

HARAMAYA UNIVERSITY

DIRECTORATE FOR POST GRADUATE PROGRAM

**PREVALENCE, ASSOCIATED RISK FACTORS, AND ANTIMICROBIAL
SENSETIVITY TEST OF *STAPHYLOCOCCUS AUREUS* ISOLATED
FROM BOVINE MASTITIS IN BABILE DISTRICT EASTERN
HARARGHE ZONE, ETHIOPIA**

MSC THESIS

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HARAMAYA UNIVERSITY, ETHIOPIA

**HARAMAYA UNIVERSITY
DIRECTORATE FOR POST GRADUATE PROGRAM**

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HARARGHE ZONE, ETHIOPIA**

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Program Directorate

HARAMAYA UNIVERSITY

**In partial Fulfillment of the Requirements for the Degree of
MASTER OF SCIENCE IN VETERINARY EPIDEMIOLOGY**

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DEDICATION

This thesis manuscript is dedicated to my parents, my mother Hindiya Abdula and my brother Mr. Sh/abduselam Mohammed-Kemal whose encouragements have always been with me and which played a key role in guiding my life in general and in the successful completion of this work in particular.

STATEMENT OF THE AUTHOUR

By my signature below, I declare and affirm that this Thesis is my own work. I followed all ethical and technical principle of the scholarship in the preparation, data collection, data analysis and completion of thesis thesis. My scholarly matter that is included in the thesis has been given recognition through citation.

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LIST OF ACRONOMS

BAP	Blood Agar Plate
BWAO	Babile Woreda Agricultural Office
CMT	California Mastitis Test
CPS	Coagulase positive sthaphylococcus
CSA	Central statistical Authority
FBD	Food Borne Disease
IMI	Intramammary Infection
MRSA	Methicillin-resistant <i>S. aureus</i>
MSA	Mannitol Salt Agar
PAB	Purple Agar Base
PCA	Plate Count Agar
SCM	Subclinical Mastitis
SES	Staphylococcal enterotoxins
SFP	Staphylococcal Food Poisoning
TSB	Tryptone Soya Broth
TSS	Toxic shock syndrome
VRBA	Violet Red Baird Agar

BIOLOGRAPHICAL SKETCH

The author, Abdelahi Mohammed-kemal was born on 23 September 1982 in Gola oda District in burka town from his father Mohammed-kemal Ibrahim and his mother Hindiya Abdula. When reached school age, he attended elementary education at Burka elementary school and secondary school education at Bedano town. After completion of Higher education entrance qualification certificate in 2002, he joined Jimma University in 2007 with collage of Agricultural and veterinary medicine and graduated in 2011 in BVSC. He employed in Babile district rural development bureau as animal health servant until 2012. Immediately in 2012 EC he joined the school of graduate studies of Haramaya University to pursue his study leading to the degree of Master of Science in veterinary epidemiology.

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**PREVALENCE, ASSOCIATED RISK FACTORS AND ANTIMICROBIAL
SENSITIVITY TEST OF *STAPHYLOCOCCUS AUREUS* ISOLATED FROM BOVINE
MASTITIS IN BABILE DISTRICT EASTERN HARARGHE ZONE, ETHIOPIA**

ABSTRACT

Mastitis is a disease of major economic importance in dairy industry worldwide particularly in Ethiopia. A cross-sectional study was carried out from August 2021 to March 2022 to determine the prevalence and associated risk factors of bovine mastitis and antimicrobial sensitivity test of S. aureus isolates in Babile district, eastern Ethiopia. A total of 384 lactating cows managed under extensive production systems were selected from three selected kebeles of Babile district. California mastitis test (CMT) was used for screening subclinical mastitis. Likewise, physical examination was also conducted to detect clinical mastitis. Out of the total animals examined, 50.5% (194/384) had mastitis, where 5.5% (21/384) and 45% (173/384) were clinical and subclinical mastitis respectively. The quarter-level prevalence was 19.01% (292/1536), from which the clinical and subclinical form was 2% (32/1536) and 11.97% (184/1536) respectively, whereas 2.7% (42/1536) of the quarters were blind. Milk samples from positive quarters were cultured to isolate S. aureus using standard bacteriological test; accordingly, 30.9% of sample showed growth of S. aureus. Among the risk factors analyzed using a univariable logistic regression analysis, except lactation stage, all factors such as kebeles, age, parity, body condition, udder size, milk yield, tick infestation and teats injury were found to be statistically significant with the occurrence of mastitis ($p < 0.05$). However, using multivariable logistic regression analysis udder size, tick infestation and teat injury were significantly associated the occurrence of bovine mastitis in the study area while the other remaining factors were not significant. Antimicrobial sensitivity test results of the 35 S. aureus isolates showed that, 100% susceptible to chloramphenicol and gentamycin. In contrast, the highest (94.2%) resistance was observed for penicillin G followed by amoxicillin (28.57%) and Erythromycin (20%). The study confirmed S. aureus was an important cause of bovine mastitis particularly related to potential risk factors and significant number of S. aureus isolates showed resistance against some chemical drugs in the study area. Hence, it warrants serious attention for the control of mastitis considering the risk factors and alternative treatment options.

Key words: Antibiotics, Bovine Mastitis, Prevalence, Resistance, Risk Factors, S. aureus

1. INTRODUCTION

1.1. Background

Ethiopia has large potential for dairy development due to its huge cattle population and favorable climate for improved high-yielding animal breeds for milk production (CSA, 2010). The total cattle population in the country is estimated to be about 56.71 million. Out of this, the female cattle constitute about 55.45%, and the remaining 44.55% are male cattle. From the total cattle, 98.66% are local breeds and the remaining are hybrid and pure exotic breeds that accounted for about 1.19 and 0.14%, respectively (Central Statistical Agency, 2015). Accordingly, the per capita milk availability was estimated at 17 to 18 kg per year compared to the global average of 100 kg per year (Dimshasha Tolera *et al.*, 2021). On the other hand, 42% of the total cattle herds for the private holdings are milking cows. However, milk production often does not satisfy the country's milk requirements; due to a multitude of associated factors such as poor genetic performances, sub optimal feeding and diseases (Gutu Kitila *et al.*, 2021).

Bovine mastitis is a common disease of high economic importance in dairy herds worldwide (Jean Baptiste *et al.*, 2019). It is a multi-etiological disease and many of microorganisms are implicated as causes. Majority of microorganisms that are responsible for mastitis and spoilage of milk are of bacterial origin (Tesfaheywet Zeryehun and Gerema Abera, 2017). The most common bacteria involved are *Staphylococcus aureus* (*S. aureus*), *S. agalactiae*, *S. pyogenes*, *Trueperella pyogenes* (*T. pyogenes*), *E. coli*, *K. pneumoniae*, *K. oxytoca*, *Enterobacter aerogenes*, *Pasteurella* spp. (Abdalhamed *et al.*, 2018; Zhang *et al.*, 2018). *S. aureus* is the common cause of mastitis in dairy animals. More than 50 species and sub-species of *Staphylococcus* have been characterized to cause Staphylococcal mastitis. Based on the ability to coagulate rabbit plasma, staphylococci are categorized as coagulase-positive *Staphylococcus* (CPS) and coagulase-negative *Staphylococcus* (CNS). Among CPS, *S. aureus* is the frequent causative agent of clinical or subclinical mastitis in cattle (Dimshasha Tolera *et al.*, 2021).

The disease can be classified into clinical mastitis which is characterized by visible signs of illness such as swelling of udder and milk abnormalities and subclinical mastitis which have not visible clinical signs unless decrease of milk production. Subclinical mastitis (SCM) is 15 to 40

times more prevalent than clinical mastitis and infected cows become major source of infection for healthier cows (Ruegg, 2017). This happens when there is lack of implementation of preventive measures such as not wearing gloves during milking or not milking mastitis infected cows last (Abebe *et al.*, 2016). Moreover, several management practices that include Genetic selection, nutrition, dry cow therapy and lactation therapy, Acoustic pulse therapy (APT) also known as shockwave therapy utilizes the hand-held instrument that produces pulsing pressure waves. Such waves penetrate through the deeper tissues and can break scar tissue of the chronic wounds leading to revascularization (Khan *et al.*, 2021).

Bovine mastitis is one of the major diseases severely affecting animal health, milk yield, quality of milk and milk products (Dimshasha Tolera *et al.*, 2021). Mastitis is probably one of the factors that hinder the increase in net milk production yield in East Africa region compared to other part of the world (Motaung *et al.*, 2017). Cows with SCM have a reduced milk yield and produce milk of lesser quality (Bobbo *et al.*, 2017), which affects farm revenues. Although it is difficult to harmonize methods used in evaluating losses due to both forms of mastitis, the total cost per cow per year was estimated to be 338 € in Sweden in 2010 (Nielsen *et al.*, 2010), 240 € in the Netherlands between 2005 and 2009 and \$ 117.35 in the USA in 1997 (Van Soest *et al.*, 2016). Economic estimations of losses associated with mastitis in Africa are rare and not well documented (Motaung *et al.*, 2017); thus, there is an overall low motivation for its control.

Apart from the substantial economic losses associated with the disease, mastitis has serious zoonotic potential and has been associated with the increasing development and the rapid emergence of multidrug resistance strains (Christine *et al.*, 2020). *S. aureus* causes a diverse array of diseases in both human and animals. Human infections range from mild skin infections to life threatening ones; such as bacteremia, endocarditis, necrotizing pneumonia and toxic shock syndrome (TSS) (Alem Abrha *et al.*, 2020). *S. aureus* is the main cause of mastitis as well as it is the second identified bacteria causing food poisoning in humans through its ability to produce several enterotoxins in milk and milk products (Rania *et al.*, 2020). Mastitis is a problem for the dairy industry in East Africa where the prevalence of SCM was 86.2% in Uganda (Abrahmsén *et al.*, 2014), Prevalence of SCM is 76.2% in Rwanda (Jean Baptiste *et al.*, 2019), 80% in Kenya (Christine *et al.*, 2020). In Ethiopia, there are several bovine mastitis studies showing spatial variations in prevalence and risk factors. According to most recent studies, the prevalence of

mastitis ranges from 39.5 – 62.6% (Gutu Kitila *et al.*, 2021; Melak Tezera and Endris Aman, 2021; Rahmeto Abebe *et al.*, 2020; Birhanu *et al.*, 2017; Mekonnen *et al.*, 2017).

The diagnosis starts with physical clinical examination which involves palpation of the mammary gland and visual inspection of milk. In CM cases, the infected quarter may manifest inflammatory changes such as hot, red, swollen and painful. Inflammatory changes in milk include change of color such as bloody or watery milk, change in consistency which can be viscous, watery and/or clots, and change in smell of the milk (Lakew *et al.*, 2009). Indirect tests such as, California Mastitis Test (CMT), White Side Test (WST), Surf Field Mastitis Test (SFMT) can be used that are simple and do not require any complex laboratory equipment (Mahfuz *et al.*, 2020). The SCM can be known only after laboratory examination, as there are no gross inflammatory changes in the udder tissue (Rahman *et al.*, 2017). However, the California mastitis test (CMT) is used as a screening test for sub-clinical mastitis. It was carried out according to the procedure described by (Quinn *et al.*, 2002).

The mastitis caused by *S. aureus* is susceptible to a variety of antibiotics in vitro but due to the peculiar biology of staphylococci along with their adaptation to the bovine host environment, development of microabscesses and biofilm-formation, some of the antibiotic agents become ineffective clinically (Rainard *et al.* 2018). Recent study conducted in Ethiopia stated that *S. aureus* was also found susceptible to antibiotics such as trimethoprim, erythromycin, gentamicin, ciprofloxacin, and chloramphenicol, suggesting that these antibiotics have not been commonly used as the treatment of choice for mastitis in farms (Letebrhan Yimesgen *et al.*, 2021; Dimshasha Tolera *et al.*, 2021).

The absence of post milking teat dipping, lack of culling of chronically infected cows, absence of dry cow therapy, and the invariable hand milking practice among the dairy herds and the optimum temperature in most parts of Ethiopia are the predisposing factors of mastitis (Ababe *et al.*, 2016). Parity, stage of lactation, previous history of mastitis, floor type, milking hygiene, and udder injury are also the potential risk factors of mastitis (Dimshasha Tolera *et al.*, 2021).

In general, Mastitis could be prevented by implementing proper animal health management systems. However, most of the emerging dairy farms in Ethiopia lack optimum management practices and are predisposed to mastitis (Letebrhan Yimesgen *et al.*, 2021)

1.2. Statement of the Problem

Bovine mastitis is a common disease of high economic importance in dairy herds worldwide. It can reduce milk yield, increase culling rate, incur treatment cost and occasionally result in death from severe infection (Rahmeto Abebe *et al.*, 2020). It can also affect the quality of milk and it is the most widespread infectious disease in dairy cattle. Mastitis is of a particular concern for farmers in developing countries like Ethiopia where the disease hinders dairy productivity (Fufa *et al.*, 2013; Teshome Gemechu *et al.*, 2019). In Ethiopia, different recently published studies were conducted on bovine mastitis with the prevalence range from 30.5 - 64.3% (Tesfaheywet Zeryehun and Gerema Abera *et al.*, 2017; Biniam Tsegaye *et al.*, 2019; Melak Tezera and Endris Aman, 2021; Gutu Kitila *et al.*, 2021; Dimshasha Tolera *et al.*, 2021). For this reason it requires the development of methodologies of control program under the prevailing husbandry system.

In Ethiopia, various studies reported on the prevalence, etiology and risk factors of mastitis in dairy farms managed by semi-intensive and intensive systems (Seyoum *et al.*, 2017). *S. aureus* is recognized worldwide as a leading pathogen causing many serious diseases in dairy and healthcare surroundings. Accordingly, few studies were conducted in the different parts of Ethiopia on the prevalence of *S. aureus* ranging from 12.5% - 59% and its highest level of resistance which is >85% to the penicillin G and tetracycline (Letebrhan Yimesgen *et al.*, 2021; Kaleab Tesfaye *et al.*, 2021; Dimshasha Tolera *et al.*, 2021). In the last few decades, Staphylococcal food poisoning has been reported as the third cause of food-borne illnesses in the world (Weldemelak *et al.*, 2020). Moreover, antibiotic-resistant *S. aureus* isolates pose a severe challenge to both veterinary and health professions and dairy cattle producers because they have a negative impact on the treatment of Staphylococcal mastitis cases. In addition, *S. aureus* udder infection is of economic significance as it often results in increased veterinary and treatment costs as well as premature culling of affected cows (Matlale *et al.*, 2020).

