaie	2 (0.99%)	4 (1.99%)	1 (1.16%)	1(1.16%)	3 (1.0%)	5(1.7%)
St. uberis	2 (0.99%)	9(4.47%)	-	1(1.16%)	2(0.6%)	10(3.48%)
E. fesalis	1(0.49%)	5(2.48%)	-	-	1 (0.3%)	5(1.74%)
Bacillus species	2 (0.00%)	16(7.98%)	1 (1.16%)	8(9.3%)	3 (1.0%)	248.3%)
E.coli	-	13(6.46%)	-	6(6.98%)	-	19(6.6%)
Enterobacter species	-	4(2.98%)	-	-	-	6(2.0%)
Klebssela species	-	6(1.99%)	-	-	-	4(1.3%)
Micrococcus	-	5(2.48%)	-	1(1.16%)	-	6(2.0%)
C.bovis	1 (0.49%)	2(0.99%)	-	1(1.16%)	1 (0.3%)	3(1.0%)
A. pyogens	-	3(1.49%)	-	2(2.32%)	-	5(1.74%)
Total	24	177	9	77	33	254

Based of the total isolation, major contagious pathogens were *S.aureus* (29.3% and 37.2%) and *St.agalactaie* (9.45% and 9.4 %) in Adama and Akaki woredas respectively (Fig 1). In this case the predominant pathogen is S.aureus in both woredas. Interims of major environmental pathogens which are *E.coli* (4.43%and6.9%), *Klebsiella* (2.9%and0%), *Enterobacter* (1.99% and 0%), *St.uberis* (5.7% and 1.16%), *St. dysgalactaie* (2, 28% and 2.3%), *A.pyogense* (1.49% and 2.3%) Adama and Akaki respectively (Fig 2).In this case Adama has predominant isolates of major environmental pathogens comparing to Akaki.

From minor pathogens *CNS* was predominant pathogen 42(4.6%) and 20(6.9%) in Adama and Akaki woredas respectively. Other minor pathogens such as *Bacillus species*, *S.intermidius*, *Micrococcus* and *C.bovis* were not that much difference in both woredas

Figure 3.Major Pathogens causing contagious mastitis

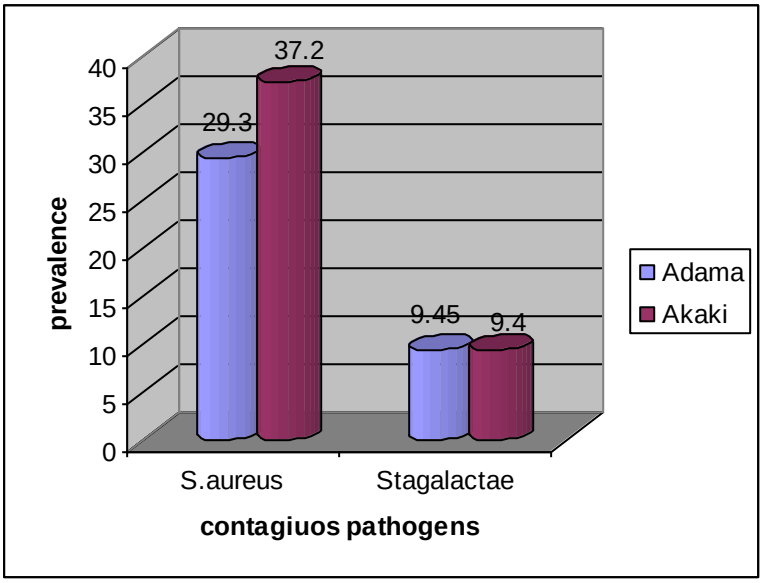


Figure 4 Major pathogens causing environmental pathogens

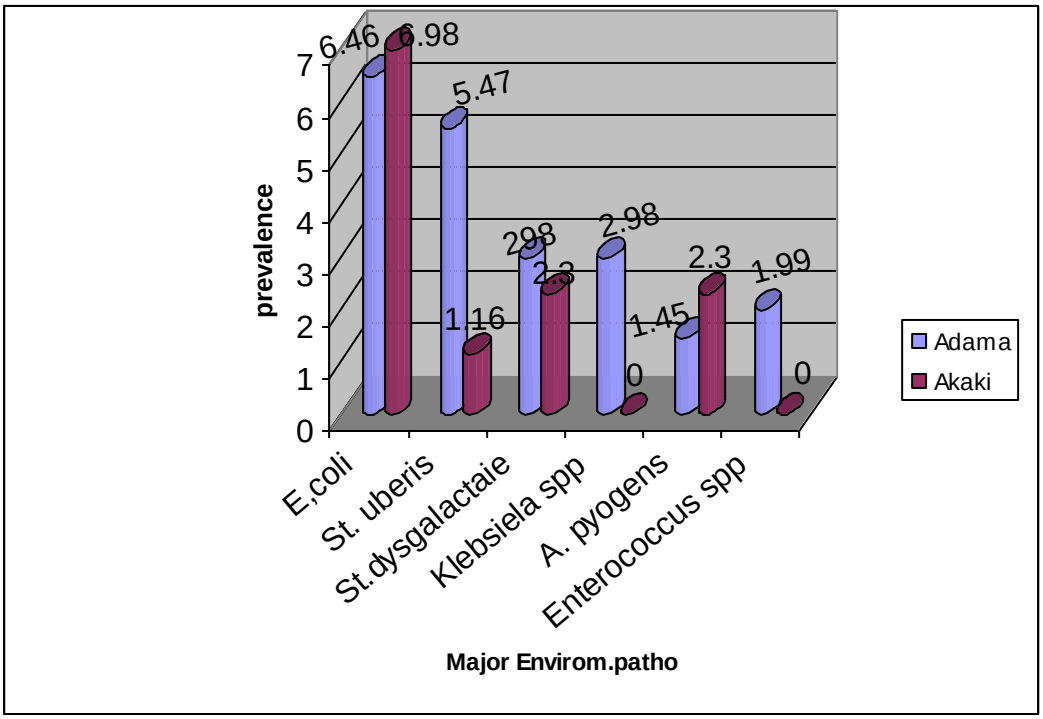
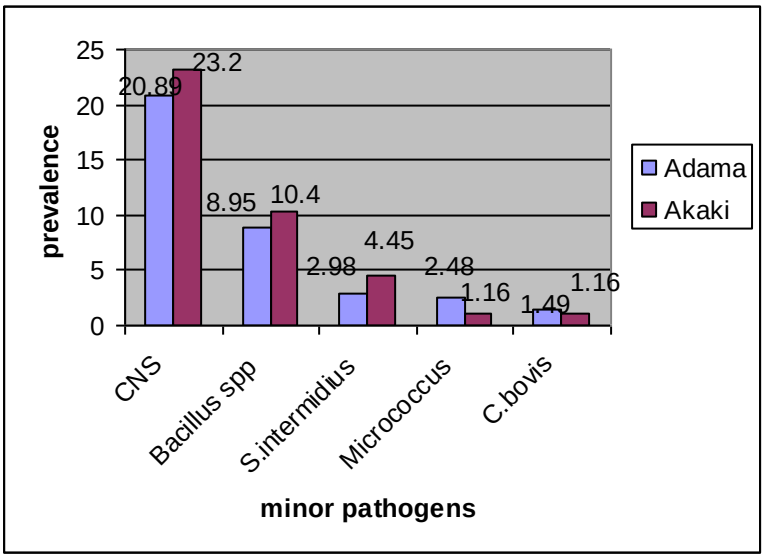


