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PREVALENCE OF BACTERIAL MASTITIS AND ITS ASSOCIATED RISK FACTORS IN ASSOSA DISTRICT, WESTERN ETHIOPIA

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Prevalence of bacterial mastitis and its associated risk factors in Assosa district, Western Ethiopia

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List of abbreviation

BGRS Benishangul Gumuz Regional State

CMT California Mastitis Test

CM Clinical Mastitis

CNS Coagulase Negative Staphylococci

IDF International Dairy Federation

IMI Intra Mammary Infections

SCC Somatic Cell Count

SCM Sub Clinical Mastitis

SPSS Statistical Package for Social Science

WBC White Blood Cell

Abstract

Mastitis is one of the most significant health problems of dairy herds as it causes physical, chemical and bacteriological changes in the milk of dairy animals resulting in inferior quality and quantity of milk with possible public health importance. The purpose of this study was helpful for stakeholders to design and to take appropriate action in order to tackle the risk of mastitis. A cross-sectional study was conducted to estimate the prevalence of bacterial mastitis and the associated potential risk factors in Assosa dairy farm. For this study, a total of 364 lactating cows were included. Data were collected by using a semi-structured questionnaire, physical examination of the udder and also Californian mastitis test was used. Different biochemical tests were also conducted to identify bacteria using appropriate media. Statistical analysis was carried out using SPSS version 20. Descriptive statistics was used to determine the prevalence of mastitis depending on clinical inspection and Californian mastitis test. Chi-square test was employed to see the impact of different risk factors on the occurrence of mastitis. Overall prevalence of mastitis was found to be 42.03% and the dominant bacterium were found to be Staphylococcus aureus (62.1%) followed by Coagulase Negative Staphylococcus (17.6%), Staphylococcus intermidius (14.4%) and Escherichia. coli (5.9%). Major exposing factors that leads to mastitis were previous exposure to mastitis, age, parity, and floor type. It is recommended that regular investigation of mastitis especially sub clinical form, should be practiced and susceptibility testing for the isolated microorganisms is very vital in controlling strategy.

Keywords/phrases: *Bacterial isolation, California mastitis test, Clinical mastitis and subclinical mastitis.*

1. INTRODUCTION

Milk and milk products have formed an important part of daily nutrition and the variety of products produced from milk has increased dramatically over the years, as modern food processing technologies have improved. Also, an increase in the global population coupled to the increasing demands for milk as an economic food and as an industrial raw food product has necessitated an increase in production by dairy farms (Javaid *et al*., 2009). Breeding goal in dairy farm is maximizing profitability by selecting animals with high production traits that remains as much as possible in herd avoiding problems. However, functionality has been endangered through years because of exhaustive selection on increasing production level and antagonistic genetic correlations between production and resistance to some diseases (Rauw *et al.,* 1998). Nowadays, profitability depends on reducing costs more than increasing income and selection is focusing on functional traits, such as fertility, diseases and calving ease (Philipsson and Lindhe, 2003).

Mastitis is induced when pathogenic microorganisms enter the udder through the teat canal, overcome the cow's defense mechanisms, begin to multiply in the udder, and produce toxins that are harmful to the mammary gland. Mammary tissue is then damaged, which causes increased vascular permeability. As a result of this, milk composition is altered: there is leakage of blood constituents, serum proteins, enzymes, and salts into the milk; decreased synthesis of caseins and lactose; and decreased fat quality (Osteras, 2000). In Ethiopia, livestock represents a major national resource and form an integral part of agricultural production system (Gebrewold *et al.,* 2000) of which dairy sector has large potential market for development and its growth is expected to continue for the next one to two decades due to the growth of income, increased urbanization and policy improvement. Thus, the development of smallholder dairy production sector in the country contributes in poverty alleviation by generating income through self-employment. Cows represent the largest proportion of the cattle population of the country. However, the milk production does not satisfy the country's requirements due to multitude factors. Risk factors associated with clinical mastitis are milking routine, type of housing, feeding, and season, as environmental effects. In addition, older cows, later first calving, first stages of lactation and cows with deep udders, week attachments, and high production are more

liable to mastitis. Health problems have negative consequences not only on animal welfare but also in economics of herds because of additional costs in veterinary medicines, reduction of production, discarded milk, and involuntary culling (Collard *et al.*, 2000). Mastitis is known to be a complex and costly disease of dairy cows that results from the interaction of the cow and environment including milking machine and microorganisms (Azmi *et al.,* 2008). Mastitis has been known to cause a great deal of loss or reduction of productivity to influence the quality and quantity of milk yield and to cause culling of animals at an unacceptable age (Vaarst and Envoldsen, 1997). Mastitis causes a reduced milk production, not only at the occurrence of the mastitis but throughout the rest of the lactation (Hagnestam *et al*., 2007), increases the risk of new cases of mastitis (Edinger *et al*., 1999; and increases the risk of culling (Schneider *et al.*, 2007). Schneider *et al*. (2007) have revealed that welfare of the cow is negatively influenced by mastitis as it can induce pain and even cause death. Mastitis is not just an issue for the cow and farmer, but also for the consumers. Consumers expect that milk comes from healthy animals seems safe but is negatively influenced by mastitis.

Subclinical mastitis is a major problem affecting dairy animals all over the world. It causes enormous losses for breeders and consequently influences the national income of a country (McDougall *et al.,* 2009). According to Sharma *et al.* (2007), mastitis is one of the most significant health problems of dairy herds as it causes physical, chemical and bacteriological changes in the milk of dairy animals resulting in inferior quality and quantity of produced milk with possible public health importance.

Therefore, conducting research on its prevalence and incidence will contribute a lot to design appropriate preventive measures and treatment regimen in the specific dairy farm. In Assosa, the disease is insufficiently investigated, and information relating to its magnitude, distribution, and risk factors is scant. Such information is important to envisage when designing appropriate strategies that would help to reduce its prevalence and effects. Thus, it is necessary to have epidemiological information about mastitis and factors associated with udder infection so as to improve dairy production and uphold quality of milk for consumers. Therefore, this research describes the results of an investigation made to elucidate the prevalence and risk factors of mastitis in lactating dairy cows in western Ethiopia.

1.1. Objectives of the study

1.1.1. General objective

The general objective of this study was to assess the prevalence of bacterial mastitis and its associated risk factors in Assosa district, Western Ethiopia.

1.1.2. Specific objectives

The specific objectives of the current study were to

- Determine the prevalence of clinical and subclinical mastitis at cow level in Assosa dairy cows.
- Isolate and characterize mastitis causing bacteria from the cow's milk in Assosa
- Determine the various risk factors associated with the occurrence of mastitis
- Identify the microbial load of milk samples.

1.2. Statement of the problem

Mastitis is one of the most important disease affecting dairy cows and it is a multi-factorial disease with worldwide distribution which incurs serious economic losses to dairy industry (De Grave and Fetrow, 1993). However, market oriented dairy production, a rapidly growing system in many African countries, is subjected to diseases of intensification including mastitis and reproductive disorders (Lemma *et al.,* 2001). A number of previous reports from different part of Ethiopia indicated that mastitis is a serious problem in dairy industry (Bishi, 1998). In Assosa, the incidence and distribution of the disease has not been studied systematically and information relating to economic loss and the overall prevalence of the disease is inadequate. The environmental condition of Assosa is assumed to be one of the representatives of kola area of Ethiopia and may favor the pathogens.

During the rainy seasons, the environment becomes muddy, humid and moisture conditions that favors for the multiplication and growth of various microorganisms and potentiate their disease producing capacity. This is one of the problems for the economic loss in the country. The findings of this study may help to prove and aware the community on the association of risk

factors with mastitis and to give due attention to this disease. This study was, therefore, initiated and to fill the gaps in the knowledge of mastitis in the study area.

Thus, in this study the following research questions were specifically assessed.

- Which type of mastitis is the most prevalent in this area?
- Which type of mastitis can be easily identified by local people?
- What are the common bacteria isolates from the study area?

1.3. Significance of the study

Mastitis is among the major contributors of disease load in Ethiopia. Particularly, in the study area mastitis is a serious problem. Therefore, the result of this study was helpful for stakeholders to design and to take appropriate action in order to tackle the risk of mastitis. The urban and rural dwellers will have an awareness on prevalence of mastitis in the study area and improving the future condition that helps them to control the distribution of mastitis in their local area. In addition to this it could be used as a baseline data for further studies in the area.

2. LITREATURE REVIEW

2.1. Definitions and general introduction of mastitis

Mastitis as a disease has received little attention in Ethiopia, especially the subclinical form. Efforts have only been concentrated on the treatment of clinical cases (Nesru, 1999). Bovine mastitis is the most important disease condition of dairy cows. Owing to the heavy financial implications involved and the inevitable existence of latent infections, mastitis is obviously an important factor that limits dairy production. Very limited researches have been done concerning on the status of bovine mastitis in Hawassa City unlike that of other areas of the country (FAO, 2003). The study area is one of the most known potential dairy areas in the country, where smallholder dairy production is practiced. Mastitis is an inflammation of the mammary gland and commonly associated with intra mammary bacterial infection. It is considered as one of the most important disease among diseases of the dairy animals Radostits *et al*. 2000. Mastitis can be defined as clinical (grossly evident changes to milk, the gland or the whole animal) or as subclinical (diagnosed using ancillary tests such as the somatic cell count). Mastitis is a multi-factorial disease, requiring exposure to a combination of environmental and pathogenic factors and with variable responses between animals.