The indiscriminate use of antibiotics has led to the development of multiple antibiotic resistances thereby rendering the antibiotic treatment ineffective. Administration of antibiotics for the feeding of animals for curative purposes or as growth promoters may also be a major factor in the selection of antimicrobial-resistant bacteria (French Food Safety Agency, 2006). Emergence of antibiotic-resistant bacteria has become a global public health concern affecting human and veterinary medicine due to the persistent circulation of resistant strains of bacteria in the environment and the possible contamination of water and food. Therefore, investigation of a wide spectrum of antimicrobial resistance of *S. aureus* isolated from cows with mastitis is crucial not only for bovine mastitis control, but also for public health (Xavier *et al.*, 2017; Kadlec *et al.*, 2019).

As mentioned here the occurrence of bovine mastitis related to *S. aureus* in different part of Ethiopia is prevalent. Many studies confirmed that *S. aureus* developed antibiotic resistances to commonly used drugs. On the other hand, in most pastoral and semi-pastoral part of Ethiopia, a large number of farmers treat their sick animals themselves or get the services from Para-veterinarians. Such practices are particularly very common in Babile district where there is limited veterinary service. This leads to increase the drug resistance and promote poor prognosis during treatment of mastitis in study area. Also in the study area, most of a time animal health technicians use only penicillin G as a drug of choice for the treatment of mastitis. However, there is no recent study which shows the current prevalence and antimicrobial resistance of *S. aureus* originated from cows' milk in Babile District, eastern Hararghe, Ethiopia. All of the previous study in and around the study area focused on intensive dairy farm. On the other hand, there is no study conducted on the prevalence and antimicrobial resistance of *S. aureus* on dairy cows kept under extensive management system where the cows are often provided with natural pasture and agricultural by-products and occasionally supplemented with imported feed. Therefore, the current study was conducted with the aim of estimating the prevalence of mastitis associated with *S. aureus*; related risk factors of clinical and subclinical mastitis of lactating cows kept under the extensive system and determination of the antimicrobial susceptibility of the isolated *S. aureus* in Babile District.

1.3. General objective

- To determine the prevalence of bovine mastitis, associated risk factors and antimicrobial susceptibility test of *S. aureus* in the study area.

1.4. Specific Objectives

- To determine the prevalence of bovine mastitis associated with *S. aureus* in Babile district
- To detect antimicrobial resistance profile of *S. aureus* isolated from milk samples of clinical and subclinical bovine mastitis in Babile District.
- To asses risk factor associated with the occurrence of bovine mastitis caused by *S. aureus* in study area.

2. LITERATURE REVIEWS

2.1. Bovine Mastitis

Mastitis is an inflammation of the udder caused by a variety of microorganisms, mostly bacteria that gain access to the interior of the mammary glands through the teat canal. Bovine mastitis is one of the most frequently encountered and important diseases of dairy cows in Ethiopia. (Getaneh and Gebremedhin, 2017). It is a result of host immune response to infectious agents affecting the udder. Normally, there is a balance of micro flora in a healthy udder. The Intramammary micro biota is composed of complex community of diverse bacteria (Andrews *et al.*, 2019). The commensal mammary micro biota present in the healthy udder plays a major role in the immune homeostasis (Derakhshani *et al.*, 2018). Therefore, a disruption in the diversity of udder microbiota (dysbiosis) can have an impact on mastitis. The normal microbiome of udder is an important factor to be kept in consideration when making diagnosis of mastitis as healthy quarters also contain some bacteria. Any assault or congenital anomaly of the udder or teat, such as teat fistula, teat spider, leaky teat, and udder wounds that exposes the udder to the external microbes or retention of milk tends to cause mastitis (Rambabu *et al.*, 2011).

2.2. Type of mastitis and clinical Relevance

Mastitis can be epidemiologically categorized into contagious and environmental mastitis and are caused by a wide spectrum of pathogens (Khan *et al.*, 2021). In addition to that, mastitis can also be classified as either clinical or subclinical form. Clinical mastitis is characterized mainly by appearances of changes in the milk such as flakes and clots and presence of signs of inflammation on the mammary glands such as swelling, heat, pain, and edema, (Blowy and P. Edmondson, 2010). as well as systemic signs on the animal including fever, rapid pulse, appetite loss, dehydration, and depression Subclinical mastitis is that which is mainly characterized by absence of visible appearance of changes in the milk or udder, but milk production decreases, bacteria are present in the secretion, and composition is altered. Hence, detection of the milk is not possible clinically but only by determining high somatic cell count (SCC) in milk or by bacterial culture (Tesfaheywet Zeryehun and Gerema Abera, 2017).

Any increase in the level of humidity and pollution in the environment of the barn also increases the load of bacterial pathogens in the environment. One of the studies has shown 74.7% prevalence of mastitis at herd-level while 62.6% prevalence at cow level. In relation to the subclinical and clinical mastitis, the former type appears to be responsible for the majority of cases (59.2%) than the latter (3.4%)(Abebe *et al.*, 2016). In General, Clinical mastitis, is less prevalent, whereas subclinical mastitis is more common and results to reduced milk production without observable clinical signs or abnormalities in the udder or milk. For this reason, subclinical mastitis is challenging to diagnose, persists longer in the herd, and is associated with higher losses compared to clinical mastitis (Christine *et al.*, 2020). The somatic cell count (SCC) in the milk should be less than 200,000 per mL in the healthy cow. Somatic cells are mostly white blood cells (WBCs), viz., infiltrated neutrophils as well as macrophages into the tissue of the mammary gland due to inflammation (Akers and Nickerson 2011). *S. agalactiae* mainly localizes in the udder and causes persistent infections with higher SCC (Kefee 2012).

2.3. Mastitis causing pathogens

Mastitis is a multi-etiological disease and some bacteria are mainly responsible for clinical, subclinical, contagious, and environmental mastitis. Majority of the pathogens that cause clinical bovine mastitis are from environmental or ubiquitous origin. In adverse, contagious agents are mainly related to subclinical infections (Abebe *et al.*, 2016; Klaas and Zadoks 2018). The most common bacteria involved are *S. aureus*, *S. agalactiae*, *S. pyogenes*, *Trueperella pyogenes* (*T. pyogenes*), *E. coli*, *K. pneumoniae*, *K. oxytoca*, *Enterobacter aerogenes*, *Pasteurella* spp. (Abdalhamed *et al.*, 2018; Zhang *et al.*, 2018).

Among these contagious pathogens *S. aureus*, *S. dysgalactiae* and *S. agalactiae*, are the predominant organisms while major environmental pathogens are members of Enterobacteriaceae particularly *E. coli*, *S. uberis*. *S. agalactiae* are the most common Gram-positive bacterium from clinical mastitis, followed by *S. aureus* while *Klebsiella* spp. and *E. coli* are the most isolated Gram-negative bacteria from clinical mastitis (Cortinhas *et al.*, 2016). *S. agalactiae* and *S. aureus* primarily spread through contact, so herd biosecurity may be considered as an important preventive measure for reduction and elimination of the reservoirs. In primiparous cows, the highest loss of milk is due to *S. aureus*, *Klebsiella* spp. as well as *E. coli*.

In older cows, significant losses are due to infections by *Streptococcus* spp., *T. pyogenes*, *S. aureus*, *Klebsiella* spp., and *E. coli*.

In general, *S. aureus*, *S. agalactiae*, and *S. uberis* are common pathogens causing mastitis, whereas *Mycoplasma bovis* and *Corynebacterium bovis* are less frequently involved (Vakkam€aki *et al.*, 2017). *Coagulase-negative staphylococci* and their role in causing mastitis should also be seriously taken into consideration. *S. agalactiae* along with various pathogens including *Prototheca* sp., *Streptococcus* spp. and *T. pyogenes* are associated with majority of the cases of mastitis. Mastitis occurs in its heaviest form when associated with infections due to coliforms, *CAMP-negative Streptococcus* spp., *T. pyogenes*, *S. agalactiae*, fungi (yeast-like) and *Prototheca* sp. *Corynebacterium* spp. (40%) and *S. aureus* (32%) were the most common isolates found by Steele and McDougall (2014) in cases of sub-clinical mastitis in New Zealand. *Prototheca* spp. are pathogenic algae and opportunistic pathogens that cause mastitis in dairy herds and possess zoonotic potential (Alves *et al.*, 2017; Dos Anjos *et al.*, 2019). *S. aureus* has been the most common pathogen identified in mastitis (McParland *et al.*, 2019). *Methicillin resistant-S. Aureus* (MRSA) CC22-MRSA-IV has been reported as intramammary pathogen by Magro *et al.* (2018).

Among all the pathogens of bovine mastitis, *S. aureus* is recognized as the most common causative agent of bovine mastitis in Ethiopia (Birhanu Misrak *et al.*, 2017 Lakew *et al.*, 2009). It is also reported that the most common contagious pathogens are *S. aureus* and *Strep. agalactiae* indicating that their presence in high prevalence could be due to lack of effective udder hygiene and poor milkers' hygiene practice during milking. The most common environmental mastitis pathogens are also coliform bacteria (Haftu *et al.*, 2012).

2.4. *Staphylococcus Aureus*

Staphylococcus aureus is a significant public health bacterial pathogen, causing mastitis in dairy animals including cattle, buffalo, sheep and goats (Abdel-Moein and zaher, 2019). More than 50 species and sub-species of *Staphylococcus* have been characterized to cause *Staphylococcal* mastitis. Based on the ability to coagulate rabbit plasma, *staphylococci* are categorized as coagulase-positive *Staphylococcus* (CPS) and coagulase-negative *Staphylococcus* (CNS).

Among CPS, *S. aureus* is the frequent causative agent of clinical or subclinical mastitis in cattle. It is also a foodborne pathogen and the leading cause of foodborne intoxications around the globe (Fetsch and Johler, 2018). *S. aureus* associated mastitis and toxins produced by *S. aureus* lead to great economic losses in dairy farms due to: (1) reduction in the milk production, (2) alteration in the composition and quality of the produced milk, (3) the need to discard the produced milk, (4) early culling of infected animals, and (5) high cost of treatment and control (Martins *et al.*, 2019).

On the other hand, according to the official European Union data from 2011, 346 foodborne outbreaks were attributed to *Staphylococcus* spp. Furthermore, *S. aureus* can produce other virulence factors such as exfoliative toxin A and B and toxic shock syndrome (TSST-1) (Rania and Amira, 2020). Although, pasteurization can destroy *S. aureus* but cannot affect their enterotoxins which are transmitted to humans and cause a public health hazard. Therefore *staphylococcus aureus* causes a diverse array of diseases in both human and animals, including dairy cows. Human infections range from mild skin infections to life threatening ones; such as bacteremia, endocarditis, necrotizing pneumonia and toxic shock syndrome (TSS). The major diseases in animals include mastitis in dairy cows, joint infections in poultry and surgical site infections equine (Alem Abrha *et al.*, 2020).

2.4.1. *Staphylococcus aureus* plasma biofilm feature

Staphylococcus aureus is a gram-positive coccus that colonizes the nasal mucosa and skin of healthy individuals. This organism can cause a wide range of diseases from skin or soft tissue infections to systemic and fatal diseases. In particular, methicillin-resistant *S. aureus* (MRSA) is a hazardous organism because of its resistance to multiple antibiotics and biofilm formation ability. It is well known that biofilm-forming bacteria can survive in the presence of high concentrations of antimicrobial agents (Ayami *et al.*, 2019). Bacterial biofilms consist of a variety of components and substances from both the bacteria (polysaccharide, peptidoglycan, and DNA) and the host (cell debris, coagulation products, and DNA). Biofilm composition varies depending on the causative organism and/or host factors. *S. aureus* possesses a specific virulence factor called coagulase, which plays a significant role in biofilm formation during *S. aureus* infections (Crosby *et al.*, 2016). So, coagulase binds to host prothrombin and forms active

staphylothrombin complexes, which converts soluble monomeric fibrinogen into self-polymerizing insoluble fibrin and activates a coagulation cascade.

2.4.2. *Staphylococcus aureus* strains spectrum

Staphylococcus aureus is the most important causative agent of intramammary infections in dairy animals with potential virulence of surface components (adhesins, capsular polysaccharides, protein A), toxins, extracellular enzymes and coagulase (Sutra L, Poutrel B., 1994). A wide array of genotypic variations has been observed with great genetic diversity in the isolates of bovine as well as caprine origin. Some of the variants are common throughout the globe as ruminant specific *S. aureus* while others are geographic related in the literature (Jørgensen *et al.*, 2005). Inflammatory respondent metabolic pathways (BoLA-DRA, GLYCAM1, FCER1G, B2M, CD74, NFKBIA and SDS), milk constituent associated (CSN2 and CSN3) and immunity related (B2M and CD74) are also specific strains of *S. aureus* of dairy mastitis (Filipe *et al.*, 2019).

2.4.3 Pathogeneses of *S. aureus*

Bacterial pathogens can recognize, respond and adapt to the harsh environmental conditions that prevail in mammalian hosts during infection. Despite the host's immune response and antibacterial treatment, it helps to invade, calm, and survive within the host (Masse, 2019). *Staphylococcus aureus* produces a variety of enzymes, including coagulase, which can coagulate plasma, converting plasma fibrinogen to fibrin, coat bacterial cells, and inhibit nutrition. Hyaluronidase (also called diffusion factor) can break down the hyaluronic acid present in tissues and support the spread of *Staphylococcus aureus* in the host. It also produces DNase (deoxyribonuclease), an enzyme that breaks down DNA. Lipase breaks down lipids, and staphylokinase also breaks down the fibrin. It is also known that *Staphylococcus aureus* produces β -lactamase, esterase, elastase and phospholipase for drug resistance, and these enzymes promote colony formation and pathogenicity (Quinn *et al.*, 2011). Other toxic factors of *S. aureus* include leukocidin (which can cause cytolytic destruction of phagocytic cells in some animals) and toxic shock syndrome toxin (TSST). The latter can cause an overproduction of lymphokine, which can lead to tissue damage (Amjad *et al.*, 2021).