Figure 5 minor pathogens causing bovine mastitis



4.4. In vitro antimicrobial susceptibility test result

Anti microbial sensitivity test was done for all the isolates in both study areas, the resuls are presented in (Table12 and13). Isolates of *S.aureus* showed high resistance of Penicillin (65.3% and 75%) Polymyxine (89% and 80%), Bacitracine(100% and 82%), Amoxaciline (62% and 75%) and sensitive to *S.aureus* were Gentamycne (100% and 92%), Kanamycin (90%and 95%), Chloramphenicol (92% and 65%), Streptomycine (54% and 80%),Vancomycine (80% and 85%) in Adama and Akaki woredas respectively.

Isolates of *S.intermidius* were sensitive almost all antimicrobial disks applied in both woredas. CNS isolates were highly resistant Penicillin (50% and 70%), Bacitracidine (85% and 90%) and Polymyxine (80% and 75% and sensitive chlramphenicol (100% and 100%) Vancomycine (70.2% and 81%) in Adama and Akaki woredas respectivly. *S. agalactaie* showed highest resistance for amoxicillin (100% and 50%), Polymy xcine 92% and 90%) and highly sensitive for Gentamycine (100% and 100%), Vancomycine (84.2% and 100%), Penicillin (80% and 84.6%), Chloramphenicol (100% and 95%) and Streptomycin (52% and 72%) in Adama and Akaki woredas respectively.

St.dysgalctia was highly sensitive to almost all antimicrobial disk applied in both woredas except for amoxicillin 80% highly resistant in Adama woreda. Isolate of *St.uberis* was resistant to Bactracine70%), amoxicillin (65%) and sensitive to all other antimicrobial disks applied in Adama woreda and highly sensitive almost all antibiotic disks in Akaki woreda, whereas the *E.coli* isolates were sensitive to all antimicrobial disks except penicillin, amoxicillin, Bactracidine and polymixine in both woredas. *Bacillus species* showed highly sensitive almost all antimicrobial disks applied except to the penicillin resistant in Akaki woreda and Bacteracidine in both woredas.

Table 16 .Result of antimicrobial susceptibility test in Adama

Table 17.Result of Antimicrobial susceptibility test in Akaki

5. DISCUTION

5.1. Prevalence of bovine mastitis

The variability in the prevalence of bovine mastitis is due to interaction of several factors mainly of management, environment and factors related to animal and causative organism. The over all prevalence of clinical mastitis based on CMT and clinical examination at cow level in this study was 5, 9% and 3.0% in Adama and Akaki woredas respectively. The prevalence of clinical mastitis in Adama (5.9%) was close to the result obserbed by Bishi (1998) 5.3%, Mungube(2001) 3.6% and Gizat(2004) 3.9% . In Akaki the prevalence of clinical mastitis (3.0%) was close to the study of Getahun (2004) 2.6% worked in central highland Ethiopia and Kivaria, (2002) 3.2% study in Tanzania in small holder dairy farms. The prevalence of subclinical mastitis based on CMT at cow level in the present study 33.6% and 25.0% in Adama and Akaki woredas respectively. In this case prevalence of sub clinical mastitis in Adama was similar to the finding of Bishi (1998), who reported 34.3%, Abayneh(2001) 34.6% worked in Addis Ababa and its surrounding. Gizat (2004) 34.4%. The prevalence of subclinical mastitis in Akaki 25.5% which is similar to Getahun (2004) 22.3%, Demelash *et al* (2005) 23% and lower prevalence to Kerro and Tareke (2003) reported 62, 9. In this study as well as in other similar studies, over whelming case of mastitis were sub clinical compared to clinical mastitis kerro and tareke (2003), workeneh *et al* (2002), Hussien (1999), kivaria(2002) and some other studies. In Ethiopia, the sub clinical form of mastitis was neglected and efforts have been concentrated on the treatment of clinical cases, account high economic loss due to sub clinical mastitis (Kerro and Tareke., 2003). According to Radositis *et al.,* (2000) the percentage of an infected quarter showed 30% and a cow 15% reduction in milk yield. As usual the owners of small holder dairy farms in the study areas were not well informed about the invisible loss from sub clinical mastitis .Since dairy forming is mostly a sideline business in them.

5.2. Bacterial isolation:

In the present study *S. aureus* was the predominant pathogen (29.3% and 37.2%) compared of all isolates in Adama and Akaki woredas respectively. In a survey conducted in Jamaica Zinegser *et al* (1991) , also isolated S.aurues to the result of (27%), and in India Barbudhe *et al* (2001) reported 23.2% these results were similar to the finding of Adama in present study .The result for Akaki woreda in the present study(37.2) was similar to the study observed by Haile(1995)reported 38.8%and Workeneh *et al* (2003) 39%..The result of both woredas in present study is much higher than that of Bishi (1998) repoted 9%, Hussien (1999) reported 10.6% studied in Addis Ababa and Gizat (2004) reported 17% in Gondar. How ever this current study much lower than that of Kerro and Tareke (2001) *S.aureus* accounted for 40.5% of isolated in southern Ethiopia and Hundura *et al* (2005) accounted 44.4% in Sebeta. The relative high prevalence of *S.aureus* in this study could be associated with lack of effective udder washing hand washing before milking, drying separate clothes, post milking teat diping and disinfection in milking routine of area.