According to Lemma *et al*. (2001) of the major diseases of crossbreed cows in Addis Ababa milk shed mastitis was the second most frequent disease next to reproductive diseases. According to Mungube *et al.* (2005) estimated the economic losses from mastitis in the urban and per urban areas of Addis Ababa, Ethiopia, to be US $ 58 per cow per lactation. The prevalence of clinical and sub clinical mastitis in Ethiopia range from 1.2 to 21.5% and 19 to 46.6%, respectively (Hussein *et al.*, 1997, Bishi 1998, Kassa *et al.*,1999, Lemma *et al.*, 2001, Workineh *et al.*, 2002, Kerro and Tareke 2003). However, most of these studies were carried out in Addis Ababa and its surroundings, capital of the country and fail to represent the occurrence of mastitis under different management and environmental situations in other regions of the country. The research was done in Alage area were indicates that subclinical mastitis is characterized by changes in

milk composition e.g. somatic cell count (SCC); leukocytes and epithelial cells), and changes in milk pH and ion concentration, without clinical signs of inflammation (Guidry, 2007**).**

2.2. Types of mastitis

2.2.1. Subclinical mastitis

Subclinical mastitis is characterized by changes in milk composition e.g. somatic cell count (SCC); leukocytes and epithelial cells), and changes in milk pH and ion concentration, without clinical signs of inflammation (Guidry, 2007). In the healthy lactating mammary gland, the milk SCC is often < 100,000 cells/mL of milk, while the SCC can increase to > 1,000,000 cells/ml of milk during subclinical mastitis. The major factor affecting the SCC at the herd and individual level is the presence of intra mammary infections (IMM) (Radostits *et al.*, 2007).

2.2.2. Clinical mastitis

Clinical mastitis is characterized by visual clots or discolorations of the milk, often in combination with tender and swollen udder, sometimes in combination with fever, loss of appetite etc.

2.3. Epidemiology of Mastitis in Ethiopia

2.3.1. Prevalence of mastitis

Different studies conducted in different parts of Ethiopia showed variable prevalence of mastitis depending on the type of farm and managements systems. Biffa *et al.* (2005) conducted a study on mastitis of 974 lactating dairy cows in Southern Ethiopia as, 34.9% (340) had mastitis; 11.9% (116) clinical, and 23.0% (224) subclinical mastitis respectively. Mastitis prevalence in dairy farms of Holeta town, Central Ethiopia at cow level was 71.0% (76/107), out of which 22.4% (24/107) and 48.6% (52/107) were clinical and subclinical, respectively. The Holeta study also revealed the quarter level prevalence of mastitis as 44.9% (192/428); from this the clinical form was 10.0% (43/428) and the subclinical was 34.8% (149/428) (Mekibib *et al.*, 2010).

Mulugeta and Wassie (2013) also carried out a research on Prevalence of bovine mastitis in and around Wolaita Sodo, Southern Ethiopia. From the total of 349 lactating cows examined, 103 (29.5%) were positive for mastitis. Of these, 9 (2.6%) and 94 (26.9%) were found to be positive for clinical mastitis and subclinical mastitis, respectively.

According *to* Zeryehun *et al*. (2013), a total of 499 cross-breed cows from 38 dairy farms were examined for mastitis detection and out of which 373 (74.7%) cows were found to be affected with clinical and sub clinical mastitis based on the clinical diagnosis and CMT. Prevalence of bovine mastitis in smallholder farms in and around Gondar was too high (Nibret *et al*., 2011). This shows that bovine mastitis is an important dairy health problem in this area. The prevalence of clinical and sub clinical mastitis in the study area was 0.93 and 31.67%, respectively with an overall prevalence of 32.6%.The overall prevalence of mastitis in lactating cows (cross breed 58.33% and local bred 33.33%) in Sidamo zone was 42.71%, which agreed with the findings of Fekadu (1995) in Chaffa valley in Northern Ethiopia (39.65%) and Kerro Dego and Tareke (2003) in Southern Ethiopia (40.40%) on bovine mastitis.

The overall prevalence of mastitis 42% at cow level was comparable to the findings of Kerro and Tareke (2003), Workineh *et al.* (2002), Bishi (1998), Mungube *et al.* (2004) and Fekadu (1995) who reported 40.4% in southern Ethiopia, 38.2% in Adami-Tulu central Ethiopia, 39.8% in and around Addis Ababa, 46.6% from central highlands of Ethiopia and 39.7% in Chaffa valley in north eastern Ethiopia, respectively. However, it is relatively lower than the study of Mekibib *et al.* (2010), Sori *et al.* (2005), Lakew *et al.* (2009) and Zeryehun *et al.* (2013) who got 71.1% from Holeta, 52.8% from Sebeta, 64.6% from Assela and 74.7% around Addis Ababa respectively. The finding was higher than previous reports of mastitis in some parts of Ethiopia, Getahun *et al.* (2008) and Bitew *et al.* (2010) who reported mastitis with prevalence of 24.9 and 28.2% in their respective studies in Selalle, and Bahir Dar.

This variation is may be due to mastitis is a complex disease involving interactions of various factors such as manage mental and husbandry, environmental conditions, animal risk factors, and causative agents (Radostitis *et al.,* 2007).

The prevalence of sub clinical mastitis reported during the study was 36.7 % at cow level which was in close agreement with prevalence reported by Alemu *et al.* (2013), Abaineh and Sintayehu (2001), Sori *et al.* (2005) and Lakew *et al.* (2009), with respective percent of 37.2, 34.6, 36.7 and 38.1% while the prevalence of clinical mastitis at cow level (5.3%) was in agreement with the finding of Enyew (2004) with recorded prevalence of (3.9%) from Bahir Dar, Ethiopia. However, the present result was lower than Zeryehun *et al.* (2013) (19.6%) around Addis Ababa and Delelesse (2010) (10.3%) around Holeta area. In general, subclinical mastitis has been reported to be higher than clinical mastitis owing to the defense mechanism of the udder, which reduces the severity of the disease (Hussien *et al.,* 1997).

2.3.2. Etiology of mastitis

Although about 20 to 35% of clinical mastitis cases are of unknown etiology (Wellenberg *et al.,* 2002), it is widely accepted that bovine mastitis is mainly bacterial in origin. It can be classified as contagious or environmental. In the former case, it is caused by organisms such as *S. aureus*, *Strep. dysgalactiae and Strep. agalactiae*, which are all adapted to survive in the udder, causing subclinical infections. Environmental pathogens like *Enterobacteriacae* like *E. coli* are not well adapted to survive within the udder and, instead, they multiply rapidly following invasion, evoke a swift immune response and are eliminated (Bradley, 2002).

The main etiological agents responsible for mastitis infections can be divided into different groups of organisms depending on the source of the organism involved. These include contagious pathogens, environmental bacteria, opportunistic bacteria and other organisms that less frequently cause mastitis less frequently (Philpot and Nickerson, 1999). Contagious microorganisms are usually found on the udder or teat surface of infected cows and are the primary source of infection between uninfected and infected udder quarters, usually during milking.

The organisms that fit into this category include: *Staphylococcus aureus* (coagulase-positive staphylococci), *Streptococcus agalactiae* and the less common sources of infection caused by *Corynebacterium bovis* and *Mycoplasma bovis (*Philpot and Nickerson., 1999). According to Quinn *et al.* (1999) a large number of Gram-positive and Gram-negative species are in a cow's environment and they cause clinical or subclinical infections in the udder and fall into a descriptive category known as environmental mastitis pathogens such as *Streptococcus uberis, Streptococcus equinus, Enterococcus faecalis and Enterococcus faecium* are Gram-positive species.

Gram-negative species include *Escherichia coli, Klebsiella spp., Enterobacter spp., Serratia spp. and Pseudomonas spp*. Environmental pathogens require moisture, favorable pH and organic material for survival and they enter the gland through the teat canal. Environmental pathogens reside in soil, bedding materials, manure and other organic matter. Therefore, efforts at prevention or control of environmental mastitis should focus on cleanliness of a cow's workplace and cleanliness of a cow. Mastitis caused by environmental organisms is essentially opportunistic in nature and becomes established if the immune system of the host is compromised or if sanitation and hygiene is not adequately practiced (Schukken *et al.,* 2005).

Mekonnen and Tesfaye (2010) revealed that contagious bacteria like Coagulase negative *staphylococci (*CNS*), S. aureus, S. agalactiae, S. dysgalactiae* and environmental microorganisms like coliforms *(Escherichia coli* and *Enterococcus faecalis* were found to be the major etiology of mastitis in market oriented smallholder dairy farms in Adama, Ethiopia. According to Nibret *et al.,* (2011) bacterial isolates in the milk of dairy herds in and around Gondar, Ethiopia were *Staphylococcus aureus, Streptcoccus agalactiae, Streptcoccus dysgalactiae, Streptcoccus uberis, Escherichia coli, CNS, Micrococcus species, Bacillus cereus, Corynebacterium bovis and Actinomyces pyogens.*

2.3.3. Diagnosis of mastitis

2.3.3.1. Qualitative examination of milk

According to Quinn *et al*. (1994) changes in color of milk can be caused by the presence of blood (red or brownish) or pus (yellow). The consistency may be increased, resulting in thicker, "sticky" milk, or it may be more than usually watery. Flakes and clots are always abnormal. The smell of the secretion may also be altered as a result of mastitis.