Depending on the strain, *S. aureus* can release some toxins that are major virulence factors. These toxins act on cell membranes containing superantigens, exfoliating toxins, and some two-component toxins such as alpha toxins, beta toxins, gamma toxins, delta toxins and Panton Valentine's toxins and leukocidin (PVL) (Kashif *et al.*, 2019). *Staphylococcus aureus* has a characteristic virulence which is called Protein A, which plays an important role in strategies for evading immunity, is immobilized on the *staphylococcus*-peptide-glycanpenta-glysin bridge using transpeptidase sortase. Protein A is able to bind to fragments of the crystal region (Fc) of IgG antibodies (γ -immunoglobulins). This phenomenon is due to the fact that Protein A binds to an IgG antibody produced against the target microorganism and reacts with the corresponding antigen usually present in the patient sample to perform an aggregation test in which a visible aggregation reaction can be observed. The *Staphylococcus aureus* strain is known to produce pigments such as staphyloxanthin and gold carotenoid pigments (Amjad *et al.*, 2021).

2.5. Epidemiology

Mastitis is a worldwide problem affecting mainly lactating animals but during their dry periods as well. It affects a wide range of hosts; different breeds, age, parity, lactation stage, and milk yield categories (Rebeka *et al.*, 2019). Even if the degree varies, mastitis exists under different environmental conditions and poor management systems (Klaas and Zadoks, 2018).

2.5.1. Transmission of *Staphylococcus aureus* in udder infections

The main reservoirs of *S. aureus* are infected mammary glands, ducts, and papillary lesions. These bacteria also found on the skin, nose and teat passages. The bacteria spread to uninfected areas through the lining of the teat cups, milker's hands, towels and fruit flies. *Staphylococcus aureus* does not persist on healthy teat skin, but tends to colonize damaged skin and teat lesions (Petersson *et al.*, 2010). The body reproduces the infected lesion, increasing the likelihood of teat colonization and subsequent udder infection. Heifers infected during calf pregnancy are an important reservoir that can be passed on to uninfected *S. aureus* herds. There has been much controversy over the route of infection with *S. aureus* in early prenatal heifers, but it is possible that the cause is a calf that was fed on a mother infected with *S. aureus* (Amjad-Islam *et al.*, 2021).

2.5.2. Associated Risk Factors

Mostly all microorganisms are involved in mastitis but some factors, pendulous udder with long teats, larger size of teat orifice in high yielding cows, traumatic injuries may play an important role as inciting factor of mastitis in dairy cows. Poor management, nutrition, and hygiene, are mostly associated with mastitis (Rahman *et al.*, 2017). The rate of intramammary infections (IMI) in breeding age and pregnant heifers is much higher. Several potential heifer mastitis risk factors have been identified which include- increased age at first calving, milk leakage (Yang *et al.*, 2015), calving in early summer, increased herd somatic cell count (SCC), presence of organisms on the skin, feeding calves with mastitis milk, contact among calves and with adult cows, absence of antibiotic therapy to heifers, poor milking practices and poor housing conditions (Oliveira *et al.*, 2015). Blood in the milk, udder and teat edema are also considered to be important risk factors. The presence of IMI before calving increases the risk of infection during lactation (Yang *et al.*, 2015). Transition or periparturient period is a high-risk time for modern dairy cows. IMI at calving has increased the risk of clinical mastitis within the first week of lactation. Mastitis before parturition and within first week after calving has increased the risk of further cases of mastitis and culling during first 45 days of lactation (Kour *et al.*, 2020). However, milk production often does not satisfy the country's milk requirements due to a multitude of associated factors such as poor genetic performances, sub optimal feeding and diseases (Gutu Kitila *et al.*, 2021).

2.5.2.1. Host factor

For contagious pathogens, adult lactating cattle are most at risk for infection, either while lactating or during the dry period. Stage of lactation is one of the intrinsic factors that determine the level of infection. Particularly, the early stage of lactation is more prone to mastitis occurrence than the remaining stage of lactation (Rahmeto *et al.*, 2016). Age of cow is also a factor that is associated with the case of mastitis where commonly aged cows are more liable to mastitis than others. Parity has a direct relationship with mastitis occurrence. The presence of mastitis increases with increasing parity number (Rahman *et al.*, 2017). The likelihood of mastitis is higher in multiparous cows having more calvings compared with primiparous cows. This might partly be associated with the position of the udder in older cows that let exposure of

the teat and udder to injury and pathogens easily so that makes it to be the most susceptible one to mammary infections (Rahmeto *et al.*, 2016). Breed of a cow is also another factor that determines presence or absence as well as the level of mastitis. Mostly high producing cows are more exposed to mastitis than low-level milk producers.

2.5.2.2. Environment factor

The primary source of environmental mastitis pathogens is the cows' environment. The bedding materials used for housing cattle may contaminate fomites, skin, and teat lesions and could be a potential source of mastitis-causing pathogens. It appeared that the floor was a potential source for mastitis organisms, particularly for the environmental pathogens, to enter the udder through the teat orifice (Rahmeto *et al.*, 2016). Bedding and bedding management contribute to udder health and milk microbial quality. A clean, dry adequately bedded stall maintains cow cleanliness, inhibits microbial growth, and their transfer to a teat (Gutu Kitila *et al.*, 2021).

2.5.2.3. Agent Factor

Mastitis is a complex disease with multi-etiology. A wide range of pathogens including bacteria, viruses, fungi, and the toxins of these agents can cause the disease. Most of the agents that cause mastitis are opportunistic environmental pathogens that are more favored during a poor hygienic condition in the cow's environment as well as related to the milkier. Others are contagious pathogens that are systemic and may finally get access to the udder and localize themselves there and cause infection. Mastitis is caused by a wide spectrum of pathogenic agents that penetrate the teat canal and multiply in the udder cistern (Oliveira *et al.*, 2015). The inflammation severity depends on the causative agent. The etiology of mastitis is in a continuous changing spree, with the evidence of new microbial species being incriminated (Shaheen *et al.*, 2016).

2.5.2.4. Management

The presence of mastitis was significantly influenced by herd size (Tesfaheywet and Gerema, 2017). There is a high chance of occurrence of mastitis in herds under intensive management. In intensively managed large herds, the higher chance of mastitis occurrence might be associated with increased exposure of the cows for mastitis pathogens in their environment due to high stocking density, dirty ground, poor ventilation, and high humidity (FAO, 2014). Cows with

poor body conditionleaking milk, or previous udder infections had a significantly higher risk of clinical mastitis. Milking a cow infected with mastitis and/or an infected teat, at last, is the best technical management to reduce the risk of transmission of the disease from infected one to the others.

2.5.3. Prevalence status of resistant *S. aureus*

The prevalence of MRSA was higher in America than Europe. Different studies reported that prevalence rates of greater than 70% in South Korea and Vietnam, and less than 50% in Portugal, Greece, and Italy (Chukwunonso *et al.*, 2018). In Egypt, a study shows prevalence of *S. aureus* was 17.2%. In another study, 70– 73% of *S. aureus* strains isolated from various foods were resistant to β -lactam such as Penicillin and Ampicillin (BeyeneTakele *et al.*, 2017). In study done in South Africa the prevalence of MRSA was 5.7–7% in commercial farms. In other African country, the prevalence of MRSA was higher Nigeria 28.57%, in Morocco 15% and low prevalence in Kenya 7.8% were recorded (Lozano *et al.*, 2016). In Ethiopia, overall prevalence of *S. aureus* with different study showed that 15.3% originating from raw cows' milk, 25% from swabs of milkers' hand, 20% from swabs of milking bucket, and 10% from swabs of drying towel (Regasa Sema *et al.*, 2019). The higher prevalence of MRSA were reported in Ethiopia (60.3%).

2.6. Diagnosis of mastitis caused by *S. aureus*

Conventional diagnostic tests for mastitis are usually qualitative with lesser specificity and sensitivity, whereas advanced tests are quantitative, highly specific and sensitive (Hussein *et al.*, 2018; Chakraborty *et al.*, 2019). The SCM can be known only after laboratory examination, as there are no gross inflammatory changes in the udder tissue (Rahman *et al.*, 2017). Besides causing huge losses to milk production, the subclinically affected animals remain a continuous source of infection to other herd mates. Early diagnosis of mastitis is essential to prevent excess economic losses. The CM can easily be detected by physical examination of udder and or systemic signs of inflammation, whereas diagnosis of SCM is more problematic since the milk appears normal. Various physical and chemical changes of milk and cultural isolation of organisms are used for diagnosis of SCM. Although bacteriological culture of milk samples is

the gold standard method for identifying mastitis but it does not provide a measure of the degree of inflammation associated with the infection. Therefore, indirect tests such as, California Mastitis Test (CMT), White Side Test (WST), Surf Field Mastitis Test (SFMT) can be used that are simple and do not require any complex laboratory equipment (Mahfuz *et al.*, 2020). Advanced molecular techniques based on phenotyping as well as genotyping methods offer rapid and specific identification methods for diagnosis of mastitis causing pathogens up-to species and subspecies level. It is important to determine the species of bacteria for proper selection of antibiotic for therapeutic purpose and for selecting proper processing method to produce dairy products specifically. Systems of identification (automated), viz., VITEK identification cards are available for this purpose providing results of identification of bacteria with stability (khan *et al.*, 2021). Another study stated that all dairy cattle which are admitted to any veterinary hospital should be managed in a way that they are having intramammary infection even when there is no overt clinical mastitis (Kandeel *et al.*, 2018).

2.7. Treatment of mastitis due to *staphylococcus aureus*

Lysostaphin is a potent *staphylococcus*-degrading enzyme containing a peptidase that can specifically cleave the polyglycine bridge specific to the cell wall of *S. aureus*. Lysostaphin activity is measured by its ability to lyse *Staphylococcus aureus* cells. It is influenced by enzyme concentration, pH, temperature, ion and salt concentration. *Staphylococcus aureus* is enveloped in a thick layer of peptide glycans, and lysostaphin destroys the layer of peptide glycans, causing lysis and cell death. Peptide glycans impart strength and rigidity to the cell walls of gram-positive microorganisms, grow and divide, maintain cell shape, and prevent osmotic lysis of *Staphylococcus aureus*. Recombinant lysostaphin (rLYS) is a zinc metal enzyme that hydrolyzes the glycylglycine bond of a peptide glycan to a pentaglycine bridge on the cell wall of *Staphylococcus aureus* (Cardoso *et al.*, 2019).

On the other hand, treatment of choice for *S. aureus* mastitis includes antimicrobials such as β -lactams, tetracyclines, and aminoglycosides. glycopeptides, fluoroquinolones, and lincosamides are also reserved for the treatment of methicillin-resistant *S. aureus* (MRSA) and multidrug-resistant (MDR) *Staphylococcus* species. This notwithstanding, there are reports of β -lactams, aminoglycosides, and macrolides resistance among *S. aureus* (Matlale *et al.*, 2020).

2.7.1. Antimicrobial Resistance of *S. aureus*

Resistance of *S. aureus* to several antimicrobials complicates the treatment of these pathogenic bacteria, which is considered an increasing challenge. Methicillin-resistant *S. aureus* (MRSA) strains can cause nosocomial infections and consequently high mortality in humans (Abdelazeem *et al.*, 2020). The indiscriminate use of antibiotics for the treatment of animal and human diseases as well as preservatives for milk has led to the development of multiple antibiotic resistances, thereby rendering the antibiotic treatment ineffective. *S. aureus* has been reported to frequently show multiple antimicrobial resistance patterns (Letebrhan Yimesgen *et al.*, 2021). Intramammary and systemic administrations are common methods for the treatment of mastitis around the world. The long history of using antibiotics in the dairy farms to treat infectious diseases, especially mastitis and this un-responsible application led to developing the resistance of bacteria to the antibiotics. Methicillin-resistant *S. aureus* (MRSA) has been characterized by the presence of *mecA* gene, which can resist the β -lactam antibiotics (jahan *et al.*, 2015). As different investigation conducted in Ethiopia showed that, high level of resistance was observed to common drugs such as penicillin and tetracycline range from 88.89%-94% and 30%-74% PenicilinG and Tetracycline respectively (Letebrhan Yimesgen *et al.*, 2021; Alem Abreha *et al.*, 2021), and of panicilin G and tertracycline respectively (Lakech Tibebu *et al.*, 2021).

2.8. Control strategy of *S. aureus*

In general Bovine mastitis control includes early detection and treatment of existing udder infection and prevention of new udder infection by blanket dry cow therapy, post milking teat disinfection, identification and culling of chronically infected cows and the routine maintenance of the milking machine (Tadele Tolosa and Feyissa Begna, 2021). Specific to *S. aureus* control autogenous vaccination is very crucial. These vaccines are the preparations having specific strains of bacteria obtained from mastitis suffered by animals and used to immunize the herd for protection against further new udder infections with the same strain of bacteria. There are evidences which shows that the use of autogenous *S. aureus* vaccines enhances antibody titer in vaccinated animals as compared to non-vaccinated herd and reduce the risk of both clinical and sub-clinical mastitis (Cheng, 2020).

2.9. Economic Significance

Mastitis is the most economically important disease of dairy cattle, accounting for 38% of the total direct costs of the common production diseases and is a global problem as it adversely affects animal health, quality of milk and economics of milk production and every country including developed ones suffer huge financial losses. It is the most important deadly disease of dairy animals is responsible for heavy economic losses due to reduced milk yield (up to 70%), milk discard after treatment (9%), cost of veterinary services (7%) and premature culling (14%). India is the highest milk producer in the world but the per capita availability of milk still remains half of the world average, demanding strategic intervention. One of the reasons for low productivity is poor animal health, particularly, mastitis which is single largest problem in dairy animal in terms of economic losses in India. It is proved by the reports that the annual economic losses due to bovine mastitis was increased 114 folds in about 4 decades from 1962 (INR 529 million/annum) to 2001 (INR 60532 million/annum) (Subha Ganguly, 2014).

Mastitis is a problem for the dairy industry in East Africa where the prevalence of SCM was 86.2% in Uganda (Abrahmsén *et al.*, 2014), 75.9% in Tanzania (Karimuribo *et al.*, 2008), and 62% in Ethiopia (Mekonnen *et al.*, 2017). For this reason mastitis is probably one of the factors that hinder the increase in net milk production yield in East Africa region (Motaung *et al.*, 2017). Both clinical and subclinical mastitis causes loss of milk as it has to be discarded apart from the cost of treatment and other expenses. Subclinical mastitis causes three times more production losses than clinical mastitis; thus, is responsible for significant losses of 60-70% of the total economic losses associated with all mastitis due to infections (De Vliegher *et al.*, 2012; Sinha *et al.*, 2014). Sinha *et al.* (2014) performed study on dairy animals to assess the incidence and economics of subclinical form of bovine mastitis in Central Region of India. The losses due to mastitis were approximately 49% due to loss of value of milk and 37% owing to veterinary expenses. The cost of treating an animal includes cost of medicine (31%) and services (5.5%). Losses were comparatively higher in cross-bred cattle due to their high production potential that was affected during mastitis period (Khan *et al.*, 2021).

