PREVALENCE OF STAPHYLOCOCCUS IN BOVINE SUB CLINICAL MASTITIS AND HYGIENIC MILK HANDLING AND PRACTICE IN SELECTED DAIRY FARMS OF MAAYAA CITY, EASTERN ETHIOPIA

MSc THESIS

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DECEMBER 2023

HARAMAYA UNIVERSITY, ETHIOPIA

Prevalence of Staphylococcus in Bovine Subclinical Mastitis and Hygienic Milk Handling and Practice in Selected Dairy Farms of Maayaa City, Eastern Ethiopia

A Thesis Submitted to the College of Veterinary Medicine,

Postgraduate Program Directorate

HARAMAYA UNIVERSITY

In Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE IN VETERINARY PUBLIC HEALTH

Rumia Omer Mohammed

DECEMBER 2023

Haramaya University, Ethiopia

HARAMAYA UNIVERSITY

POSTGRADUATE PROGRAM DIRECTORATE

We here by certify that we have read and evaluated this thesis prepared, under our guidance, by Rumia Omer Mohammed, entitled "**prevalence of Staphylococcus in bovine subclinical mastitis and hygienic milk handling and practice in selected dairy farms of Maayaa City, Eastern Ethiopia**" we recommend that it can be submitted as fulfilling thesis requirement.

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As members of the board of examiners of the MSc thesis open defense examination, we certify that we have read and evaluated the thesis prepared by Rumia Omer Mohammed and examined the candidate. We recommend that the thesis be accepted as it fulfills the requirement for the degree of Master in Veterinary Public Health (MVPH).

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DEDICATION

This manuscript is dedicated to my beloved father Omer Mohammed, my mother Fatuma Ahmed and all of my lovely families for their unlimited support in different manner.

STATEMENT OF THE AUTHOR

First, 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 this thesis. My scholarly matter that was included in the thesis has been given recognition through citation.

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BIOGRAPHICAL SKETCH

Rumia Omer, the author of this paper was born in 1996 G.C in Tullo Woreda West Hararghe Zone in Oromia Regional State of Ethiopia. When she reached school age, she attended elementary and junior education at Hirna primary School and with comprehensive secondary school education at Doba high school. She joined Alage Agricultural Technical, vocational and educational college (AATVET) in 2011 and in 2013 graduated Diploma in Animal Health and employed as expert in Tullo woreda and she joined to Jimma University in 2016 with college of veterinary medicine and graduated in 2020 in Bachelor of Veterinary Science (BVSc) and again in 2021, she joined regular M.Sc. program in the Post Graduate Program Studies of Haramaya University to pursue her study for Degree of Master of Science in Veterinary Public Health (MVPH).

ACKNOWLEDGEMENT

First of all, I thanks to Allah, for giving me the life, patience, wisdom and made it possible to begin and finish this work successfully.

I would like to thanks to my major advisor Prof. Adem Hiko and co-advisor Dr. Sisay Girma for their valuable comments, advice, and support from their knowledge, sustained guidance and constructive comments at all stages during the research work and write up the thesis. They shared with me their rich professional experience and were cooperative from the beginning of proposal writing to the completion of the thesis work, without them the success of this work would not have been achieved.

Most importantly, I appreciate and thanks to Haramaya University, Collage of veterinary medicine and laboratory coordinator Mr. Abdallahi Abdurehman, laboratory staffs and all my teachers for their tireless efforts in assisting me in providing with all facilities to complete my work in time and support during my study.

Furthermore, it is my great pleasure to express my sincere appreciation to my family: my father Omer Mohammed, my mother Fatuma Ahmed, my brothers Shamsudin Omer and Beyan Omer. I would also like to appreciate and thank all my classmates for all the wonderful times we shared during our stay together. The support and encouragement received from all my friends and relatives are also gratefully acknowledged.

Finally, I would like to thank Haramaya University for creating conductive learning environment on the campus with all the needed services.

LIST OF ACRONYMS AND ABREVETION

BAP	Blood Agar Plates
BC	Bacteriological Cure
BMSCC	Balk Milk Somatic Cell Count
CMT	California Mastitis Test
CNS	Coagulase Negative *Staphylococcus*
CPS	Coagulase Positive *Staphylococcus*
MSA	Mannitol Salt Agar
NAP	Nutrient Agar Plates
PCR	Plymerase Chain Reaction
SCC	Somatic Cell Count
SCM	Subclinical Mastitis
SCV	Small Colony Variant
SEs	*Staphylococcus* Enterotoxins
SFP	*Staphylococcus* Food Poisoning

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ABSTRACT

Staphylococcus is causing the majority of mastitis cases in different dairy regions around the world. A cross-sectional study was conducted from June 2022 to November 2023 to isolate Staphylococcus aureus and Coagulase-negative Staphylococcus from bovine subclinical mastitis, estimate prevalence, and assess hygienic milk handling practices in selected dairy farms in Haramaya district, Eastern Ethiopia. Milk sample were collected using a purposive sampling technique and analyzed using STATA software. A semi-structured questionnaire survey and observation, a California mastitis test, bacterial isolation, and identification were conducted during the study. A total of 379 dairy cows from nine (9) dairy farms were screened for subclinical mastitis by the California mastitis test. The prevalence of subclinical mastitis in the study areas was based on the California mastitis test. 379 (51.45%) were positive for subclinical mastitis. The prevalence of 112 (29.55%) of Coagulase Negative Staphylococcus was higher than that of Staphylococcus aureus 83 (21.9%). Among the risk factors studied, subclinical mastitis due to Staphylococcus was significantly higher ($P < 0.05$) in older cows (94.3%) than younger cows (58.6%), in cows during late lactation (91.9%), and in cows that gave more than 5 births (87%). Respondents with an age group ranging from 25 to 50 years and a higher educational level were aware that drinking raw milk is a possible source of Staphylococcus. In addition, this is an opportunity to create awareness about the prevention and control of zoonosis among actively involved groups of society. An observational assessment of the milk handling practices on dairy farms showed that about 55.5% of them did not wash their hands before milking. The study showed that the hygienic status of the udder and houses of lactating cows in the majority of dairy farms in the study area were poor, and the prevalence of subclinical mastitis and Staphylococcus species was high. This suggests that more work on awareness creation is required in societies with lower educational levels compared to those with higher educational levels. Based on the results, creating awareness, careful hygienic milking practices, and regular health monitoring should be practiced to reduce the prevalence of subclinical mastitis and Staphylococcus infection.

Keywords: *Hygiene, Lactating Cow, Milk, Prevalence, Subclinical Mastitis, Staphylococcus*

1. INTRODUCTION

In Ethiopia, livestock represent a major national resource and form an integral part of the agricultural production system. The country has the largest livestock population of any African country, with an estimated 65 million heads of cattle and cows, representing the largest proportion of indigenous cattle in the country (CSA, 2020). Milk produced from these animals provides an important dietary source for the majority of rural as well as a considerable number of urban and peri-urban populations. However, milk production often does not satisfy the country's requirements due to a multitude of factors, among which a disease of the mammary glands known as mastitis is among the various factors contributing to reduce milk production (Berhanu *et al*., 2017). Despite thorough studies, subclinical mastitis continues to be the most costly and potentially contagious disease for the dairy business and consumers globally, irrespective of the animal category (Ayano *et al.,* 2013). Worldwide, dairy cows' health is impacted by the condition known as bovine mastitis (Atalla *et al.*, 2010). In Ethiopia, it is also one of the most expensive dairy cattle diseases (Avall-Jaaskelainen *et al*., 2013). Both the amount and quality of the milk are negatively impacted (Barbier *et al*., 2010).

Mastitis can be divided into two types, clinical and subclinical, based on its symptoms. The clinical form can be identified by one or more of the following symptoms in the udder: edema, discomfort, redness, and heat can be associated with changes in milk. Systemic diseases in general may also be present. The subclinical form, on the other hand, is known as "the silent form" because there are no visible signs (Bochniarz *et al.,* 2013). From cases of bovine mastitis, more than 140 distinct bacteria have been isolated. The most frequent mastitis-causing bacteria are either environmental pathogens, such as *Escherichia coli, Enterococcu*s faecalis, *Streptococcus dysgalactiae*, and *Streptococcus uberis*, or contagious pathogens, such as *Staphylococcus species*, *Streptococcus agalactiae, Corynbacterium bovis*, and *Mycoplasma spp* (Etinosa *et al*., 2016).

The majority of mastitis cases in various dairy regions of the world are caused by Staphylococcus. The skin and mucosal surfaces of both humans and animals are commensal with Staphylococcus *species* (Lee and Yang, 2021). But they have also been linked to human diseases such

as dermatitis, toxic shock syndrome, and food poisoning. Its strains are categorized into two main groups, *coagulase-positive staphylococci* (*CPS*) and *coagulase-negative staphylococci* (*CNS*), based on their capacity to coagulate plasma (Fijalkowski *et al*., 2014). *S. Aureus* is the most dangerous pathogen among *CPS* since it can intoxicate people and animals through food and cause a wide range of illnesses (Frey *et al*., 2013).

According to Kadariya *et al*., (2014), the other groups are to blame for a variety of opportunistic diseases in both people and animals. *S. Aureus* is primarily linked to subclinical and clinical mastitis in dairy cows among the *Coagulase Positive Staphylococcus species* (Pyzik *et al*., 2019). In most nations with a dairy industry, *Staphylococcus aureus* is the most typical causal agent of mastitis in cows. *Staphylococcus* food poisoning (SFP), one of the most common food-borne illnesses, although typically not life-threatening, is brought on by ingesting *Staphylococcus* enterotoxins (ses), which are mostly generated by *CPS*, especially *S. Aureus* (Fisher *et al.*, 2018).

It is the third most commonly reported foodborne pathogen on the globe and a major cause of worry for public health worldwide. It is asserted to be one of the main agents of clinical and subclinical mastitis in nursing cows, which results from mammary gland infection. Research on the epidemic characteristics of *S. Aureus* in raw milk has garnered considerable interest due to the pathogenic microorganisms' contamination of raw milk, notably the toxins produced by *S. Aureus*, as well as the major problem of medication resistance. Most often, *CNS*-induced mastitis is asymptomatic or exhibits very modest clinical symptoms. For whatever reason, primiparous cows and heifers are more prone to developing *Coagulase-negative Staphylococcus* mastitis. The majority of the cows with *CNS* mastitis demonstrated some clinical signs, but in most cases the signs were mild (Umaru *et al*., 2016).

Sometimes there was also a minor swelling of the affected areas, but most of the time it was merely alterations in the look of the milk, such as clots and flakes. The identification of this kind of mastitis is challenging and necessitates the methodical administration of the California mastitis test in dairy production herds due to the lack of clinical symptoms and changes in milk appearance (Bharathy *et al*., 2015).

Although more than 15 different species of *CNS* have been found to be responsible for mastitis in dairy cow, (Schmidt *et al*., 2015) found that *S. Chromogenes, S. Xylosus, S. Simulans, S. Epidermidis, S. Haemolyticus*, and *S. Hyicus* are the most frequently isolated from bovine mastitis. Subclinical mastitis is also brought on by *Coagulase-negative Staphylococus species*, but the symptoms are less severe. *Staphylococcus aureus* and *CNS* typically live on the healthy skin of healthy animals' teats, vagina, coats, and nostrils, as well as on the hands of milkers; from these locations, they colonize the teat canal, penetrate the secretary tissue, and cause infections. They are hence frequently referred to as opportunistic pathogens (Li *et al*., 2019).