2.3.3.2. California Mastitis Test (CMT)

This practical test was developed in the 1950's during a California testing program; it gives a measure of the SCC of the sampled milk. The reagent (3% sodium lauryl sulphate is often used) is a detergent which ruptures somatic cells in the milk, thereby releasing DNA. This forms a precipitate with other serum components, fat particles and the CMT reagent, causing visible gelling of the milk. A pH-indicator (for example bromcresol purple) may be added to the reagent. The test procedure is simple and straightforward: after the stripping milk is discarded, a few streams of (fore) milk from each quarter are milked into four plastic dishes set on a paddle. The paddle is then tipped nearly vertically to drain excess milk. An equal volume of the reagent is added from a plastic squeeze bottle and the two components mixed by swirling (Quinn *et al.*, 2002).

The early detection of disease is very important because in early stages it is amenable to treatment. Physical examination of udder helps in detecting cases where changes have occurred. The California mastitis test is most commonly used and has proved to be very efficient. Clinical mastitis is recognized by an abnormal milk, gland swelling and/ or illness. Sub clinical mastitis is characterized by normal milk and requires indirect tests such as Somatic Cell Count (SCC) that includes white blood cells (WBC) and occasionally sloughed epithelial cells. The California mastitis test (CMT) is the commonly used screening test for sub clinical mastitis. Culturing for microbial examination of both individual cow and bulk milk samples are used in the identification of pathogens.

2.3.3.3. Culture method

The surest way of diagnosing mastitis is by directly isolating and identifying any pathogenic microorganisms which may be present in the milk. This can be achieved by cultural methods and a number of additional determinative tests. To obtain correct results and avoid contamination and hence bias, it is important to work as securely and as accurately as possible under the circumstances. Similarly, the procedure of routine mastitis testing should be standardized and work protocols instituted (Sears, 1993 and Quinn *et al.,* 1994).

2.3.4. Treatment regimen for mastitis

2.3.4.1. Treatment of clinical mastitis in practice

Treatment of mastitis should be targeted towards the causative bacteria whenever possible, but in acute situations, treatment is initiated based on herd data and personal experience. Rapid or on-farm bacteriological diagnosis would facilitate the selection of the most appropriate antimicrobial. Treatment protocols and drug selection for each farm should be made by veterinarians familiar with the farm (Sawant *et al.,* 2005; Wagner and Erskine, 2006).

The use of on-farm written protocols for mastitis treatment can promote judicious use of antimicrobials (Raymond *et al.,* 2006; Passantino, 2007). Therapeutic response of the cows can be monitored using individual somatic cell count data if available, or using the California Mastitis Test, and with bacteriological samples in herds with contagious mastitis. In general, the use of narrow-spectrum antimicrobials is preferable. Prudent use guidelines have been developed which also include antimicrobial treatment of mastitis (Anonymouse, 2003; Passantino, 2007). First choice antimicrobials for treating mastitis caused by streptococci and penicillin-susceptible staphylococci are β-lactam antimicrobials, particularly penicillin G. Broad-spectrum antimicrobials such as third or fourth generation cephalosporin's should not be used as first alternatives for mastitis, as they may increase emergence of broad spectrum β-lactam resistance.

Systemic treatment is recommended in clinical mastitis due to *S. aureus* and in severe cases of coliform mastitis, preferably in combination with IMM treatment (Barkema *et al.,* 2006). Too short a duration of standard treatment is probably an important reason for poor cure rates in mastitis therapy. A longer treatment improves cure rates, and duration of treatment should generally be extended in mastitis caused by *S. aureus* and *Streptococcus uberis* (Oliver *et al.,* 2004; Deluyker *et al.,* 2005). Clinical mastitis should be treated for at least three days; this recommended treatment duration is longer than label treatments in many countries. All mastitis treatment should be evidence based i.e., the efficacy of each product and treatment length should be demonstrated by scientific studies (Cockcroft and Holmes, 2003).

2.3.4.2. Treatment of Subclinical mastitis

Treating subclinical mastitis with antimicrobials is generally not economical during lactation because of high treatment costs and poor efficacy. In a study with a large number of subclinical mastitis cases (Wilson *et al.,* 1999) the overall bacteriological cure rate for antimicrobial treatment was 75% and that for no treatment 68%. The marginal benefit applied for streptococcal mastitis only; in mastitis due to *Staphylococcus aureus,* antimicrobials were equal to no treatment. Treatment of subclinical mastitis will not affect the incidence of mastitis in the herd unless other preventive measures are taken. In herd problems caused by very contagious bacteria such as *S. aureus* or *Streptococcus agalactiae* treatment of subclinical mastitis is advised (Wagner and Erskine, 2006).

2.3.5. Pathogenesis of mastitis

Inflammation of the mammary gland predominantly occurs via the teat canal except in the case of tuberculosis, leptospirosis and brucellosis where the method of spread may be haematogenous. The development of mastitis can be explained in terms of three stages as invasion, infection and inflammation. The invasive stage refers to the time in which pathogens move from the teat end to the milk through the teat canal. The infection stage is the stage in which the pathogens multiply rapidly and invade the mammary tissue. The stage of inflammation is the stage with varying degrees of clinical abnormalities of the udder and with systemic effects from mild to per acute as well as gross and subclinical abnormalities of the milk **(**Radostits *et al.,* 2007).

2.3.6. Basic facts for control of mastitis

Philpot and Nickerson, (1999) indicated that milking practice is of paramount importance as this is common route of infection. The udder should be prepared before milking by washing the teats, followed by disinfection and drying with clean paper towels. If the teat area is dripping with water from run-off of areas that were heavily soiled it could lead to pathogens gaining access to the teat canal. Milker's hands should also be disinfected to prevent the transfer of pathogens. Post milking treatment is also important and all cows should be treated with a teat dip disinfectant to reduce the risk of infection. A strategy to control mastitis must be practical and economical.

The primary goal would be to reduce the rate of new infections and the duration of current. Infections within a herd. It would also be essentially important to maintain normal udder health ensuring that the natural immune response in the cow can resist and fight disease while still producing the required level of milk. Control strategies need to target every fact and process of dairy farming and can begin with maintaining good hygiene practices in the environment. The holding yards or stalls should be kept clean and dry. The water supply should be adequate and free of coliform bacteria and equipment should be maintained and sanitized between milking.

The welfare of animals is becoming increasingly important in modern dairy production as consumers become more concerned about the manner in which farm animals are treated. The Farm Animal Welfare Council in the UK has defined "the five freedoms" of animals, which highlight issues relating to the treatment and management of animals. The advantage of implementing such quality control measures within the herd would ensure that dairy cows are free of a stressful environment, injury, pain, hunger and discomfort, which in turn would promote a healthy immune system and udder health in general (Sandgren and Ekman, 2005). The control of mastitis has been successfully achieved through the establishment of effective herd health control programs (Erskine *et al.,* 2002). Antimicrobial agents are the main therapeutic tools for the treatment and control of mastitis.

2.4. Mastitis and its potential associated risk factors

2.4.1. Parity

(Demelash*et al.,* 2005, Gizat *et al.,* 2008, Rahman *et al*., 2009, Matios *et al*., 2009 and Molalegn *et al*., 2010) indicated that the higher parity numbers the more the prevalence of mastitis. According to Steeneveld *et al*., (2008) in multiparous cows, the risk of developing clinical mastitis (CM) increases with increasing parity. To pay for the costs of rearing it is very important that the first-parity cow is healthy, and able to produce good quality milk, which also will enhance longevity. Unfortunately, first-parity cows have been shown to have as high, or higher, incidence of udder disorders in early lactation as older cows (Valde *et al.,* 2004).This can be detrimental to her future life due to reduced milk production (Hagnestam *et al.,* 2007), increased risk of new cases of mastitis (Edinger *et al.,* 1999) and increased risk of culling (Schneider *et al.,* 2007). According to Skrzypek *et al.* (2004) the level of SCC has been reported to be influenced by parity and SCC increases with advanced parities.

2.4.2. Udder conformation and prevalence of mastitis

Mastitis occurs when microorganisms enter via the teat opening or duct and are able to overcome the immune system, multiply and establish within the teat canal and the mammary tissue. Invasion of the udder most likely occurs between milking periods. This is when microorganisms are present on the outer surface of the udder, on milking machines, or on the hands of workers. The opening of the teat canal has sphincter muscles that provide a physical barrier from the outside and is able to maintain a tight closure of the opening (Philpot and Nickerson, 1999). In addition, the teat canal is also lined with keratin which is a waxy substance derived from squamous epithelial cells. The keratin not only acts as a barrier between invading organisms and the gland cistern, but also contains bacteriostatic anti- microbial agents (Sordillo and Streicher, 2002).

This physical barrier can be compromised through trauma incurred, or microorganisms can simply be propelled through the teat canal during the use of milking machines (Philpot and Nickerson, 1999). These anatomical factors are the first line of defense against colonization and form part of the innate or non-specific immune response in the mammary gland (Oviedo-Boyso *et al.,* 2007). According to Girma (2010) and Sori *et al.* (2005) animals with pendulous udder showed higher incidence of mastitis than cows with non-pendulous udder and there was an association between the two categories this is because of more exposure to the environmental pathogens and injurious materials.

2.4.3. Stages of Lactation

The prevalence of mastitis was significantly higher at 6-10 months after calving than 1-5 months after calving (Rahman *et al.,* 2004). The highest prevalence of sub clinical mastitis occurred during the 4th months of lactation whiles the lowest during 5th or more than 5th months of lactation (Rahman *et al*., 1997). As Gizat *et al*. (2008) revealed stage of lactation was found to be significant with the occurrence of mastitis.

Risk of new environmental streptococcal infection is influenced by stage of lactation, parity, nutrition, and immunity in addition to factors that increase teat end exposure. The importance of the dry period in control of environmental streptococcal IMI cannot be over emphasized (Green *et al.*, 2002).