2.9.1. Low milk yield

The loss of yield depends on certain factors of great importance like severity of mastitis, nature of causative agent, stage of lactation at the time of mastitis. Losses are severe in primiparous cows due to clinical mastitis caused by *Staph. Aureus*, *E. coli* with *Klebsiella*. However, the maximum loss of production in multiparous is caused by *Streptococcus* spp., *Staphylococcus aureus* and others pathogens (Mbindyo *et al.*, 2020). The loss of production is higher in multiparous cows than primiparous. Clinical mastitis occurring before peak production stage/yield causes more extensive loss as compared to rest of the lactation and loss of milk yield is persistent throughout the lactation. According to a study, this yield loss for multiparous could be 300-400 kg (4–6% of lactation) while 200-300 kg in primiparous animals. The magnitude of yield loss in 30% cases of clinical mastitis reached up to 950-1050 kg per lactation. Whereas, in subclinical mastitis the losses incorporated are 80 kg/lactation (1.3%) and 120 kg/lactation (1.7%) in primiparous and multiparous respectively (Amjad-Islam *et al.*, 2021).

2.9.2. Altered milk composition and Discard of milk

The mastitis milk is low in fats and casein due to reduction in the synthetic capacity of secretory tissues of the udder parenchyma. The reduction in the fats up to 3-22 kg (1.5–7.5%) and casein protein contents up to 0 to 15 kg (0 to 8.5%) of the milk and higher SCC incurs the penalty to the producers in premium payment. Milk of the cow is discarded after mastitis diagnosis or while cow is being treated with antibiotics due to presence of antibiotic residues in milk during withdrawal period. The length of the withdrawal period depends on the drugs used and production system (i.e. conventional or organic). This discarded milk cost higher per unit than the milk not produced due to feed costs inclusions (Amjad *et al.*, 2021).

2.9.3. Veterinary and medicinal cost

The veterinarian cost for treatment fee, travel and labor charges. On an average a handsome expenditure of \$444 in clinical mastitis case are charged. This includes (128\$) directly in term of diagnostic (10\$), medicinal expenditure (\$36), discarded milk (\$25), Veterinary charges (\$41), extra labor (\$4) while death losses (\$32). The indirect costs are (\$316) which include (\$125) through future production losses and (\$182) for culling and replacement and whereas

reproductive losses are (\$9). Therefore, to take an accurate decision to control the mastitis depends on understanding of economic impact of mastitis (Rollin *et al.*, 2015). Mastitis is among the main reason in cattle for the use of antibiotics and this exposure of animal to antibiotics is main reason behind the development of antibiotic resistant bacteria and antimicrobial residues in milk which are of a great public health concern (Naseer *et al.*, 2020).

2.9.4. Culling of animal

The risk of culling and mortality rate is increased with clinical mastitis. Similar to milk loss the increased culling also augments the hidden cost. Involuntary culling is associated with replacement costs and is an important component of total mastitis cost. Economic cost also increases as cows

3. MATERIAL AND METHODS

3.1. Study Area

The study was conducted in Babile district, eastern Hararghe Zone, Ethiopia. Geographically, Babile district is located at 561km away from the capital city of Ethiopia (Addis Ababa) at 8°39'5999" N latitude and 42°24'5999" E longitude and the elevation is ranges from 1450- 1700 meters above sea level (Figure-1). The weather conditions characterized by semi-arid and arid climate with average annual temperature and the annual rain falls is ranged from 24 to 28 °C and 410 to 800mm respectively. Agro pastoral and pastoral is the main occupation of the population of the area. According to Babile District agricultural office annual report (Babile District, 2016), the livestock population estimated as 76,161 cattle, 11,470 sheep, 20,644 goats, 7,393 donkey, 15,430 camel and 21,114 poultry.

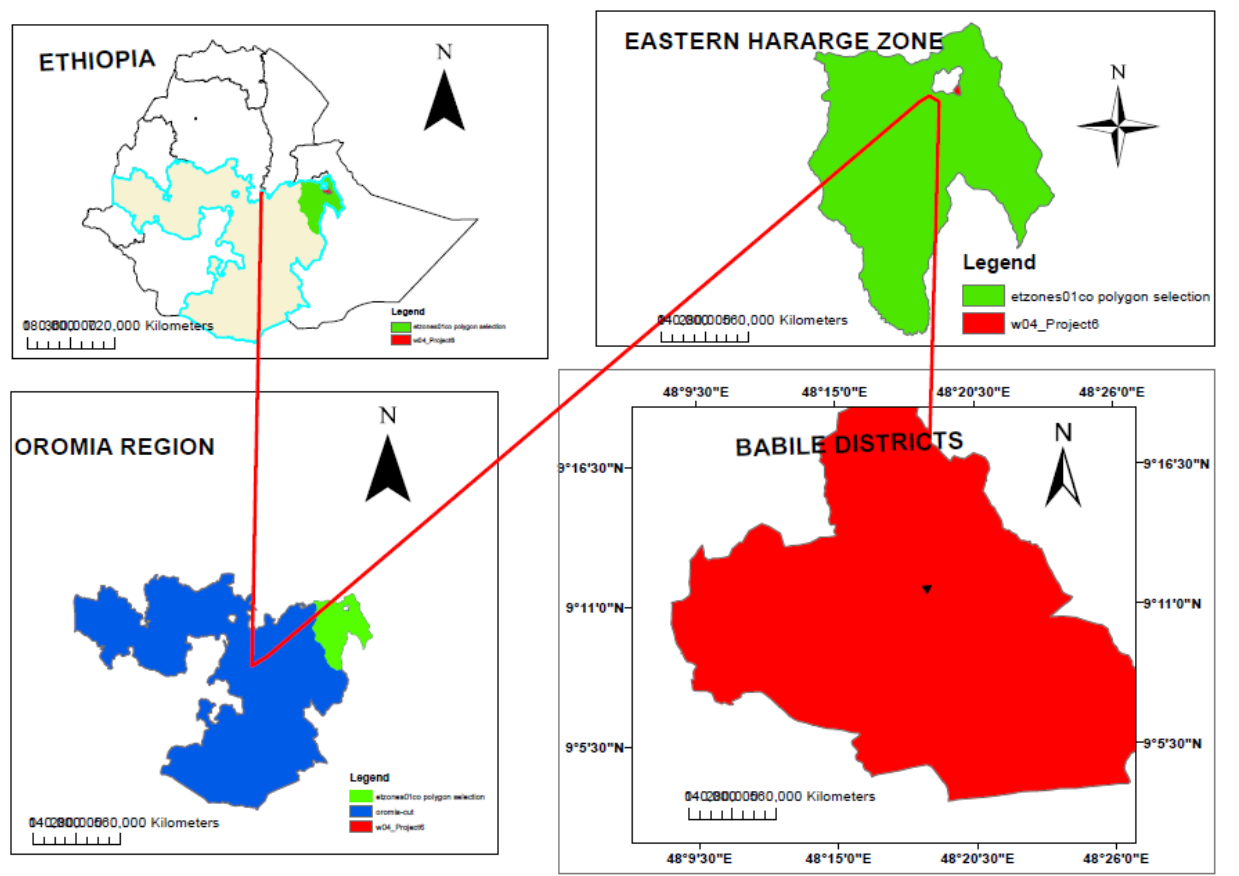


Figure-1: Map of study Area

Source: From GIS

3.2. Study population

The study populations were those lactating Zebu cows (indigenous cattle) found in the Babile District and managed under an extensive management system. All age categories of cows and the number of parities were included. Age of animals was determined from birth records and categorized as young-adult (3–6 years), adults (6 to ≤ 10 years), and old (> 10). Stage of lactation was categorized as early (1–4 month), middle (> 4 –8 month), and late (> 8 month to the beginning of dry period). Parity was categorized as few (with ≤ 3 calves), moderate (4–7 calves), and many (> 7 calves). Body condition score was also categorized as good medium and poor (Biniam Tsegaye *et al.*, 2019; Tesfaheywet Zeryehun and Gerema Abera, 2017; Berhanu misrak *et al.*, 2017; Haben Fesseha *et al.*, 2021). On the other hand, the semi-intensively managed cattle grazed freely on pasture but received supplementary feeds in the morning and evening when they were also included. All cows were hand milked twice daily, in the morning and evening.

3.2.1. Inclusion criteria

All age categories of indigenous lactating Zebu cow (*bos indicus*) under extensive and semi-intensive system were included in this study.

3.3. Sample size determination

The sample size was determined by using the formula given by Thrusfield (2005) with 95% confidence interval (CI) and 5% desired absolute precision. The total sample size using the formula given here below was 384.

$$n = \frac{Z^2 P_{\text{exp}} (1-P_{\text{exp}})}{d^2}$$

Where, n = required sample size.

P_{exp} = expected prevalence of *S. aureus* occurrence in bovine milk.

Z= z statistic for level of confidence which is 1.96.

d=desired absolute precision of 5%.

Accordingly, a total of 384 lactating cows were selected from selected three kebeles of Babile district to determine the prevalence of bovine mastitis and assess the associated risk factors related to its occurrence.

3.4. Study design and sampling strategy

A cross-sectional study was conducted from August 2021 to March 2022 to determine the prevalence and associated risk factors of bovine mastitis. Cows were examined directly using physical examination technique to detect clinical mastitis and indirectly using CMT for the detection of subclinical mastitis. The households and study animals were selected using systematic random sampling.

Currently there are twenty two kebeles (Kebele refers to the smallest administrative unit under district) in Babille district. From twenty two kebeles found in Babile district, three kebeles, namely Ifa, Tula and Sheek, were purposively selected from the district on basis of road accessibility (to transport the sample easily without any problem) and electric availability (to store the collected samples properly in the refrigerator). Using systematic random sampling techniques, the households were chosen from each of the selected kebeles. The study animals within the households were also selected using a systematic random sampling technique.

Equal proportion of the sample size was divided in to three selected kebeles belonging to Babile District as mentioned below.

- ✚ Ifa with number of sample Size -128
- ✚ Tula with the number of sample -128
- ✚ Sheek Abdi with the amount of sample -128

3.5. Screening procedure for subclinical mastitis

3.5.1. California mastitis test (CMT)

The CMT were used as a screening test for sub-clinical mastitis. It was carried out according to the procedure described by Quinn *et al.*, (2002). A squirt of milk, about 2 ml from each quarter

was placed in each of four shallow cups in the CMT paddle. An equal amount of the commercial CMT reagent was added to each cup. A gentle circular motion was applied to the mixtures in a horizontal plane for 15 s. The CMT results were scored as 0 (negative), trace, 1 (weak positive), 2 (distinct positive) and 3 (strong positive) based on gel formation. All CMT scores of 0 and trace were considered as negative while CMT scores of 1, 2, and 3 will be considered as indicators of sub clinical mastitis. Positive cows were defined as having at least one quarter with CMT score of 1 +.

3.5.1. Clinical examination

Physical examination for evidence of clinical mastitis was conducted in all lactating cows that were sampled in the study area. The udders of the selected cows were examined visually and by palpation for any presence of clinical mastitis. During examination, attention was paid to cardinal signs of inflammation, blindness, injuries, milk clots, symmetry, size, consistency of udder quarters, swelling and abnormal milk (Radostits *et al.*, 2007). A cow was considered to have clinical mastitis if it fulfilled at least two of the clinical findings, (1) pain reaction upon palpation, (2) changes in color and consistency of milk (blood-tinged milk, watery secretions, clots, pus) and (3) change in consistency of the udder (Lakew *et al.*, 2009).

3.6. Associated risk factor determination procedure

Structured questionnaires and observer check list were developed and used to identify the associated risk factors responsible for the occurrence of bovine mastitis. Information on cow attributes such as breed, age, body condition, parity number, lactation stage, teat or udder condition (lesion, fibrosis, atrophy), production system, floor type, milking hygienic practice, tick infestation of udder or teat, presence of blind teat, milk condition (watery, bloody, pussy) was collected at the time of sampling using data recording sheet. The age, lactation stage, and parity numbers were recorded from animal owners, milker and observation. In addition, the livestock attributes like herd size, production type, and status of barn floor were also considered in the questionnaire.

3.7. Milk sample collection, handling, storage and transporting

Procedure for collection of milk was according to Quinn *et al.* (2002). Milk sampling and screening were performed for each quarter. The time chosen for milk sampling was before milking. Teats that identified as positive for clinical and subclinical mastitis were considered for milk sample collection. First, after the hands were cleaned by detergents and clean water, the udder and teats were washed with tap water and dried. If there was a considerable amount of dirt, it was properly removed. The teat on the far side of the udder was cleaned first and then those on the near side. Then the teat ends were vigorously swabbed with cotton soaked in 70% ethyl alcohol prior to sampling. For bacteriological culture, 10 mL of mastitis milk (clinical and subclinical) were collected aseptically from affected teats separately into horizontally hold sterile test tube after discarding the first 2-3 milking streams (Moges *et al.*, 2011). The 10 ml test tube was used to collect milk sample. The examination of udder and milk and sample collection was performed in this way. The samples were placed in racks for ease of handling and transported in an icebox to the Microbiology Laboratory at College of Veterinary Medicine, Haramaya University within 2 to 3 hours; where the samples were stored at 4 °C for a maximum of 24 hrs until inoculation on a standard bacteriological media was done.

3.8. Isolation and identification of *Staphylococcus aureus*

3.8.1. Enrichment Medium

Bacteriological examination was done according to the Quinn *et al.*, (2002) and National Committee for Clinical Laboratory Standards (NCCLS, 2014). Refrigerated milk samples were warm at room temperature (25° C) for about an hour and then mixed by shaking in order to disperse bacteria and fat. Samples were allowed to stand for a while for foam to disperse before just inoculation. A loopful of milk sample was streaked on blood agar base enriched with 7% defibrinated sheep blood (Oxoid, UK) using the quadrant streaking method for each quarter. Blood agar plates were incubated aerobically at 37° C. The plates were checked for growth after 24 hr, 48 hr, and 72 hr. to rule out slow growing bacteria. The plates were examined for growth, morphological features, such as colony size, shape, and color, and hemolytic characteristic. Suspected colonies were sub-cultured on mannitol salt agar plate (Oxoid, UK) were examined

for growth and golden yellow fermentation which was considered as a presumptive identification of *S. aureus*. Yellow colony on mannitol salt agar was sub-cultured on nutrient agar for further examination. After pure colonies were obtained, Gram-stained smears were done for primary identification of bacteria to genus level, such as Gram reaction (Gram positive and Gram negative), and cellular morphology (coccus or rods).