However, there have been reports of clinical mastitis cases that frequently require antibiotic therapy, even though intramammary infections brought on by the *CNS* are typically self-limiting (Akindolire *et al*., 2015). According to Anamika *et al.*, (2015), penicillin antimicrobials are efficient against *CNS* infections. *Coagulation-negative Staphylococcus* was once thought to be a natural component of the skin's microbiota. Since these infections have started to outweigh other mastitis etiological factors in several countries, the role of the *CNS* in bovine mastitis has expanded. There is an urgent need to draw attention to the threat that *CNS* poses to both human and animal health given the severity of mastitis issues, the extensive use of antibiotics in dairy cattle, the quantity of animals, and the consumption of milk products. There is little understanding of the causes, progression, and persistence of *CN*S mastitis. Bovine *CNS* strains are little understood, and what little is known about them is mostly based on phenotypic characteristics (Schmidt *et al*., 2015).

Bovine mastitis is thus a worldwide issue because it not only compromises the health of animals but also lowers milk supply and raises the cost of medical care, all of which ultimately result in significant financial losses for the dairy sector. Both the quality and the amount of milk are negatively impacted. *Staphylococcus species* was the primary cause of many microorganisms that were responsible for mastitis. *S. aureus* is more pathogenic than *Coagulase negative Staphylococcus*. *S. Aureus* is an important human and animal pathogen that can cause a wide range of illnesses, including septicemia, osteomyelitis, and superficial skin and soft tissue infections (Li *et al*., 2019). Although associated with animal feeds such as improper handling of straw and other poor hygiene practices, Staphylococcus was the primary cause of these bacteria

in many cases of subclinical mastitis that were dominated by *CNS*. There is little understanding of how subclinical mastitis develops, spreads within a herd, and remains. Is a significant zoonotic bacteria that can seriously infect both humans and animals. *S. Aureus* is responsible for about 40.0% of mastitis cases in various nations.

A few studies have been done in Ethiopia to estimate the prevalence of mastitis (Ananya *et al*., 2015), but very little is known about the disease of mastitis, especially subclinical mastitis. There isn't enough research demonstrating how common mastitis and *S. Aureus* in dairy cows are in Ethiopia. For instance, central Ethiopian samples from Holeta (Ayano *et al*., 2013) and Bishoftu Town (Birhanu *et al*., 2017) both included 13.8% *S. Aureus*, 11.7% *S. Epdermides*, and 44.9% *S. aureus*, respectively. However, there were little studies on the incidence of *S. Aureus* and *CNS* from sub-clinical mastitis in Eastern Ethiopia. Additionally, thorough and methodical investigation and collection of data on the disease's state are required for the control and prevention of such a significant disease in the dairy industry.

Objectives of the Study

General Objective

To estimate the prevalence of *Staphylococcus* in bovine subclinical mastitis and assess hygienic milk handling and practice in selected dairy farms in the study area.

Specific Objectives

- To estimates the prevalence of subclinical mastitis caused by *Staphylococcus aureus* and *Coagulase negative staphylococcus* in dairy cows.
- To assess the potential risk factors for Staphylococcus species associated with bovine subclinical mastitis in the study area.
- To assess on hygienic milk handling and practice of raw cow’s milk.

2. LITERATURE REVIEW

2.1. Overview of Subclinical Mastitis

Mastitis can be described as an inflammation of the mammary gland parenchyma, which might decrease milk production and change the makeup of the milk. Mastitis falls into two primary categories: The first is clinical mastitis, which shows symptoms in the milk or on the animal. The other is subclinical mastitis, which doesn't manifest any outward symptoms in the udder unless diagnostic techniques are used (Ayano *et al*., 2013). Inflammation of the mammary gland without obvious gross lesions on the udder or its secretion when there are pathogenic bacteria and an unusually high amount of somatic cells in the milk are both examples of subclinical mastitis. It is the most common and economically damaging illness in dairy cow nationwide (Oguttu *et al.,* 2018).

It is 3–40 times more common than clinical mastitis and causes the greatest overall losses in most dairy herds. It is a multi-etiologically complex disease that consists of infectious and noninfectious agents as potential risk factors (Birhanu *et al.,* 2017). The prevalence of subclinical mastitis in cows increases with increased milk production, unhygienic management practices, and an increasing number of lactations (Saidi *et al.,* 2013). There are no visible changes in the udder or milk, but it reduces milk production and adversely affects milk quality. Early detection of subclinical mastitis can be done by various indirect and direct tests. Subclinical mastitis in dairy cattle is a major and silent problem that causes higher economic losses to the farmers. It scores top among the diseases that cause significant loss to owners and is one of the main reasons of low production and poor quality milk (Sumon *et al*., 2017).

2.1.1. Causative Agents of Subclinical Mastitis

The presence of pathogenic bacteria in the milk, higher SCCs, and a reduction in milk production from the diseased udder are the main signs of infectious sub-clinical mastitis (Sayeed *et al*., 2020). *S. dysgalactiae* was the most common (50.00%) organism isolated from instances of subclinical mastitis in cows. Numerous additional organisms, including as *Actinomyces pyogenes*, *Pseudomonas aeruginosa*, *Nocardia asteroides*, *Clostridium perfringens*, as well as *species of yeasts*, *Mycobacterium*, *Mycoplasma*, *Pastuerella*, and *Prototheca*, may also be linked

to it. Only a few common bacterial infections, including the *Staphylococcus species*, *Streptococcus species*, *Coliforms*, and *Actinomyces pyogenes*, are responsible for the vast majority of cases (Romero *et al*., 2018).

2.1.2. Economic Importance of Subclinical Mastitis

The most expensive issue facing the dairy business is mastitis. (Mungube *et al*. 2005) state that because of its higher prevalence and longer-lasting negative consequences than clinical mastitis, subclinical mastitis is regarded as the most economically significant type of mastitis. The ensuing economic losses include direct losses like (1) a temporary or permanent decline in milk production; (2) a deterioration in milk quality in subclinical instances because of an increase in somatic cells; and (3) a complete rejection of milk in clinical mastitic cases or because of antibiotic residues. Indirect losses are also comprised of the following: (1) early replacement costs and premature dairy cow culling; (2) poor cow selling prices; (3) mastitis prevention costs; and (4) additional veterinary and treatment expenses (Nibret *et al*., 2013).

For instance, production losses from subclinical mastitis (SCM) have been estimated to cost 38 US dollars per lactation per cow in Ethiopian crossbred dairy cows, with subclinical mastitis accounting for more than 90% of the overall loss in milk output. According to Romero *et al.,* (2018), SCM caused milk production losses per farm in Colombia that ranged from 1.3% to 13.5% and had an estimated economic impact of about USD $800.000 annually. is the third most commonly reported foodborne pathogen in the world and a major cause of worry for public health globally. Research on the S. aureus epidemic features is necessary because raw milk can be contaminated with harmful bacteria, notably the toxins produced by *S. aureus*, and there is a severe problem with medication resistance (Umaru *et al.,* 2016).

2.1.3. Public Health Risk of Subclinical Mastitis

They have a significant impact on a variety of diseases in the veterinary field, including septicaemia in poultry, exudative epidermitis in pigs, canine pyoderma, and bovine mastitis (both caused by *Staphylococcus aureus* and *Coagulase-negative Staphylococcus species*). The most dangerous pathogen among *CPS* is because it can intoxicate people and animals through food and cause a wide range of illnesses According to (Becker *et al.,* 2014). The other group, the *CNS*, is

to blame for a variety of opportunistic infections in both people and animals. Normally, *S. aureus* and *CNS* live on the healthy skin of healthy animals' teats, vagina, coats, and nostrils, as well as on the hands of milkers. From these locations, they colonize the teat canal, invade the secretory tissue, and cause infections. Hence, they are often called as opportunistic pathogens are a significant zoonotic virus that can seriously infect both humans and animals. *S. aureus* is responsible for about 40.0% of mastitis cases in various nations.

The *CNS* can cause food poisoning and is linked to nosocomial infections in neonatal critical care units, although it is less virulent than *S. aureus* because it has a narrower variety of virulence factors (Beyene *et al*., 2017). Although with less severe symptoms, *coagulase-negative Staphylococcus* species can also cause clinical or subclinical mastitis. In addition to *S. aureus*, *CNS* has significantly increased the rate of mastitis in recent years (Bharathy *et al*., 2015). Because there are numerous species in this group, *CNS* was previously thought to be a contaminant in clinical specimens (Cemil *et al*, 2016). Due to the recent appearance of numerous novel species, particularly those associated with infections brought on by foreign medical devices and infections in patients who have impaired immune systems, this perception is shifting (Chaje *et al.,* 2015). Due to the widespread use of antibiotics in the diagnosis, treatment, and management of subclinical mastitis, antibiotic residues in milk are a further public health concern. Antibiotic residues in food can trigger severe reactions in people who are allergic to them and, at low concentrations, can sensitize healthy people. The producers are responsible for minimizing the danger of antibiotic residues appearing in milk and meat by following the advised withholding times (Debaraj *et al*., 2016).

2.2. *Staphylococcus*

2.2.1. Characteristics of Mastitis Caused by *Staphylococcu*s Group

The prevalence of contagious diseases, has decreased, which has led to a sharp fall in the average bulk milk SCC (BMSCC) throughout the majority of Ethiopian nations. Even with low BMSCC, *S. aureus* is still a significant mastitis-causing infection on many farms (Mpatswenumugabo *et al* , 2017). The degree of difficulty in treating existing infections, which results in both recurring clinical episodes and the transfer of bacteria to herd mates, is a significant factor in why *S. aureus* prevalence in herds continues to be high. Bacteriological cure (BC) of *S. aureus* mastitis during

lactation is often poor, both in subclinical and clinical instances (Youssif *et al.*, 2020), and is mostly influenced by the length of the infection, the kind of bacteria involved, and the length of the therapeutic regimen (Mureithi *et al.*, 2016).

Additionally, according to (Björk *et al.*, 2014) some strains of *S. aureus* have a better likelihood of cure, a lower persistence, and a stronger clinical manifestation than others. It is possible to choose which subclinical *Staphylococcus*-infected cows will receive therapy based on the likelihood that they will recover. According to popular thinking, the *CNS* is a group of bacteria that protects the udder from serious diseases while causing minor, subclinical inflammation (Etinosa *et al*., 2014). On the other hand, they are now referred to as "emerging mastitis pathogens" (Ghias *et al*., 2016) and their significance in the etiology of animal illnesses has been steadily growing. The progression of *CNS* mastitis is characterized by a small number of variables. The illness is typically asymptomatic, mild, and occasionally self-curable. We are unaware of any data about the degree of pathogen eradication following recovery.