The result of coagulase negative staphylococcus (CNS) in present study isolated 20.8% and 23.2% in Adama and Akaki woredas respectively. These both results are much lower than the finding of Bishi (1998), Hussein (1999) and Gizat (2004) reported 54%, 42% and 46%. How ever much higher than the result of Miline *et al* (2002) reported 10%. CNS regarded as minor pathogen and normally considered as normal inhabitants of bovine udder.(Gentilini *et al.*,2002)..

The isolates St. Agalactaie in this study was 9.45% and 9.3% in Adama and Akaki woredas respectively. These results are lower than compared to the finding of Kerro and Tareke (2001) who reported, higher isolation rate of 13.1% and Bishi (1998) reported 27% in Addis Ababa.

Regarding Environmental St. uberis , St.dysgalactaaie , and E.coli were isolated in present study (5.58% and 1.16%), (2.48 and 2.3%) and (6.46% and 6.9%) in Adama and Akaki

woredas respectively. St.uberis isolated in Akaki (2.3%) was similar to the with report of Gizat (2004) in Gondar and Bishi (1998) in Addis Ababa 1.48% and 1.9% respectively. This pathogen isolated in Adama(5.58%) closely similar finding of Kerro and Tareke (2003**)** who reported 5.1% and much lower than that of Miltenburg *et al* (1996) reported 12.1%. Isolates of *St. dysgalactaie* in both woredas were higher where compared to Bishi (1998) who reported 0.5%in Addis Ababa and was lower finding compare to that of Kerro and Tareke (2003) reported 5.6% in southern Etiopia. Regarding of E.coli in present study isolated 6.46% and 6.9% in Adama and Akaki woredas respectively. This isolate is predominant among environmental pathogens in present study. The prevalence of environmental *E.coli* , may be associated with poor farm cleaness and poor of stable areas . In this study also environmental pathogens were isolated in similar proportion in both woredas however a common understanding that with increasing that with increasing herd size, manure disposal and sanitation problem less to build up to bacterial population (coliform and environmental streptococcus) in the cows immediate environment in both study areas. Isolation of bacillus species from mastitis cases has been reported Radositis *et al.,* (1994). Haile (1995) reported 11.5% of *Bacillus species*, which is similar with the current result (8.9%) and (10.4%) in Adama and Akaki respectively.

5.3. Antibiotics sensitivity test

The total of 59(29.3%) and 32(37.2%) S.aureus, 6(2.98%) and 5(4.65%) S.intermidius , 42(20.8%) and 20 (23.2%) CNS, 19(9.45%) and 9(9.3 %%) St.agalactaie, 10(5.58%) and 1(1.16%) St.uberis , 6(2.98%) and 2(2.3%) St.dysgalactaie, 18(8.9%) and 9(10.4%) Bacillus species, 12(6.46) and 6(6.9%) in Adama and Akaki woredas respectively to nine antibiotics applied in this study. S.aureus sensitive to gentamycine (100% and 92%), Chloramphenicol (92% and 65%), Kanamycine (90%and 95%), Vancomycine (78% and 82%) highly resistant to Bacteracince (100% and 100%), Polymixin (89% and 80%),penicillin (65%and 89%) and Amoxaciline (62% and 75%), this result is closed to the study Edward *et al.,* (2002) who is reported Bacitracine highly resistant for S. aureus 94% and Amoxaciline 74 %, According to San martin *et al.,* (2007) report S.aureus resistant to Amoxaciline(60%) and penicillin (68%). (Gentilini, 2002) reported in Argentina S.aureus highly susceptible for Gentamycine (90%)

and Chloamphenicol. The report of Kang, (2007) *S.aueus* and the streptococcal species including CNS were highly resistant to penicillin, which is similar to present study in both woredas that may due to the long term use of B-Lactam antibiotics in IMI therapy bacterial resistance to B-Lactam mechanism include production of B-Lactamases and the production of a low –affinity penicillin binding protein.

Accoding to Sanmartin *et a*l (2007). The corresponding values for CNS strain were resistant to penicillin 56% and Amoxicilin 42%,.this result is similar to the present study which was CNS isolates resistant to Amoxacilin 72% and 35% in Adama and Akaki woredas respectivly St.agalacttaie in this study showed sensitive to Gentamicin 100% and 100% , Chloramphenicol 100% and 95% , penicillin 80% and 85% , Kanamicin 72%and 50% , Vancomicin 84% and 100% and resistance to Amoxicilin 100% and 50% and Polimixin 91%and 90% in Adama and Akaki woredas respectively. These results are similar to that the result of Shakantal *et al* (2003) more sensitive antibiotics for *St.agalactaie* were Gentamycin (100%) and Chloaphenicol (75%).

In this study *E.coli* isolates was sensitive to chloraphenicol (100%) in both woredas, Kanamycine (75% and 78%) Gentamycine 92% and 80%, Streptomycin 65% and 78% and Vancomycyn 95% and 100%., where as resistant to penicillin (79% and 65%), Bactracine (80% and 92%). These results are closed to the report in India (ICAR) Shakuntala *et al* (2003) *E.coli* sensitive to Chloramphenicol(100%) and Gentamycine (50%)

In general when compairing to over all efficacy (on all Isolates) Gentamycine, Kanamycine, Chloamphenicol and Vancomycine were the most effective antibiotics in both woredas. Fallowing to that Streptomycin, penicillin, Polymyxine were effective antibiotics .The least effective antibiotics was Bactracine in both woredas.

6. CONCLUTION and RECOMENDAION

Mastitis a multifactor disease involving three bio systems the cow, the infectious agent and the environment this no single factor should be regarded as the most important in the disease process and the combined effect of various factors are decisive in disease incidence.

This study has indicated the occurrence of moderate and high prevalence of subclinical mastitis at cow, herd and quarter level and *S.aureus* was predominant pathogens in clinical and subclinical mastitis in both woredas.

The stage of lactation, parity number, and presence of teat lesion were the most important risk factor affecting the prevalence of sub clinical mastitis in cow level and milking mastitic cow at last in herd level of both woredas

It is observed that the study sites low level of management such as improper milking procedure uncleaned dairy houses and poor housing system, inadequate veterinary cheek up and supervision, absence of culling of chronic shedders, absence of dry cow therapy and absence of post teat dipping..

S.aureus was the predominant isolate which is highly resistant to Penicillin, Polymixin, Bactracine and Amoxiciline in both woredas, this resistance might be due to continuous use of this drug for any clinical case,

The study also indicates that antibiotic therapy was used indiscriminately without any concern to the consequence, hence drug resistance bacteria have been recorded .There fore no single prevention and control system can provide a means to minimize the over all prevalence of disease.