3.8.2. Selective plating on culture media

The selective medium used for isolation of *S. aureus* was Mannitol Salt Agar (MSA). A loopful of inoculum from blood agar plates were streaked on MSA and were incubated for 48 h at 37 °C. Characteristic appearance of yellow colonies on MSA was considered to be presumptive *S. aureus*. The pure cultures were streaked on Nutrient agar and incubated for 24 h at 37 °C to get a pure culture. The pure isolated in the nutrient slant were preserved and maintained at +4 °C for characterizing the isolates by biochemical tests (Quinn *et al.*, 2002).

3.8.3. Gram's Stain Test

All suspect cultures of *S. aureus* were subjected to Gram's stain and observed under a light microscope for Gram's reaction, size, and shape and cell arrangements. The Gram's stain smears from typical colonies that were show Gram-positive cocci occurring in bunch, grapelike irregular clusters were taken as presumptive *S. aureus*.

3.8.4. Biochemical Tests for *Staphylococcus aureus*

3.8.4.1. Catalase test

Pure culture of the isolates was picked up by bacteriological loop from the agar plate and mixed with a drop of 3% H₂O₂ on a clean slide for catalase test. When the organism was positive, bubbles of oxygen were liberated within a few seconds. Those positive cocci were considered as *Staphylococci* (Quinn *et al.*, 2002).

3.8.4.2. Mannitol salt Agar (Mannitol fermentation)

The colonies were confirmed by Gram's staining reaction, catalase positive and oxidase negative were selected and streaked on Mannitol salt agar plate and incubated at 37 °C and examined after

24-48 hrs for growth. The presence of growth and change of pH in the medium (red to yellow) were regarded as presumptive identification of *S. aureus* or coagulase positive *S. aureus*. Phenol red pH indicator detected the acidic metabolic product of mannitol. Fermentation of mannitol by *S. aureus* was also causes yellow discoloration of the medium within 24 hrs of incubation (Quinn *et al.*, 2002).

3.8.4.3. Coagulase test

The coagulase tests were used as both slide and tube coagulase tests. The presumptively identified *S. aureus* from mannitol salt Agar were sub-cultured to nutrient agar plate and after 24 hours culture colonies of *S. aureus* were picked by bacteriological loop and placed on clean slide with a small drop of distilled water and emulsified. The test suspension was also be 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 coagulase positive *S. aureus* (Quinn *et al.*, 2002).

3.9. Antimicrobial Susceptibility Test

Antimicrobial susceptibility test was performed on isolates of *S. aureus* from bovine milk using the disc diffusion according to the Bauer and Kirby method using Muller-Hinton agar (MHA) (Oxoid, CM0405B, UK). The isolates were tested for 7 antibiotics which had been reported as being commonly in use in the study area. Accordingly, Penicillin G (10 units), chloramphenicol (30 µg), Sulfamethoxazole (25 µg), amoxicillin (10 µg), cefoxitin (30 µg) in place of methicillin (5 µg), erythromycin (15 µg), and Gentamycin (30µg) were tested. The procedures recommended by National Committee on Clinical Laboratory Standards for antimicrobial susceptibility testing were followed. Three to five isolated colonies of isolated *S. aureus* were transferred to 5 ml of 0.85% saline water. Turbidity was measured using densitometry and adjusted to 0.5 McFarland. After measuring the turbidity, sterile cotton tipped swab was dipped into the suspension and then Mueller-Hinton agar plate was inoculated by rotating 60°. Each inoculated plate, seven antibiotics discs were applied using a disc dispenser (Oxoid). The plates were incubated at 37 °C for 16 - 18 hours and the zone of inhibition were measured and compared to standards as per the manufacturer's recommendation. Results were recorded as susceptible, intermediate and resistant according to the size of the zone of growth inhibition (CLSI document, 2008). *S. aureus* ATCC 25923 was used as quality standard control strain in each run.

3.10. Data Management and Analyses

The data generated during the sample collection and from the questionnaire were entered into the Microsoft Excel spread sheets and was later analyzed by using STATA version 13 software. The effect of risk factors with possible association of the disease was analyzed using crude odds ratio. The associations between dependent and independent variables were tested, and $p < 0.05$ was taken as statistically significant (Snedecor and Cochran, 1989).

4. RESULTS

4.1. Prevalence of Clinical and Subclinical Mastitis

A total of 384 cows (1536 quarters) were examined for the detection of mastitis in this study. The overall cow level prevalence of mastitis was 50.5% (194/384; 95% CI: 45.4%-55.6%); of which 5.5% (21/384; 95% CI: 3.4%-8.2%) were clinically sick based on clinical examination and 45% (173/384; 95% CI: 40.0%-50.2%) had subclinical mastitis using CMT test (table 1).

Table 1. The percentage of clinical and subclinical mastitis

Type of mastitis	Number of animals	Number of	Prevalence	
	Examined	animals affected	(%)	95% CI
Clinical Mastitis (CM)	384	21	(5.5)	3.4-8.2
Subclinical Mastitis (SCM)	363	173	(45.1)	40.0-50.2
Overall	384	194	(50.5)	45.4-55.6

From the total of 1536 quarters examined, the overall prevalence to the quarter level bovine mastitis was 19.01% (292/1536). 2.7% (42/1536) of the teats were blind. The remaining 78.2% (1202/1536) of udder were negative for mastitis. Accordingly, the prevalence of clinical and subclinical mastitis at quarter level was 2% (32/1536) and 11.97% (184/1536) respectively (table 2).

Table 2. Prevalence of bovine mastitis at quarter level

Quarter	Total Number	Number	Prevalence
	Examined	Positive	(%)
Right Front	384	62	16.15
Left Front	384	62	16.15
Right Hind	384	69	17.97
Left Hind	384	99	25.78
Total	1536	292	19.01

4.2. Mastitis Risk Factors

4.2.1. Prevalence with respect to risk factors

4.2.1.1. Univariable Logistic Regression Analysis

Prevalence of mastitis related to specific risk factors was determined as the proportion of affected cows out of the total examined. Among the risk factors analyzed using a univariable logistic regression analysis, except lactation stage all factors such as kebeles, age, parity, body condition, udder size, milk yield, tick infestation and teats injury were found to be having statistically significant association with the occurrence of mastitis ($p < 0.05$) as indicated in table 3. On multicollinearity test, age was found to be collinear to parity and udder size with that of milk yield. Thus, in the multivariable analysis age and milk yield were removed from the analysis (table 3).

Table 3. Result of a univariable logistic regression analysis

Risk factor/ Variable	Level/ Category	Number Examined	Number Positive	Prevalence	COR (95% CI)	P-value
Kebele	Abdulkadir	129	54	41.86	Ref.	
	Ifa	127	71	55.91	1.76 (1.07-2.89)	0.025*
	Tula	128	69	53.91	1.62 (0.99-2.66)	0.054
Age	Young-adult (3-6 yrs)	142	71	50.00	Ref.	
	Adult (7-≤10yrs)	178	81	45.51	0.84 (0.54-1.30)	0.424
	Old (> 10yrs)	64	42	65.63	1.91 (1.04-3.52)	0.038*
Parity	Few (≤3calves)	257	120	46.69		
	Moderate (4-7calves)	92	50	54.35	1.36 (0.84-2.19)	0.208
	Many (> 7 calves)	35	24	68.57	2.49 (1.17-5.30)	0.018*
Body condition	Good	172	74	43.02	Ref.	
	Medium	134	69	51.49	1.41 (0.89-2.21)	0.141
	Poor	78	51	65.38	2.50 (1.44-4.36)	0.001*

Lactation stage	Early (1-4 month)	154	82	53.25	Ref.	
	Middle (>4-8 months)	127	59	46.46	0.76 (0.48-1.22)	0.2958
	Late (>8 months)	103	53	51.46	0.93 (0.56-1.53)	0.778
Udder size	Small	168	61	36.31	Ref.	
	Medium	112	53	47.32	1.58 (0.97-2.56)	0.067
	Large	104	80	76.92	5.85 (3.36-10.18)	<0.001*
Milk yield	Low	144	47	32.64	Ref.	
	Medium	138	65	47.10	1.84 (1.13-2.98)	0.013*
	High	102	82	80.39	8.46 (4.64-15.42)	<0.001*
Tick infestation	No	242	91	37.60	Ref.	
	Yes	142	103	72.54	4.38 (2.79-6.88)	<0.001*
Teats injury	No	251	88	35.06	Ref.	
	Yes	133	106	79.70	7.27 (4.43-11.94)	<0.001*

* Significant

4.2.1.2. Multivariate Logistic Regression Analysis

According to multivariate logistic regression analysis factors such as udder size, tick infestation and teat injury were found to be having significant association with the prevalence of bovine mastitis in the study area. Whereas, kebeles, parity, body condition, lactation stage did not show any significant association to the prevalence of mastitis in the study area (table 4).

In the current study, statistically higher prevalence of both CM and SCM ($P < 0.01$) was recorded in cow exposed to tick infestation (72.54%) as compared to cows not exposed to tick infestation (37.60%). Cow exposed to tick infestation were 2.99 times more susceptible to mastitis than cow not exposed to tick infestation (95% CI= 1.77-5.05) (table 4). On the other hand, the difference in presence and absence of teat lesion/injury was significantly associated ($P < 0.01$) with the occurrence of mastitis. Accordingly, higher prevalence (79.70%) was observed in cows that exposed to teats injury when compared to cows not exposed to teat injury (35.06%). Cows with teat injury were 4.80 times more prone to mammary infections than cows without teat injury (95% CI= 2.78-8.28) (table 4). The odd ratio of getting mastitis was greater in cows with

pendulous udders than cows with normal udder position: 3.03 times that of the latter (95% CI =1.59-5.77). Hence, the udder size was statistically associated with the occurrence of bovine mastitis in the study area ($P<0.01$) (table 4).

Table 4. Result of a multivariate logistic regression analysis

Risk factor/ Variable	Level/ Category	Number Examined	Number Positive	Prevalence	COR (95% CI)	P-value
Kebele	Abdulkadir	129	54	41.86	Ref.	
	Ifa	127	71	55.91	1.75 (0.96-3.21)	0.070
	Tula	128	69	53.91	1.68 (0.91-3.09)	0.094
Parity	Few (≤ 3 calves)	257	120	46.69	Ref.	
	Moderate (4-7calves)	92	50	54.35	1.25 (0.69-2.25)	0.460
	Many (> 7 calves)	35	24	68.57	1.51 (0.62-3.67)	0.368
Body condition	Good	172	74	43.02	Ref.	
	Medium	134	69	51.49	0.92 (0.53-1.61)	0.783
	Poor	78	51	65.38	1.89 (0.96-3.71)	0.064
Udder size	Small	168	61	36.31	Ref.	
	Medium	112	53	47.32	1.00 (0.57-1.76)	0.996
	Large	104	80	76.92	3.03 (1.59-5.77)	$<0.001^*$
Tick infestation	No	242	91	37.60	Ref.	
	Yes	142	103	72.54	2.99 (1.77-5.05)	$<0.001^*$
Teats injury	No	251	88	35.06	Ref.	
	Yes	133	106	79.70	4.80 (2.78-8.28)	$<0.001^*$

* Significant

4.3. Bacterial Isolation and Identification

The current study showed that the overall prevalence of bovine mastitis in Babile district is 50.5%. During the course of the study, a total of 194 clinical and sub-clinical positive milk

samples were collected from 3 kebeles of Babile district and cultured on blood agar media. Accordingly, growth of different groups of bacteria was observed in 194 (100%) samples. However, since the objective was to isolate *S. aureus*, further bacteriological tests were conducted by taking only those colonies presumptive of this species and leaving others. Finally, coagulase positive *S. aureus* was detected in 30.92% (60/194). Accordingly, 8.2% (16/194) and 6.84% (20/292) of *S. aureus* were isolated from clinical mastitis at cow level and quarter level respectively, while 22.68% (44/194) and 19.17% (56/292) of *S. aureus* were isolated from subclinical mastitis at cow level and quarter level respectively (table 5). In general the overall prevalence *S. Aureus* isolated from both clinical and subclinical mastitis were 30.9% and 25% at cow level and at quarter level respectively.

Table 5. Prevalence of isolated *S. aureus*

Isolated <i>S. aureus</i>	Cow level prevalence	Quarter level prevalence
Clinical mastitis	16/194 (8.2%)	20/292 (6.84%)
SCM	44/194 (22.68%)	56/292 (19.17%)
Total prevalence	60/194 (30.92%)	76/292 (26%)

4.4. Antimicrobial sensitivity test result

Antimicrobial sensitivity test results of the 35 COPS isolates showed that, 100% susceptible to chloramphenicol and gentamycin. Similarly, highest susceptibility was recorded to ceftiofur 91.4% (32/35) but 8.5% (3/35) of ceftiofur were resistance to isolated coagulase positive *S. aureus*. In contrast, the highest (94.2%) resistance development was observed for penicillin G followed by amoxicillin 28.57% (10/35) and Erythromycin 20% (7/35). However, 71.4% (25/35) and 80% (28/35) were susceptible to Amoxicillin and Erythromycin respectively. In the present study 77%, 11.4% and 11.4% of isolated coagulase positive *S. aureus* showed susceptible, intermediate, and resistance to sulfamethazole respectively. In the current study, 34/35 (97.2%) of isolated *S. aureus* was resistant only one drug while 1/35 (2.8%) were multidrug resistance (table 6).

Table 6. Antibiotic Sensitivity Test Result

Antibiotics	Resistance in (%)	Intermediate (%)	Susceptible in (%)
Chloramphenicol	0	0	100
Gentamycin	0	0	100
Cefoxitin	8.5	0	91.4
Erythromycin	20	0	80
Sulfamethazolidine	11.4	11.4	77
Amoxicillin	28.27	0	71.43
Penicillin G	94.2	2	5.7

5. DISCUSSION

In the present study the overall prevalence of bovine mastitis at cow level were 50.5%; of which 45% and 5.5% were subclinical and clinical mastitis respectively. This finding was similar with 50.4% overall prevalence in Rwanda (Mpatswenumugabo *et al.*, 2017). The cow-level prevalence of this study was also comparable to three previous studies conducted in Ethiopia: 52.9% in and around Areka town (G/Michael *et al.*, 2013), 53.25% in Dire Dawa town (Biniam *et al.*, 2015); 54.2% in southern Ethiopia (Rahmeto Abebe *et al.*, 2020) and 56.5% in and around Batu (Duro and Taddele, 2011). However, it was higher than those reported in other studies conducted in selected areas of Bench Maji Zone, Southwest Ethiopia (30.2%) (Teshome Gemechu *et al.*, 2019); in and around Adama town central Ethiopia (31.5%) (Kaleab Tesfaye *et al.*, 2021) and in Bishoftu town (40.1%)(Birhanu *et al.*, 2017).

