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Biomedicine and Nursing

MARSLAND PRESS
Multidisciplinary Academic Journal Publisher

Study on the prevalence and associated risk factors of bovine mastitis in and around dessie town, south wollo, northeastern Ethiopia

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Abstract: Mastitis is a disease of major economic importance in dairy industry worldwide, particularly in developing countries. Even though mastitis is reported from different parts of Ethiopia, information concerning this disease in and around Dessie town is limited. Hence, this cross- sectional study was conducted from November 2016 to April 2017 in and around Dessie town, Ethiopia, to estimate the prevalence and risk factors, and isolate the major bacterial pathogens of bovine mastitis at cow level. Out of the total 180 lactating dairy cows clinically examined and tested by CMT, the overall prevalence of mastitis was 27.2%; where 3.3 % and 23.9 % cows were found with clinical and sub clinical mastitis, respectively. Generally, except study area (p- value > 0.05) almost all other risk factors such as farm, breed, age, parity, lactation stage and hygienic condition considered in this study were significantly associated (p- value < 0.05) with the overall prevalence of mastitis and bacterial isolates. Of 49 milk samples collected from 6 clinical and 43 sub clinical mastitic cows, and cultured for bacteriological examination, 85.7% (n=42) yielded bacteria. The isolation rate of Escherichia coli, Staphylococcus aureus and coagulase negative staphylococcus from mastitis milk samples in this study were 77.6 %, 61.2% and 16.3 %, respectively. All risk factors considered in this study such as farm, areas, breed, age, parity, lactation stage and hygienic condition were not significantly associated (p- value > 0.05) with the isolation rate of Escherichia coli, Staphylococcus aureus and coagulase negative staphylococcus from mastitic milk samples. All bacterial isolates were tested for their antimicrobial susceptibility patterns and showed varied responses. The majority of Escherichia coli isolates were resistance against Cloxacillin and Erythromycin but susceptible to Streptomycin, Ampicillin and Sulfamethoxazole-Trimethoprim. All Staphylococcus auerus isolates were susceptible for kanamycin but showed intermediate resistance for Enrofloxacin, Ciprofloxacin, Chloramphinicol and Streptomycin. All coagulase negative staphylococcus isolates were resistant for Bacitracin, Penicillin G and tetracycline but susceptible for Kanamycin and Gentamycine. In general, the presence of mastitis and emergence of multidrug resistant bacteria in the study area could indicate their potential risks on dairy production and public health. Based on the results obtained, recommendations are forwarded for further studies, and prevention and control of mastitis in the study area.
[Mulunesh Yenew, Habtamu Addis. **Study on the prevalence and associated risk factors of bovine mastitis in and around dessie town, south wollo, northeastern Ethiopia.** *Biomedicine and Nursing* 2020;6(3): 59-76]. ISSN 2379-8211 (print); ISSN 2379-8203 (online). http://www.nbmedicine.org. 7. doi:10.7537/marsbnj060320.07.

Key words: Coagulase Negative Staphylococcus, Escherichia coli, Mastitis, Staphylococcus aure

1. Introduction

Ethiopia has the largest cattle population in Africa, where cows represent the biggest portion of cattle population of the country. Around 20.7% of the total cattle heads are milking cows (CSA, 2014). The milk harvested from these animals serves an important dietary source for most of the rural, urban and periurban population (CSA, 2010). However, the dairy sector has not been fully exploited and milk production is below its potential failing the demands of the population in the country due to several constraints. Infectious diseases, such as mastitis, represent serious potential constraints to further development of dairy production and alleviating poverty in developing countries including Ethiopia (Mohamed *et al*., 2004; Avinash *et al*., 2012; Matope, 2013).

Mastitis is an inflammation of the mammary gland and udder tissue, and is a major endemic disease of dairy cattle that follows a number of factors including the cow, the pathogen and the environment (Radostits *et al*., 2007). Besides health disorders of the mammary gland, mastitis can also cause significant losses in milk yield, alterations in its quality, fertility disorders and even systemic diseases (Fekadu,1995; Souto *et al*., 2010; Awale *et al.,* 2012). Moreover, causative agents of mastitis with zoonotic potential may represent a health risk for human populations via the food chain. It is considered as the most complex

disease because of its multi-factorial causation (Nibret, 2009).

Mastitis is universally classified as clinical and subclinical mastitis (Mungube, *et al*., 2004; Radostits *et al*., 2007). Clinical mastitis is characterized mainly by appearances of changes in the milk, presence of inflammatory signs on the mammary glands and systemic signs. While subclinical mastitis produces no visible appearance of changes in the milk and/or udder (Radostits *et al*., 2007; Blowy and Edmondson, 2010).

Mastitis, being a complex and multietiologic disease, many microorganisms are implicated as causes. A wide range of pathogens including viruses, bacteria, fungi and their toxins can cause mastitis. Among these the majority of microorganisms that are responsible for mastitis and spoilage of milk are of bacterial origin and include *S. aureus*, *Streptococcus agalactiae (Strep. agalactia),* other *Streptococcus species* , CNS, *E. coli*, *Micrococcus species*, *Corynebacterium species*, *Bacillus species*, *Pasteurella species, Klebsiella species*, *Mycoplasma species* and *Nocardia species* (Quinn *et al.,* 2002; Radostits *et al*., 2007; Ondiek *et al*., 2013). Of these bacterial pathogens *Streptococcus spp*, *S. aureus, CNS* and *E. coli* are the most frequent causes of mastitis in dairy cattle (Barkema *et al*., 1998; Olde Riekerink *et al*., 2008).

Several studies conducted in different corners of Ethiopia indicated that over all prevalence of mastitis ranges from 13.7% to 81.1% **(**Biffa *et al*., 2005; Sori *et al*., 2005; Mekibib, 2010; Dugma *et al*., 2013; Zenebe *et al*., 2014); and several pathogenic bacteria associated with mastitis have been isolated including *Strep. agalactia, Strep. uberis, Strep. dysgalactiae, S. aureus, E. coli* and others (Workineh *et al*., 2002; Kerro and Tareke, 2003; Biffa *et al*., 2005; Hunderra *et al*., 2005; Mungube *et al*., 2005; Almaw *et al*., 2008; Getahun *et al*., 2008; Bitew *et al*., 2010; Razi *et al*., 2012). However, information concerning the prevalence, and major risk factors and bacterial etiologies of mastitis in and around Dessie town is limited.

Therefore the present study was conducted with the following objectives;

- ✓ To determine the prevalence of bovine mastitis in and around Dessie town.
- ✓ To find out the prevalence of *E.coli* and *staphylococcus* species from bovine mastitis.
- ✓ To assess the major risk factors associated with the occurrence of the bovine mastitis.
- ✓ To determine the antimicrobial resistance profiles of prevalent isolates.

Litrature Review

2.1. General Description of Mastitis

Mastitis is one of the most devastating and continuous threat to dairy industry; which is multifaceted disease that occurs as acute, chronic and sub-clinical forms. It is a complex disease of lactating animals which is characterized by inflammation of the mammary parenchyma and udder tissue (Memon *et al,* 2013; Zenebe *et al.,* 2014). Infectious agents, animal and environmental factors play important role in determining the incidence and establishing of the infection in the udder (Hussain *et al.,* 2013; Kulkarni and Kaliwal, 2013; Ali *et al.,* 2014; Tripura *et al.,* 2014). It usually occurs as an immune response to pathogenic microorganisms present in the teat canal and as a result of chemical, mechanical and thermal injury to the udder. The majority of infections are caused by various types of micro organisms mainly by bacterial pathogens. *Streptococci* species, *CNS*, coliforms as well as *S. aureus* are reported as predominant infectious agents in cases of mastitis (Jubb and Kennedy, 1997; Tenhagen *et al*., 2009). Other bacterial agents like *Corynebacterium bovis*, *Pseudomona aeruginosa*, *Coagulase negative staphylococcus*, *S. chromogens*, *S. epidermitis*, *S. hyicus*, *S. simulans*, *S. hamolyticus, S. xylosus Klebsiella* species, Mycoplasma species, *d*ifferent species of yeasts and fungi are also isolated and reported from mastitis (Radostits *et al.,* 2007).