Based of the above conclusion the following points are forwarded as recommendation:

- Antibiotic therapy should be provided based on the principle of antimicrobial therapy and regular testing of antimicrobial susceptibility of mastitis causing pathogens should be carried out giving special emphasis to new formulation of drugs;

- As a routine practice milking should be carried out hygienically including proper udder management practice includes; teat washing and drying using separate towel, use of strip cup detects clinical mastitis, post milking teat dip, precipitous cows should be milked first and clinical mastitis and chronic shedders last. This could help in be minimizing the contagious mastitis;

- Strong extension package that involves training on farm hygiene, health care and feeding, culling chronically infected animals and dry cow therapy should be enforced.

- Mastitis control strategy should be initiated and promoted in study areas.

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8. ANNEX

.Annex 1. Questionnaire Survey

Owners Name: _____Address_____

Date of sample Collection_____

1. Cow History
   - Breed_____Age_____Calvingdate _____
   - Teat Lesion Yes_____No_____
   - MilkQuality Watery_____BloodTinged_____Clots/Flakes___ Normal_____
   - Sample Collected from: FR___FL____HR___HL_____

- CMT Score: HR___HL____FR___FL_____
- Stage of lactation Biggning_____Middle_____End_____
- Parity number 1-3 _____ abave 3_____
- Herd size 1-5 _____abave 5_____

2. Milking Practice

- Do you wash udder before milking? yes____ no____
- Do you wash hand between milking? yes____ no____
- Do you use separate cloth for drying teat? yes____ no____
- Do you practice milking mastitic cows last? yes____ no____

3 Barn floor statuses

- Housing Closed _____ semi closed _____open _____
- Floor: concrete_____stone_____soil_____slopy_____leveled__
- Roof: metal sheet_____ grass_____
- Wall: concrete_____mud_____other_____Drenage
- Drainage: Good_____ Bad _____
- General hygiene: Good_____ Bad _____

4. Mastitis Situation

- Previous mastitic problem in barn Yes_____ No_____
- Person treated mastitic cow Vet Profision _____ My self _____
- Problem of cure mastitic cow Yes _____ No_____

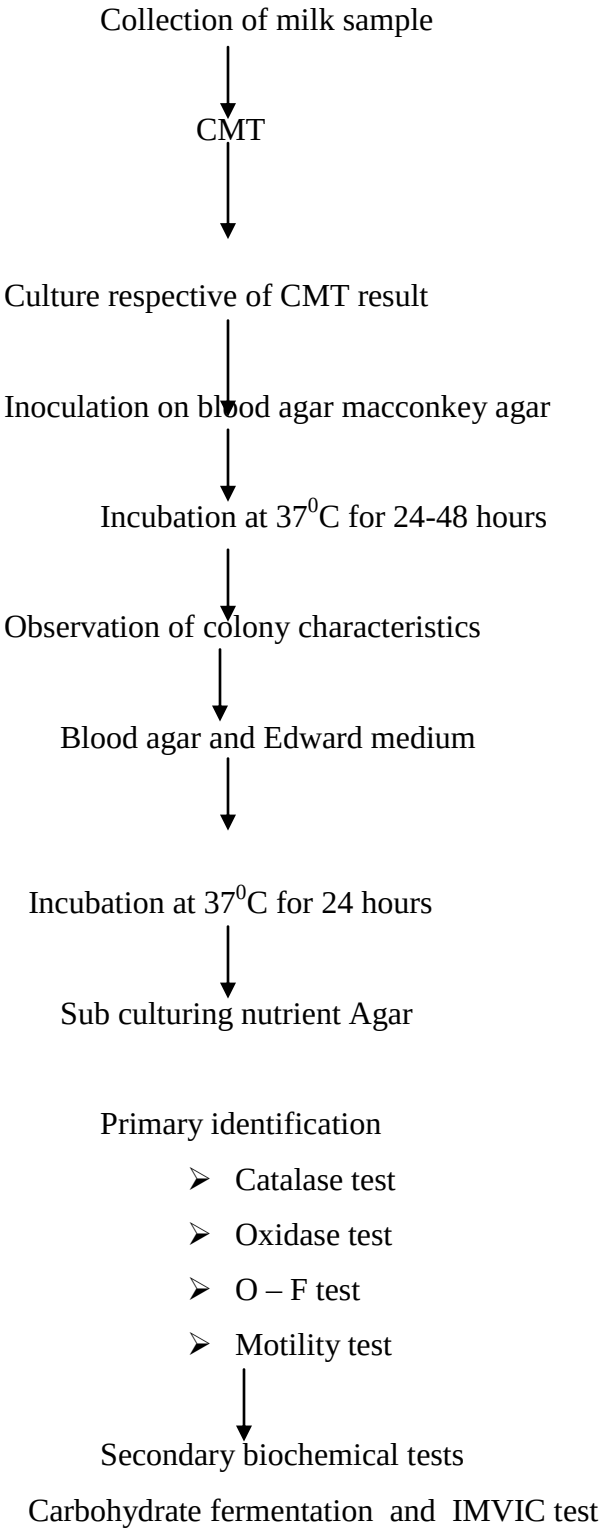
Annex 2.

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: Schalm *et al*. (1974) and Quinn *et al* (1994)

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



Annex 4 Photos of isolation and identification of bacteria from mastitic milk in the study areas