In contrast, the present prevalence is lower than a previous report in Hawasa milk shed (62.6%) (Abebe *et al.*, 2016); North-West Ethiopia (62%)(Mekonnen *et al.*, 2017); in selected district of eastern Hararghe (64.3%)(Tasfahiyiwot zarihun and Gerema Abera 2017) and in and around Haramaya District (63.2%)(Beniam tasfaye *et al.*, 2019). The variability in prevalence of bovine mastitis between the current and the previous studies could be due to the difference in the management systems, improper use of antibiotics, agro climatic areas and differences in study methods and instruments employed by the investigators.

In the present study, 5.5% prevalence of clinical mastitis (CM) was observed. This was comparable with a recent published study (6.8%) in Kenya (Christine *et al.*, 2020). In our country, this finding was also in line with previous studies so far by Tesfaheywet Zeryehun and Gerema Abera, (2017); Gutu Kitila *et al.*, (2021) and Rahmeto Abebe *et al.*, (2020). However, the present finding was lower than the previous study (30%) elsewhere in Ethiopia (Mekibib *et al.*, 2010; Kaleab Tesfaye *et al.*, 2021).

Higher prevalence of subclinical mastitis (SCM) (45%) was observed in this study, which is by far higher than the occurrence of clinical cases (5.5%). This finding was slightly similar with two previous studies in Rwanda 50.4% by Mpatswenumugabo *et al.* (2017), and 43.1% Jean Baptiste Ndahetuye *et al.* (2019). In our country, this was in line with study done by Biniam Tsegaye *et al.*, (2019) and Rahmeto Abebe *et al.*, (2020). Higher prevalence of subclinical bovine mastitis

was recorded in this study than other studies reported by Sori *et al.*, (2005) (36.67%) and Biniam *et al.*, (2015) (44.16%). However, the present study recorded lower prevalence of subclinical mastitis than study reported by Zeryehun *et al.*, (2013) (55.1%) and Bedada and Hiko, (2011) (55.8%). The overall prevalence of subclinical mastitis at both cow and quarter level was found to be higher than clinical mastitis. This could be attributed to the little attention given to subclinical mastitis while treating clinical cases. According to Sori *et al.* (2005), subclinical mastitis was higher than clinical mastitis owing to the defense mechanism of the udder, which reduces the severity of the disease. Moreover, farmers in Ethiopia are not well informed about the silent cases of mastitis (Zeryehun *et al.*, 2013). The differences might also be contributed from the fact that clinical mastitis can easily be diagnosed and treated (Motaung *et al.*, 2017), while subclinical form has no visible abnormalities which is hardly diagnosed by the farmers and progresses to be a source of infection in the extensive farm (Abebe *et al.*, 2016).

The quarter level prevalence of mastitis in the present study was 19.01 % where 2% were clinical whereas 11.97% were subclinical. This study result was closer to the findings of Mekibib *et al.*, (2017) who report prevalence at quarter level of 16.2%. The finding was lower than study result done by Melak Tezera and Endris Aman, (2021) who reported the quarter level prevalence of 26%, whereas 57.1% and 44% of prevalence was also reported by Tesfaheywet Zeryehun and Gerema Abera, (2017) and Makbeb *et al.* (2010) respectively. The variability in the bovine mastitis at quarter level among the reported findings at different area could be attributed to difference in management of farm or farmer's management and technical know-how of the investigator. Additionally, ineffective mastitis control programmes and poor hygiene standards in the study areas might have been a key contributor (Abrahmsén *et al.*, 2014).

The finding of 2.7% blind teat in this study was comparable with 3.9% result conducted by Melak Tezera and Endris Aman, (2021). The blind quarters observed in this study might indicate the serious impact imposed by mastitis in the study area and the absence of culling that should have served to remove a source of mammary pathogens for the cows. Besides, unable to screen subclinical mastitis and late or complete absence of treating clinical cases could possibly leads to blindness of mammary quarter. It can be hypothesized that blind mammary quarters contribute to high subclinical mastitis and loss of milk production with a subsequent impact on food security Melak Tezera and Endris Aman, (2021).

In the present study it was also found that cows with more pendulous udders were the most prone to mammary infections. The likelihood of getting mastitis was 3.03 times greater in cows with pendulous udders than cows with normal udder position. This finding was in agreement with study done by Nahom Belay *et al.*, (2022); Abebe *et al.*, (2016) and Tesfaheywet Zeryehun and Gerema Abera, (2017). As reported by Girma *et al.* (2012) and Sori *et al.*, (2005), animals with pendulous udders had a higher incidence of mastitis than cows with non-pendulous udders. It has also been noted that cows with pendulous udders turn out to be the most prone to mammary infections. Pendulous udders open the teats and udders to injury, and microbes readily adhere to the teats and gain entry to gland tissue (Abebe, *et al.*, 2016).

Likewise, there was a statistical association between tick infestation and bovine mastitis in the study area: the existence of elevated the likelihood of mastitis by 2.99 times on cow with tick infestation when compared to those with no tick infestation. This result was in line with the most recent report of Nahom Belay *et al.*, (2022). Injuries produced by ticks are believed to create direct inflammatory reactions, necrosis, and abscess formation within the mammary gland, which can cause udder damage or risk of serious secondary infections (Biffa *et al.*, 2005). In contrast, this finding was disagreed with the study conducted by Biniam Tsegaye *et al.*, (2019); Melak Tezera and Endris Aman, (2021) and Dimshasha Tolera *et al.*, (2021) who reported insignificant association of tick infestation with the prevalence of bovine mastitis in their study area. The variability between current finding and previous study was due to the different in the management, control strategy of tick within the farmer and farm manager and breed of the animal which have resistance against tick.

In this study, a strong association was also observed between teat injury ($p < 0.001$) and occurrence of bovine mastitis. The prevalence of mastitis was higher in cows with teat injury (79.70%) than cows without teat injury (35.05%). Likewise, the odds of mastitis were 4.80 times higher in cows exposed to teat injury than cows that was not exposed to with teat injury. Current study was similar with previous reports elsewhere by Biniam Tsegaye *et al.*, (2019) and Mekibib *et al.*, (2010). The higher prevalence of mastitis in cows having teat injury in this study suggests that there may be unsatisfactory udder management and health. The presence of barbed wire fence in the area and ticks infestation around the udder might be the customary causes of udder/teat injuries in the farms or lactating cows in our case.

According to multivariate logistic regression analysis factors such as parity, body condition, lactation stage did not show any significant association to the prevalence of bovine mastitis in the study area. This research showed statistically insignificant prevalence of mastitis among different lactation stages which is in agreement with previous studies conducted elsewhere by Beniam Tsegaye *et al.* (2019), Kaleb Tasfaye *et al.* (2021), Tesfaheywet Zeryehun and Gerema Abera, (2017) and Haben Fesseha *et al.* (2021). Although it was insignificant, the prevalence of mastitis was relatively higher in early and mid lactation compared to the late lactation; this might be associated with the presence of higher number of the cows with early (154) and middle lactation stage (127) compared to the late lactation stage stage (103).

The current observation showed that higher mastitis prevalence in the hind quarters (17.97%-RH and 25.78% -LH) as compared with front quarter (16.15% both RF and LF). This higher mastitis prevalence in the hind quarter was similar to earlier studies conducted by Nahom Belay *et al.*, (2022); Tesfaheywet Zeryehun and Gerema Abera, (2017) and Ababe *et al.*, (2016). Although it was difficult to give a plausible explanation for this observation, this might be due to the lower position of the hind quarters in relation to fore ones, which make them more prone to contamination or injuries leading to mastitis. Greater milk yield produced by the hind quarters may also be a reason as risk of mastitis tends to increase with increase in milk production (Kocak, 2006).

The prevalence of isolated *S. aureus* from positive mastitic bovine milk sample was found to be 30.92%. The current study was similar with previous study done in and around Adama Town (30.6%) by Kaleab Tesfaye *et al.*, (2021). However, present finding was lower than the study done in Bishoftu Town (44%) (Birhanu *et al.*, 2017), Holota town (47%) (Mekibib *et al.*, 2010) and selected areas of Bench Maji Zone, Southwest Ethiopia (59.26%) (Teshome Gemechu *et al.*, 2019). In contrast, present finding was higher than earlier study finding conducted in and around Haramaya District (24.1%) (Tasfahiyiwot zarihun and Gerema Abera, 2017), and in Toke Kutaye, Cheliya, and Dendi Districts, West Shewa Zone, Oromia (5.5%) (Dimshasha Tolera *et al.*, 2021). This high prevalence of *S. aureus* in the study area may be due to the poor management practiced in the study area and due to the treatment of clinical mastitis with antibiotics particularly using penicillin G for which *S. aureus* might have been developed resistance.

The current study showed a very high level of resistance (94.2%) developed by coagulase positive *S. aureus* isolates to penicillin G. The finding of this study was similar to the result recovered by Tebabu Lakech *et al.* (2021), Alem Abrha Kalayu *et al.* (2020) and Letebrhan Yimesgen *et al.* (2021) who reported high level of resistance development to penicillin. This finding was also higher than recently published study done in Egeypt by Abdelazeem *et al.*, (2020) who reported 64% of resistance to penicillin. *S. aureus* also developed 28.57% and 20% of resistance to Amoxicillin and Erythromycin respectively. This finding was in agreement with recent study conducted in western part of Romania (Corina *et al.*, 2022) who reported 25% of resistance to amoxicillin. This finding was lower than study conducted by Kaleab Tesfaye *et al.*, (2021) who recovered the highest 75% of resistance. This finding was higher than 5.56% of resistance observed by Letebrhan Yimesgen *et al.*, (2021). This high *S. aureus* resistance to penicillin G might be due to the fact that the beta-lactams are the drugs of choice for intramammary infections. However, the inappropriate and regular use of these medications by animal health experts and even the owner of animals in the study have contributed to the emergence of resistance. It is also known that the isolates produce a penicillinase enzyme (a type of β -lactamase) that hydrolyses the beta-lactam ring of penicillin (Letebrhan Yimesgen *et al.*, 2021).

In contrast, in the current study *S. aureus* was 100% susceptible to gentamycin and chloramphenicol. Current study was in line with the finding recorded by Letebrhan Yimesgen *et al.*, (2021) who reported 100% susceptible *S. aureus* to gentamycin and chloramphenicol. Tassew *et al.*, (2015) and Dimishasha Tolera *et al.*, (2020) also reported 100% susceptibility of *S. aureus* to gentamycin in Konbolcha and West Shewa respectively. Infected animals and associated products have been supposed to be a potential source of community acquired MRSA. Recently, the isolation of MRSA in animal and human has become a worldwide concern. Though highest susceptibility (91.4%) was recorded to cefoxitin, the current study revealed that resistance to cefoxitin was 8.5% which is indicator of MRSA occurrence. In this investigation the highest susceptibility rate and the lowest prevalence of resistance of cefoxitin was comparable with previous study conducted in south Africa (Matlale *et al.*, 2020) and Letebrhan Yimesgen *et al.*, (2021) who reported low prevalence of resistance was observed by *S. aureus* to cefoxitin. The reason of MRSA occurrence is due to occurrence of sub-clinical mastitis in dairy farms and this transmits to raw milk and by this the opportunity of MRSA transmission from the

milking halls and from infected milkiers' hands into the raw milk, other human and animal. In addition, the presence of substandard hygienic procedure practiced by milkers is the most important probable reasons for occurrence of MRSA (Tibebu Lakech, *et al.*, 2021).

6. CONCLUSION AND RECOMMENDATIONS

The present study recorded an overall prevalence of 50.5%, which might entail that mastitis was a major health problem of dairy cows which undoubtedly will have drawback on productivity of dairy industry. Particularly the prevalence of subclinical mastitis was higher than clinical form in the study areas (45.1%) which might mean owners of lactating cows are only concerned with clinical form of mastitis and often are unawareness of the status of subclinical infection in the study area. In current study, the high prevalence of *S. aureus* was observed which indicate the organism is a major cause of mastitis in dairy animals in the study area. Resistance development by *S. aureus* to antimicrobials, such as penicillin G, might complicate the treatment of these pathogenic bacteria, which is considered an increasing challenge. On the other hand, *S. aureus* was also found susceptible to antibiotics, such as gentamicin, and chloramphenicol, suggesting that these antibiotics have not been commonly used as the treatment of choice for mastitis in the study area. Tick infestation, teat injuries and udder size were predictors of bovine mastitis in this study. Therefore, based on the above conclusion the following recommendations are forwarded:-

- ✚ Awareness creation among farmers about the problem of tick on the lactating cow and proper prevention of tick infestation and avoiding of teat injury should be enhanced through animal-health extension services.
- ✚ Implementation of regular screening of subclinical mastitis and provision of treatment to positive cases on dairy farms must be there.
- ✚ Continuous surveillance of the emerging and antimicrobial resistant species using molecular studies is warranted.
- ✚ As a short-term solution, cows with clinical mastitis should be treated with gentamicin or chloramphenicol antibiotics to cure and minimize the risk of transmission to other healthy cows.
- ✚ Application of an in-vitro antibiotic susceptibility test prior to the use of antibiotics in both treatment and prevention of intramammary infections should be implemented.
- ✚ Genotypic analysis to accurately identify *S. aureus* species, detection of genes responsible for antimicrobial resistance must be conducted in the future.

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8. APPENDIX

Blood Agar Base (infusion Agar)

Directions:

Suspend 40.0 grams in 1000 ml distilled water.

Heat to boiling to dissolve the medium completely.

Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 minutes cool to 45-50 °C and aseptically add 5% v/v sterile defibrinated blood. Mix well and pour into sterile petri plates.

Uses:

For isolation and cultivation of many fastidious pathogenic microorganisms after addition of blood.

Standard Formula

Ingredients	Grams/liter
Meat infusion#	10.0
Tryptose	10.0
Sodium chloride	5
Agar	15.0

Final PH (at 25 °C) 7.3 ± 0.2

Formula adjusted, standardized to suit performance parameters.