In Ethiopia, mastitis has long been known however, the information on the magnitude, risk factors and causative agent of the disease is inadequate in some area. Such information is important when designing appropriate strategies that would help to reduce its prevalence and effects (Abera *et al*., 2012).

2.2. Types of Mastitis

Mastitis is a frustrating, costly and extremely complex disease that results in a marked reduction in the quality and quantity of milk). Depending on the clinical manifestation mastitis is universally classified as clinical and subclinical mastitis (Bartelet *et al.,* 1992).

2.2.1. Clinical mastitis

Clinical mastitis is characterized by the production of abnormal milk with or without secondary symptoms such as swollen quarters, elevated body temperature and/or other systemic signs (Schroeder *et al*., 2010). It is observed in less than 5% of animals in a well-managed dairy herd. On the farm, clinical mastitis is usually detected by the examination of any visible changes to the normal appearance of milk, which could include a color change, a consistency change, or the presence of flakes, clots, and/or blood. The udder producing this milk may become swollen, red, hot, and hard and there may be also fever, rapid heart rate and loss of appetite**.** In chronic cases there may be local fibrosis and atrophy of mammary tissue (Radostits *et al.,* 2007; Schroeder

et a.l, 2010). Cows that developed clinical mastitis suffered an immediate drop in production and will not regain previous production levels during the 60 days following the clinical onset. Although clinical infections are rarely apparent prior to calving, routine observation for abnormal swelling is important. It can be recognized in pre- and post-calving secretions, colostrums or milk by the presence of gargot (clots and flakes), abnormal texture or discoloration. A case of mastitis is considered severe when systemic signs of an inflammatory response are apparent including fever, anorexia and shock (Christos, 2011).

2.2.2. Sub clinical mastitis

In subclinical mastitis, unlike clinical one, no visible abnormalities are appreciated either the milk or the udder. It is generally characterized by change in milk composition (somatic cell count (SCC), changes in milk pH and ion concentration) with no clinical signs of gross inflammation or milk abnormalities (Radostits *et al.,* 2007). A sudden rise in milk somatic cell count observed in normal milk from normal udders may indicate the presence of subclinical mastitis. Animals which have subclinical mastitis are usually not producing milk to their full potential and can serve as a potential source of infection to healthy udders (Radostits *et al.,* 2007; Schroeder *et a.l*, 2010). In excess of 50% of lactating animals in a herd can have subclinical mastitis at any given time (Kabaria *et al*, 2014).

In dairy industry, the Sub clinical mastitis is important because this form is more prevalent than the clinical form, it usually precedes the clinical form, stay for long duration, difficult to detect, reduces milk production without any noticeable signs, adversely affects milk quality (Beshti *et al*., 2010), constitutes a reservoir of microorganisms that can affect other animals within the herd due to its contagious nature. Besides causing huge losses to milk production, the sub clinically affected animals remain a continuous source of infection to other herd mates and human (Kathiriya *et al*., 2014).

2.3. Diagnosis of Mastitis

It is essential to diagnose mastitis at the initial stage of infection to initiate the treatment as early as possible before the bacteria are anchored in the mammary gland, transmitted to other animal and human, and severely affect the health and production potential of animals. Monitoring udder health performance is impossible without reliable and affordable diagnostic methods. Various methods, based on physical and chemical changes of milk/udder and isolation of organisms, are generally used for diagnosis of mastitis (Santos *et al*., 2004; Lame *et al*., 2009; Britten, 2012).

Clinical mastitis is most commonly diagnosed by proper examination of the animal for any signs of abnormalities/inflammatory lesions in the mammary gland and qualitative examination of milk for any visible changes in color, the presence of flakes and clots, blood or pus, change in consistency and abnormal smell (Reneau, 2001; Quinn *et al*., 2002; Radostits *et al*., 2007; Mulugeta and Wassie, 2013).

Since visible abnormalities are not apparent both in the milk or the udder, indirect tests are required to detect subclinical mastitis. The most frequently used diagnostic methods for diagnosis of subclinical mastitis are SCC (somatic cell count) and bacteriological culturing of milk. Currently, other methods such as measurement of N-acetyl- β D glucosaminidase (NAGase), lactate dehydrogenase activity (LDH), electric conductivity (EC) on milk, are also used less frequently (Reneau, 2001; Quinn *et al*., 2002).

Early diagnosis of mastitis is vital because changes in the udder tissue take place much earlier before they become apparent. So, easy, cheap, rapid and accurate "cow-side" mastitis test, like CMT, must be used by farmers and veterinarians on farm to diagnose and treat mastitis in its early stages; before its propagation in the herd (Viguier, 2009; Razi *et al*., 2012; Kayesh *et al*., 2014).

2.4. Economic Impact of Mastitis

Mastitis remains the most important and common diseases that cause economic loss in dairy industry worldwide (Bedacha *et al.,* 2011). Generally, sources of economic loss include reduced milk production, animal replacement due to culling, discarded milk due to antibiotic treatment, cost of treatment, veterinary service, and extra labor cost to care for the animal. It also affects the milk quality in terms of decrease in protein, fat, milk, sugar (lactose) contents and increase in somatic cell count (Sharif and Muhammed, 2009). In study conducted by Bennett *et al*. (1999) the total economic impact of clinical mastitis is estimated to be £119 per cow-case in Great Britain. More than $130 million is lost by the Australian dairy industry ($A200/cow/year) every year due to mastitis. In the USA mastitis causes a loss of over 1.7 billion dollars a year alone. In Ethiopia studies conducted to quantify milk production losses associated with mastitis is limited. The economic impact of mastitis on milk production losses accounted for 78% of the total losses in dairy industry (Schepers and Dijkhuizen, 1991).

2.5. Public Health Importance of Mastitis

With mastitis there is a danger that the bacterial contamination of milk from affected cows may render it unsuitable for human consumption by causing food poisoning and provides a mechanism of spread of disease to humans through consumption of raw milk. Raw (unpasteurized) milk has been found to harbour and participate in spreading out of various illnesses. The most common and principal bacterial infection

associated with ingestion of milk and milk products are caused by *Mycobacterium bovis, Brucella abortus, Staphylococcus aureus, Listeria monocytogenes, Campylobacter jejuni, Salmonella, Staphylococci species,* and *E. Coli* (Heeschen, 2012). Considering the absence of visible changes, milk of cows with subclinical mastitis is accidentally mixed into bulk milk, and can enters into food chain and be dangerous to humans. The presence of residue in milk following treatment of mastitis is another major public health concerns that adversely affects the dairy industry, the practicing veterinarian and the safety of milk for human consumption. Consumption of antibiotic contaminated milk results in allergic responses, changes in intestinal flora and development of antibiotic resistant pathogenic bacteria (Sharif and Muhammed**,** 2009)

2.6. Treatment of Mastitis

The success of bovine mastitis therapy depends on the etiology, clinical presentation and antimicrobial susceptibility of the etiological agent among other factors **(**Miltenburg *et al*, 2007). Therapy failure in the management of mastitis could result from pathological changes that occur in the udder, etiology related factors, pharmacokinetic properties of the antimicrobial drugs, poor animal husbandry and inadequate veterinary services. The most common route of administration of antimicrobials in mastitis is the intra mammary (IMM) route. Systemic treatment is recommended in clinical mastitis due to S. *aureus* and in severe cases of coliform mastitis, preferably in combination with IMM treatment (Hierton and Berry, 2005. The systemic route of administration has been suggested to be more efficient than IMM for the treatment of clinical mastitis as antimicrobials theoretically have better penetration of the udder tissue by this route**.** Antibiotic therapy is usually prescribed when clinical symptoms of mastitis are presented. If detected early, it is very effective in curing and controlling the spread of contagious pathogens. Culling is another method of control especially when dealing with chronically infected animals. This eliminates the potential source of infection at the expense of purchasing a replacement animal (Tomita, 2001).