2.4.4. Age of cows

According to the Ethiopian researchers the study conducted in different part of Ethiopia by different authors, (Mungube *et al.*, 2004, Demelash *et al*., 2005 and Regassa *et al*., 2010b) indicated that age considered as potential risk factor to the prevalence of mastitis. As the age of cow advances the prevalence rate become higher (older cows were more affected by mastitis than younger cows), with prominent statistical variation ($p<0.05$).

2.4.5. Hygiene scoring

According to researches the environment in which dairy cows are kept has a decisive effect on their health and welfare. A clean and comfortable shelter represents the key to maintaining the dairy cows' health and longevity. The shelter's hygiene level can be evaluated through several assessment systems based on the quantification of the manure pollution in different body regions of the cows (Chaplin *et al.*, 2000). It is stated that the majority of these systems failed as practical hygiene monitoring tools at farm level, apart from their value in scientific research (Cook, 2002). For a scoring system to be useful both for veterinarians and farmers, the significance of manure contamination in different body areas must be understood and the pollution level compared to an established standard, derived either from the contamination level of the same farm in time, or from the data obtained in some similar farms (Cook, 2002).

For the hygiene scoring to be taken seriously, the farmer must understand what the costs of keeping animals in a dirty environment area. For dairy cows the outcomes of low hygiene are the high risk of mastitis and the worsening of lameness. The relation between shelter hygiene, clean cows and low number of somatic cells in mixed milk were indicated in several studies (Barkema *et al.*, 1999; Barkema and Schukken, 2003;). The hygiene scoring system was elaborated by Cook (2002) in order to quantify the hygiene level in the farm and for the assessment of the improvements which have to be made in hygiene management. This system is considered a remedial tool of the existing deficiencies.

Schreiner (2003) indicated that manure on teat ends is an environmental hazard (determinant) for the cause of mastitis when bacteria in manure enter the mammary gland. A cow's chances of mastitis increase with the number of herd teats covered with manure (all four teat ends vs. one teat end), the frequency of contamination (negligible versus every day) and duration of time (negligible versus most of the day). For a cow lying in slurry, the environmental hazard is obviously manure. At the herd level, choices in bedding type, slurry removal from alleys or housing type may be global hazards that affect the frequency of environmental mastitis. Risk is a measure of the likelihood of occurrence and the magnitude of the consequences of an adverse outcome such as mastitis. In veterinary medicine, there is scant information to quantify the risk of environmental mastitis.

Researchers report incidence, prevalence, associations or likelihood ratios but seldom quantify impact. For example, a researcher may report cows with dirty udders are 1.5 times more likely to have major pathogens isolated from their milk samples than cows with cleaner udders (Schreiner, 2003). Others report a positive association between dirty udders and hind limbs and individual cow somatic cell counts (Reneau *et al.*, 2005). According to Molalegn *et al.* (2010) and Matios *et al.* (2009) the cow's hygiene significantly affects the prevalence of mastitis.

3. Materials and Methods

3.1. Study period and area

The study was conducted from July 2017 to September 2018 on prevalence of bacterial mastitis and its associated risk factors in Assosa district, western Ethiopia. Assosa is found in Benishangul Gumuz Regional State of Assoa zone, located 662 km to the west of Addis Ababa, the capital city of Ethiopia. The area is bounded with Homosha district to the west, Bambasi district to the east and Wonebera district to the north. Its altitude ranges from 1650 to 2980 (m.a.s.l). It receives an annual rainfall ranging from 1000 - 1200 mm and an annual temperature range of 13-33°C. The area is categorized under kola agro ecological zone Aki (2015).

3.2. Study design

A cross-sectional study design was conducted to assess the prevalence of bacterial mastitis and its associated risk factors among the selected dairy farms of Assosa district, Western Ethiopia. In this study, physical examination of the udder, screening of agents using the California mastitis test (CMT) and bacteriological examination were undertaken. The study areas were purposively selected to determine mastitis occurrence and associated risk factors.

3.3. Sample size and Sampling method

The sample size was determined according to Thrusfield (2005) formula with expected prevalence of 39% Aki (2015).

$n=\frac{1.96^2 Pexp(1-Pexp)}{d^2}$.In this formula, n is the required sample size, 1.96 is the value of Z at the 95% confidence level, Pexp is the expected prevalence of mastitis, and d is the desired absolute precision which is 5%. Accordingly, for the purpose of this study a total of 364 lactating cows were included. These lactating cows were selected randomly from a total animal populations.

3.4. Study animals

The study animals were all lactating dairy cows found in Asossa and the dairy cows were selected by simple randomly sampling method. The study animals were lactating dairy cows of different conditions with different age groups, lactation stages and parity. A total of 364 milk samples were collected from 364 lactating cows from dairy cows of Assosa, district.

3.5. Data collection methods

3.5.1. Questionnaire

A semi-structured questionnaire was developed and all information related to the study objectives was recorded. The data collected includes the type of dairy breed, age, parity, and lactation stage. Udder and milk abnormalities (injuries, swelling, milk clots and abnormal secretion) were also recorded. Depending on clinical examination and CMT results, were categorized as either positive or negative. Positive cases were further categorized as clinical and sub-clinical mastitis. According to Henok, (2010), age of the animals were determined from birth records characteristics and categorized as young (>3 to 6 years), adults (>6 to ≤10 years), and old (>10 years). As Ashenafi, (2008), stage of lactation was categorized as early (1^{st} to 3^{th} month), mid (4^{th} to 6^{th}month), and late (7^{th} to 9^{th} month) and dry (above 9^{th} month).

According to Bedacha and Mengistu, (2011), parity was categorized as few (with ≤3 calves), moderate (4–7 calves) and many (>7 calves). Data relating to previous history of udder exposure to infection and causes of abnormalities to the mammary were obtained from clinical records of the farms and interviews with the owners of the animals.

3.5.2. Physical examination of the udder

The udders of the study cows were first examined visually and then through palpation to detect possible fibrosis, cardinal signs of inflammation, visible injury and swelling of the supra-mammary lymph nodes were identified.

3.5.3. Sample collection, Transportation and Storage

Milk samples collection was conducted according to the procedures recommended by national mastitis council (NMC, 1990). The udder, especially the teats were cleaned and dried before milk samples collection. Dust particles or other filth was removed by cleaning the surface of the teats and udder with a dry clean towel. The teats were washed with tap water and dried. Then the teats were wiped thoroughly with 70% ethyl alcohol. Milk samples were collected by standard milk sampling techniques from all lactating cows with clinical and sub clinical mastitis. To reduce contamination of the teat ends during sample collection, the near teats were sampled first followed by the far once. Approximately, 3ml of milk sample was collected from each cow and put into sterile cup of universal bottle. Samples were placed in ice box and transported to the Assosa Veterinary laboratory.

The Californian mastitis reagent was used to screen the milk samples with subclinical and clinical cases. After screening of the milk sample with CMT all the positive samples were clearly labeled with the cows identification number using permanent marker on the Petri dish plate and in the laboratory samples were cultured immediately or stored at $+4^0$C for a maximum of 24 hour until cultured on a standard bacteriological media ((Biru, 1989; NMC, 1999).

3.5.4. California Mastitis Test (CMT)

The Californian mastitis reagent was used to screen the milk samples with subclinical and clinical cases. Briefly, milk sample was dropped in to each of the strip cups on the CMT paddle and an equal amount of 3% CMT reagent was added to each cup and mixed gently. The mixing reaction was interpreted based on the thickness of gel formed by CMT reagent and milk mixture and scored as 0(negative), T (trace), 1(weak positive), 2(distinct positive) and 3(strong positive). Finally, samples with CMT score of 1 or above were judged as positive for sub clinical mastitis; otherwise negative (Quinn *et al.*, 1999) (Annex, II). After screening of the milk sample with CMT all the positive samples were clearly labeled with the cows identification number using permanent marker on the Petri dish plate and in the laboratory samples were cultured immediately or stored at $+4^0$C for a maximum of 24 hour until cultured on a standard bacteriological media ((Biru, 1989; NMC, 1999).

3.5.5. Media and Biochemical Tests used for Bacteriological examination

Bacteriological examination was done according to the NMC (1990) and National Committee for Clinical Laboratory Standards (NCCLS) (1997). All CMT and clinically positive samples were analyzed microbiologically as described by National Mastitis Council, (1999). Positive samples were cultured on MacConkey agar plate, mannitol salt agar plates and purple base gar (Annex iii). Bacterial growth was observed and recorded after 24 to 48 hours of incubation at 37°C. Cultures were considered to be positive when bacterial growth was observed on the culture plates and negative when no bacterial growth was observed on the culture plates. Bacterial isolates were identified on the basis of colony characteristics, gram stain, and biochemical tests (Quinn *et al.*, 1994). Biochemicals tests used for this study were catalase, coagulase and oxidase test.

Mannitol Salt Agar

A loop ful of milk sample was streaked on Mannitol Salt Agar and the inoculated plates were incubated aerobically at 37^0C for 24 to 36 hours, after which presence or absence of bacterial growth, colony morphology and color were recorded on primary culture. On the mannitol salt agar plate the presence of growth and change of pH in the medium (red to yellow color) were regarded as confirmative identification of *Staphylococci. Staphylococci* species produced yellow colony and yellow medium that indicates mannitol fermentation other bacterial species could not ferment mannitol.