#Equivalent to Beef heart peptone.

For in vitro Diagnostics

Country of origin- India.

2. MANNITOL SALT AGAR (MSA)

(CHAPMAN MEDIUM), (EUR PHARM)

For the isolation of and enumeration of pathogenic staphylococci from clinical samples and other materials. Suspend 111 grams of the medium in one liter of distilled water were mixed well and dissolved by heating with frequent agitation. Boil for one minutes until complete dissolution. Finally the boiled medium were sterilized in autoclave at 121 °c for 15 minutes. It were cooled at 45-50 °c. Then it was mixed well and dispense into plates.

Compositions

Formula in grams per liter of distilled water

Ingredients:	Grams/liters
Sodium chloride	75.0
D-Mannitol	10.0
Pancreatic Digest of casein	5.0
Peptic Digest of animal tissue	5.0
Beef extract	1.0
Phenol red	0.025
Bacteriological Agar	15.0

Country of origin India

2. NUTRIENT AGAR MEDIUM

Directions:

Suspend 37.0 grams in 1000ml purified /distilled water.

It was heated to boiling to dissolve the medium completely. It was sterilized by autoclaving at Δ 115 $^{\circ}\text{C}$ for 30 minutes or alternatively at 15 Ibs Pressure (121 $^{\circ}\text{C}$) for 15 minutes or as validated cycle. Δ Corresponds to Ibs pressure.

Standard formula

Ingredients	Grams/liters
Peptone	10.00
Beef Extracts	10.00
Sodium chloride	5.00
Agar	12.00
PH after sterilization	7.3 $\pm$ 0.1

Formula adjusted, standardized to suit performance parameters.

APPENDIX-2

QUESTIONNAIRES (Individual animals)

General Information

District_____, Kebeles_____, Name of Animal owners_____, phone number_____

Put(x) mark in space provided for closed ended questions.

1. What is breed of the animal/cow?

2. Age of the animals/cow grouped under

Young_____, Adult_____ any others_____

3. What is the lactation stage of the cow? Early_____, Mid_____, Late_____

4. What is parity of the animals/cow is?

5. Is the udder washed before milking? Yes _____, No _____

When yes, always_____ only when they are dirty_____, No _____

6. with what product? Only water_____, detergents_____, Disinfectant_____, other, what?_____

7. Body condition of animal/cow poor_____, good_____

8. Are treated cow clearly identified? Yes_____No _____

9. Are treated cows are separated from milking cows? Yes_____, No _____

10. Is there any dry cow therapy? Yes_____, No _____

11. Is your cow have any tick? Yes_____, No _____

12. Do you have tick control activity? Yes_____, No
 13. Is there any blind teats? Yes_____ in number____ No
 14. Is there any teats injuries? Yes _____, No
 15. Are there any house for lactating cow? Yes _____, No
 16. What is type of housing?
 17. What is type of floor?
- Bad concrete _____, good concrete_____, muddy soil____and any other
18. Milking mastitis cow lastly? Yes _____, No
 19. Hand pre washing with detergent? Yes_____, No
 20. Is the cow fungal pathogen positive? Yes _____, No
 21. Is the cow bacterial positive? Yes_____, No
 22. Do you isolate cow during milking when you know that they are infected with pathogen?
 23. Quarters are disinfected after milking a cow? Yes _____, No
 24. Fresh cows are separated from the rest of the group? Yes _____, No
 25. Are treated cows separated from postpartum cows? Yes _____, No
 26. What is treatment outcome of cow?

ANNEX-2: PRODUCTION SYSTEM MASTITIS

1. Is it closed herds for at list one years? YES _____, No

If it is not being closed herds answer the following questions

2. Were mastitis records reviewed when the cow were bought? Yes _____, No
3. How many lactating cow does the herd have?
4. Was a culture for mastitis pathogen made at the arrival of the animals? Yes _____, No
5. Was a mastitis test for mastitis made when cows entered in lactation? Yes _____, No
6. If you bought animals what is their origin? A farm _____, several farms _____ Auction
7. Are cows taken to expositions or other events where they are mixed with other events where they are mixed with others animals? Yes _____, No
8. Cows are culled if they have more than _____ cases of mastitis by lactation.
9. Cows are culled if they get more than _____, lactations.
10. Cows are culled when they show high chronic somatic cell count? Yes _____, No
11. Do you isolate the cow during milking when you know that they are infected with pathogen? Yes _____, No
12. Quarter are disinfected after milking a cow? Yes _____, No
13. Are treated cows clearly identified? Yes _____, No
14. Fresh cows are separated from of the group? Yes _____, No
15. Are treated cows separated from milking cows? Yes _____, No
16. Are treated cow separated from postpartum cows? Yes _____, No
17. Frequency of mastitis occurrence in the herd? Yes _____, No
18. How do you manage this condition in your herd?
19. At what age is most susceptible to the mastitis?

20. What are the clinical signs that they observe for them to conclude that the condition affecting the animals is mastitis?

21. Parity of animal (number of time she had calved down)

22. Do you isolate cow with mastitis? Yes _____, No

MILKING PROCEDURE

23. Type of milking: Manually_____, Mechanical

24. The milker's have been trained in the last years?

25. What is the sex of the milker's? F_____, M

26. Milking with calf?

27. Milking protocols are written?

28. Do you apply oxytocin before milking?

29. How frequently do you milk your cows?

30. Are first lactation cows separated from older lactating cow?

31. What is total number of adult cows (all lactation, milking and dry) cows on your her?

32. What is the number of milking cows?

33. How often do you scrape out the dirty bedding from your milking cows?

34. Are dry cows (far- off) pre fresh cows (close-up) kept in separate group?

Treatment and procedure

35. What is response of mastitis to treatment? Good _____, poor

Appendix 3- important laboratory procedure

Reagents Used in Gram Staining

- Crystal Violet, the primary stain
- Iodine, the mordant
- A decolorizer made of acetone and alcohol (95%)
- Safranin, the counterstain

Gram staining procedure

1. Take a clean, grease free slide.
2. Prepare the smear of suspension on the clean slide with a loopful of sample.
3. Air dry and heat fix
4. Crystal Violet was poured and kept for about 30 seconds to 1 minutes and rinse with water.
5. Flood the gram's iodine for 1 minute and wash with water.
6. Then ,wash with 95% alcohol or acetone for about 10-20 seconds and rinse with water.
7. Add safranin for about 1 minute and wash with water.
8. Air dry, Blot dry and Observe under Microscope.

Interpretation

The Grams stain smears from typical colonies that were show Gram-positive cocci occurring in bunch, grapelike irregular clusters were taken as presumptive *S. aureus*.

BIOCHEMICAL TEST

Catalase test

Procedure of Catalase Test

Tube Method

1. Pour 1-2 ml of hydrogen peroxide solution into a test tube.

2. Using a sterile wooden stick or a glass rod, take several colonies of the 18 to 24 hours test organism and immerse in the hydrogen peroxide solution.
3. Observe for immediate bubbling.

Slide Method

1. Use a loop or sterile wooden stick to transfer a small amount of colony growth in the surface of a clean, dry glass slide.
2. Place a drop of 3% H₂O₂ in the glass slide.
3. Observe for the evolution of oxygen bubbles.

Positive- formation of bubble within few second whereas, Negative is has not formation of bubble.

 ***Staphylococcus* (catalase positive)**

Coagulase test

Procedure and Types of Coagulase Test

Slide Test (to detect bound coagulase)

1. Place a drop of physiological saline on each end of a slide, or on two separate slides.
2. With the loop, straight wire or wooden stick, emulsify a portion of the isolated colony in each drops to make two thick suspensions.
3. Add a drop of human or rabbit plasma to one of the suspensions, and mix gently.
4. Look for clumping of the organisms within 10 seconds.
5. No plasma is added to the second suspension to differentiate any granular appearance of the organism from true coagulase clumping.

Tube Test (to detect free coagulase)

1. Dilute the plasma 1 in 10 in physiological saline (mix 0.2 ml of plasma with 1.8 ml of saline).
2. Take 3 small test tubes and label as T (Test), P (Positive Control) and N (Negative Control). Test is 18-24 hour broth culture, Positive control is 18-24 hr *S. aureus* broth culture and Negative control is sterile broth.
3. Pipette 0.5 ml of the diluted plasma into each tube.

4. Add 5 drops (0.1 ml) of the Test organisms to the tube labelled “T”, 5 drops of *S. aureus* culture to the tube labelled “P” and 5 drops of sterile broth to the tube labelled “N”.
5. After mixing, incubate the three tubes at 35-37 Degree Celsius.
6. Examine for clotting after 1 hours. If no clotting has occurred, examine at 30 minutes intervals for up to 6 hours.

Interpretation of Coagulase Test

Positive- Those forming Clumping of cocci were taken as coagulase positive *S. aureus* (Quinn *et al.*, 2002).

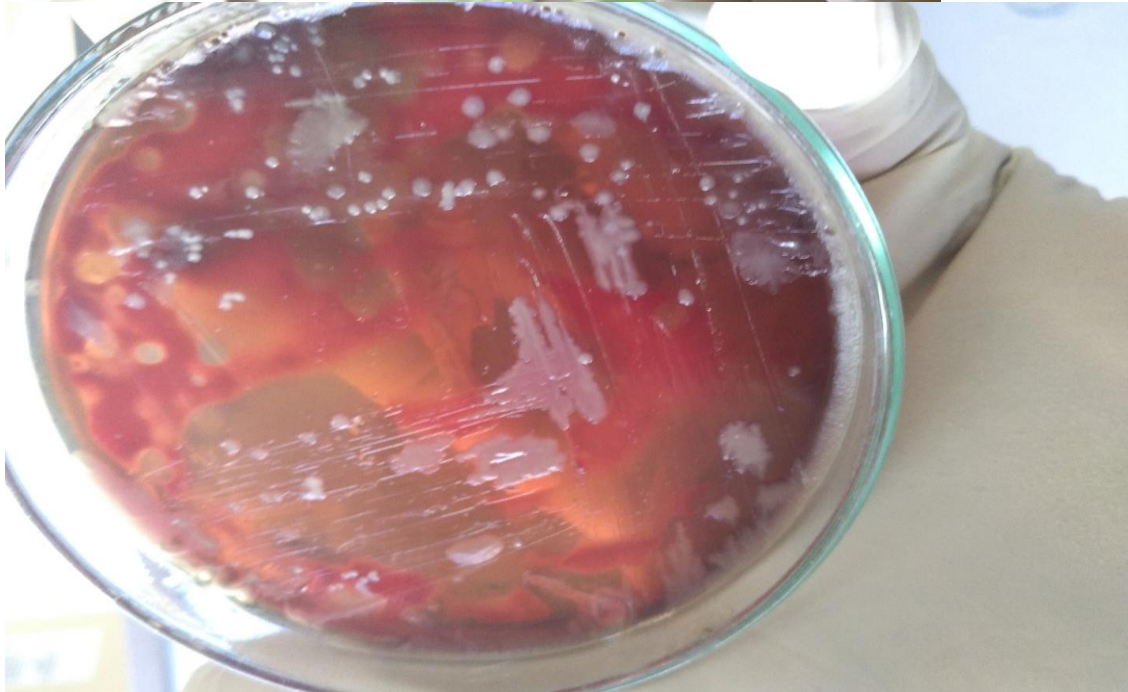
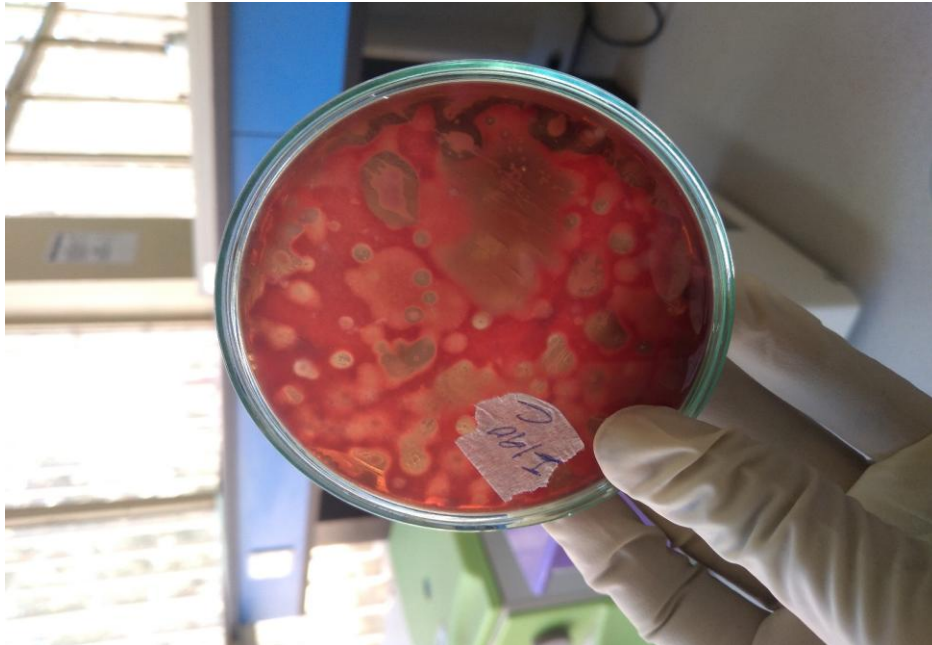
1. Diagnosis of of SCM / CMT