When comparing *S. haemolyticus* subtypes from milk and teat apices showed that the intramammary infection-causing agent most likely originates from the teat skin, suggesting that *CNS* species present in the animal environment (skin) differ from those found in milk. Mastitis is typically caused by a single species of *CNS*; however, (Morgensternet *et al*, 2016) discovered two strains in milk from cows with the condition. However, it can also cause serious or even fatal infections in people, companion animals, and food-producing animals. Even though they occasionally produce asymptomatic colonization in healthy people, *Coagulase-positive staphylococci* (*CPS*) are typically harmful. There are currently 52 species and 28 subspecies in the group (Neeraj et al., 2017). Molecular techniques can be used to discriminate between the forty or *Staphylococcus CNS* species that are currently recognized. While some of them are indicative of bovine mastitis, not all of them are (Nunes *et al*, 2015*). S. chromogenes*, *S. xylosus, S. simulans, S. epidermidis, S. hemolyticus*, and *S. sciuri* are the most frequently isolated bovine *CNS* strains (Osman *et al*, 2016). There are nine species of *CPS* that are commonly recognized in veterinary bacteriology, including *Staphylococcus schleiferi* subsp. *Coagulans*, *Staphylococcus intermedius*, *Staphylococcus pseudointermedius*, *Staphylococcus delphini*, and *Staphylococcus hyicus*. *Streptococcus cornubiensis*, *Streptococcus lutrae*, and *Streptococcus agnetis*. Some

species, such as *S. hyicus* and *S. agnetis*, are classified as coagulase variable, which means that different strains of these species may be coagulase positive or negative.

According to(Vitali *et al,* 2014) *S. chromogenes, S. sciuri* and *S. haemolyticus* were among the other major species recovered from Polish cows' milk. A limited amount of information, rarely supported by genetic data, has been presented to date in articles on several *CNS* species that cause mastitis. We can infer that *S. chromogenes* infections are less likely to develop antibiotic resistance and hence respond better to antibiotic treatment. However, *S. chromogenes* infections (Moser *et al*, 2013) can be clinical in form or endure in the udder (Kudinha *et al.*, 2012) and *S. simulans* infections can also endure. According to (Fry *et al*, 2014) *S. chromogenes* is a frequent cause.

2.2.2. Differences and Similarity between *Coagulase Negative Staphylococcus* and *S. aureus*

The similarities and differences between *CNS* and *S aureus* mastitis are unclear. It is important to examine the claim made by (Becker *et al,* 2014) that *CNS* and *S. aureus* are similar mastitis etiological agents. Somatic cell counts (SCC) are typically increased by *S. aureus* more than *CNS*, but the pattern for SCC (SCC being elevated for a long time) is comparable. Furthermore, both *CNS* and *S. aureus* cause equal harm to the udder tissue. The ability of *CNS* to persist in the udder in a manner comparable to that of *S. aureus* in the so-called small colony variation (SCV) phenotype is both intriguing and concerning (El-Jakee *et al.*, 2013).

2.2.3. *Staphylococcus* Virulence Factors that Play a Role during Mastitis in Dairy Cattle

The ability of the pathogen to invade the host (by breaking through the barriers of skin and mucosal surfaces), colonize the host, and successfully multiply inside the organism (and evade the subsequent steps of the immune response) are the three main factors that determine the development and outcome of bacterial infections. These characteristics are linked to the pathogenicity or virulence factors of *Staphylococcus*. The functional classes of *Staphylococcu*s virulence factors can be broken down into three categories: adhesion mediators, immune response inhibitors that originate from secreted proteins, and bacterial cell structure components. Toxins are created during the stationary phase of bacterial development, and microbial surface

components recognize sticky matrix molecules during the logarithmic growth phase (Xu *et al,* 2016).

S. aureus produces a number of virulence factors that can contribute to the pathogenesis of mastitis in various ways (Li *et al*., 2019). *S. aureus* virulence factors can be generally divided into two classes: surface-localized structural elements that act as virulence factors and secreted virulence factors. These two types of virulence factors work in concert to help the pathogen get past the host's defenses and invade mammary glands. Membrane-bound factors (collagen binding protein, fibrinogen binding protein, elastin binding protein, and penicillin binding protein), cell wall-bound factors (protein A, lactamase, and protease), and cell surface-associated factors (capsule and slime) are a few of the surface-localized structural elements that act as virulence factors (Bexiga *et al*, 2014).

It is challenging to genetically determine the pathogenicity and virulence of *Staphylococcus*. Some virulence factors experience ongoing variations in their expression. In the beginning, virulence factors were only discovered to be part of pathogen genomes. Skin *Staphylococcu*s can be providers of virulence genes for *S. aureus* as it has been shown that they may also be coded by commensal bacteria. Genomic islands (formerly known as pathogenic islands and islets) are structures in the accessory genome and the core genome that can encode virulence factors. Global regulators like the accessory gene regulator (AGR) and the *Staphylococcus* accessory regulator (SAR) control the expression of these factors in relation to environmental variables (Fisher *et al*, 2018).

Contrary to *S. aureus*, *coagulase-negative Staphylococcus* lack well-established virulence factors. *Coagulase-negative staphylococcus* have not been identified to possess any of the primary virulence factors or toxins of *S. aureus* and it is apparent that the formation and persistence of different pathways lead to *Staphylococcus* infections, which are frequently linked to external objects. *S. pseudintermedius* shares several characteristics with *S. aureus* that make it virulent. The assumption that some host factors also have a substantial role in the development and outcome of the disease is supported by the fact that virulence factors are frequently identified in isolates from healthy and diseased animals (Silva *et al*., 2014).

2.2.3.1. Plasmids and transposons

Most *Staphylococcu*s carry a variety of plasmids, some of which can be exchanged by conjugation with other *Staphylococcus* or *S. aureus* of different species (Bexiga *et al,* 2014; Bhattacharyya *et al,* 2016). According to (Beuron *et a*l, 2014) this appears to be a key route for the dissemination of antibiotic resistance determinants, particularly aminoglycoside and beta-lactam resistance. In *Coagulase-negative staphylococcus* transposons can transfer resistance genes between plasmids and from plasmids to chromosomal sites.

2.2.3.2. Bacteriophages

For *Coagulase-negative Staphylococcus* there are bacteriophages tailored just like for *S. aureus*. Modern genetic typing techniques, such as pulsed-field gel electrophoresis of chromosomal digests or PCR-based methods, have replaced earlier attempts to build a phage typing system comparable to that used to categorize *S. aureus* (Cepas *et a*l, 2019).

2.2.3.3. Surface Proteins

Several cell wall proteins of *Staphylococcus* have been described, and specific bacterial binding mediated by these proteins to extracellular matrix molecules (i.e., fibrinogen, fibronectin, vitronectin, laminin, and collagen) has been observed (Srednik et a*l,,* 2017). However, the importance of these protein interactions in the pathogenesis of *Coagulase-negative Staphylococcus* colonization or infection remains to be demonstrated conclusively. Recently, electron microscopy has revealed a fimbria-like protein structure that may play a role in the attachment of *Coagulase-negative Staphylococcus* to foreign materials in the host (Silva *et al,* 2014). Several proteins have been demonstrated to play a role in the pathogenesis of *S. saprophyticu*s infections, in contrast to *S. epidermidis*. The invasion of the organism has been related to a urease, and a protein called hemagglutinin and surface fibrillar proteins have been linked to attachment to the urinary tract epithelium (Mahato *et al*, 2017).

2.2.3.4. Capsular Polysaccharides

Although there is still a dearth of knowledge regarding the chemical makeup and precise functions of the polysaccharides on the surface of *Coagulase-negative Staphylococcus* it is almost certain that these compounds play major virulence factors in the attachment and/or persistence of bacteria on foreign materials (Yu *et al*, 2017).

2.2.4. Transmission between Humans and Dairy Cows

Staphylococcus virulence factors can have an impact on people in two different ways. In the beginning, close interaction with animals can promote interspecies transfer and *Staphylococcus* adaptation from animals to humans. Second, gadgets infected with animal strains of *Staphylococcus* or foods of animal origin like milk and milk products might indirectly transmit the disease. The transmission of *Staphylococcus* mastitis strains from dairy cows to humans is uncommon. However, it was noted that such a transfer is probable (Tremblay *et al*, 2014). Additionally(Oguttu *et al*, 2017) discovered identical strains of *CNS* (*S. epidermidis*) in milk and on milkers' hands, raising the possibility that humans are the source of the bacteria that cows are exposed to. Major pathogen infections that are treated with *CNS* often accompany. The idea introduced, e.g., by (Frey *et al*, 2013) that the *CNS* harbors drug resistance genes for other bacteria, such as *S. aureus* or *Streptococcus* species, is very disturbing. Ingestion of contaminated raw milk is the major cause of serious food-poisoning outbreaks, potentially resulting from microbial toxin production (Ayano *et al.,* 2013). Contaminated raw milk may contain hazardous microorganisms that cause milk to spoil or cause the onset of public health hazards (Neeraj *et al.,* 2017). *Staphylococcus aureus* (*S. aureus*) is one of the most important opportunistic pathogens in raw milk that can cause serious infection in humans (Saidi *et al.,* 2013).

Staphylococcus aureus has high pathogenicity due to its widespread distribution, high contamination rate, and rapid transmission. It may result in a variety of clinical signs, ranging from mild skin lesions to serious invasive infections, and it may even be fatal (Mureithi and Njuguna, 2016). But *S. aureus* can enter milk through direct excretion from a cow with clinical or subclinical *Staphylococcus* mastitis as well as through environmental contamination during the handling and processing of raw milk, posing a risk to consumers (Romero *et al*., 2018). In order

to identify the possible risk that this bacterium poses to the public health, it is crucial to constantly monitor virulent strains of *S. aureus* (Qolbaini *et al.*, 2014).

According to epidemiological research, dairy products can be contaminated with *CNS* when made with unpasteurized raw milk and handled by workers during manufacturing processes (Suja *et al*., 2017). A case study of food poisoning outbreaks linked *CNS* strains to unpasteurized milk was also conducted (Klibi *et al*., 2018). According to (Osman *et al.* 2017) the *CNS* strains isolated from food have the capacity to produce a variety of virulence factors, including Staphylococcus enterotoxins. Despite the fact that *CNS* is a very important bacteria for food production and preservation from a hygienic standpoint (Ibadin *et al*, 2017 , May *et al* ,2014) deemed the presence of CNS in food to be of public health significance due to the potential for the spread of AMRB and AMRG. The CNS of both animals and humans are thought to function as significant AMRG reservoirs (Shrestha *et al*., 2017), which may transfer and integrate into the genome of *S. aureus*, resulting in the formation of new, potentially more resistant strains (de Oliveira *et a*l., 2016).

2.2.5. Intracellular Survival of *Staphylococcus*

Bacterial survival inside the host's cells is frequently associated with the formation of the so-called small colony variants (SCV), a bacterial phenotype that some authors believe originates from stable genetic mutations (Nonnemann *et al.*, 2019). It was observed by (Markey *et al*, 2013) that infections caused by the CNS persist. Bacteria survived the first lactation inside the udders of pregnant heifers. SCVs in dairy cows are a cause of chronic, persistent mastitis and are probably more common than previously thought (Asmare *et al.,* 2017*). CPS,* such as S. *aureus,* can form the SCV phenotype, as proven by (Fowoyo *et al.* 2017), and survive inside macrophages, neutrophils, and mammary epithelial cells (Cengiz *et al*., 2015). Invasion of mammary epithelial cells by SCVs includes bacterial adherence to the epithelial cell surface, the formation of pseudopod-like structures, and engulfment within endocytic vesicles.