MacConkey Agar

A loop ful of milk sample was streaked on MacConkey agar and the inoculated plates were incubated aerobically at 37^0C for 18 to 24 hours, after which presence or absence of bacterial growth, colony morphology and color were recorded on primary culture. Bacterial isolation was identified on the basis of colony characteristics of lactose fermentation. The bacteria that appear pink to red in color and surround by zone of bile of salt precipitation was lactose fermenting bacteria. Non lactose fermenting colonies such as *Shigella* species and *Salmonella* species,

appear colorless with no zone of bile salt precipitation. *Escherichia coli* grow, colonies pink to red with bile salt precipitate surrounding colonies gram stain, and biochemical tests.

Purple base agar

Purple base agar was used for bacteria that ferments maltose and the indicator changes to yellow color. Purple base agar (PBA) with the addition of 1 % maltose was used to differentiate the pathogenic *Staphylococci*, particularly the coagulase-positive bacteria. The suspected culture was inoculated on PBA medium plate with 1% of maltose and incubated at 37^{o}C for 14-36 hours. The identification was based on the *S. aureus* rapidly ferment maltose and the acid metabolic products cause the pH indicator (bromocresol purple) to change the medium and colonies to yellow. *S. intermedius* was gives a weak or delayed reaction in the medium producing an alkaline reaction (a deeper purple) around the colonies. *S. aurues* produced yellow colony and yellow medium whereas *S. intermedius* ferment maltose slightly and only yellow colony but no color change on the medium.

Gram stain

Gram stain was a method used for all suspected cultures of bacterial species that were subjected to gram's stain and observed under a light microscope for gram's reaction, size, and shape and cells arrangements. The gram stained smears from typical colonies that showed gram-positive cocci occurring in bunched, grape like irregular clusters were taken as *Staphylococcus* species. Taking of a thin film, allowing of the film to dry in air and fix the film by passing through the bunsen flame several times. Then flood the slide with crystal violent for 30 to 60 seconds, pour of the stain, wash the remaining stain with iodine solution. And also wash off the iodine and shake the excess water from the slide and decolorize with ethanol. Finally counter stain with safranin for 30 to 60 seconds and wash with water.

Catalase Test

A loop ful of the bacterial growth was taken from the top of the colonies from the nutrient agar plate and the bacterial cells are placed on a clean microscope slide and a drop of 3% H_2O_2 was added and the colonies were positive, bubbles of oxygen was liberated within a few seconds and the colonies were negative if it did not produce bubbles. This positive cocci were Staphylococci.

Coagulase Test

It is used to differentiate *S. aureus* (positive) from *Coagulase negative Staphylococcu*s *(CNS).* The coagulase test was used both slide coagulase and tube coagulase tests using 0.5 ml of rabbit plasma. The identified *S. aureus* from Mannitol Salt Agar was sub cultured to nutrient agar plate and after 24 hours culture colonies of *S. aureus* was picked and placed on clean slide with a small drop of distilled water and emulsified it. The test suspension was treated with a drop of rabbit plasma and mixed well with a needle for 5-10 seconds. Those forming Clumping of cocci were taken as positive. The tube coagulase test was performed in sterile tubes by adding 0.5 ml of selected isolates of *Staphylococcus* grown on mannitol salt agar at 37^0C for 24 hours and which to 0.5 ml of fresh rabbit plasma was added. After mixing by gentle rotation, the tubes were incubated at 37^0C along with a negative control tube containing a mixture of 0.5 ml of sterile test tube and 0.5 ml of rabbit plasma. Clotting was evaluated at 30 minutes intervals for the first 4 hours of the test and then after 24 hours incubation the result was interpreted depending on the formation of clot for positive reaction. *Staph. aureus* and *Staph. intermidius* showed positive result otherwise considered as *CNS*.

Oxidase test

The oxidase test is used to identify bacteria that produce cytochrome oxidase, an enzyme of the bacterial electron transport chain. When present, the cytochrome oxidase oxidizes the reagent (tetramethyl-p-phenylenediamine) to (indophenols) purple color end product. When the enzyme is not present, the reagent remains reduced and is colorless. Taking of a piece of filter paper and moistened in a petri dish with one percent aqueous solution of tetramethyl –p-phenylenediamiane. The test colonies were streaked firmly across the filter paper with a glass

rod. Then disappearance of dark purple color along the streak on the filter paper was considered as Staphylococcus.

Collection of milk sample (CMT and clinical +ve (3ml))

Culture CMT +ve milk samples

Primary identification

Gram's staining (bunch of grapes with cocci)

Catalase test=3% H_2O_2 (positive)

Oxidase (negative)

3%KOH (no gel formation for gram positive Staphylococci)

Slide coagulase test (clumping factor)

Secondary identification for confirmation

Tube Coagulase test (clot +ve)

Growth on mannitol salt agar (golden yellow colony)

Purple agar base (yellow color)

Take colonies on to MSA and incubate at 37^0C for 24-48 hours, growth and change in the pH of the medium is confirmative for Staphylococcus classified as highly fermentative (*S. aureus*), weakly fermentative (*S. intermidius*) and non-fermentative (*CNS*)

Coagulase test to identify the most pathogenic CPS (*S. aureus, and S. intermidius*) from *CNS*

Coagulase positive *staphylococcus* bacteria was put on PBA media plate with 1% of maltose and incubate at 37^0C for 24-48 hours to differentiate the pathogenic *staphylococci,* particularly for the identification coagulase-positive bacteria. The identification was based on the fact that *S. aureus* rapidly ferments maltose to change the medium and colonies to yellow. *S. intermedius* gives a weak or delayed reaction.

Figure 1-10. Shows this flow chart for isolation and identification of bacteria from milk sample

3.6. Data analysis methods

The data collected were coded and entered into Microsoft excel computer software. Statistical analysis was carried out using SPSS version 20. Descriptive statistics was used to determine the prevalence of mastitis depending on clinical inspection and CMT. Chi-square(x^2) test was employed to see the impact of different risk factors on the occurrence of mastitis. Statistically significant association between variables is considered to exist if the p value is < 0.05.

3.7. Ethical considerations

The animals were treated with kindness and took proper care by minimizing discomfort, distress or pain. We assumed that all the procedures which cause pain in human beings may cause pain in study animals. The ethical clearance letter was obtained from Jimma University, College of Natural Sciences Research and Ethical Review Board and samples from animals were collected by professionals in area of Veterinary Medicine.

4. Result

A total of 364 lactating milking cows examined during the time of the study at Asossa dairy farm. Of these total 364 examined lactating cows, 153 (42.03%) cows were found to be positive for mastitis (Table 1). Out of this, 52 (14.3%) was clinical mastitis and 101 (27.73%) was subclinical mastitis cases. The prevalence of mastitis in the district was 42.03% (Table 1).

Table 1 The prevalence of mastitis on cow level (n=364) in Assosa (2018).

Types of Mastitis	Total number of positive	Prevalence rate %	$\chi 2$ 320.25	P-value p = 0.000**
Clinical	52	14.3		
Sub clinical	101	27.73		
Total	153	42.03		

The isolates displayed diverse morphological and biochemical results based on laboratory study (Table 1). The most frequent isolate from the milk sample was *Staphylococcus aureus* (62.1%) followed by *coagulase negative Staphylococcus* (17.6%), *S. intermidius* (14.4%) *and Escherichia coli* (5.9%).

Table 2 Morphological and Biochemical characteristics of bacteria isolated from milk samples of cows.

Suggested genera of isolated identity	Number of total isolates	Gram reaction	Catalase test	Coagulase test	Oxidase test	Motility	Colony characteristics	Isolate code
Staphylococcus Spp *S. aureus* *S. intermidius* *CNS*(*Coagulase* *Negative* *Staphylococcus)*	144	+ ve	+ ve	+ ve only *CNS* -ve	-ve	Non motile	Cocci	01 02 03
Enterobacteriace Spp *Escherichia coli*	9	-ve	-ve	ND	-ve	Motile	Rod	04

ND (Not Determined)

The prevalence of mastitis was measured among different age groups of lactating cows where young (1.62%), adult (98.1%), and old (92%) were positive. The prevalence associated with age group was found statistically significant (P= 0.000). The prevalence was found higher in the adult age group than the young age group (Table 3).

Table 3 Prevalence of mastitis based on different age groups.

	Status of mastitis					
					$\chi 2$	P-value
Age	Total No of Examined	Clinical (%)	Sub-clinical (%)	Over all (%)	324.74	p = 0.000**
Young	209	1(0.48)	3(1.44)	4(1.62)		
Adult	105	29(27.62)	74(70.48)	103(98.1)		
Old	50	8(16)	38(76)	46(92)		
Total	364	38(10.4)	115(31.6)	153(42.03)		

Age in years 3-6 (young) 6-10(mid-age) and (>10) (old age). ** highly significance difference

The prevalence of mastitis associated with parity in dairy farms were 14(6.5%), 110(91.6%) and 29(96.7%). In 1-3, 4-6 and greater than or equal to 7 parity respectively. The prevalence of mastitis measure based on the parity of cows was higher in many parity than few parity number of cows. The study shows that there was statistical significant (P<0.05) between prevalence of mastitis associated with parity (Table 4).

Table 4 Prevalence of mastitis associated with parity in the dairy farms.

	Status of mastitis					
					$\chi 2$	P-value
parity	Total No of Examined	Clinical (%)	Sub-clinical (%)	Over all (%)	351.86	p = 0.000**
Few	214	5(2.5)	9(4.2)	14(6.5)		
Moderate	120	40(33.3)	70(58.3)	110(91.6)		
Many	30	11(36.7)	18(60)	29(96.7)		
Total	364	56(15.4)	97(26.6)	153(42.03)		

Number of parity few= (<3), moderate = (4-6) and many (>7)

The prevalence of mastitis on early, mid, late and dry stage of lactation was (30.4%), (53%), (50%) and (30.4%), respectively. In the study area the result showed that the disease is insignificantly associated with stage of lactation (P>0.05) (Table 5).