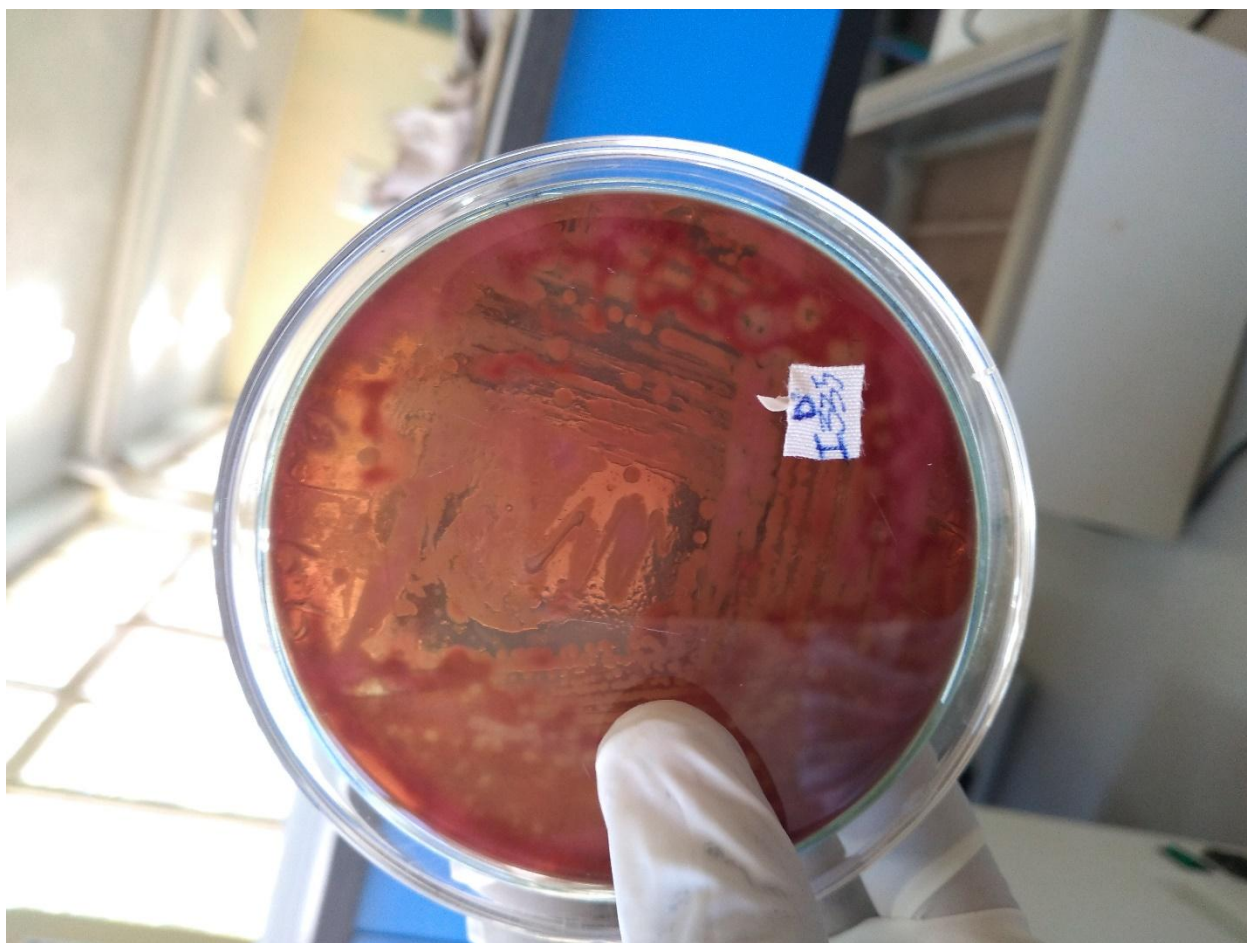
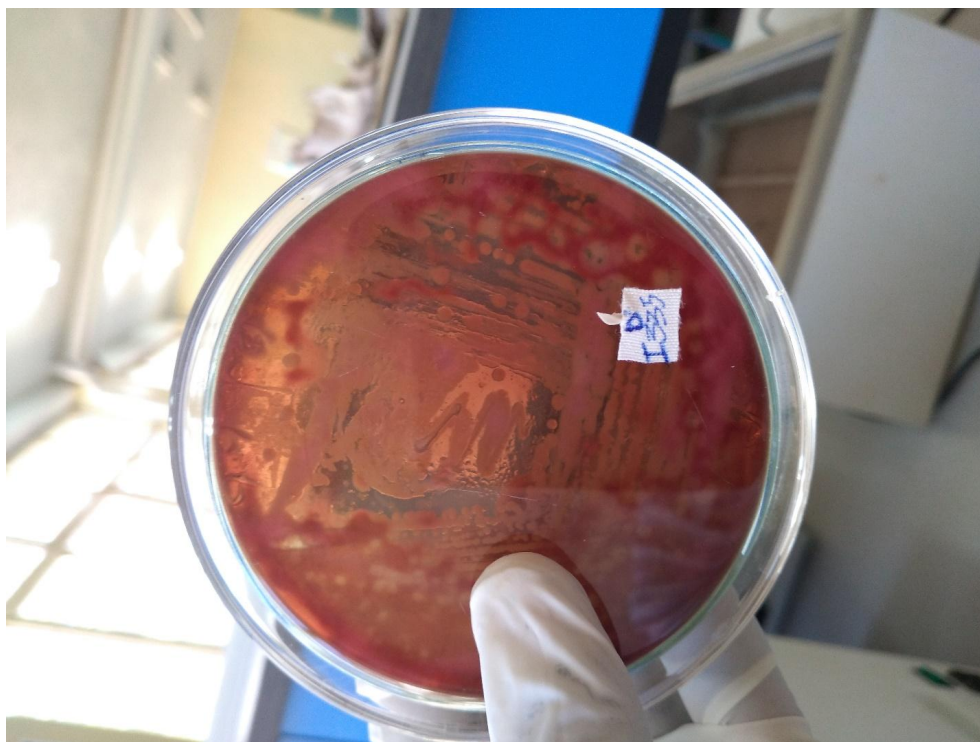


Clinical Examination of Udder



2. Enrichment medium





3. Selective medium



Golden yellow colony on MSA

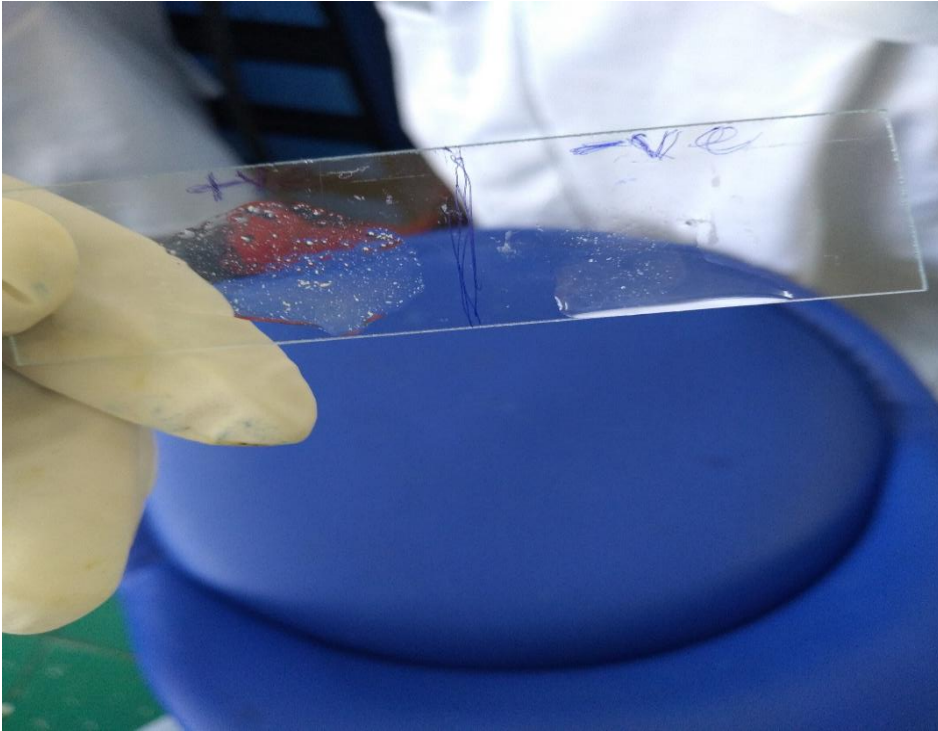


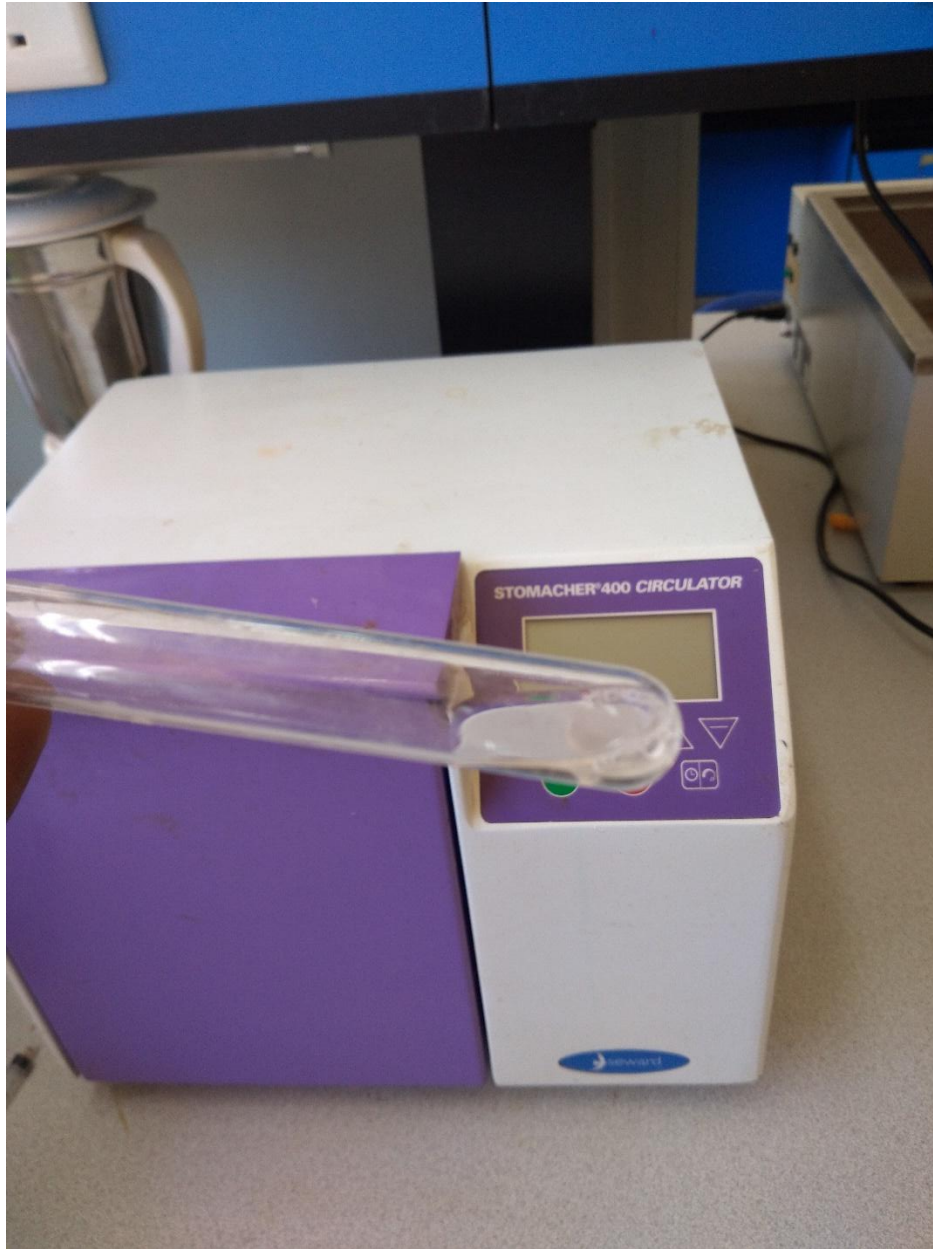
Pure culture on nutrient agar medium

4. Biochemical test



Slide coagulase test





tube coagulase test

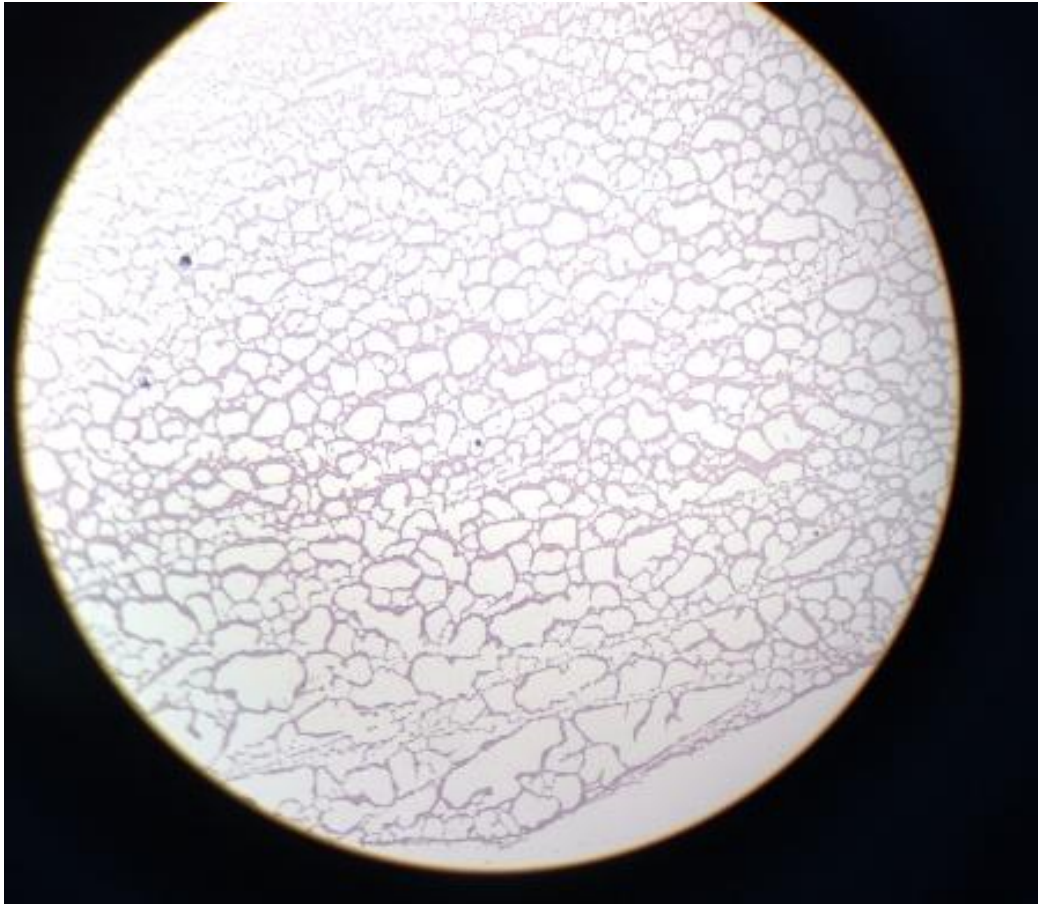
Catalase Test





5. Microscopic morphology of staphylococcus

Morphology of bacteria



Grape like shape of cocci

7. Antibiotic susceptibility test