Toxic SCV strains then secrete the pore-forming α-toxin and escape to the cytoplasm. Bacterial leucocidins are secreted to evade lysosomal destruction (Kaliwal *et al.,* 2011). Intracellular survival of *S. epidermidis* is likely to be connected to its biofilm production ability, but the SCV

problem has not been examined regarding CNS in bovine mastitis. The *S. aureus* SCV formation is connected to changes in the expression of the global regulator Agr and mutations in genes coding for the electron transport chain proteins (Zmantar *et a*l, 2011).

2.2.6. Treatment

Because most infections due to *coagulase-negative staphylococcus* are nosocomial in origin, it is not surprising that they have become increasingly resistant to multiple antibiotics over the years. Intravenous treatment is generally required, at least initially, because most infections are systemic and associated with foreign materials. If the organism is susceptible to well-absorbed oral antibiotics, therapy can sometimes be completed by the oral route once the infection has been controlled and the foreign material removed (Nibret *et al.,* 2011). Anti-staphylococcus penicillins (such as methicillin and oxacillin) are the first-line agents for the treatment of susceptible *Staphylococcus* infections. They are potent and rapidly bactericidal, and they have demonstrated effectiveness against difficult-to-eradicate infections such as endocarditis (Bachaya *et al.,* 2011). First- and second-generation cephalosporins (cefazolin, cefuroxime) generally have excellent activity against methicillin-susceptible *Staphylococcu*s and can be substituted in appropriate clinical situations. Unfortunately, more than 80% of clinical strains are now resistant to methicillin (Otto *et al,* 2012). Methicillin-resistant strains are invariably resistant to cephalosporins.

2.3. Prevalence of Subclinical Mastitis and *Staphylococcu*s

2.3.1. Prevalence of Subclinical Mastitis in the World

As shown in Table 1, SCM is globally distributed and occurs in the dairy industry. It ranged from 42.5% in Iran (Hashemi *et al.*, 2011). A high prevalence of 87.9% (Kasozi *et al.*, 2014) in Uganda, 86% in Indonesia (Qolbaini *et al.*, 2014), and 85.33% in Nigeria (Shittu *et al.*, 2012) were reported.

Table 1: Prevalence of bovine subclinical mastitis globally

Country	Location	Total No. Examined	Percentage of SCM (%)	Reference
Algeria	Jhenaida Bangladesh	100	67.9	Sayeed *et al*, 2020
Algeria	Fayoum district	1153	38.5	Youssif *et al.*, 2020
Colombia	Colombia	12000	55.2	Romero *et al.*, 2018
Rwanda	Rubabu and nambibu distinct	123	50.4	Mpatswenumugabo *et al.*, 2017
India	Madhya Pradesh	550	27.81	Maheshwari *et al.*, 2016
Kenya	Thika subcountry	172	64	Mureithi and Njuguna, 2016
Uganda	Kiboga district	124	87.9	Kasozi *et al.*, 2014
Indonesia	KUNAK Bogor	102	86	Qolbaini *et al.*, 2014
Algeria	the center region of Algeria	140	28.57	Saidi *et al.*, 2013
Thailand	Khon Kaen province	285	36.14	Jarassaeng *et al.*, 2012
Nigeria	Savannah Region	300	85.33	Shittu *et al.*, 2012
Iran	Fars province, south of Iran	1545	42.5	Hashemi *et al.*, 2011
New S.Wales	New South Wales	382	49.5	Plozza *et al.*, 2011
Algeria	Haryan (Hindia)*	2057	7.33	Sharma and Sindhu, 2007.

*Buffalo

2.3.2. Prevalence of Subclinical Mastitis Reported in Ethiopia

The prevalence of subclinical mastitis ranged from 16.1% (Birhanu *et al*., 2017) to 62% in Ethiopia.

Table 2. The Prevalence of bovine subclinical mastitis in Ethiopia

Location	Total No. Examined	Percentage of SCM (%)	Reference
Wolmara district	384	44.8	Abdeta and Gemechisa, 2020
Mecha district	344	21.8	Yimam *et al.*, 2020
Bishoftu Town	262	16.1	Birhanu *et al*. 2017
Wellega	532	27.7	Gutu *et al.*, 2021
North West	167	62	Mokennin *et al.*, 2017
Assosa	368	28.34	Melak et al., 2021
Holata district	546	41.2	Ayano *et al.*, 2013

2.3.3. *Staphylococcus* Report from Ethiopia

Different studies have shown that SCM is mainly caused by gram positive bacteria including CNS and *S. aureus (*Hegde *et al*., 2013). Thus as shown in Table 3, the frequently reported from SCM are *S. aureus* and CNS from subclinical mastitis in Ethiopia.

Table 3. Prevalence of from subclinical mastitis *Staphylococcus* species

Country	Location	Total Animal Examined	Percentage of subclinical mastitis (%)	Staphylococcal species	%	Reference
Ethiopia	Holeta	546	41.02	*S. aureus*	13.8	Ayano *et al.,* 2013
Ethiopia	Holeta	546	41.02	*S. epdermides*	11.7	Ayano et al., 2013
Ethiopia	Bishoftu	262	16.1	*S. aureus*	44.9	Birhanu *et al*. 2017
Ethiopia	H.Univ.	210	71.4	*S. aureus*	66	Tefa *et al.,* 2015
Ethiopia	H.University	50	48	*S. aureus*	33.3	Alemu and Abraha, 2017
Ethiopia	E.Hararghe. Zone	247	51.8	*CNS*	34.2	Zeryehun and Abera, 2017
Rwanda	Rubabu	123	50.4	*CNS*	51.5	Mpatswenu *et al.,* 2017
Rwanda	Rubabu	123	50.4	*S. aureus*	20.6	Mpatswenu *et al*., 2017
Kenya	Thika	172	64	*S. aureus*	35.5	Mureithi, 2016
New S. Walse	NewS.Walse	382	49.5	*S. agalactae*	34	Plozza *et al.,* 2011
Algeria	C. region	140	28.57	*S. aureus*	40	Saidi *et al.,* 2013
India	India	2057	91.3	*Staphylococcus*	38.81	Sharma and Sindhu, 2007
Bangladesh	Bangladesh	158	51	*Strept.* spp.	20.99	Sumon *et al.,* 2017
Bangladesh	Mymensingh	240[a]	51	*S. aureus*	18.33	Sumon *et al*., 2017
Bangladesh	Mymensingh	240	51	*CNS*	10	Sumon *et al*., 2017
Egypt	Fayoum	350	19.9	*S. agalactae*	31.52	Youssif *et al*., 2020
Egypt	Fayoum	350	19.19	*S. aureus*	52.5	Youssif *et al.,* 2020
Egypt	Fayoum	1153	38.5	*S. aureus*	66.66	Youssif *et al*., 2020

[a]Quarter

3. MATERIALS AND METHODS

3.1. Study Area

A cross-sectional study was conducted on the selected dairy farms in Maayaa City, Eastern Ethiopia. Haramaya District is located in the Eastern Hararghe Zone of the Oromia region of Ethiopia. The area is located 14 km west of the Harare region, 508 km east of Addis Ababa, and 35 kilometers from Dire Dawa. The estimated animal population in the area is about 63,723 cattle, 13,612 sheep, 20,350 goats, 15,978 donkeys, 530 camels, and 42,035 chickens. Topographically, it is situated at an altitude of 1600 to 2100 m above sea level, which puts the area into the category of a highland with a mean annual temperature and relative humidity of 18°C and 65%, respectively. Haramaya district is located at 42 ° 01′ E and 9° 24′ N at an altitude of 1950 meters above sea level. It has 9 dairy farms based on animal population in the dairy farms categorized in to two groups' small holder and intensive type or sample source of farms.

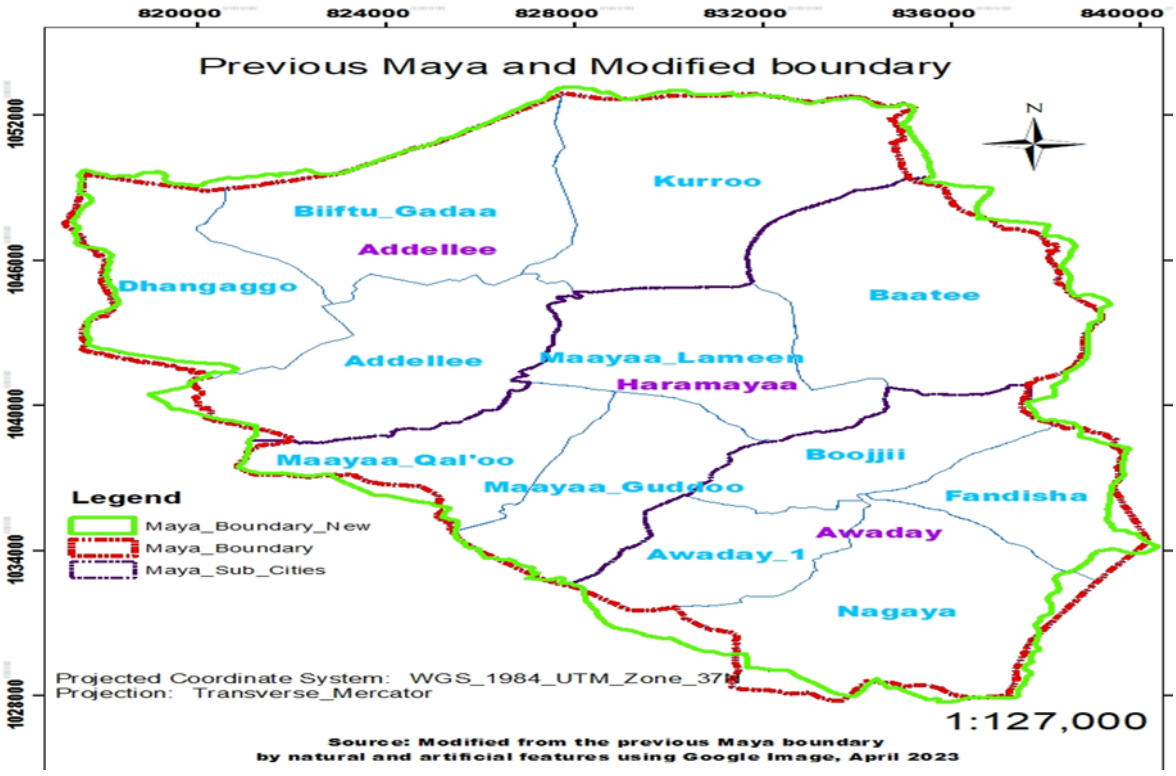

Figure 1. Map of Maayaa City

3.2. Study population

The study populations were those lactating Holstein cross-breed cows from nine farms documented in Haramaya University, Adele, Maayaa city, and Awaday town. All age-lactating categories of cows and the number of parities were included. All of the animals were kept indoors and supplemented by products of the brewery, molasses and hay. The farms were intensive productions where dairy animals were kept indoors at zero grazing.