Table 5 Prevalence of mastitis based on stage of lactation.

	Status of mastitis				$\chi 2$	P-value
Stage of lactation	Total No of examined	Clinical (%)	Sub-clinical (%)	Over all (%)	6.37	0.095
Early	147	15(10.2)	30(20.4)	45(30.6)		
Mid	130	22(16.9)	47(36.1)	69(53)		
Late	64	13(20.3)	19(29.7)	32(50)		
Dry	23	2(8.7)	5(21.7)	7(30.4)		
Total	364	52(14.3)	101(27.73)	153(42.03)		

Stages of lactation in months 1-3(early), 4-6 (mid), 7-9 (late) and above 9(dry)

The prevalence of mastitis on poor and good milk hygiene was (42.77%) and (45.66%) respectively. The result of the study showed in the area that the disease is insignificantly associated with milk hygiene (P>0.05) (Table 6). This means according to this study in the milk hygiene is not a risk factor for the occurrence of mastitis.

Table 6 prevalence of mastitis associated with milk hygiene in the dairy farms.

	Status of mastitis					
					$\chi 2$	P-value
Milk hygiene	Total No of Examined	Clinical (%)	Sub-clinical (%)	Over all (%)	0.147	0.70
Poor	241	38(15.77)	65(27)	103(42.77)		
Good	123	22(17.89)	28(22.77)	50(91.6)		
Total	364	60(16.5)	93(25.55)	153(42.03)		

The prevalence of mastitis associated with floor type in dairy farms muddy (50%), natural (32.07%), and Concrete (27.1%). The prevalence of mastitis measure vary with floor type based on the floor type of dairy cows was higher in muddy type floor than natural and concrete type of dairy cows. The study shows that there was statistically significant difference ($P< 0.05$) between mastitis associated with floor type (Table 7).

Table 7 Prevalence of mastitis based on different type of floor in Assosa (2017).

	Status of mastitis					
					$\chi 2$	P-value
Type of floor	Total No of examined	Clinical (%)	Sub-clinical (%)	Over all (%)	17.93	p = 0.000**
Muddy	200	35(17.5)	65(32.5)	100(50)		
Natural	153	16(10.5)	34(22.2)	50(32.7)		
Concrete	11	1(9.1)	2(18.1)	3(27.2)		
Total	364	52(14.3)	101(27.73)	153(42.03)		

The prevalence of mastitis associated with previous exposure to mammary gland infection in dairy farms were (97.8%) and non-exposed (8%). The prevalence of mastitis measure based on the previous exposure to mammary gland infection of dairy cows was higher than non-exposure to mammary infection of dairy cows. The study shows that there was statistical significant ($P<0.05$) between mastitis associated with previous exposure to mastitis (Table 8).

Table 8 prevalence of mastitis associated with previous exposure to mastitis.

	Status of mastitis					
					$\chi 2$	P-value
PETM	Total No of Examined	Clinical (%)	Sub-clinical (%)	Over all (%)	283.96	0.000**
Yes	138	53(38.4)	82(59.4)	135(97.8)		
No	226	7(3.1)	11(4.9)	18(8)		
Total	364	60(16.5)	93(25.55)	153(42.03)		

PETM ((previous exposure to mastitis) and ** (highly significance difference)

The bacterial species isolated from CMT positive samples that grow on culture medium were *Staphylococcus aureus*, *Staphylococcus intermidius, CNS* (*Coagulase Negative Staphylococcus)* and *Escherichia coli* (Table 9). From a total 364 milk samples (52 clinical cases and 101 subclinical cases) were culturally positive and 211 (57.97%) yield no bacterial growth. The highest prevalent bacteria was found to be *Staphylococcus aureus* (62. 1%) followed by *CNS* (17.6%), *Staphylococcus intermidius* (14.4%) *and Escherichia. coli* (5.9%).

Table 9 Isolated bacterial species from CMT positive.

Types of bacteria isolated	Clinical Mastitis%	Sub clinical mastitis%	Total	proportion %
Staphylococcus aureus	35(22.9)	60(39.2)	95	(62.1)
Staphylococcus intermidius	8(5.2)	14(9.2)	22	(14.4)
CNS(*Coagulase Negative Staphylococcus)*	5(3.3)	22(14.3)	27	(17.6)
Escherichia coli	4(2.6)	5(3.3)	9	(5.9)
Total	52(34)	101(66)	153	(100)

5. DISCUSSION

In this study area the overall prevalence of mastitis was found to be 42.03%. Out of this, 52 (14.3%) was clinical mastitis and 101 (27.73%) was subclinical mastitis cases. In Ethiopia the efforts have been concentrated on the treatment of clinical cases, but the subclinical forms of mastitis received little attention Kerro and Tareke (2003), but the reality that found on the ground of this study was the subclinical mastitis prevalence is more prevalent than the clinical one, if so the strategy of mastitis prevention should be reestablished using the findings of the different studied did in the country.

The result of this study showed that the overall prevalence of mastitis in lactating dairy cows in Assosa was 42.03%, which agreed with the findings of Quinn *et al.* (2002), in Chaffa valley in Northern Ethiopia (39.65%) and Quinn *et al.* (2003), in Southern Ethiopia (40.40%) on mastitis. The variability in the prevalence of mastitis between findings could be the complexity of the disease which involves interaction of several factors, mainly the difference in management of the farms, breeds, environment and factors related to causative agent, variation in veterinary service coverage and awareness of the owner toward the disease, and technical know-how of the researchers Radostitis *et al.* (2007).

Significantly higher prevalence of mastitis (92%) in older than young adult cows (1.62%) was in agreement with Fufa *et al.* (2013), who reported increased the prevalence of mastitis with advancing age. Parity and age had also showed an effect on the occurrence of mastitis. The prevalence was higher in animals with higher number of births followed by cows with parity of 4 to 6 and those with parity of 1 to 3. The study finding is agree with finding of Biffa *et al.* (2005), who reported that increased prevalence of mastitis with age and parity. This is in agreement with our current finding of significantly higher prevalence of mastitis in cows with many calves (96.7%) than with few calves (6.5%). Moreover, cows with advanced parity become more productive, so it can be assumed that as the parity of cow advances and the age increases cows become prone to mastitis.

This observation is in agreement with the reports of Biffa *et al.* (2005), Mungube *et al.* (2004) and Kerro and Tareke (2003) from the country. The association between floor type and high prevalence of mastitis recorded in our study is consistent with the findings of Abera *et al.* (2010). In the study stages of lactation indicated that statistically insignificant. This result is not coincided with Biffa *et al.* (2005), who reported lactation stage has significant effect on the prevalence of mastitis in Ethiopia. The result showed that the disease is insignificantly associated with milk hygiene (P>0.05). The prevalence of mastitis measure based on the floor type of dairy cows was higher in muddy type of floor than natural and concrete type of dairy cows. Previously infected cows were at greater risk of being re-infected, suggesting that repeated challenges of the mammary tissues with micro-organisms coupled with other stress factors could put the glands at greater risks of re-infection. More cows which had experienced mastitis problem before, were found to be positive to clinical or/and sub clinical form of mastitis at current investigation than non-exposed ones. This is comparable with the findings of Mekonnen and Tesfaye, (2010) who indicated cows with previous exposure to udder infection were more likely to be re-infected than those never exposed. This might be attributed to possibility of previously exposed cows remained at carrier state and impotency of drugs used for mastitis treatment in the study area.

A total 153 CMT positive samples was grow on the culture. Out of this 144(94.1%), the gram positive cocci bacteria were 9(5.9%), gram negative rods. With regard to the bacteriological analysis of milk samples, the study revealed that from the 153 CMT positive milk samples the most prevalent bacteria isolates were *Staphyloccus aureus* (62.1%), followed by *Coagulase Negative Staphylococcus (CNS)* (17.6%), *Staphylococcus intermidius* (14.4%) *and Escherichia coli* (5.9%). In this finding, *S. aureus* was the predominant pathogen that constituted 62.1% of all isolates. The high level isolation of *S. aureus* (62.1%) in this study almost coincided with the finding of Lakew *et al.* (2009), who reported in Asella, southern Ethiopia 39.4%. *S. aureus* is adapted to survive in the udder and usually establishes mild sub clinical infection of long duration from which it is shaded through milk serving as sources of infection for other healthy cows and transmitted during the milking process Radostits and Blood, (1994). Thus, the organism has been assuming as a major importance as a cause of mastitis.

The present findings, *Staphylococcus intermedius* (14.4%) is higher than Brihanu *et al.*, (2013) who reported (7.14%), but lower than the 38.4% report of Argaw and Tolosa, (2008). Coagulase negative *staphylococcus*, which contribute about 17.6% of the isolates and agreed with the report of Sori *et al.* (2005), in and around Sebeta, Ethiopia with prevalence 14.93%. In this study, *E. coli* (5.9%) was the lowest of all isolated bacteria from mastitis positive samples, but *E. coli* was matched with reports of Mengistu, (1986), who found 3.14% in Bahir-dar, Ethiopia. The study indicated that mastitis is a prevalent disease in dairy cows in Asossa district and the findings suggested that mastitis was a limiting factor for decreasing of milk production. In this study the risk factors age, parity, floor type and previous exposure to mastitis showed statistically significant association with the occurrence of mastitis and they are major exposing factors that leads to mastitis.