2.7. Prevention and Control

Bovine mastitis is an endemic disease that cannot be completely eradicated. The wide ranges of microorganisms that can cause this disease, and the ubiquity of these organisms, make complete eradication unlikely. Optimum control therefore lies in first understanding the epidemiology of the disease and the causal agents and then implementing an integrated control strategy. The control of mastitis has been successfully achieved through the establishment of effective herd health control programs. Early diagnosis of mastitis with reliable tests facilitates successful treatment and control. The main control principles include: sound husbandry practices and sanitation, post milking teat dip, treatment of mastitis during non-lactating period, and culling of chronically infected animals (Sharif and Muhammad, 2009)

Successful control of contagious mastitis pathogens is focused on reducing exposure of teats to pathogens found in milk that originated from infected cows. Control of environmental mastitis can be achieved by reducing the number of bacteria to which teat is exposed, increasing immune resistance of the cow; pre milking teat dipping with a germicidal. Animal environment should be as clean and dry as possible. Antimicrobials are routinely used for treatment of dairy cattle affected with clinical and subclinical infections. The teat canal remains open up to 2-3 hours after milking to resume its normal confirmation. This is the reason for providing feed and water immediately after milking to encourage animals to remain standing and the reason for having freshly cleaned and bedded stalls when the cows do lie down (Hillerton and Berry, 2005). Calf suckling must be avoided at all costs in dairy animals to reduce damages of the udder during suckling. Proper ventilation and good sanitation at the farm building is necessary to decrease the exposure of pathogens to the mammary gland. Other general practices used to prevent contagious and environmental mastitis include dry cow treatment and milking of infected animals last. (Shakala *et al*, 2013).

Proper washing, drying and cleaning of milker's hand and milking utensils, provision of dry bedding, immediate removal of dung and urine can also minimize the spread of disease. Recently, the National Mastitis Council of USA and Canada expanded the ten-point plans: establishment of goals for udder health, maintenance of a clean, dry and comfortable environment, proper milking procedures, proper maintenance and use of milking equipment, good record keeping, appropriate management of clinical mastitis during lactation, effective dry cow management, maintenance of bio-security for contagious pathogens and culling of incurable and chronically infected cows, regular monitoring of udder health status and periodic review of the mastitis control program **(**NAAS,2013**).**

Materials And Methods

3.1. Study Area

This study was conducted from November 2016 to April 2017 in and around Dessie town, South Wollo Zone, Amhara region, North Eastern Ethiopia. Dessie town is found about 401 km north-east of Addis Ababa and located at a latitude of 11° 07′ 59.81″ N and a longitude of 39° 37′ 59.83″ E. It has an elevation

ranging from 2,400 -2,550m above sea level. The annual rainfall of the area ranges from 1,100 to 1,200 mm. The mean annual minimum and maximum temperatures are 11.7^{0}C and 24^{0}C, respectively. The mean annual relative humidity is about 60% and the area experiences a bi-modal rainfall patterns with a short rainy season which occurs from February to March and long rainy season which starts at the end of June and ends at early November (CSA, 2014).

3.2. Study Animals and Design

A cross- sectional study was carried out from November, 2016 to April, 2017 to determine the prevalence, risk factors and isolate major bacterial pathogens of bovine mastitis at cow level based on clinical examination and CMT test. The study animals were comprised of lactating dairy cows found in and around Dessie town without discrimination of their breed, age, lactation stage and parity. Random sampling method was employed to select dairy farms and lactating cows for this study. Seven dairy farms with herd size range from 13 to 43 were included depending on owner willingness, transportation access and the time of their milking practice.

3.3. Sample Size Determination

The sample size for this study was calculated following the formula described by (Thrustfeild 2005);

$$n=\frac{(1.96)^2 p_{exp}\,(1-p_{exp})}{d^2}$$

Where, n = sample size, z = statistic for a level of confidence

d = required absolute precision, p = expected prevalence

Due to lack of previous research on bovine mastitis in the study area an expected prevalence of 50% was taken to estimate the sample size. Accordingly, the minimum sample size was calculated to be 384. However, due to financial and transportation constraints and owners willingness only 180 randomly selected lactating dairy cows were examined.

3.4. Sampling Technique and Sample Collection

3.4.1. Physical examination of the udder

The udder was first examined visually and then thoroughly palpated to detect any possible changes like atrophy, fibrosis, cardinal signs of inflammation, visible injury and swelling of the supra mammary lymph nodes. Rectal temperature of those cows with clinical mastitis was taken check systemic involvement. Information related to the previous health history of the mammary quarters and cause of blindness was obtained from the owners and case record. Other data regarding to age, parity, lactation stage, housing conditions and previous history of mastitis were collected and recorded properly from farm records and by interviewing the farm owner, managers and workers. Following clinical examination, clinical mastitis was diagnosed at the quarter level based on visible and palpable signs (Kivaria *et al*, 2007).

3.4.2. Milk sample collection

A total of 180 milk samples (6 from clinical mastitic and 174 from apparently health cows) were collected from the randomly selected dairy cows. Samples were taken from the cow which was not treated before with either intra mammary or systemic antimicrobial agents. Udder halves were cleaned and disinfected prior to sampling with 70% Ethyl alcohol and dried with sterile towel. The first 3 squirts of milk were discarded and approximately 5 ml of milk was taken in a sterile tube for CMT and bacteriological examinations. The Milk samples were taken from individual cows by mixing quarters in sterile bottle during morning and evening milking time. Milk samples collected in test tubes were labeled and sealed properly, wrapped with par film (to avoid contamination) and transported in ice box immediately to Wollo University school of Veterinary Medicine, Microbiology laboratory. Milk samples collected from apparently health cows were checked for the presence of subclinical mastitis by using CMT test (Quinn *et al*., 2002).

3.4.3. California mastitis test

The California mastitis test (CMT) was conducted to diagnose the presence of subclinical mastitis and this screening test was performed according to the procedure given in Quinn *et al*. (2002). Each sample was mixed by vortex mixer and 1 ml of milk from each sample was placed in each of four CMT paddles and an equal amount of the CMT reagent was added. A gentle circular motion was applied in a horizontal plane. Samples that show gel formation within a few seconds were scored and recorded as positive. And Samples that scored negative were assumed healthy. The result was scored based on the gel formation and categorized as negative when there was no gel formation and positive if there was gel formation as +1, +2, or +3 depending on the intensity of reaction (gel formation). Then CMT positive milk samples were further processed for bacterial isolation and identification (Quinn *et al*., 2004; Britten, 2012)

3.4.4. Bacteriological culturing

3.4.4.1. Isolation and identification of Escherichia coli

One ml of milk samples collected from clinically mastitic cows and CMT positive milk samples were inoculated into 9 ml peptone water and incubated at 37°C over night. A loopful of the enriched sample were inoculated on MacConkey Agar (MCA) (HIMEDIA, India) and incubated for 24 hrs at 37 °C.

Pink colored colonies appeared on MCA were picked from MCA, and streaked on Eosin Methylene Blue Agar medium (EMB) and incubated at 37 °C for 24 hours. Colonies with characteristic green metallic sheen growth were suspected as *E. coli* (Quinn *et al*., 2002). Gram's staining was performed as per procedures described by Merchantand (1969) to determine the size, shape and arrangement of bacteria. The organisms revealed Gram negative, pink colored with rod shaped appearance and arranged in single or in pair were considered as *E. coli.* Colony morphology and colors on MCA and EMB agar plates together with the Gram stain procedure were used as initial identification of *E. coli* colonies. Such colonies were taken from EMB into nutrient agar for further biochemical examination. Standard biochemical tests were used as confirmation of identification (Brenner *et al*., 2005). Biochemical tests used in this study for characterization and confirmation of *E. coli* include Catalase, oxidase, Citrate utilization, oxidation-fermentation and triple sugar iron agar test (Simmons, 1960; Cheesbrough, 1985; Vanderzant and Splittstresser, 1992; Snell *et al*., 1999; (MacFaddin, 2000; Quinn *et al*.,2002).