3.3. Study Design and Sample Size Determination

A cross-sectional study was carried out from June 2022 to April 2023 on apparently healthy lactating dairy cows. The sample size was calculated using the average 44.5% expected staphylococcal prevalence from SCM calculated from three reports (33.3% *S. aureus* and 66% *CPS* from Haramaya University (Tafa *et al.,* 2015; Alemu *et al.,* 2017) and 34.2% *CNS* from selected districts of Eastern Hararghe Zone (Zeryehun *et al.*, 2017), all from Eastern Ethiopia, based on the formula given by Thrusfield (2008). The 95% confidence interval and 5% level of precision were also used. The total sample size using the formula given here below was 379.

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

Where n = required sample size, p = expected prevalence, and d=desired absolute precision.

The number of study animals selected from each farm was determined based on the proportion of the cattle population on each farm. Accordingly, 379 lactating cows were sampled from nine farms: one (1) Haramaya University dairy farm, two (2) dairy farms from Adele, three (3) dairy farms in Haramaya towns, and three (3) Awaday towns.

3.4. Inclusion and Exclusion Criteria

Only apparently health lactating cows with no any clinical sign of mastitis were included in the study sample. Up on purposively sampling, apparently health lactating cows were included. However, animal showing any one sign of clinical mastitis, animal under treatment and new animal entered in the farm were excluded from the study.

3.5 Sampling Technique

A total of 379 samples were collected from June 2022 to April 2023 from the Haramaya district nine dairy farms. 10ml milk sample were collected using a purposive sampling technique. By using gloves, the milk samples from purposively selected nine dairy farms of Maayaa city were taken directly from the four teats of lactating cows of each purposively selected nine dairy farms during study period. Then the animal were followed through CMT (California Mastitis Test) to examine sub clinical mastitis for possible bacterial isolation during the milking process. 262, 161, 63 and 114, milk sample were taken from each dairy farms at Aweday, Haramaya town, Haramaya university and Adelle dairy farms respectively based on size of dairy farms the animals.

3.6. Collection of Milk Samples

The udder and especially the teat were cleaned of dirt with a textile cloth moistened with distilled water. After that, the teat apex was cleaned with a cotton swab moistened with antiseptic solution. Approximately 10 ml of milk was collected from subclinical (CMT positive) mastitic cows into horizontally held sterile test tube after discarding the first 2-3 milking streams and transported to Haramaya University Microbiology Laboratory for microbiological analysis in an ice box maintaining temperature at 4 ^{0}C±1 ^{0}C. Milk samples for bacteriology were collected aseptically.

3.7. Questionnaire Survey and Observation

Semi-structured questions were used to assess the management and hygienic practices of dairy farms. The farm owners, milking personnel, and farm attendants from selected farms were interviewed face-to-face about the way they handle and manage farms, milk, and milk products. Generally, farm or animal owners, milk collectors, and farm attendants were interviewed while sampling. Consequently, hygienic practices employed in the farms, such as house cleaning, udder cleaning, hand washing practices, milking utensils, collecting vessels (buckets), hygiene, and other conditions that affect the hygienic quality of raw milk, were assessed. For the questionnaire survey, sample size was calculated using the formula given by Arsham (2005): $N = 0.25/SE^2$, where N = sample size and SE (standard error) = 5%. The required sample size for the questionnaire survey was 100, but to add a 10% non-response rate, a total of 110 individuals are needed to assess the management and hygienic

practices of raw milk cows on each selected dairy farm. The personal observations included hygienic practices in the dairy cows and the personal hygiene of dairy house workers.

3.8. Isolation and Identification of Staphylococcus Species

In this study, a California mastitis test and bacterial isolation were conducted following standard procedures. The California mastitis test (CMT) was carried out following the procedure for screening subclinical mastitis. Briefly, a drop of the CMT reagent (4% NaOH in distilled water and 1% bromothymol blue) was put on the 4 cups of the CMT paddle, to which an equal amount of 5 ml of milk from the respective quarters of the cow was added and gently mixed by rotating the paddle in a horizontal plane for 20–30 seconds. The test result was interpreted based on the thickness of the gel formed by CMT reagent and milk mixture as 0 and trace for negative and +1, +2, and +3 for positive. Cows were considered positive for CMT, when at least one-quarter turned out positive (Sumon *et al.,* 2017).

Bacterial culture and identification were conducted aseptically and collected by standard milk sampling techniques. The samples were inoculated aseptically onto sterile blood agar plates (BAP) enriched with 7% heparinized sheep blood, incubated at 37°C for 24–48 hrs under aerobic culture conditions, and examined for the presence of *Staphylococcus.* Colonies of *Staphylococcus* species were identified based on their morphological aspects (creamy, greyish, white, or yellow colonies) and hemolytic pattern on the surface of BAP.

Presumed staphylococcal colonies were sub-cultured on nutrient agar plates (NAP) and incubated at 37°C for 24–48 hrs to get pure culture. Pure cultures of a single colony type from the NAP were inoculated into nutrient slants and incubated at 37°C for 24–48 hrs under aerobic culture conditions; the pure isolates in the nutrient slant were preserved and maintained at 4°C for further analysis. The isolation and identification of *Staphylococcus* species were performed from pure isolates grown on NAP. The shape and arrangement of these colonies were detected after performing Gram staining. The catalase tests were conducted and the *Staphylococcus* species were assumed to be the colonies that produced gas bubbles.

The colonies that were identified by Gram staining and catalase tests were sub-cultured on Mannitol Salt Agar (MSA) plates and incubated at 37°C examined after 24–48hrs for growth and change in the color of the medium. The presence of growth and change of pH in the media (red to yellow color) was regarded as confirmative identification of the salt-

tolerant *staphylococci*. The fermentation of mannitol by *S. aureus* causes yellow discoloration of the medium. Colonies that develop weak or delayed yellow color after 24 hrs of incubation were regarded as *S. intermedius* and colonies that failed to produce any change on the medium were considered as *S. hyicus* and *CNS.*

3.9. Data Management and Analysis

Data collected were coded accordingly, entered into a Microsoft Excel 2007© spread sheet, and analyzed using STATA software. Descriptive statistics were used to summarize the collected data. The prevalence of subclinical mastitis and *Staphylococcus* species was calculated using percentages. The associations in the occurrence of *Staphylococcus* among subclinical mastitic dairy cows were assessed using statistical tests such as the Chi square test and logistic regeression which were done by considering a 95% confidence interval (CI) and a 5% level of significance. A P-value less than or equal to 0.05 was considered statistically significant.

4. RESULTS

4.1. Prevalence of subclinical mastitis and the *Staphylococcus*

The overall prevalence of subclinical mastitis among the studied dairy cows was 51.45% (Table 4). There is a significant difference in prevalence among site, breed, age, and parity (P<0.05). Considering the study sites, a higher (98.4%) prevalence was observed at Haramaya University (P<0.001). According to the age of cows, a high prevalence (93.6%) in the old age of cows and a high prevalence (80%) in the late lactation stage were observed when compared with the early lactation stage. Regarding the parity of cows, significantly higher (80.9%) were observed in the cows with more than 5 parity (p<0.001).

Table 4. Over all prevalence of subclinical mastitic among dairy cow

Considered factor	Variable categories	Total No of Examined	No(%) positive	$\chi 2$	P-Value
Site	Awaday	262	136(51.9)		
	Haramaya town	161	110(68.3)	49.789	0.000
	Haramaya University	63	62(98.4)		
	Addelle	114	71(62.3)		
Type farm	Intensive	325	198(60.9)	1.534	0.215
	Small holder	275	181(65.8)		
Age of cow	3-5 years	260	116(44.6)		
	6-8 years	246	175(71.1)	154.25	<0.001
	>8 years	94	88(93.6)		
Lactation .Stage	1-3 months	197	128(64.9)		
	4-6 months	278	151(54.3)	0.731	0.694
	>6 months	125	100(80)		
Parity(births)	1-3 births	317	173(54.5)		
	4-5 births	178	121(67.9)	68.467	<0.001
	>5 births	105	85 (80.9)		
Total		600	379 (63.17)		

As shown in Table 5, an overall prevalence of 33.0% *Staphylococcus* was observed among dairy cow. The prevalence ranged from 18.46% in cow of age 3-5years to 62.77% in those age >8 years. Significant difference ($p < 0.05$) was observed based on studied site, cow's age and the parity (births), but not in other studied variables.

Table 5. Over all prevalence of *Staphylococcus* among dairy cow

Considered factor	Variable categories	Total No of Examined	No(%) positive	$\chi 2$	P-Value
Studied Site	Awaday	262	86 (32.82)		
	Haramaya town	161	47 (29.19)	9.06	0.028
	Haramaya University	63	31 (49.21)		
	Addelle	114	34 (29.28)		
Type farm	Intensive	325	117 (36.0)	2.88	0.089
	Small holder	275	81 (29.45)		
Age of cow	3-5 years	260	48 (18.46)		
	6-8 years	246	91 (36.99)	64.29	<0.001
	>8 years	94	59 (62.77)		
Lactation .S	1-3 months	197	66 (33.5)		
	4-6 months	278	85 (30.58)	1.95	0.37
	>6 months	125	47 (37.6)		
Parity(births)	1-3 births	317	73 (23.03)		
	4-5 births	178	67 (37.64)	39.47	<0.001
	>5 births	105	58 (55.24)		
Total		600	198 (33.00)		

4.2. Prevalence of *Staphylococcus* in subclinical mastitic

An overall *staphylococcus* prevalence among the studied mastitis dairy cow was 51.45%. (Table 6) where prevalence were similar (P= 0.74), significant difference in the prevalence among studied risk factors were observed (P<0.05). Considering the study sites, the lower (41.82%) prevalence were observed at Maayaa city (P= 0.034). Regarding the farm types, significantly high (57.58%) were observed (P=0.013).

Table 6. Over all prevalence of *Staphylococcus* among subclinical mastitis dairy cow

Considered factor	Variable categories	Total No of Examined	No(%) positive	$\chi 2$	P-Value
Site	Awaday	136	82 (60.3)	8.48	0.034
	Haramaya town	110	46 (41.82)		
	Haramaya University	62	32 (51.61)		
	Addelle	71	35(49.30)		
Type farm	Intensive	198	114(57.58)	6.23	0.013
	Small holder	181	81(44.75)		
Age of cow	3-5 years	116	61(52.59)	54.31	0.000
	6-8 years	175	61(34.86)		
	>8 years	88	73(82.95)		
Lactation .S	1-3 months	128	54(42.19)	38.33	0.000
	4-6 months	151	63(41.72)		
	>6 months	100	78(78)		
Parity(births)	1-3 births	173	59(34.1)	43.32	0.000
	4-5 births	121	72(59.5)		
	>5 births	85	64(75.29)		
Total		379	195(51.45)		

Overall *Staphylococcus aureus* prevalence among the studied mastitis dairy cows was 21.9%. (Table 7), where prevalence was similar (P = 0.082), a significant difference in prevalence among the studied risk factors was observed (P<0.05). Considering the study sites, the lower (14.08%) prevalence was observed at Addelle (P = 0.035). Regarding the farm types, significantly high values (27.78%) were observed in intensive farms (P = 0.004).