Photo 1

1-CMT reagent
2- CMT paddle

Photo 2 CMT positive milk sample

Photo 3-Blood agar

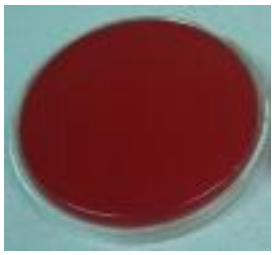


Photo 4 mixed colonies
On blood agar media

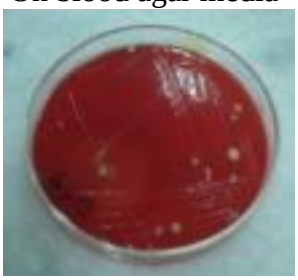


Photo 5 S.aureus on
blood agar media

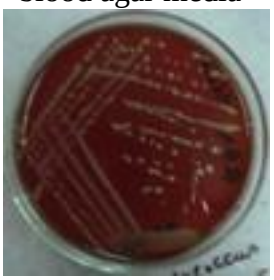


photo 6- nutrient agar

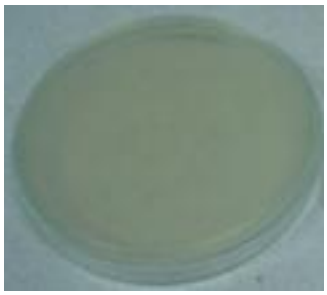


Photo 7 St.agalactaie
Nutrient agar media

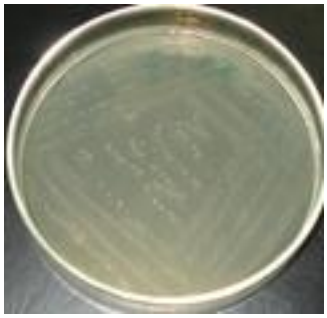


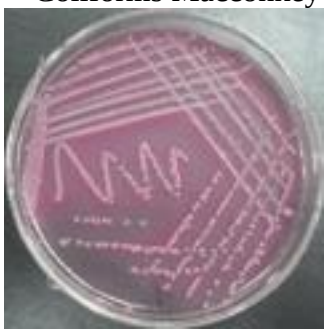
Photo 8 CNS on
nutrient agar media

Photo 9 Bacillus species
On nutrient agar media

Photo 10 St.aurues on
nutrient agar media

Photo 9 Macconkey agar C

Photo10 lactose fermentative. Coliforms Macconkey agar



Non fermentative Gram $^{-ve}$ bacteria on Macconkey agar

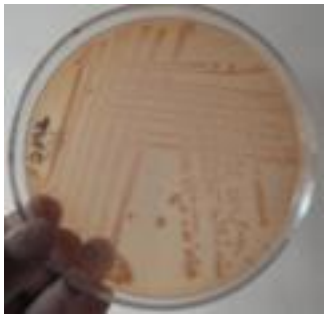


Photo 11 Edwards medium

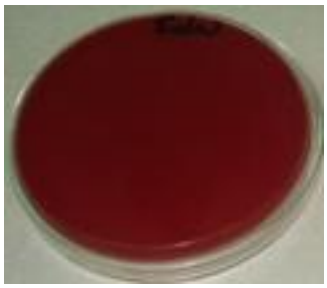
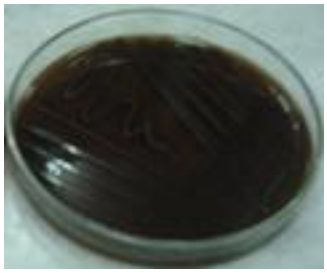


Photo 12 St.uberis on Edwards medium



Poto 13 E. fesalis on Edwards midium

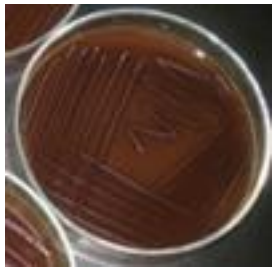


Photo 14 Eiosin methylen Blue agar medium

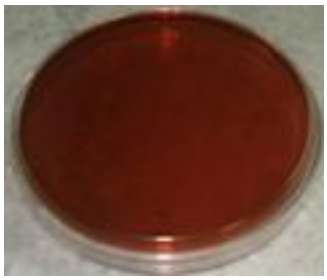
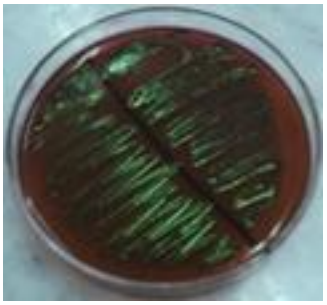
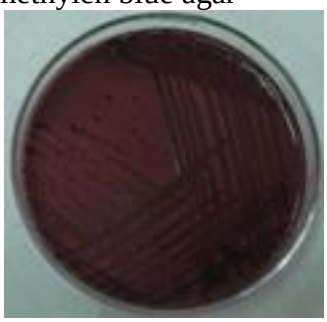


Photo 15 Ecoli on Eiosin methylen blue medium



(Metallic sheen)

Photo 16 E.coli on Eiosin methylen blue agar



(Metallic sheen)

Photo 17 Purple Agar Base

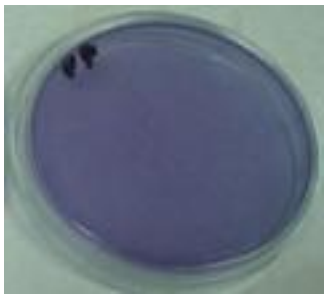


Photo 18 Manitol salt agar

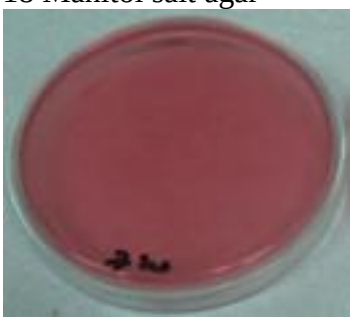


Photo 19 Innoculated Staphylococcus species on purple agar base

A. not fermented In 24 hrs incubation | B.fermented in 24hrs incubation | C. Fermented in 24 in 24hr incubation

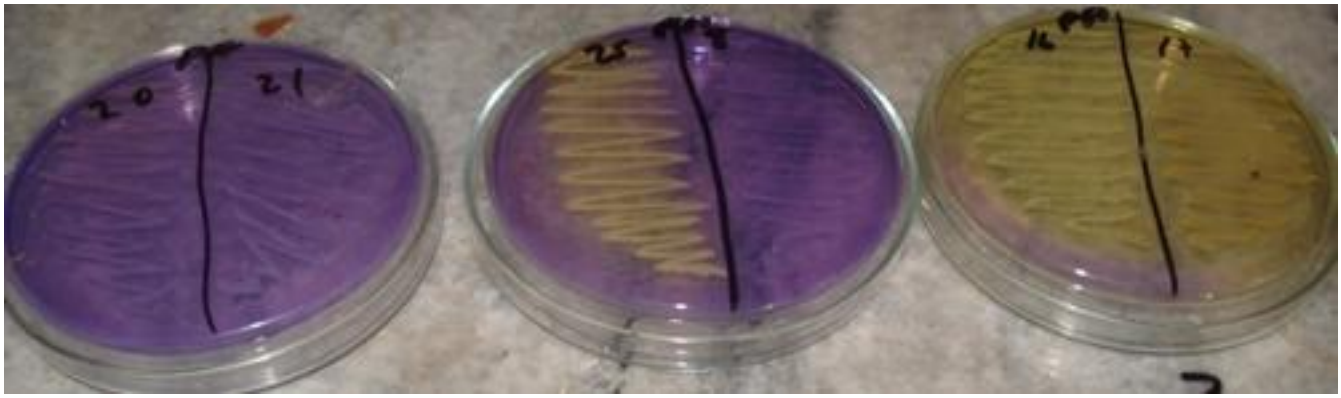


Photo 20 Inoculated Staphylococcus species on Manitol salt agar

A.not femented in 24 hrs Incubation | B.fermented in24hrs Incubation | C. fermented in 24 hrs Incubation