3.4.4.2. Isolation and Identification of Staphylococcus

One ml of milk samples collected from clinically mastitic cows and CMT positive milk samples were inoculated into 9 ml peptone water and incubated at 37°C over night. A loopful of the enriched sample was streaked aseptically on Mannitol Salt Agar (MAS) (HIMEDIA, India). Plates were incubated at 37°C for 24-48hr. The plates were examined for the presence of discrete colonies showing typical golden yellowish or white color colonies with yellow color of MSA. Gram's staining was performed to determine the size, shape and arrangement of bacteria. The organisms revealed Gram positive, colored with purple cocci (spherical) shaped appearance and arranged in chain suspected as *staphylococcus.* Colony morphology and colors on MAS agar plates together with the Gram stain procedure were used as initial identification of staphylococcus colonies. Presumed colonies were then sub-cultured on nutrient agar plates and incubated at 37°C for 24 hr to do further biochemical tests. The purified *Staphylococcus* isolates were identified through different biochemical tests: catalase test, oxidation- fermentation (O-F) test, Triple sugar iron test (TSI), hemolysis, slide coagulase test, DNAse test (Deoxyribonucleic acid) (Quinn *et al*., 2002). All media were prepared and used according to the manufacturer's specification.

Antibiotics susceptibility test

All isolates of *E. coli, S. aureus* and *CNS* were screened for their in vitro antimicrobial susceptibility using the agar disk diffusion method (Bauer *et al.* (1966). Because of limitation, all isolates of *E.coli* were tested for their susceptibility against Erythromycin, Streptomycin, Cloxacillin, Ampicillin and Sulfamethoxazole-Trimethoprim. Ciprofloxacin, enrofloxacin, Kanamycine, Chloramphinicol and Streptomycine were used for *S. aureus.* The susceptibility of all CNS isolate were also tested for Gentamycine, Bacitracin, kanamycine, Tetracycline and Pencillin G.

Four to five well-isolated colonies of each organism from nutrient agar plates were transferred into tubes containing 5 ml of normal saline solution until it achieved 0.5 McFarland turbidity standards, and then a sterile cotton swab was dipped into the adjusted suspension within 15 minutes and excess broth was purged by pressing and rotating the swab firmly against the inside of the tube above the fluid level. The swab was then spread evenly over the entire surface of the plate of Mueller-Hinton agar (Oxoid Ltd., Basingstoke, and Hampshire, England) to obtain uniform inoculums. The plates were then allowed to dry for 3 to 5 minutes. Antibiotics impregnated discs were then applied to the surface of the inoculated plates with sterile forceps. Each disc was gently pressed down onto the agar to ensure complete contact with the agar surface. Even distribution of discs and minimum distance of 24 mm from center to center were ensured and from the edge of the plates to prevent overlapping of the inhibition zones. Five antibiotic discs were placed in each petri-dish. Within 15 minutes of the application of the discs, the plates were inverted and incubated at 37°C. After 18 to 24 hours of incubation, the plates were examined, and the diameters of the zones of complete inhibition to the nearest whole millimeter were measured by digital caliper. The clear zone (inhibition zones of bacterial growth around the antibiotic disc diameter for individual antimicrobial agents was then translated into Sensitive (S), Intermediate (I), and Resistant (R), categories according to the interpretation table of the Clinical and Laboratory Standard Institute (CLSI, 2014).

Data Management and Analysis

Data collected from each study animal and laboratory work results were coded appropriately and enter in Microsoft excel spread sheet 2007. Then analyses were performed using Statistics Package for Social Science (SPSS) version 20. Association of specific variables (risk factors) breed, parity, age, stage of lactation and hygiene were performed by using Pearson chi-square (X^2). X^2 and *p -values* were calculated and *p -values* ≤ 0.05 and $X^2 \geq 3.84$ was considered as statistically significant. And the prevalence was calculated by dividing the number of CMT positive animals to the total number of animals examined times 100%.

Results

Overall Prevalence of Mastitis and Isolated Pathogens

Out of the total 180 lactating dairy cows clinically examined and tested by CMT, the overall prevalence of mastitis was 27.2% (n=49); where 3.3 % (n = 6) and 23.9 % (n = 43) cows were found with clinical and sub clinical mastitis, respectively. The prevalence of mastitis was higher in farm 1 100% (n=9) compared to others. Higher prevalence of mastitis was recorded in cows during their early 52.7 % (n=29) and late stages of lactation 29.7 % (n=11) than their mid stage of lactation 10.2 % (n=9). Cows giving birth to more than 6 calves had the highest prevalence of mastitis 50 % (n=12) than cows having less than 6 calves. The prevalence of mastitis was found to be higher in cows at the age group of > 6 years 69.4 % (n =25) than others. In addition, higher prevalence of mastitis was recorded in cross breed 31.4 % (n=43) than local breeds of cow 14.0 % (n=6).

More prevalence of mastitis was found in cows managed in poor 44% (n=22) and good hygienic condition 36.7% (n=22) than those kept under better hygienic condition 7.1 % (n=5). The overall prevalence of *E. coli, S. aureus* and *CNS* isolated in this study were 21.1% (n=38), 16.7% (n=30) and 4.4% (n=8), respectively. Generally, except study area (*p-value* > 0.05) almost all other risk factors such as farm, breed, age, parity, lactation stage and hygienic condition considered in this study were significantly associated (*p- value* $\leq$ 0.05) with the overall prevalence of mastitis and bacterial isolates. However, breed had no significant effect (*p – value* > 0.05) on the prevalence of *CNS* and *S. aureus*. Similarly, parity and farm were also not significantly associated (*p – value* > 0.05) with the prevalence of *CNS*. The frequency of occurrence and overall prevalence of mastitis, *E. coli, S. aureus* and *CNS* identified in this study are shown in table 1 and 2.

Relative Occurrence of Bacterial Isolates and their Antimicrobial Susceptibility Pattern Out of the total 49 milk samples, collected from both clinical and sub clinical mastitic cows, cultured for bacteriological examination, 85.7% (n=42) yielded bacteria.

The isolation rate of E. coli, S. aureus and CNS, from mastitis milk samples in this study were 77.6 % (n=38), 61.2% (n=30) and 16.3 % (n=8), respectively. All risk factors; age, breed, lactation stage and parity were not significantly associated (p- value > 0.05) with the isolation rate of E. coli, S. aureus and CNS from mastitic milk samples (table3). However, farm and hygienic conditions had significant effect (p-value < 0.05) on the occurrence of E. coli and S. aureus respectively. The relative occurrence of E. coli, S. aureus and CNS in CMT positive milk samples in this study is shown in table.

Table 1: The Overall Prevalence of Mastitis, *E. coli*, *S. aureus* and *CNS* among Farm, Study area and hygienic condition.