Table 7. Prevalence of *Staphylococcus aureus* among subclinical mastitis dairy cow

Considered factor	Variable categories	Total No of Examined	No. (%) positive	χ^2	P-Value
Site	Awaday	136	37(27.21)		
	Haramaya District	110	18(16.36)		
	Haramaya University	62	18(29.03)	8.58	0.035
	Addelle	71	10(14.08)		
Sample Source	Intensive	198	55(27.78)	8.37	0.004
	Small holder	181	28(15.47)		
Age of Cows	3-5 years	116	31(26.72)		
	6-8 years	175	26(14.86)	9.66	0.008
	>8 years	88	26(29.55)		
Lactation Stage	1-3 months	128	26(20.31)	6.96	0.031
	4-6 months	151	26(17.22)		
	>6 months	100	31(31)		
Parity(births)	1-3 births	173	27(15.61)		
	4-5 births	121	34(28.10)	7.51	0.023
	>5 births	85	22(25.88)		
Total		379	83(21.9)		

The overall *Coagulase Negative Staphylococcu*s prevalence among the studied mastitis dairy cows was 29.55%. There is a significant difference in prevalence among lactation stages, parity, and age of cows ($P<0.05$). Considering the study sites, the lower (22.58%) prevalence was observed at Haramaya University ($P = 0.236$) (Table 8).

Table 8. Prevalence of *Coagulase Negative Staphylococcus* among subclinical mastitis dairy cow

Considered factor	Variable categories	Total No of Examined	No(%) positive	$\chi 2$	P-Value
Site of Sample Collection	Awaday	136	45(33.09)		
	Haramaya	110	28(25.45)	4.24	0.236
	District				
	Haramaya	62	14(22.58)		
	University				
	Addelle	71	25(35.21)		
Sample Source	Intensive	198	59(29.80)	0.012	0.912
	Small holder	181	53(29.28)		
Lactation Stage (months)	1-3 months	128	28(21.88)		
	4-6 months	151	37(24.5)	20.09	0.000
	>6 months	100	47(47)		
Parity(births)	1-3 births	173	32(18.5)		
	4-5 births	121	38(31.4)	26.45	0.000
	>5 births	85	42(39.4)		
Age of Cows (years)	3-5 years	116	30(25.8)		
	6-8 years	175	35(20)	32.48	0.000
	>8 years	88	47(53.4)		
Total		379	112(29.55)		

Association of *Staphylococcus aureus* and *Coagulase Negative Staphylococcus* among the studied mastitis dairy cow was observed on some studied risk factors. (Table 9) where prevalence were similar (P= 0.079), significant difference in the prevalence among studied risk factors were observed (P<0.05). Considering the age of cows, the higher (29.55%) of *Staphylococcus aureus* and (53.4%) of *Coagulase Negative Staphylococcus* prevalence were observed in the age of cows greater than 8 years (P<0.001). Regarding the lactation stage, higher (31%) of *Staphylococcus aureus* and (47%) of *Coagulase Negative Staphylococcus* prevalence were observed at late lactation stage (p< 0.001).

Table 9. Association of Staphylococcus species by study factors among subclinical mastitis dairy cow.

Considered factor	Variable categories	Total No of Examined	No(%) positive *S. aureus*	No(%)Positive CNS	$\chi 2$	P-Value
Site of sample collection	Awaday	136	37(27.21)	45(33.09)	14.05	0.029
	Haramaya town	110	18(16.36)	28(25.45)		
	Haramaya University	62	18(29.03)	14(22.58)		
	Addelle	71	10(14.08)	25(35.21)		
Age of Cows	3-5 years	116	31(26.72)	30(25.8)	58.32	<0.001
	6-8 years	175	26(14.86)	35(20)		
	>8 years	88	26(29.55)	47(53.4)		
Lactation Stage(months)	1-3 months	128	26(20.31)	28(21.88)	39.32	<0.001
	4-6 months	151	26(17.22)	37(24.5)		
	>6 months	100	31(31)	47(47)		
Parity(births)	1-3 births	173	27(15.61)	32(18.5)	46.79	<0.001
	4-5 births	121	34(28.10)	38(31.4)		
	>5 births	85	22(25.88)	42(39.4)		
Sample Source	Intensive	198	55(27.78)	59(29.80)	9.75	0.008
	Small holder	181	28(15.47)	53(29.28)		
Total		379	83(21.9)	112(29.55)		

4.3. Logistic regression

As shown in Table 10, the logistic regression of the overall prevalence of *Staphylococcus* by study factors among subclinical mastitic dairy cows shows a significant difference in prevalence ($p < 0.05$).

Table 10. Logistic Regression of an overall prevalence of *Staphylococcus* by study factors among subclinical mastitic dairy cow.

Considered factor	Coef.	SE	Z	P>|Z|	95% CI
Study site	0.878	0.16	5.43	<0.001	[0.56,1.19]
Age of Cow	0.85	0.16	5.29	<0.001	[0.53,1.16]
Lactation Stage	0.39	0.15	2.64	0.008	[0.101, 0.69]
Parity births	0.48	0.15	3.21	<0.001	[0.785, 0.19]

As shown in Table 11, except parity of births, the Logistic Regression of an overall prevalence of by study factors among subclinical mastitic dairy cow shows significant difference in the prevalence ($p < 0.05$).

Table 11. Logistic Regression on the prevalence of study factors among subclinical mastitic dairy cow

Considered factor	Coef.	SE	Z	P>|Z|	95% CI
Study site	0.68	0.12	5.59	<0.001	[0.44. 0.92]
Age of Cows	0.909	0.12	7.08	<0.001	[0.65, 1.16]
Lactation Stage	0.64	0.12	5.26	<0.001	[0.403, 0.882]
Parity births	0.27	0.116	0.23	0.816	[0.2, 0.254]

Except parity of births (p=0.359) as shown in Table 12, the Logistic Regression of an overall prevalence of *Coagulase Negative Staphylococcus* by study factors among subclinical mastitic dairy cow shows significant difference in the prevalence ($p < 0.05$).

Table 12. Logistic Regression on the prevalence of *Coagulase Negative Staphylococcus* study factors among subclinical mastitis dairy cow

Considered factor	Coef.	SE	Z	P>|Z|	95% CI
Study site	0.65	0.12	5.11	<0.001	[0.406, 0.912]
Age of Cow	0.74	0.13	5.68	<0.001	[0.487, 1]
Lactation Stage	0.512	0.12	4.06	<0.001	[0.264, 0.76]
Parity births	0.112	0.12	0.92	0.359	[0.352, 0.127]

As shown in Table 13, the Logistic Regression of an overall prevalence of *Staphylococcus aureus* and *Coagulase Negative Staphylococcus* by study factors among subclinical mastitis dairy cow show significant difference in the prevalence ($p < 0.05$).

Table 13. Logistic Regression on the prevalence of S. *aureus* and *Coagulase Negative Staphylococcus* by study factors among subclinical mastitis dairy cow

Considered factor	Coef.	SE	Z	P>|Z|	95% CI
Study site	0.79	0.15	5.25	<0.001	[0.498, 1.093]
Age of Cow	0.77	0.15	5.13	<0.001	[0.481, 1.076]
Lactation Stage	0.402	0.144	2.78	0.005	[0.118, 0.685]
Parity births	0.402	0.143	2.8	0.005	[0.684, 0.12]

Logistic Regression Odds Ratio of overall *Coagulase Negative Staphylococcus* study factors among subclinical mastitis dairy cows show a significant difference in the parity of births in the prevalence ($p< 0.05$), and there was no significant difference in the prevalence for the other studied factors ($p> 0.05$) (Table 16).

Table 14. Logistic Regression Odds Ratio of overall *Staphylococcu*s by study factors among subclinical mastitis dairy cow

Considered factor	Odd ratio	SE	Z	P>|Z|	95% CI
Study site	0.572	0.124	2.57	0.01	[0.373, 0.876]
Age of Cow	0.936	0.2	0.29	0.769	[0.604, 1.45]
Lactation Stage	1.75	0.385	2.57	0.01	[1.14, 2.701]
Parity births	3.75	0.816	6.08	<0.001	[2.45, 5.75]

Logistic Regression Odds Ratio of overall *S. aureus* by study factors among subclinical mastitis dairy cows were not show significant difference in the prevalence ($p> 0.05$) (Table 15).

Table 15. Logistic Regression Odds Ratio of *S. aureus* by study factors among subclinical mastitis dairy cow

Considered factor	Odd ratio	SE	Z	P>|Z|	95% CI
Study site	0.624	0.157	1.86	0.06	[0.38, 1.025]
Age of Cow	0.675	0.176	1.5	0.133	[0.405, 1.12]
Lactation Stage	1.15	0.307	0.53	0.59	[0.68, 1.94]
Parity births	2.01	0.528	2.69	0.07	[1.2, 3.37]

Logistic Regression Odds Ratio of overall *Coagulase Negative Staphylococcus* study factors among subclinical mastitis dairy cows show a significant difference in the parity of births in the prevalence ($p< 0.05$), and there was no significant difference in the prevalence for the other studied factors ($p> 0.05$) (Table 16). *Staphylococcus* study factors among subclinical mastitis dairy cows.

Table 16. Logistic Regression Odds Ratio of *Coagulase Negative Staphylococcus* by study factors among subclinical mastitis dairy cow

Variable	Odd ratio	SE	Z	P>|Z|	95% CI
Study site	0.769	0.178	1.13	0.25	[0.488, 1.212]
Age of Cows	1.298	0.325	1.04	0.296	[0.795, 2.121]
Lactation Stage	1.796	0.453	2.32	0.02	[1.095, 2.944]
Parity births	2.79	0.678	4.24	<0.001	[1.739, 4.499]

4.4. Community practice and attitude on milk borne zoonosis and contamination of the milk

Out of 110 respondents toward educational status more of respondents 52(47.7%) were elementary, 24(21.8%) high school, 18(16.4%) illiterate and 16(14.5%) were college and above. Most of the respondents 76(69%) male and 34(30.9%) female were interviewed. According to the age of the respondents more of them 57(51.8%) were 25-50 years, 32(29%) 11-24 years and 29(19%) were 50-75 years (Table-17).

Table 17. Socio demographic characteristics of respondents (30 milkers and 80 consumers)

Factors	Values	Frequency	Percentage (%)
Educational status	Illiterate	18	16.4
	Elementary	52	47.3
	High school	24	21.8
	College and above	16	14.5
Sex	Male	76	69
	Female	34	30.9
Age	11-24 years	32	29
	25-50 years	57	51.8
	51-75 years	21	19

The knowledge of milk-borne zoonosis and the risk of milk contamination of respondents toward sex, age, and knowledge status were analyzed. According to the sex of respondents, males (57.5%) were more knowledgeable than females (21.76%), and respondents between the ages of 25 and 50 (43.74%) were more understanding about milk-borne zoonosis. Regarding the knowledge status of respondents, all in high school and above were well knowledgeable about milk-borne zoonosis. Consider the sex of respondents: male 65 (85.53%) were more knowledgeable about the risk of milk contamination than female 23 (67.65%), and respondents between the ages of 25 and 50 were more knowledgeable about the risk of milk contamination than the others (Table 18).