6. CONCLUSION AND RECOMMEDATIONS

6.1. Conclusion

The study indicated that mastitis is a prevalent disease in dairy cows in Asossa district and the findings suggested that mastitis was a limiting factor for decreasing of milk production. The sub clinical mastitis was a devastate problem in milking cows of Assosa and it is occurred in local breed of lactating dairy cattle. Major exposing factors that leads to mastitis were previously exposure to mastitis, age, parity, and floor type have the association with mastitis. The *Staphylococcus aureus* was the predominant one that causes mastitis in the study area. This implies that mastitis has an over looked impact on dairy development and food security in the area.

6.2. Recommendations

- Awareness should be created to ensure early diagnosis and regular screening of cows for subclinical mastitis together with the treatment of clinical cases.
- Mastitis control and prevention strategies should be designed and implemented with consideration of the age, parity of cows and previously exposure to mastitis so as to reduce its influence on milk production and food security.
- Adequate housing with proper sanitation and regular screening for early detection and treatment,
- Susceptibility testing of the isolated microorganisms is recommended before treatment.
- Culling of old aged and repeatedly infected cows should be done on regular planned basis.

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Annex I Checklist of semi structured questionnaire were given for the owners of the Dairy cows to estimate the prevalence of bacterial mastitis in Assosa district.

Generally, information of the respondent

1. Kebeles _____ date_____
2. Name _____ age _____ sex_____
3. marital status _____
4. History of the dairy cows

   I) breed: locally ☐ cross ☐

   II) Age group:

   1-4 year ☐ 5-8 year ☐ above 8 year ☐

   Young Adults Old

   III) Stage of lactation

   Early stage ≤ 3 month: ☐

   Middle stage 4-6 month: ☐

   Late stage 7-9 month: ☐

   Dry stage above 9 month: ☐

   IV) Parity:

   Few: (<3calves) ☐

   Moderate: (4-6calves) ☐

   Many: (>7 calves) ☐
5. Previous history of (mastitis I) infected: yes: ☐ No: ☐

   II) Pregnancy: yes: ☐ No: ☐

   III) Mastitis status clinical: ☐ non clinical: ☐

   IV) Previous the cows are treated: yes: ☐ No: ☐

   6. Way of milking hygiene: good: ☐ poor: ☐

7. Teat infestation Present: ☐absent:☐

I) teat lesion: present:☐ absent: ☐

II) The nature of teat: blind: ☐not blind:☐

8. Gross milk quality watery:☐blood containing:☐

Normal:☐clots: ☐ flakes:☐

9. The sample collection from: Hinder right: ☐ Hinder left:☐

Front right: ☐ Front left:☐

10. Milking practice

I) Do you wash your hand before milking? Yes: ☐No:☐

II) Do you wash your hand in between milking? Yes: ☐No:☐

III) Do you wash the udder? Yes:☐No:☐

11. The housing of the dairy cows

I) Does it: Concrete: ☐muddy:☐ natural floor:☐

II) Manure removal: Daily:☐weekly ☐monthly ☐

12, mastitis situation

I) is there previous prevalence of mastitis problem yes ☐ No ☐

II) Can you differentiate or distinguish: Yes ☐ No☐

III) Do you treat the mastitis case when the disease occurred? Yes☐ No ☐

IV) Present treatment of mastitis will be mode by professional ☐ my self ☐

13. CMT (California mastitis test): Reactive ☐ Non-Reactive ☐

Annex II Interpretation for the California Mastitis Test

CMT score	Interpretation	Visible reaction	Total cell count
0	Negative	Milk fluid is normal	0-200,000 (0-25% neutrophils)
T	Trace	Slight precipitation	(1.5-5)x105(30-40neutrophils**)**
1	Weak positive	Distinct precipitation but not gel formation	(4-15)x105(40-60% neutrophils)
2	Distinct positive	Mixture thickens with gel formation	(8-50)x10(60-70% neutrophils)
3	Strong positive	Strong gel that is cohesive with a convex surface	>5,000,000(70-80% neutrophils)

Source: Quinn *et al.* (1999)

ANNEX III Media and Biochemicals used for Bacteriological examination

Nutrient Agar

A loop ful of milk sample was streaked on sterile nutrient agar, composed of (g/l) powder1.0; Yeast extract 2.0; Peptone 5.0; Sodium chloride 5.0; Agar15. The inoculated plates were incubated aerobically at 37^0C for 24 to 48 hours, after which presence or absence of bacterial growth, colony morphology and color were recorded on primary culture. Cultures were considered to be positive when bacterial growth was observed on the culture plates and negative when no bacterial growth was observed on the culture plates. Bacterial isolates were identified on the basis of colony characteristics, gram stain, and biochemical tests.

Mannitol Salt Agar

A loop ful of milk sample was streaked on Mannitol salt Agar, composed of (g/l) powder 1.0; Peptone 10.0; Mannitol 10.0; Sodium chloride 7.5; Phenol red 0.025; Agar 15.0. The inoculated plates were incubated aerobically at 37^0C for 24 to 48 hours, after which presence or absence of bacterial growth, colony morphology and color were recorded on primary culture. On the mannitol salt agar plate the presence of growth and change of pH in the media (red to yellow color) were regarded as confirmative identification of *Staphylococci*. Phenol red pH indicators are detected to the acidic metabolic product of the mannitol fermentation. The fermented mannitol by *S. aureus* were causes yellow discoloration of the medium and colonies that develop weak or delayed yellow color after 24 hours of incubation were *S. intermedius* and CNS (Quinn *et al*., 2002; Mahon and Manusekis, 1995). Staphylococci species produced yellow colony and yellow medium that indicates mannitol fermentation other bacterial species could not ferment mannitol.

MacConkey Agar

A loop ful of milk sample was streaked on MacConkey agar, composed of (g/l) powder 1.0; Peptone 17.0; Lactose 10.0; Sodium chloride 5; Neutral red 0.03; Agar 13.5. The inoculated plates were incubated aerobically at 37^0C for 24 to 48 hours, after which presence or absence of bacterial growth, colony morphology and color were recorded on primary culture. Bacterial isolation was identified on the basis of colony characteristics of lactose fermentation. The bacteria that appear pink to red in color and surround by zone of bile of salt precipitation was lactose fermenting bacteria. Non lactose fermenting colonies such as *shigella* species and *salmonella* species, appear colorless with no zone of bile salt precipitation. *Escherichia coli* grow, colonies pink to red with bile salt precipitate surrounding colonies gram stain, and biochemical tests.

Purple base agar

Purple base agar contains peptone special 10.00, beef extract 1.00, sodium chloride 5.00, bromocresol purple 0.02, agar 15.00. The bacteria that ferments maltose and the indicator changes to yellow color. Purplebase agar (PBA) with the addition of 1 % maltose was used to differentiate the pathogenic *Staphylococci*, particularly the coagulase-positive bacteria. The suspected culture was inoculated on PBA media plate with 1% of maltose and incubated at 37^oC for 24-48 hours. The identification was based on the *S. aureus* rapidly ferment maltose and the acid metabolic products cause the pH indicator (bromocresol purple) to change the medium and colonies to yellow. *S. intermedius* was gives a weak or delayed reaction in the medium producing an alkaline reaction (a deeper purple) around the colonies (Quinn *et al*., 2002). *S. aurues* produced yellow colony and yellow medium whereas *S. intermedius* ferment maltose slightly and only yellow colony but no color change on the medium.

Catalase Test

A loop ful of the bacterial growth was taken from the top of the colonies from the nutrient agar plate and the bacterial cells are placed on a clean microscope slide and a drop of 3% $H_2O_2$was added and the colonies were positive, bubbles of oxygen was liberated within a few seconds and the colonies were negative it did not produce bubbles. This positive cocci were Staphylococci.

Coagulase Test

The free coagulase secreted by *S. aureus* reacts with coagulase reacting factor (CRF) in plasma to form a complex, which is thrombin. This converts fibrinogen to fibrin resulting in clotting of plasma. *S. aureus* produces two forms of coagulase: bound and free. It is used to differentiate *S. aureus* (positive) from *Coagulase Negative Staphylococcu*s (CNS).The coagulase test were used for both slide coagulase and tube coagulase tests using 0.5 ml of rabbit plasma. Slide coagulase test is done to detect bound coagulase or clumping factor and tube coagulase test is done to detect free coagulase. The identified *S. aureus* from mannitol salt Agar were sub cultured to nutrient agar plate and after 24 hours culture colonies of *S. aureus* was picked and placed on clean slide with a small drop of distilled water and emulsified it. The test suspension was treated with a drop of rabbit plasma and mixed well with a needle for 5-10 seconds. Those forming Clumping of cocci were taken as positive.

The tube coagulase test was performed in sterile tubes by adding 0.5 ml of selected isolates of *Staphylococcus* grown on mannitol salt agar at 37^0C for 24 hours to 0.5 ml of fresh rabbit plasma. After mixing by gentle rotation, the tubes were incubated at 37^0C along with a negative control tube containing a mixture of 0.5 ml of sterile and 0.5 ml of rabbit plasma (Hebert *et al*., 1988). Clotting was evaluated at 30 minutes intervals for the first 4 hours of the test and then after 24 hours incubation. The reaction was considered positive, if any degree of clotting from a loose clot to a solid clot that is immovable when the tube is inverted (tilted) was visible within the tube and no degree of clotting would be taken as negative.