Risk factor		No.	Mastitis	Clinical mastitis	Subclinical mastitis	Bacterial growth	*S. aureus*	*CNS*	*E.coli*
Farm	1	9	9 (100%)	1(11.1%)	8(88.9%)	9(100%)	8(88.9%)	1(11.1%)	8(88.9%)
	2	33	17(51.5%)	1(3.0%)	16(48.5%)	15(45.5%)	8(24.2%)	4(12.15%)	15(45.5%)
	3	33	3(9.1%)	0(0.0%)	3(9.1%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)
	4	37	2(5.4%)	2(5.4%)	0(0.0%)	2(5.4%)	1(2.7%0	0(0.0%)	2(5.4%)
	5	35	11(31.4%)	2(5.7%)	9(25.7%)	10(28.6%)	9(25.7%)	1(2.9%)	7(20.0%)
	6	16	2(12.5%)	0(0.0%)	2(12.5%)	2(12.5%)	1(6.2%)	1(6.2%)	2(12.5%)
	7	17	5(29.4%)	0(0.0%)	5(29.4%)	4(23.5%)	3(17.6%)	1(5.9%)	4(23.5%)
	total	180	49(27.2%)	6(3.3%)	43(23.9%)	42(23.3%)	30(16.7%)	8(4.4%)	38(21.1%)
	p-value		0.000	0.000	0.000	0.000	0.000	0.163	0.000
	χ^2		50.360	55.896	55.896	56.877	50.283	9.190	51.676
Area	Dessie	112	31(27.7%)	4(3.6%)	27(24.1%)	26(23.2%)	17(15.2%)	5(4.5%)	25(22.3%)
	Boru	35	11(31.4%)	2(5.7%)	9(25.7%)	10(28.6%)	9(25.7%)	1(2.9%)	7(20.0%)
	Haike	33	7(21.2%)	0(0.0%)	7(21.7%)	6(18.2%)	4(12.1%)	2(6.1%)	6(18.2%)
	total	180	49(27.2%)	6(3.3%)	43(23.9%)	42(23.3%)	30(16.7%)	8(4.4%)	38(21.1%)
	p-value		0.629	0.714	0.598	0.598	0.255	0.814	0.863
	χ^2		0.926	2.116	1.027	1.027	2.732	0.411	0.294
Hygienic condition	poor	50	22(44.0%)	1(2.0%)	21(42.0%)	19(38.0%0	11(22.0%)	5(10.0%)	19(38.0%)
	good	60	22(36.7%)	3(5.0%)	19(31.7%)	21(35.0%)	18(30.0%)	3(5.0%)	17(28.3%)
	Better	70	5(7.1%)	2(2.9%)	3(4.3%)	2(2.9%)	1(1.4%)	0(0.0%)	2(2.9%)
	total	180	49(27.2%)	6(3.3%)	43(23.9%)	42(23.3%)	30(16.7%)	8(4.4%)	38(21.1%)
	p-value		0.000	0.000	0.000	0.000	0.000	0.031	0.000
	χ^2		24.051	27.007	27.007	26.984	20.407	6.933	24.448

N.B: χ^2 = chi- square, No. = number of samples collected

3. All bacterial isolates were tested for their antimicrobial susceptibility patterns and showed varied responses. In the present investigation, the majority of E. coli isolates were susceptible to Streptomycin (100%), Ampicillin (57.8%) and Sulfamethoxazole-Trimethoprim (57.8%) but resistance against Cloxacillin (78.9%) and Erythromycin (57.9%). The majority of S. aureus isolates showed intermediate effect for Enrofloxacin (46.7%), Ciprofloxacin (78.9%), Chloramphinicol (78.9%) and Streptomycine (50%). However all S. aureus isolates were susceptible for kanamycin (100%). Similarly, all CNS isolates were susceptible for Kanamycin (100%) and Gentamycine (100%), but resistance for Bacitracin (100%), Penicillin G (100%) and tetracycline (100%). The antimicrobial susceptibility patterns of E. coli, S. aureus and CNS in this study is summarized in table 4.

Table 1: The Overall Prevalence of Mastitis, E. coli, S. aureus and CNS according to lactation stage, parity, breed and age.

Risk factor		No.	Mastitis (%)	Clinical mastitis	Subclinical mastitis	Bacterial growth	*Staph. aureus*	CNS	*E. coli*
Age	≤3yrs	61	8(13.1%)	1(1.6%)	7(11.5%)	6(9.8%)	4(6.6%)	0(0.0%)	6(9.8%)
	4-6yrs	83	16(19.3%)	2(2.4%)	14(16.9%)	14(16.9%)	11(13.3%)	3(3.6%)	13(15.7%)
	>6	36	25(69.4%)	3(8.3%)	22(61.1%)	22(61.1%)	15(41.7%)	5(13.9%)	19(52.8%)
	Total	180	49(27.2%)	6(3.3%)	43(23.9%)	42(23.3%)	30(16.7%)	8(4.4%)	38(21.1%)
	p-value		0.000	0.000	0.000	0.000	0.000)	0.005	0.000
	χ^2		41.166	41.171	41.171	36.872	21.835	10.533	27.812
Breed	Zebu	43	6(14.0%)	2(4.7%)	4(9.3%)	4(9.3%)	3(7.0%)	0(0.0%)	4(9.3%)
	Cross	137	43(31.4%)	4(2.9%)	39(28.5%)	38(27.7%)	27(19.7%)	8(5.8%)	34(24.8%)
	Total	180	49(27.2%)	6(3.3%)	43(23.9%)	42(23.3%)	30(16.7%)	8(4.4%)	38(21.1%)
	p-value		0.025	0.035)	0.035	0.013	0.51	0.105	0.030
	χ^2		5.021	6.693	6.693	6.217	3.819	2.628	4.720
Parity No	≤3 calves	75	13(17.3%)	1(1.3%)	12(16.0%)	10(13.3%)	5(6.7%)	3(4.0%)	9(12.0%)
	4-6 calves	81	24(29.6%)	2(2.5%)	22(27.2%)	21(25.9%)	16(19.8%)	4(4.9%)	21(25.9%)
	>6	24	12(50.0%)	3(12.5%)	9(37.5%)	11(45.8%)	9(37.5%)	1(4.2%)	8(33.9%)
	Total	180	49(27.2%)	6(3.3%)	43(23.9%)	42(23.3%)	30(16.7%)	8(4.4%)	38(21.1%)
	p-value		0.006	0.007	0.007	0.004	0.001	0.958	0.001
	χ^2		10.224	14.093	14.093	11.289	13.456	0.086	13.456
lactation stage	≤3 m	55	29(52.7%)	4(7.3%)	25(45.5%)	25(45.5%)	17(30.9%)	6(10.9%)	22(40.0%)
	4-6m	88	9(10.2%)	2(2.3%)	7(8.0%)	9(10.2%)	8(9.1%)	0(0.0%)	9(10.2%)
	>6 m	37	11(29.7%)	0(0.0%)	11(29.7%)	8(21.6%)	5(13.5%)	2(5.4%)	7(18.9%)
	Total	180	49(27.2%)	6(3.3%)	43(23.9%)	42(23, 3%)	30(16.7%)	8(4.4%)	38(21.1%)
	p-value		0.000	0.000	0.000	0.000	0.003	0.001	0.000
	χ^2		31.006	33.120	33.120	23.556	11.934	13.456	18.149

N.B: χ^2 = chi- square, No. = number of samples collected Table 2: The relative occurrence of *E. coli*, *S. aureus* and *CNS* in CMT positive milk in this study.