Photo 21 Ununoculated
OF test

Photo 22. Result oxidative and fermentative test

Second from left is oxidative, all others fermentative

Picture 23. Catalase positive test

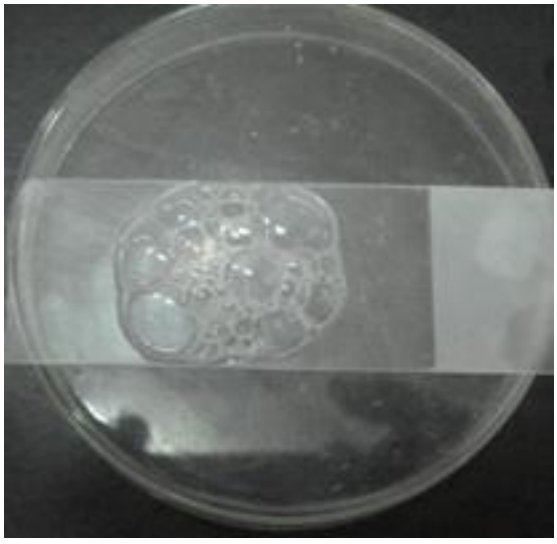


Photo 24 Rabbit plasma for coagulase test

Coculase positive Staph(CPS). Coagulase negative Staph(CNS).

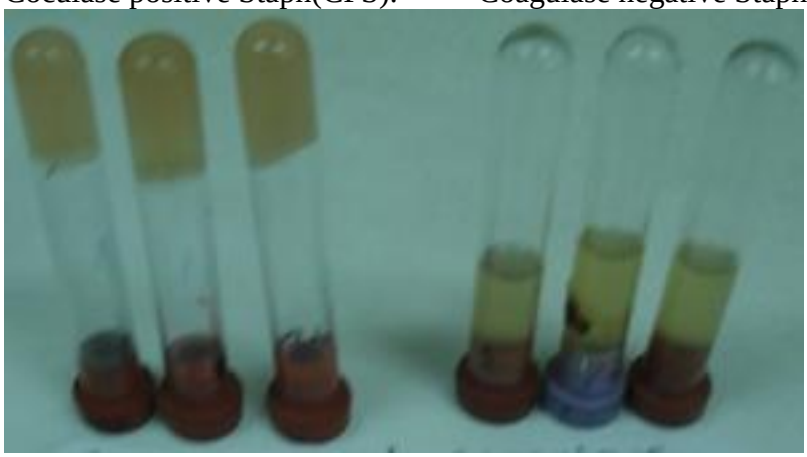


Photo 25. CAMP test

Photo 26. Uninoculated TSI

Photo 27. Positive test A/A gas prod.

Ptoto 28 Uninoculated Simon citrate

Photo29 Posetive citrate test

Photo 30 uninoculated

MR-VP positive test

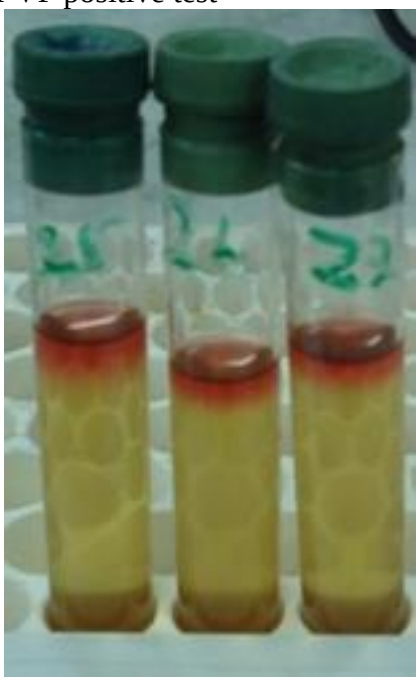


Photo 31uninoc Ulated test.SIM

Photo 32 Indol positive test

Motilility and Indole Positive test

Photo33.Antibiotics disks for Sensitivity test

Photo 34.Mc Farland slandered

Photo 35. measurezone inhibition

Photo36.Antibiotics sensitivity of St.aureus

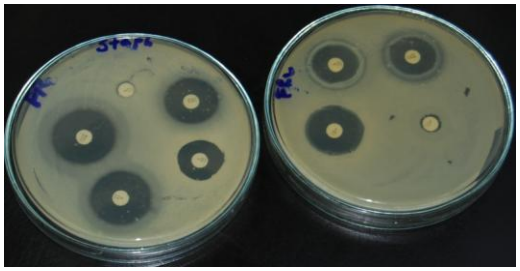
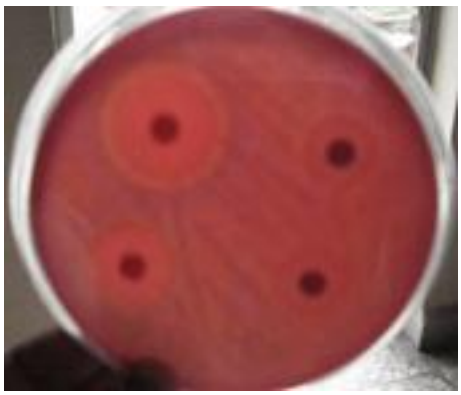