Table. 18. Knowledge on the risk of milk contaminating and milk borne zoonosis

Considered Questionnaires	Variable	Values	Total No of Interviewed	No(%) respondents who know
Do you Know Milk borne Zoonosis?	Sex of Respondents	Female	34	21(61.76)
		Male	76	57(75)
	Age of Respondents	11-24 years	32	22(68.75)
		25-50 years	57	43(75.44)
		51-75 years	21	13(61.9)
	Knowledge of Status	Illiterate	18	6(33.33)
		Elementary	52	32(61.54)
		High School	24	24(100)
		College and Above	16	16(100)
Total			110	78(70.91)
Do you Know Risk of Milk Contamination?	Sex of Respondents	Female	34	23(67.65)
		Male	76	65(85.53)
	Age of Respondents	11-24 years	32	24(75)
		25-50 years	57	47(82.46)
		51-75 years	21	17(80)
	Knowledge of Status	Illiterate	18	12(66.67)
		Elementary	52	36(69.23)
		High School	24	24(100)
		College and Above	16	16(100)
Total			110	88(80)

Practice in control of milk-borne zoonotic and implementation of hygiene were compared among the sex, age, and knowledge status of the respondents. Regarding the sex of respondents, more females (31, 91 .18%) are drinking raw milk than males (67,88.16%), and respondents between the ages of 25 and 50 are 85.96% high in consuming raw milk. Considering the knowledge status of respondents, all illiterate elementary and high school respondents were drinking raw milk rather than boiling it. More educated respondents boiled the milk before consuming it, and less educated respondents did not. There is a

significant difference in the knowledge of respondents toward the boiling of milk before consumption (Table 19).

Table. 19. Practice in control of milk borne zoonotic and implementation of the hygiene

Considered Questionnaires	Variable	Values	Total No of Interviewed	No(%) respondents who say "yes"
Do you drinking raw milk?	Sex of Respondents	Female	34	31(91.18)
		Male	76	67(88.16)
	Age of Respondents	11-24 years	32	31(96.88)
		25-50 years	57	49(85.96)
		51-75 years	21	18(85.71)
	Knowledge of Status	Illiterate	18	18(100)
		Elementary	52	52(100)
		High School	24	24(100)
		College and Above	16	4(25)
Total			110	98(89.09)
Do you boiling milk before consuming?	Sex of Respondents	Female	34	3(8.82)
		Male	76	8(10.53)
	Age of Respondents	11-24 years	32	1(3.13)
		25-50 years	57	7(12.28)
		51-75 years	21	3(14.29)
	Knowledge of Status	Illiterate	18	0(0.00)
		Elementary	52	0(0.00)
		High School	24	0(0.00)
		College and Above	16	11(68.75)
Total			110	11(10)

5. DISCUSSION

In the present study, the overall prevalence of bovine subclinical mastitis was 51.45%. The result was in disagreement with (Mureithi and Njuguna, 2016) in Kenya and in Eastern Ethiopia, who reported prevalences of 64% and 62%, respectively and 71.4% in Eastern Ethiopia (Tefa *et al*., 2015), 67.9% in Aljeria (Sayeed *et al.,* 2020), 38% in Egypt (Youssif *et al.,* 2020), 28.34% in Assosa 27.7% in Wellega 21.8% in Mecha district (Yimam *et al.,* 2020), and 16.1% in Bishoftu (Birhanu *et al.,* 2015). The present study also showed a prevalence of 51.45% for subclinical mastitis, which was much higher than the findings of 7.33% in Algeria (Sharma and Sindu, 2007), 16.1% in Bishoftu (Birhanu *et al.,* 2017), and 28% in Algeria (Saidi *et al.,* 2013), and much lower than the reports of 87.9% in Algeria (Kasozi *et al.,* 2014), 86% in Indonesia (Qolbaini *et al.,* 2014), and 85.33% in Nigeria (Shittu *et al.,* 2012). Risk factors that influence the occurrence of subclinical mastitis were delineated as animal, pathogen, and environmental risk factors, which could contribute to the differences in subclinical mastitis prevalence (Abdeta and Gemachisa, 2020).

The current study showed that a different prevalence of bovine subclinical mastitis was recorded among the samples of origin in the study area. The highest prevalence of subclinical mastitis (67.6%) was recorded in Awaday, followed by 33 (53.2%) in Haramaya University, 32 (45%) in Adele, and the lowest prevalence (49 (44.5%) in Haramaya town. There is statistically significant variation between the different samples of origins and positivity of subclinical mastitis. The difference may be due to greater experience in drying off, the potential effect of level of milking hygiene, herd size and cleanliness, and the application of sanitary measures in these farms. This finding is in disagreement with the report of (Zeryehun and Abera, 2017) who reported there was no statistically significant variation among the origins of the samples and the positivity of subclinical mastitis.

In the current study the overall *Staphylococcus* prevalence among the studied mastitis dairy cow was 51.45%. Considering the study sites, the lower (41.82%) prevalence was observed at Haramaya district. Regarding the farm types, significantly high (57.58%) was observed in intensive farming system.). Regarding to the age of cows, lactating stage and parity of births the prevalence of *Staphylococcus* was discussed in the current study. According to this, high prevalence (82.95%) in older age, (78%) in late lactation stage and (75.29%) were observed

in cows have greater than 5 births. Overall *Coagulase Negative Staphylococcus* prevalence among the studied mastitis dairy cow was 29.55%. There is significant difference in the prevalence among lactation stage; parity and age of cows were observed. Considering the study sites, the lower (22.58%) at Haramaya University. Higher prevalence (47%) was observed in late lactation stage and similarly high (53.4%) in older age.

Association of *Coagulase Negative Staphylococcus* among the studied mastitic dairy cow was observed on some studied risk factors. Considering the age of cows, the higher (29.55%) of and (53.4%) of *Coagulase Negative Staphylococcus* prevalence were observed in the age of cows greater than 8 years. Regarding the lactation stage, higher (31%) of and (47%) of *Coagulase Negative Staphylococcus* prevalence were observed at late lactation stage. In the current study, the prevalence (29.55%) of *Coagulase Negative Staphylococcus* was higher than that of *Staphylococcus Aureus (*21.9%). This finding was lower than the reports of (Murethi *et al.,* 2016) who reported 35.5% of *Staphylococcus aureus* in Kenya, 33.3% in Ethiopia (Alemu and Abraha *et al*, 2017), and 40% in Algeria (Saidi *et al.,* 2013). And also, the finding of the present study is lower than the reports of (Mpatswenu *et al.* 2017), (Birhanu *et al ,2017),* and (Youssif *et al.,* 2020), who reported 51.5% of *Coagulase-negative staphylococcus* in Rwanda, 44.9% in Ethiopia, and 66.7% in Egypt, respectively, and much higher than 10% of *Coagulase-negative staphylococcus* in Bangladesh (Sumon *et a*l, 2017), 13.8% of *Staphylococcus aureus* in Holeta *(Ayano et al., 2013), 18.33% of S. aureus* in Bangladesh (Sumon *et al.,* 2017), and 20.6% of S. *aureus in* Rwanda (Mpatswenu *et al.,* 2017).

The study showed that there were significant statistical associations between the prevalence of subclinical mastitis and the age and parity of animals, where the risk of mastitis increases with age and parity. The present result was in agreement with the observation of (Tefa *et al ,* 2015) who stated that parity and age are significantly associated with infection rates. Similarly (Birhanu *et al.* 2017) in Bishoftu town (Zeryehun and Abera *et al* ,2017) in Eastern Ethiopia have reported that cows with a large number of cows had a higher prevalence of mastitis, while similar to the findings of the current study, the prevalence of subclinical mastitis was reported to increase with age in studies conducted in Colombia (Romero *et al.*, 2018) and Nigeria (Shittu *et al.,* 2012). The higher prevalence in older cows in the present study might be due to prolonged periods of exposure to the infecting organisms and predisposing factors like stress of lactation, which favor dilation of the teat

canal due to repeat milking, thereby facilitating the entry of pathogens into the teat canal to cause subclinical intramammary infection (Sumon *et al.,* 2017). In this study, the prevalence of *Staphylococcus* was significantly higher in late lactation as compared to early and mid-lactation, as noted by (Mureithi *et al.* 2016) and (Mpatswenu *et al.* 2017), who state that an increased prevalence of mastitis was encountered as lactation stage advanced. On the contrary, (Youssif *et al.* 2020) and (Saidi *et al.,* 2013) reported that cows at the early stage of lactation are more susceptible to subclinical mastitis.

The Logistic Regression of an overall prevalence of *Staphylococcus* by study factors among subclinical mastitis dairy cow shows significant difference in the prevalence. Except parity of births, the Logistic Regression of an overall prevalence of *Staphylococcus aures* by study factors among subclinical mastitis dairy cow shows significant difference in the prevalence. Except parity of births, the Logistic Regression of an overall prevalence of *Coagulase Negative Staphylococcus* by study factors among subclinical mastitis dairy cow shows significant difference in the prevalence. The Logistic Regression of an overall prevalence of *Staphylococcus aureus* and *Coagulase Negative Staphylococcus* by study factors among subclinical mastitis dairy cow show significant difference in the prevalence.

Logistic Regression odds Ratio of overall *Staphylococcus* by study factors among subclinical mastitis dairy cow show significant difference among the site of selection, lactation stage and parity of births and, not significant difference in the prevalence on age. Logistic Regression Odds Ratio of overall *S. aureus* by study factors among subclinical mastitis dairy cows were not show significant difference in the prevalence. Logistic Regression Odds Ratio of overall *Coagulase Negative Staphylococcus* by study factors among subclinical mastitis dairy cows show significant difference in the parity of births in the prevalence and there were no significant difference in the prevalence on the other studied factors.

In the current study, the prevalence of *Staphylococcus* species among lactating cows with subclinical mastitis was 82.5%. The prevalence of 92 (44%) of *Coagulase Negative Staphylococcus* was higher than that of *Staphylococcus Aureus* 78 (37%). This finding was in agreement with the reports of(Murethi *et al.,* 2016) who reported 35.5% of *Staphylococcus aureus* in Kenya, 33.3% in Ethiopia (Alemu and Abraha *et a*l 2017), and 40% in Algeria (Saidi *et al.,* 2013). But the finding of the present study is lower than the

reports of(Mpatswenu *et al*., 2017), (Birhanu *et al,2017),* and (Youssif *et al.,* 2020), who reported 51.5% of *Coagulase-negative Staphylococcus* in Rwanda, 44.9% of *Staphylococcus aureus* in Ethiopia, and 66.7% of *Staphylococcus aureus* in Egypt, respectively, and much higher than 10% of *Coagulase-negative Staphylococcus* in Bangladesh (Sumon *et al.,* 2017), 13.8% of *Staphylococcus aureus* in Holeta (Ayano *et al.,* 2013), 18.33% of *Staphylococcus aureus* in Bangladesh (Sumon *et al.,* 2017), 20.6% of *Staphylococcus aureus* in Rwanda (Mpatswenu *et al.,* 2017), and 34.2% of *Coagulase negative Staphylococcus* in Ethiopia (Zeryehun and Abera *et al* , 2017).