Table 3. The risk factors of bacteria

Risk factors		No.	Bacteria (%)	*E.coli*	*S.aureus*	*CNS*
Farm	1	9	9(100%)	8(88.9%)	8(88.9%)	1(11.1%)
	2	17	15(88.2%)	15(88.2%)	8(47.1%)	4(23.5%)
	3	3	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)
	4	2	2(100%)	2(100%)	1(50.0%)	0(0.0%)
	5	11	10(90.9%)	7(63.6%)	9(81.8%)	1(9.1%)
	6	2	2(100%)	2(100%)	1(50.0%)	1(50.0%)
	7	5	4(80.0%)	4(80%)	3(60.0%)	1(20.0%)
	total	49	42(85.7%)	38(77.6%)	30(61.2%)	8(16.3%)
	P- value (χ^2)		0.002 (20.631)	0.024 (14.541)	0.081 (11.256)	0.686 (3.931)
Area	Dessie	31	26(83.9%)	25(80.6%)	17(54.8%)	5(16.1%)
	Boru	11	10(90.9%)	7(63.6%)	9(81.8%)	1(9.1%)
	Haike	7	6(85.7%)	6(85.7%)	4(57.1%)	2(28.6%)
	Total	49	42(85.7%)	38(77.6%)	30(61.2%)	8(16.3%)
	p- value (χ^2)		0.849(0.328)	0.436(1.662)	0.288(2.547)	0.551(1.191)
Breed	zebu	6	4(66.7%)	4(66.7%)	3(50.0%)	0(0.0%)
	cross	43	38(88.4%)	34(79.1%)	27(62.8%)	8(18.6%)
	total	49	42(85.7%)	38(77.6%)	30(61.2%)	8(16.3%)
	p- value (χ^2)		0.155(2.02)	0.495(0.465)	0.547(0.363)	0.248(1.334)
Age	≤3 yrs	8	6(75.0%)	6(75.0%)	4(50.0%)	0(0.0%)
	4-6yrs	16	14(87.5%)	13(81.2%)	11(68.8%)	3(18.8%)

Risk factors		No.	Bacteria (%)	*E.coli*	*S.aureus*	*CNS*
	>6yrs	25	22(88.0%)	19(76.0%)	15(60.0%)	5(20.0%)
	total	49	42(87.5%)	38(77.6%)	30(61.2%)	8(16.3%)
	p –value (χ^2)		0.638(0.898)	0.909(0.190)	0.663(0.822)	0.391(1.877)
	≤3calves	13	10(76.9%)	9(69.2%)	5(38.5%)	3(23.1%)
	4-6 calves	24	21(85.5%)	21(87.5%)	16(66.7%)	4(16.7%)
Parity	>6 calves	12	11(91.7%)	8(66.7%)	9(75.0%)	1(8.3%)
	total	49	42(85.7%)	38(77.6%)	30(61.2%)	8(16.3%)
	p-value (χ^2)		0.541(1.230)	0.259(2.698)	0.129(4.096)	0.607(0.997)
	≤3 months	29	25(86.2%)	22(75.9%)	17(58.6%)	6(20.7%)
	4-6 months	9	9(100%)	9(100%)	8(88.9%)	0(0.0%)
Lactation stage	>6months	11	8(72.7%)	7(63.6%)	5(45.5%)	2(18.2%)
	total	49	42(87.5%)	38(77.6%)	30(61.2%)	8(16.3%)
	p-value (χ^2)		0.221(3.021)	0.144(3.876)	0.126(4.136)	0.335(2.188)
	Poor	22	19(86.4%)	19(86.4%)	11(50.0%)	5(22.7%)
	Good	22	21(95.5%)	17(77.3%)	18(81.8%)	3(13.6%)
Hygiene	Better	5	2(40.0%)	2(40.0%)	1(20.0%)	0(0.0%)
	total	49	42(85.7%)	38(77.6%)	30(61.2%)	8(16.3%)
	p-value (χ^2)		0.006(10.245)	0.081(5.032)	0.013(8.677)	0.416(1.752)

Table 3: Antimicrobial susceptibility patterns of *E. coli*, *S. aureus* and *CNS* in this study.

Bacterial isolate	Sensitivity	Antimicrobial drugs tested												
		CX	E	AP	TS	S	ENF	CIP	K	C	PG	T	GM	BA
E. coli	Sus.	21.1 % (n=8)	0	57.8 % (n=22)	57.8 % (n=22)	100% (n=38)	-	-	-	-	-	-	-	-
	I	0	42.1 % (n=16)	21.1 % (n=8)	21.1 % (n=8)	0	-	-	-	-	-	-	-	-
	R	78.9% (n=30)	57.9 % (n=22)	21.1 % (n=8)	21.1 % (n=8)	0	-	-	-	-	-	-	-	-
	Total	100 % (n=38)	100 % (n=38)	100% (n=38)	100% (n=38)	100 % (n=38)	-	-	-	-	-	-	-	-
S. aureus	Sus.	-	-	-	-	50% (n=15)	26.7% (n=8)	21.1 % (n=8)	100% (n=30)	21.1 % (n=8)	-	-	--	-
	I	-	-	-	-	50% (n=15)	46.7% (n=14)	78.9% (n=22)	0	78.9% (n=30)	-	-	-	-
	R	-	-	-	-	0	26.7% (n=8)	0	0	0	-	-	-	-
	Total	-	-	-	-	100% (n=30)	100% (n=30)	100% (n=30)	100% (n=30)	100% (n=30)	-	-	-	-
CNS	Sus.	-	-	-	-	-	-	-	100% (n=10)	-	0	0	100 % (n=10)	0
	I	-	-	-	-	-	-	-	0	-	0	0	0	0
	R	-	-	-	-	-	-	-	0	-	100 % (n=10)	100 % (n=10)	0	100 % (n=10)
	Total	-	-	-	-	-	-	-	100% (n=10)	-	100% (n=10)	100 % (n=10)	100% (n=10)	100 % (n=10)

N.B: Sus = susceptible, *I = intermidate, *R = Resistance, * AP = Ampicillin (25 µg), *CIP = Ciprofloxacin (5µg), *K = Kanamycine (30µg), *C = Chloramphinicol (30µg), *S = Streptomycin (10µg), *PG = PenciliinG (10 units), *T = Tetracycline (10µg), * TS = Trimethoprim + Sulfamethoxazole (1.2µg+23.75µg), *GM = Gentamycine (10µg). CX = Cloxacillin (5µg), *ENF = Enrofloxacin (5µg), * BA = Bacitracin (10 units),* E=Erythromycin (15µg).

5. DISSCUSSION

In this study, out of the total 180 lactating cows examined clinically and tested by CMT, 27.2% (n=49) were found to be positive for mastitis. The result recorded in this study agrees with the previous reports of Bitew *et al.* (2010) and Mekibib *et al.* (2010) who recorded mastitis in dairy cows with an overall prevalence of 28.2% at Bahir Dar and its surrounding and 25.22% in Holeta, respectively. However, the finding of this study is lower than the results of Chtikobo (2010), Siddiquee *et al.* (2013), Lakew *et al.* (2009), Fekadu (1995) who reported mastitis with an overall prevalence of 58.6%, 55.1%, 64.4% and 45.5%, and in and around Nyagatare (Rwanda), Banglandish, Asella, and Soddo, respectively. Lower prevalence of mastitis was also reported by Getahun *et al.* (2008), Nesru *et al.* (1997) and Biffa *et al.* (2005) who noted a prevalence rate of 22.3%, 19% and 23.0%, respectively, in different parts of Ethiopia. The variation in the prevalence of bovine mastitis among

this and other reports could be due to differences in production type considered, herd size and management of the farms, and parity, age, lactation stage and breeds of the animal (Radostits *et al*., 2007; Girma, 2010). Generally, this study showed that the prevalence of mastitis was significantly associated between/among the different farms (*p- value* =0.000), age (*p- value* = 0.000), breed (*p- value* = 0.025), parity number (*p- value* = 0.006), lactation stage (*p- value* = 0.000) and hygiene (*p- value* = 0.000) but not with area (*p- value* = 0.629).

In this study both clinical and sub clinical mastitis were found at a prevalence of 3.3 % (n = 6) and 23.9 % (n = 43), respectively. This is comparable with the previous findings of Enyew (2004) (3.9%), Bitew *et al.* (2010) (3%) and Moges *et al.* (2011) (0.93%) who reported low prevalence of clinical mastitis compared to sub clinical mastitis in their studies. However, higher prevalence of clinical mastitis was recorded by Mekibib *et al.* (2009) (22.4 %) in Holeta and Sori *et al.* (2005) (16.11%) in and around Sebeta. The prevalence of sub-clinical mastitis recorded in this study, (23.9%), was also comparable with the report of Bitew *et al.* (2010) (25.2%), Getahun *et al*. (2008) (22.3%) and Biffa *et al*. (2005) (23.0%) but lower than the findings of (40.7%) and Argaw and Tolosa (2008) (89.5%) from different parts of Ethiopia. In most reports including the present study, clinical mastitis is far lower than subclinical mastitis. This could be attributed to the reason that compared to clinical mastitis little attention is given to subclinical mastitis by farms and veterinarians when it comes to treatment, as the infected animal shows no obvious symptoms and secrets apparently normal milk. Moreover, efforts have been concentrated on the treatment of clinical cases and farmers, especially small holders, are not well informed about invisible loss from sub clinical mastitis (Almaw *et al*., 2008).