Photo 37. Antibiotic sensitivity of Bacillus species

Photo 38. Antibiotics sensitivity of St. agalactaie



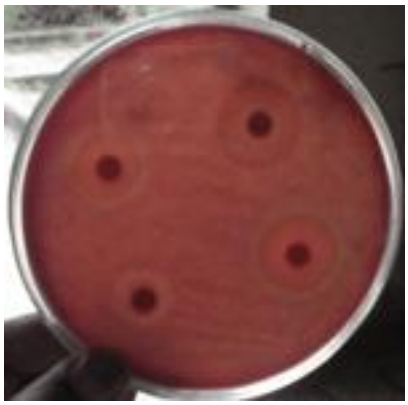


Photo 39. Antibiotics sensitivity of CNS

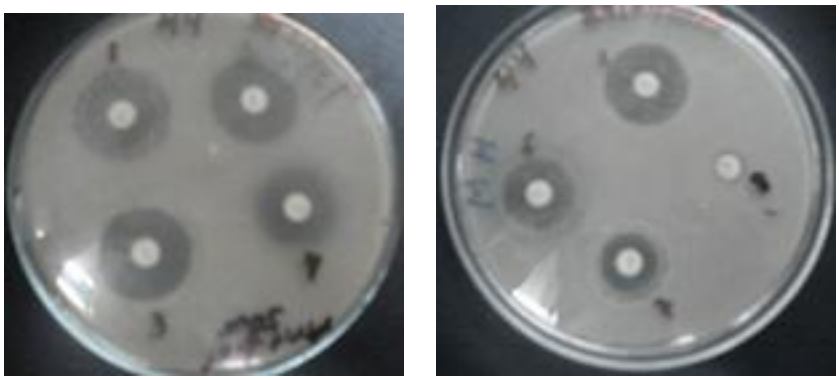
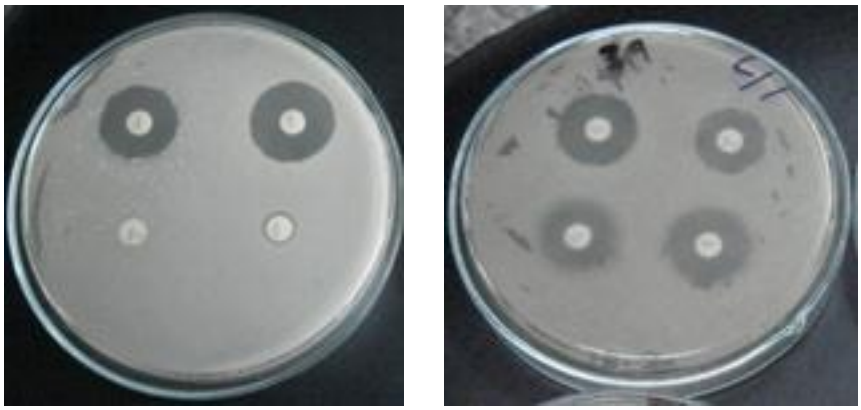
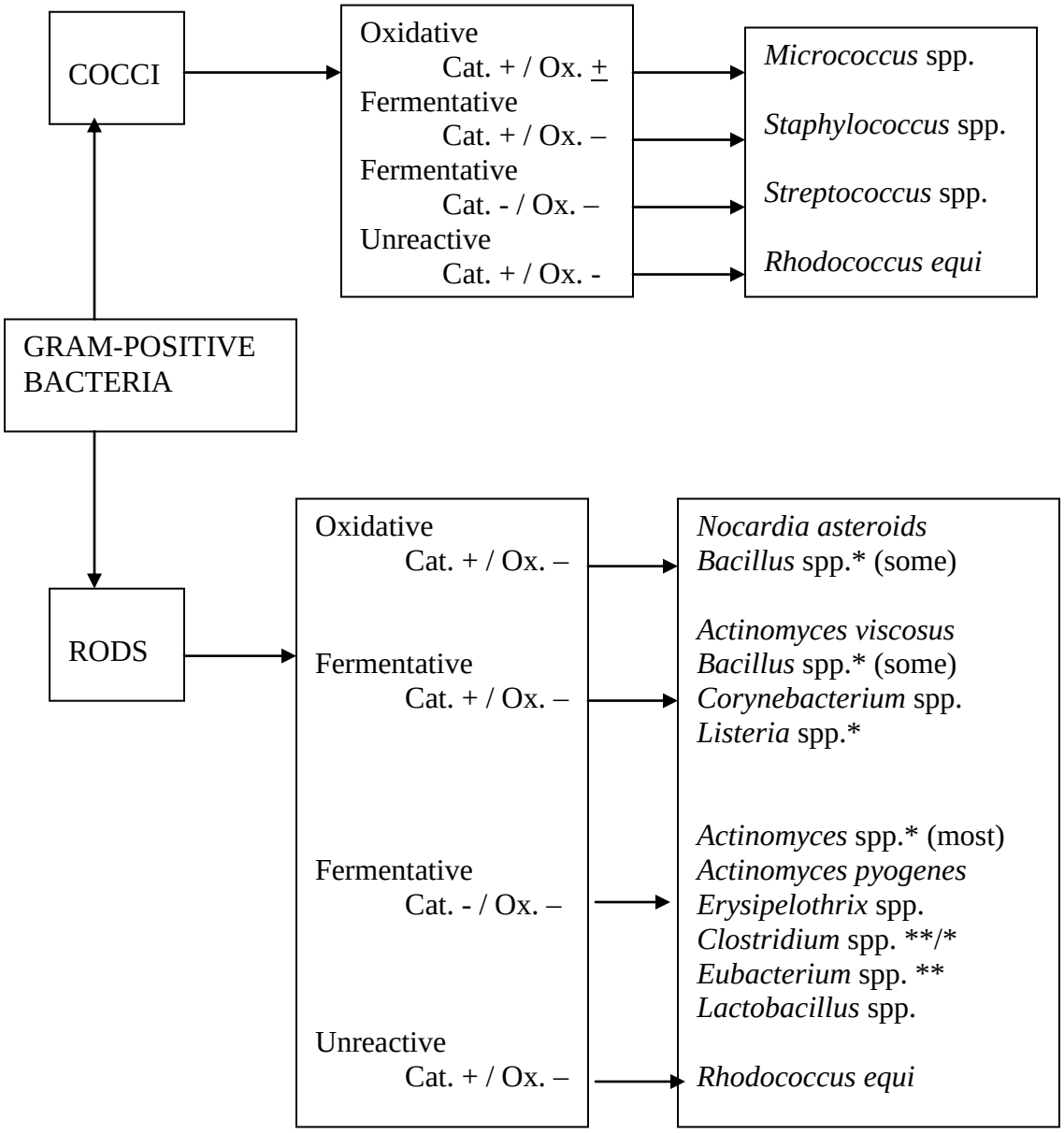