Oxidase

The oxidase test is used to identify bacteria that produce cytochrome oxidase, an enzyme of the bacterial electron transport chain. When present, the cytochrome oxidase oxidizes the reagent (tetramethyl-p-phenylenediamine) to (indophenols) purple color end product. When the enzyme is not present, the reagent remains reduced and is colorless. Takinga piece of filter paper andmoistened in a petridish with one percent aqueous solution of tetramethyl –p-phenylenediamiane. The test colony were streaked firmly across the filter paper with a glass rod. Then disappearance of dark purple color along the streak on the filter paper was considered as Staphylococcus.

Gram stain

Gram stain was composed of crystal violet, gram's iodine (mordant), ethanol 95% and counter stain (carbon fuchsine / safranin) and it is a method used for all suspected cultures of bacterial species were subjected to gram's stain and observed under a light microscope for gram's reaction, size, and shape and cells arrangements. The grams stained smears from typical colonies that showed gram-positive cocci occurring in bunched, grape like irregular clusters were taken as *Staphylococcus* species (Quinn *et al*., 2002).

Taking of a thin smear or film and allowing of the film to dry in air and fix the film by passing through the bunsen flame several times. Then flood the slide with crystal violent for 30 to 60 seconds and pour of the stain and wash the remaining stain with iodine solution. And also wash off the iodine and shake the excess water from the slide and decolorize with ethanol. Finally counter stain with safranin for 30 to 60 seconds and wash with water.

ANNEX IV Figures for isolation and identification of bacteria from milk sample

Figure 1 Nutrient Agar media and microbesdispense on it.

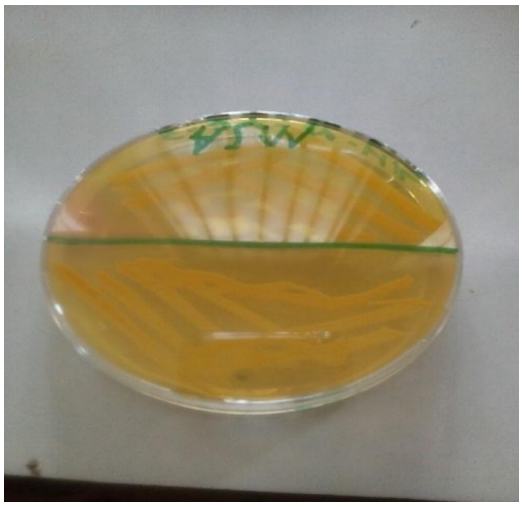

Figure 2 Mannitol Salt agar and microbesdispense on it.

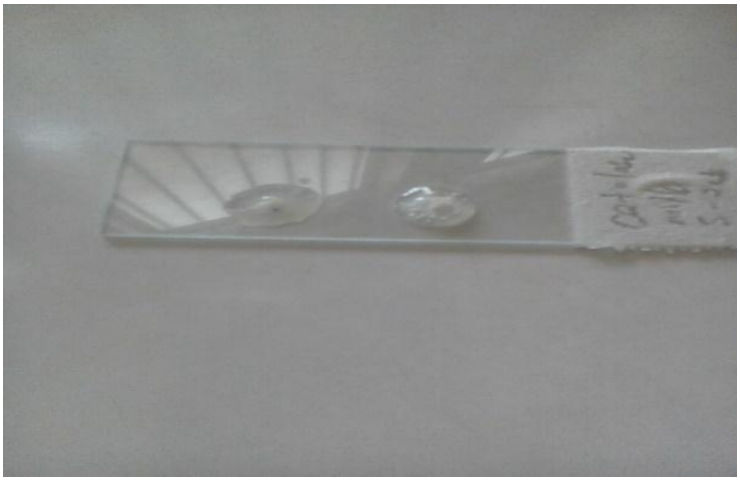

Figure 3 Catalase test for isolation of *Staphylococcus Species.*

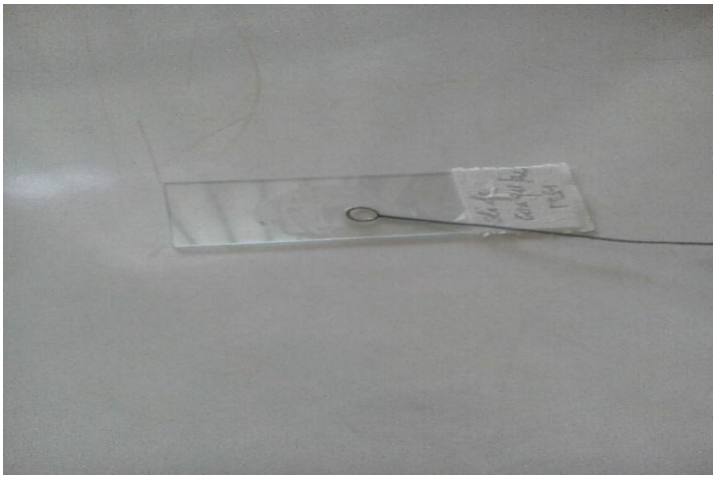

Figure 4 Slide coagulase test for isolation of*Staphylococcus Species.*

Figure 5 Tube coagulase test for isolation of *Staphylococcus Species.*

Figure 6 Oxidase Test for isolation of *Staphylococcus Specie.*

Figure 7 Purple agar base for differentiation of maltose fermenter and non-fermenter.

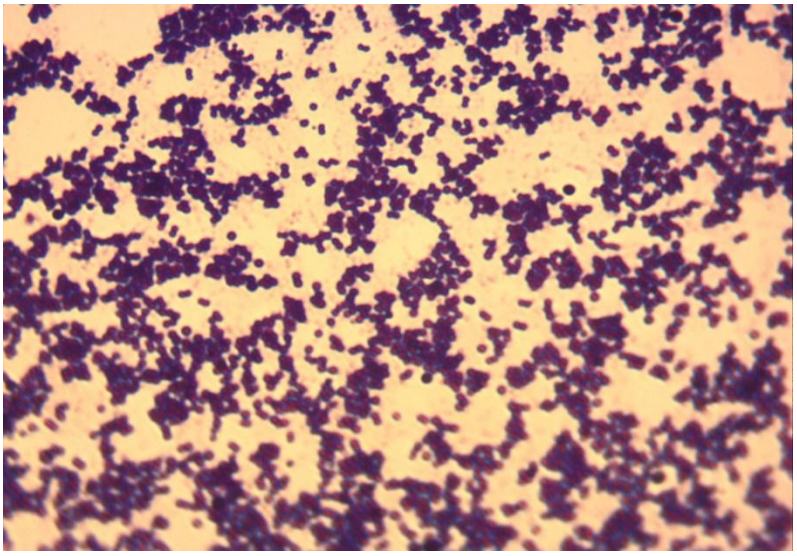

Figure 8 Staph 08

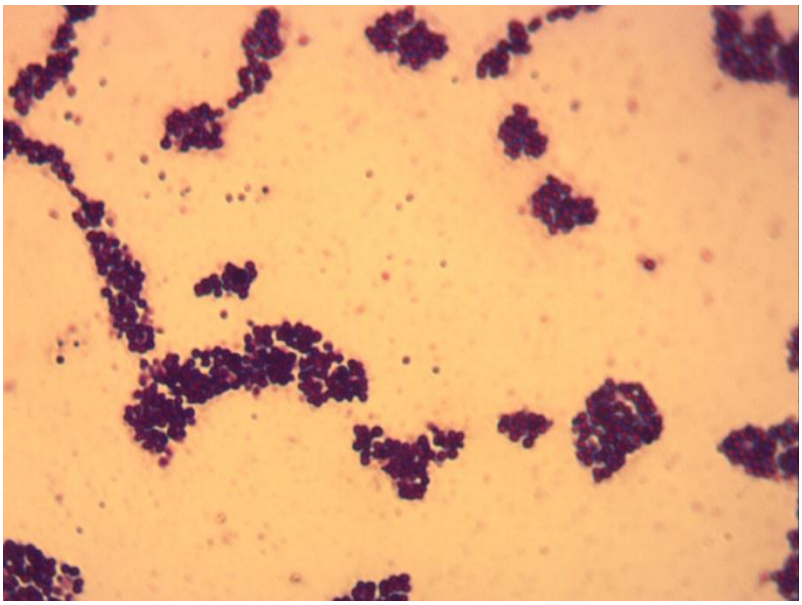

Figure 9 Staph 11

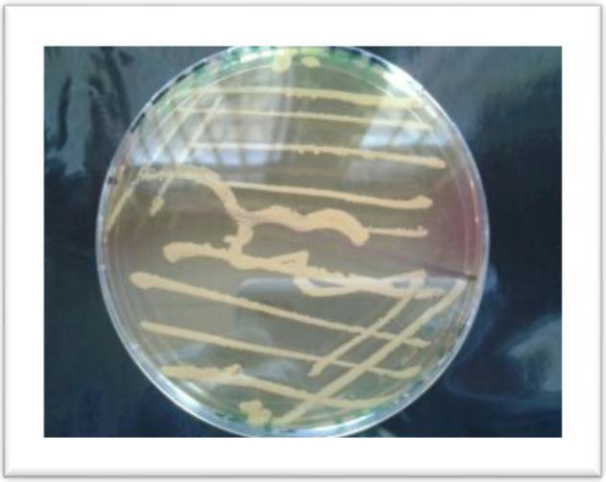

Figure 10 MacConkey agar and microbesdispense on it.

DECLARATION

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

Name: Asmare Adamu Boru

Signature: _____

Date of Submission: 10/12/2018

This has been submitted for examination with our approval as University advisor

Delelegn Woyessa (Assoc. Prof) _____