The increased prevalence of mastitis with age and parity reported in the current study is comparable with the previous studies (Biffa *et al*., 2005; Tamirat, 2007; Mekibib *et al.,* 2010; Haftu *et al*., 2012). This high prevalence of mastitis could be due to repeated and increased exposure of animals to one of the agents which causes mastitis through their life time. In addition, the prolonged duration of infection and increased susceptibility of cows to pathogenic organisms in udder and relaxed sphincter muscles of teats could also increase the prevalence of mastitis with age and parity (Girma, 2010; Moges *et al.,* 2011; Zeryehun *et al.,* 2013). The higher susceptibility of cross breed cows than zebu (local) cows observed in this study is comparable with findings of other studies such as Almaw *et al*. (2009) and Sori *et al*. (2005) in and around Sebeta. This might also be due to variation of breeds in their genetic potential to disease resistance and adaptability to environments. Moreover, the anatomical size of the udder in cross breed cows is larger that can easily be contaminated and exposed to different pathogens.

The higher prevalence of mastitis found in this study was in accordance to the previous studies of Biffa *et al. (*2005); Mulugeta and Wassie (2013) and Tamirat (2007). This could be due to the fact that cows are in their peak milk production period that may favour the development of infection by retaining the milk in the mammary glands (Radostits *et al*., 2007 and Tamirat, 2007). Their udder during this time is also larger and more prone to exposure to pathogens. In addition, hormonal changes include increasing a five-fold of blood cortisol concentration and 17 β-oestradiol levels occur during the periparturient period (Weber *et al,*2004; Lippolis *et al,* 2006 and Pylorala *et al* 2008) and at the day of parturition (Lamote *et al*,2004; Weber *et al*,2004 and Burton *et al*,2005) might also suppress the immune system of dairy cows leading to increase incidence of mastitis. Cows at farms with poor hygiene condition 44%(n=22) are highly affected than those with good and better hygiene practices (Sori *et al*., 2005; Lakew *et al*., 2009). This might be due to the reason that, absence of udder washing, milking of cows with common milkers' and using of common udder towel, contamination of udder with faces and mud could increase the transmission and risk of infection.

From the total 49 mastitic milk samples, 85.7 % (n=42) were bacteriological positive and three bacterial species, *E.coli*, *S. aureus* and *CNS*, were isolated. In the current study *E.coli* was the predominant bacterial isolate in this study with over all prevalence of 21.1% (n=38) and comparable with the findings of Rajeev (2010) (26%) and Amit (2012) (27.3%). However, the result of this study was higher than the findings of Mekonnen and Tesfaye (2010), Hawari and Al-dabbas (2008), Kivaria and Noordhuizen (2007), Alemu *et al*. (2013), Matios *et al*. (2009), Sori *et al*. (2005), Mekibib *et al*. (2010) and Getahun *et al*. (2008) who reported *E. Coli* with over all prevalence of 7.5%, 15.6%, 14.1%, 11.6%, 7.5%, 0.75%, 4.6% and 0.5%, in different parts of Ethiopia, respectively. *E. coli* was isolated from 77.6 % (n=38) of mastitis milk samples. The isolation of *E. coli* in the present and other studies could be related to poor level of hygienic and milking practices in farms, and with the natural habitat of *E. coli* which can survive in faecal particles, dust and water for weeks and months (Quinn *et al.,* 2002), and can easily get access into mammary gland and trigger inflammatory processes that may lead to clinical and subclinical mastitis during stressful conditions (Quinn *et al.,* 2002; Radostitis *et al.,* 2006). *E. coli* is an environmental contaminant and widely used as an indicator of fecal

contamination (Quinn *et al.,* 2002; Radostitis *et al.,* 2006).

The overall prevalence of *S. aureus* 16.7% (n=30) in this study is almost in agreement with the findings of Almaw (2004) (16.67%) and Bitew et *al*. (2010) (15.5%) from different parts of the country. However, it is lower than the reports of Abebe *et al*. (2013), Million *et al*. (2015), Workineh *et al*. (2002), Melesse *et al*. (2012), Mesele *et al*. (2012) and Kerro and Tareke (2003), who reported 20.3%, 26.20%, 39.2%, 19.6%, 48.6%, and 40.3%, respectively, from different parts of the country. *S. aureus* was isolated from 61.2% (n=30) of mastitis milk collected and cultured in this study. The overall prevalence of *CNS* isolated in this study was 4.4% (n=8), respectively. This result is lower than findings of Molalegn *et al.* (2010) and Belina *et al*. (2016) who reported 51.9% and 37.7%, respectively. The isolation rate of *CNS* from mastitis milk samples in this study was 16.3 % (n=8). *Staphylococci* Spp are normally found on skin and mucous membranes, and as environmental contaminants. Any infections of *Staphylococci* are opportunistic and associated with trauma, immune-suppression, inter-current infections and other stress factors (Quinn *et al.,* 2002). *S. aureus* remains one of the most important causes of contagious clinical mastitis, and the most frequently isolated pathogen in subclinical mastitis cases worldwide (Radostits *et al*., 2007). Its ubiquitous presence in dairy herds is potentially due to its ability to cause chronically recurring infections, and to its resistance to antimicrobial treatment (Quinn *et al.,* 2002; Radostits *et al*., 2007). In addition, *CNS* are of great interest because they are currently the most commonly isolated microorganisms in cows and heifers in herds, and are currently considered emerging pathogens of bovine mastitis (Pyöräla *et al*., 2009)

In the present investigation, the antimicrobial sensitivity test indicated that the responses of the various milk bacterial isolates to different antimicrobial agents were variable. Among the isolated pathogens, the majority of *E. coli* isolates exhibited susceptibility to Streptomycin (100%) followed by Ampicillin (57.8%) and Sulfamethoxazole-Trimethoprim (57.8%). However, most of the isolates of *E. coli* were resistant to the two tested drugs; Cloxacillin (78.9%) and Erythromycin (57.9%). All isolates of *S. aureus* isolated in this study showed sensitivity for kanamycin (100%). However, the majority of *S. aureus* isolates showed intermediate effect for Enrofloxacin (46.7%), Ciprofloxacin (78.9%), Chloramphinicol (78.9%) and Streptomycine (50%). In addition, all CNS isolates were susceptible for Kanamycin (100%) and Gentamycine (100%), but resistance for three drugs; Bacitracin (100%), Penicillin G (100%) and tetracycline (100%). Even though, some variation existed the antimicrobial sensitivity test results bacterial isolates for the tested drugs is a general agreement with the findings of Abera *et al*. (2010), Abebe *et al*. (2013), Biniam *et al*. (2014), Zefie*et al*. (2014), Ousman *et al.* (2014), Fitsum (2014), Alemayehu *et al*. (2015) and Million *et al*. (2015). The variations in susceptibility of the isolated pathogens to the different drugs may be attributed to the prevailing differences on frequency and type of antibiotic treatments employed at the smallholder level. A previous report indicated that Antimicrobial resistance may arise either spontaneously by selective pressure or due to antimicrobial misuse by humans or overuse in feeding or treatment of animals by farmers (Schroeder *et al*., 2002).