Photo 40. Antibiotics sensitivity of E. coli

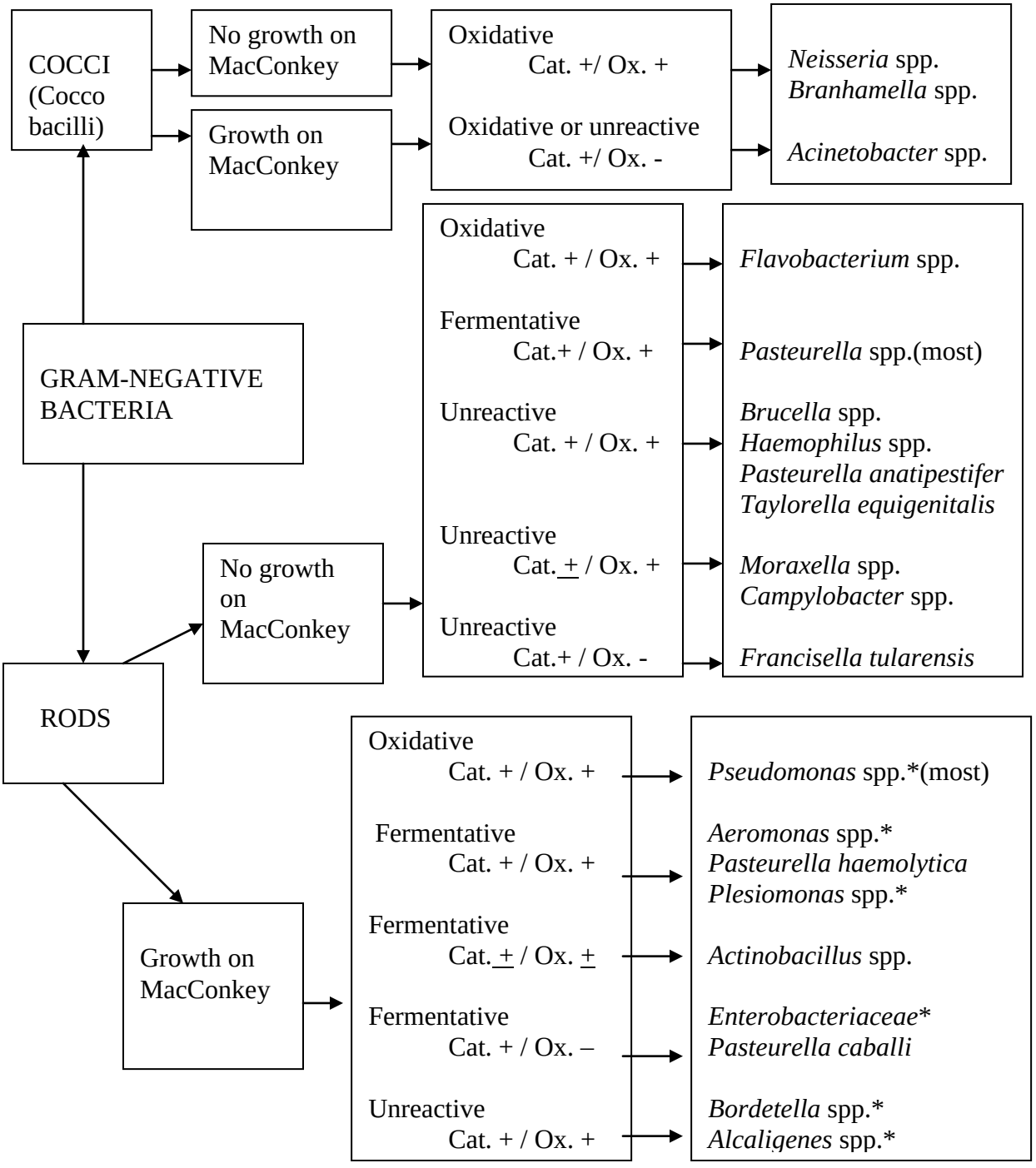


Annex 5: Primary identification of Gram-positive bacteria



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

Annex 6: Primary identification of Gram-negative bacteria



(Cat. =Catalase; Ox. =oxidase; += positive reaction; - = negative reaction; ± = variable,= motile)* Source: (Quinn et al., 1999)

Annex 7: 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 aeruginosa* 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 370C for 18 –24 hours. A positive

CAMP test is indicated by an arrow–head of complete haemolysis. The group B streptococci produce a diffusible metabolite that complete the lysis of the red cells, only partially haemolysed by the beta –haemolysin of the staphylococcus.

Annex 8: Secondary identification test

Indole test (Quinn *et al*., 2002)

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.*, 2002)

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*., 2002)

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*., 2002)

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

.

Citrate utilizations

Simmon citrate agar (Difco, U.S.A) slope surface was streaked with the suspected bacterial colonies and incubated at 37^0c for 24 hours. Typical reaction for citrate utilization and possibility was declared by the change of the medium from green to blue color (quinn *et al.*, 2002)

Annex 9: Differentiation of mastitis causing Staphylococcus species and Micrococcus species

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 10: Differentiation of mastitis causing Streptococcus species.

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

Annex 11: Differential test used for Bacillus species.

Bacillus 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 12: Differential tests used for Corynebacterium and Actinomyces species

Corynebacterium and *A. pyogenes*	Catalase test	Hemolysis	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 13: 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^-	-	+
Cit. 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 14: 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^{o}C for 15 minutes. Cool the base to 45 to 50^{o}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^{o}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^{o}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^{o}C); at approximately 50^{o}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^{o}C). At less than 60^{o}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^{0}C). Cool to 45 to 50^{0}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 121^0C 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 121^0C). 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 121^0C 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 115^0C for 20 minutes. Cool to 50^0C; 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 121^0C for 15 minutes. Cool to 60^0C 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 121^0C for 15 minutes. Cool to 50^0C and add 100 ml of urea agar base. Mix thoroughly and dispense aseptically in sterile tubes. Cool tubed medium in a slanted position so that deep butts are formed.

14. Muller Hinton Agar (BBL®, Becton Dickinson, 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.

Annex 15. Procedures to conduct antibiotic susceptibility test (Carter, 1997)

- Preparation of the inoculums

  Inoculation of a distinct colony in to 5ml of nutrient broth and incubated 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 inoculums) 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). Incubate the plates invented aerobically for 16 to 18 hours at 35^{0}C but not 37^{0}C.

- Interpretation

Zone of Inhibition 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
Tetracycline TE30	30µg	≤14	15-18	≥19
Erythromycin E15	15µg	≤ 13	14-17	≥18
Penicillin G10 for staphylococci	10U	≤20	21-28	≥29
PenicillinG10	10U	≤11	12-21	≥22
Vancomicin	30 mcg	9 ≤	10-11	≥ 12
Chloramphenicol	30 mcg	12≤	13-17	≥ 18
Polymyxin B PB30	300U	≤8	9-11	≥12
Amoxilin		≤13	14-17	≥ 18
Gentamicine(CN 10)	10mcg	12≤	-	≥13
Kanamycin K30	30µg	≤13	14-17	≥18
Bactracine (B 10)	10 U	≤ 8	9- 12	13≥