Conclusion And Recommendations

Mastitis remains to be the most common economically damaging and zoonotic potential disease. In Ethiopia, mastitis has long been known in different dairy production systems, and reported by various studies with diverse etiological agents and risk factors. Even though the overall prevalence of mastitis recorded in this study was relatively lower as compared with other reports in different areas, both clinical and subclinical mastitis were identified. In this study *E.coli*, *S. aureus* and *CNS* were isolated from mastitic milk samples. All risk factors considered in this study were important potential risk for the overall prevalence of mastitis, *E.coli*, *S. aureus* and *CNS*. This study also discovered the emergency of bacterial isolates resistant to the various antimicrobial agents. Most of *E. coli* isolates were resistance for Cloxacillin and Erythromycin. More importantly, all *CNS* isolates showed resistance for Bacitracin, Penicillin G and tetracycline. In general, the results of this study, presence of mastitis and emergence of multidrug resistant pathogens could be among the major constraints of dairy production in the study areas.

Based on the above conclusions the following recommendations are forwarded:

- Further bacteriological and molecular studies must be conducted to characterize the major etiological agents of mastitis.
- Further and subsequent studies should be undertaken to investigate the risk factors that contribute to the occurrence of mastitis.
- Proper and careful milking practice and hygienic condition should be implemented in the farm.
- Regular monitoring of infection status of the udder should be undertaken and positive animals should be separated and treated early using appropriate drug.

- Identification of pathogen and antimicrobial sensitivity test must be performed before treatment of mastitis randomly with any antibiotics.

Acknowledgment

First and for most I would like to express my great gratitude to my advisor Dr Habtamu Addis or his continues assessment by giving materials and advising. Secondly I would like to express my great pleasure to thank my beloved friends for his contribution by giving computer and flash.

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8. Annexes

Annex 1: Thesis Data Collection Format.

Parameters	Sub parameter	Sample number (ID. NO)													
		1	2	3	4	5	6	7	8	9	10	11	12	13	…
Husbandry system	intensive														
	semi intensive														
	extensive														
Age	young														
	adult														
	old														
Breed	local														
	cross														
	exotic														
Parity No.	few														
	moderate														
	many														
Stage of lactation	early														
	mid														
	late														

CMT result	positive														
	negative														
Infected quarter	RF														
	RB														
	LF														
	LB														

Annex 2: General Information about the Farms.

1. Farm information

1.1. Farm name _____ name of owner _____

1.2. Location: sub city _____ kebelle _____

1.3. When established _____

2. Cow history_____

2.1. Breed: local ____ cross ____

2.2. Age: young ____ adult ____ old _____

2.3. Parity number: _____

2.4. Stage of lactation: early _____ mid _____ late _____ dry off _____

3. Management

3.1. Housing system

3.1.1. Floor: muddy _____ concrete _____

3.1.2. Draining system: very good _____ good _____ poor _____

3.1.3. Do you clean their house daily: yes _____ no _____

3.1.4. Ventilation system: very good _____ good _____ poor _____

3.2. Milking system

3.2.1. What type of milking do you use: machine _____ hand _____

3.2.2. Do you wash your hand before and after milking: yes_____ no _____

3.2.3. Do you wash the udder and teat of the cow before and after milking: yes ____ no

3.2.4. Do you use one towel for one cow: yes _____ no _____

3.2.5. Do you use any disinfectant before and after milking: yes _____ no _____

3.2.6. When do you milk mastitis cow: at fist ____ at last _____ without any order ___

3.3.7. Frequency of cleaning and disinfection

Annex 3: Composition and preparation of media used for isolation and identification *E. coli* (David, G. 2010).

MacConkey agar (sisco research laboratories. Pvt. Ltd. India) MM11

Composition g/liter: peptone 17g, agar 13.5g, lactose 10g, sodium chloride 5g, bile salts 1.50g, peptone mixture 3g, neutral red 0.003g, crystal violet.001g. Final pH 7.1 ± 0.2 at 25ºC.

Direction: Add 50.03g powder to distilled (deionized) water. Bring volume to 1 liter and mix thoroughly, gently heat and bring to boiling. Autoclave at 15 psi pressure at 121ºC for 15 minute, and dispense on patridish.

Triple sugar iron agar TSI (oxoidltd. Basigstockehamhire England)

Composition (g/liter): lab-lemco powder 3.00g, yeast extract 3.00g, peptone 20g, sodium chloride, lactose 10.00g, sucrose 10.00g, glucose1.00g, ferric citrate 0.3g, sodium thiosulphate 0.3g, phenol red 9.5g and agar 12.00g, PH =7.4±0.2 at 25° C.

Preparation: Suspend 64.6 grams of the medium in one liter of distilled water. Dissolve by heating and agitating frequently for one minute. Distribute in tubes and sterilize at 121º C for 15 minutes and cool in a slanted position, as to obtain butts of 1.5 – 2 cm depth.

Simmon's citrate agar (DIFCo' USA)

Composition (g/liter): magnesium sulfate 0.2g, ammonium dihydrogen phosphate 1g, sodium citrate 2.00g, sodium chloride 5.00g, agar15.00g, bromothymol blue 0.08g. Final pH 6.8 ± 0.2 at 25ºC.

Direction: suspends 24.4gram in one litter of distilled water and boil to dissolve completely on hotplate sterilize at121ºC for 15 minutes. Dispense in tube and put at slanted position until solidified.

Annex 4: Lists of reagent used.

Oxidiz reagent **(**0.5%-N, N, N, N tetra methy- l-p-phenylene diamine dihydrochloride)

- Grams reagent (crystal violet, Iodine, 95% ethanol, carbon fuchsine/ safranine)
- Catalase reagent Hydrogen pre oxide (3% H_2O_2)

Annex 5: Detail Procedure for Biochemical tests for isolation and identification of *E. coli*, *S. aureus* and *CNS*.

Gram stain (*Quinn *et al, 1999)

Principle: to categories bacteria as gram negative or gram positive, based on their cell wall structure. If it is gram positive stained by primary stain crystal violet while gram negative stained by counter stain carbon fuchsine.

Procedure;

- Make a thin smear.
- Allow the smear to dry in air.
- Fix the film by passing through the Bunsen flame 2 to 3 times.
- Flood the slide with crystal violet for 30-60 seconds.
- Rinse gently with tap water.
- Pour of the stain with iodine solution for 30-60 sec.
- Wash of the iodine the excess water from the slide.
- Decolorize with alcohol (95% ethanol) for 10-30 seconds.
- Counter stain with carbon fuschsine for 30-60 second and wash with water.
- Air dry and examine under oil immersion.

Result: pink and purple color was observed under microscope.

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

Principle:-oxidase test detect the presence of enzyme, cytochrome oxidase that can oxidize the substrate, Tetra methyl phenylene diamines dihiydrochloride forming colored and products indophenols (purple color). Procedure: prepare a solution of 1% Tetra methyl phenylene diamines dihiydrochloride and then a piece of filter paper is moistened in a plate with the fresh reagent and the test bacteria are streaked firmly across the filter paper with a glass rod. A dark purple color along the streak line within 10 second indicates positive reaction. ***Catalase test (*Quinn *et al., 2002)*** Principle: the enzyme catalase breaks down hydrogen peroxides to water and oxygen and resulting in visible formation of bubbles of oxygen.

Procedure:-A loop full of bacteria growth is taken from the top of colonies of the medium. The bacterial cells are placed on a clean microscopic slide and a drop of 3 % hydrogen peroxide (H_2O_2) is added. An effervescence of oxygen gas within a few seconds indicates a positive reaction.

Citrate test (*Baron *et al., 1994)

Principle: If sodium-citrate is utilized, alkaline products including sodium hydro oxide (NaOH) are produced. This is indicated by bromothymol blue, which is a blue color at alkaline PH.

Procedure: inoculates the surface of Simon citrate agar slant in a single strength and cap loosely and then incubate at 37^{o}C for 24hrs.

Result- positive = deep blue color, negative=Original green color

Triple sugar iron test (*wood land, *2006)

Principle: carbohydrate fermenting organism can change either the butt or slant and butt from red-orange to yellow that indicated by phenol red indicator. It also determines the production of gas and H_2S production.

9/19/2020