On the other hand, from the total number of dairy farms, 6 (66.7%) of them had poor udder hygiene of cows, 5 (55.5%) were not washed their hands before milking, 6 (66.7%) used common towels, and 3 (33.3%) of them were not used towels while drying the cow teats. House hygiene had a significant effect on the prevalence of *Staphylococcus* species. In the case of good house hygiene, the prevalence was 22.22%, while cows managed in poor house hygiene had a prevalence of 77.9%. In dairy farms managed in unhygienic houses, manure and wet bedding materials were not frequently removed, which in turn favored the high occurrence of *Staphylococcus*. This is in agreement with the findings of earlier works in Ethiopia that implicated poor barn hygiene in the high prevalence of mastitis (Tefa *et al.,* 2015; Birhanu *et al.,* 2017).

The knowledge of milk-borne zoonosis and the risk of milk contamination of respondents toward sex, age, and knowledge status were analyzed. According to the sex of respondents, males (57.5%) were more knowledgeable than females (21.76%), and respondents between the ages of 25 and 50 (43.74%) were more understanding about milk-borne zoonosis. Regarding the knowledge status of respondents, all in high school and above were well knowledgeable about milk-borne zoonosis ($p = 0.000$). Consider the sex of respondents: male 65 (85.53%) were more knowledgeable about the risk of milk contamination than female 23 (67.65%) ($P = 0.030$), and respondents between the ages of 25 and 50 were more knowledgeable about the risk of milk contamination than the other respondents ($p = 0.000$).

Practice in control of milk-borne zoonotic and implementation of hygiene were compared among the sex, age, and knowledge status of the respondents. Regarding the sex of respondents, more females (31, 91.18%) are drinking raw milk than males (67,88.16%), and respondents between the ages of 25 and 50 are 85.96% high in consuming raw milk.

Considering the knowledge status of respondents, all illiterate elementary and high school respondents were drinking raw milk rather than boiling it ($p = 0.000$). Respondents with an age group ranging from 25 to 50 years and a higher educational level were aware that drinking raw milk is a possible source of *Staphylococcus.* This suggests that more work on awareness creation is required in societies with lower educational levels compared to those with higher educational levels. In addition, this is an opportunity to create awareness about the prevention and control of zoonosis among actively involved groups of society. There is a significant difference in the knowledge of respondents toward the boiling of milk before consumption ($p = 0.000$).

However, *S. aureus* can enter milk through direct excretion from the udder of a cow with subclinical *Staphylococcus* mastitis, as well as through contamination from the environment during raw milk handling and processing, posing a risk to consumers (Romero *et al.*, 2018). Accordingly, poor hygienic practices during milk handling may favor the multiplication *of Staphylococcus* species. In the current study, attitudes towards the implementation of zoonotic control and milk hygiene through training experience were analyzed and recorded. Out of total respondents, 78 (70.91%) knew about zoonotic disease without receiving formal training informally. From the total respondents, 88 (80%) knew contamination was a risk through milk handling practices. Respondents with an age group ranging from 25 to 50 years and a higher educational level were aware that drinking raw milk is a possible source of *Staphylococcus*. This suggests that more work on awareness creation is required in societies with lower educational levels compared to those with higher educational levels. In addition, this is an opportunity to create awareness about the prevention and control of zoonosis among actively involved groups of society.

The fact that drinking raw milk provides a chance for microorganisms, including *Staphylococcus*, to cause human infection However, this study revealed that 87.3% of respondents also consume raw milk. Thus, it is also the potential source of *Staphylococcus* and associated infection, particularly for raw milk consumers, as indicated by the occurrence of *Coagulase-positive Staphylococcus (37%),* and *Coagulase-negative Staphylococcus (44%). The majority* of respondents (87.3%) did not boil raw milk while consuming it due to carelessness and the absence of formal training in the community. But the majority of respondents, 70.9%, know about zoonotic disease, and 80% know contamination as a risk through informal training and thought that *Staphylococcus* can be prevented through personal

hygiene and cooked raw milk; this community knowledge would have paramount importance in *Staphylococcu*s control but still needs community training under sustainable conditions. This study the knowledge of respondents toward zoonotic disease was compered according to socio demographic characteristics. Out of 110 respondents toward their educational status, 18 of them were illiterate and only 6(33.3%) respondent know about zoonosis, 52 of them were elementary and 32(61.5%) of the respondents know zoonosis. All of (100%) the respondents those trained high school and college were know about the zoonosis. There was statistically significant difference between educational status with respondents were know about zoonosis disease (P=0.000). But (87.3%) of respondents do not boiling the milk while consuming and drinking raw cow milk due to carelessness with knowledge of consuming raw milk cause source of *Staphylococcus.*

6. CONCLUSION AND RECOMMENDATIONS

The present study recorded an overall prevalence of subclinical mastitis that was high in the study areas (51.45%), which might imply that mastitis is a major health problem for dairy cows, which undoubtedly will have a drawback on the productivity of the dairy industry and hence warrants serious attention. The current study also displays that pathogenic *Staphylococcus* species are the major bacteria, along with other environmental bacteria, to be associated with subclinical mastitis. In identified *Staphylococcus species,* the prevalence of *Coagulase-negative Staphylococcu*s (29.55%) was higher than that of *Coagulase-positive Staphylococcus* (21.9%). The high prevalence of *Staphylococcu*s infection detected in dairy cows raises public health concerns since *Coagulase-positive Staphylococcus* bacteria are capable of producing heat-stable enterotoxins, which might cause *Staphylococcus* food poisoning outbreaks when ingested by humans in sufficient quantities. This could be an indicator of poor hygienic practices and the absence of regular animal health monitoring. Based on the above conclusion, the following recommendations were forwarded:

- Regular screening for the detection of subclinical mastitis and proper treatment of cows during the dry and lactation periods should be practiced.
- Careful hygienic milking practices and regular health monitoring should be practiced to reduce the reservoir of infection and contamination in the rest of the herd.
- Community training on the zoonoses risk of subclinical mastitis and implementation of hygienic practices of drinking raw milk by carelessness were recommended.
- Further research is needed to identify species and strains of *coagulase-positive* and coagulase-negative *Staphylococcus* has paramount significance in reducing and preventing the pathogen effect on the dairy industry.

7. REFERENCES

Abdeta D. and Gemechisa B. 2020. A Study on the Prevalence of Subclinical Mastitis in Lactating Cows and Associated Risk Factors in Wolmara District, Oromia Regional State, Ethiopia. *Biomed J Sci & Tech Res* 28(2)-2020.

Alemu, S., and Abraha, A. 2017. Prevalence of Bacteria Associated with Subclinical Mastitis in Haramaya University Dairy Cattle, Goat and Sheep Farms. *East African Journal of Veterinary and Animal Sciences, 1(2), 61–66.*

Akindolire Muyiwa Ajoke, Olubukola Oluranti Babalola, Collins Njie Ateba. 2015. Detection of Antibiotic Resistant from Milk: A Public Health Implication. *Int. J. Environ. Res. Public Health*, 12:10254-10275

Anamika vyas, Megha Sharma, Sanjeev Kumar, Mrityunjay Kumar, Sudhir Kumar Mehra. 2015. A comparative study of oxacillin screen agar, oxacillin disc diffusion and Cefoxitin disc diffusion, oxacillin E-test method for routine screening of methicillin resistant Staphylococcus aureus. *Int J Cur Res Rev,* 7(10):55-60.

Ananya Mohanta, Pranab Behari Mazumder.2015. Detection of Staphylococci in raw milk and milk products and evaluation of their antibiotic sensitivity: a report from Southern Assam, India. *IOSR Journal of Environmental science, Toxicology and Food Technology*, 9(1):17-22.

Asmare AA, Kassa F. 2017. Incidence of dairy cow mastitis and associated risk factors in Sodo town and its surroundings, Wolaitia zone, Ethiopia. *Slovak J Anim Sci.* 50:77-89.

Atalla H., Gyles C., Mallard B., 2010. Persistence of small colony variants (S-aureus SCV) within bovine mammary epithelial cells. *Veterinary Microbiology*143, 319-328.

Avall-Jaaskelainen S., Koort J., Simojoki H., Taponen S., 2013. Bovine-associated CNS species resist phagocytosis differently. *BMC Veterinary Research* 9.

Ayano A. A., Hiriko F., Simyalew A.M., and Yohannes A. 2013. Prevalence of subclinical mastitis in lactating cows in selected commercial dairy farms of Holeta district. *J. Vet. Med. Anim. Health,* 5(3): 67-72.

Bachaya, H. A. Raza, M. A. Murtaza, S. and Akbar. I. U. R. 2011. Subclinical bovine mastitis inMuzaffar Garh district of Punjab (Pakistan), *Journal of Animal and Plant Sciences*, vol. 21, no. 2, pp. 16–19.

Barbier F., Ruppe E., Hernandez D., Lebeaux D., Francois P., Felix B., Despreza., Maiga A., Woerther P-L., Gaillard K., Jeanrot C., Wolff M., Schrenzel J., Andremont A., Ruimy R., 2010. Methicillin-Resistant *Coagulase-Negative Staphylococci* in the Community: High Homology of SCCmec IVa between Staphylococcus epidermidis and Major Clones of Methicillin-Resistant Staphylococcus aureus. *Journal of Infectious Diseases* 202, 270-281.

Becker K, Heilmann C, Peters G. 2014. *Coagulase negative Staphylococci*. Clinical Microbiology Review, 27: 870-926.

Beuron DC, Cortinhas CS, Botaro BG, Macedo SN, Gonçalves JL, Brito MAVP. 2014. Risk factors associated with the antimicrobial resistance of isolated from bovine mastitis. *Pesqui Vet Bras*. 34:947–52.

Bexiga R, Rato MG, Lemsaddek A, Semedo-Lemsaddek T, Carneiro C, Pereira H, 2014. Dynamics of bovine intramammary infections due to coagulasenegative staphylococci on four farms. *J Dairy Res.* 81:208–14.

Beyene Takele, Halefom Hayishe, Fikru Gizaw, Ashenafi Feyisa Beyi1, Fufa Abunna, Bedaso Mammo. 2017. Prevalence and antimicrobial resistance profile of Staphylococcus in dairy farms, abattoir and humans in Addis Ababa, Ethiopia. *BMC Res Notes*, 10:171.

Bharathy Sukumar, Gunaseelan Lakshmanasami, Porteen Kannan, Bojiraj Munnisamy. 2015. Prevalence of Staphylococcus aureus in raw milk: can it be a potential public health threat. *International Journal of Advanced Research*, 3(2):801-806.

Bhattacharyya D, Banerjee J, Bandyopadhyay S, Mondal B, Nanda PK, Samanta I. 2016. First report on Vancomycin-resistant in bovine and Caprine Milk. *Microb Drug Resist.* 22:675–81.

Birhanu M, Leta S., Mamo G and Tesfaye S. 2017. Prevalence of bovine subclinical mastitis and isolation of its major causes in Bishoftu Town, Ethiopia. *BMC Res Notes* (2017) 10:767

Björk S, Båge R, Kanyima BM, et al. 2014 Characterization of coagulase negative staphylococci from cases of subclinical mastitis in dairy cattle in Kampala, Uganda. *Ir Vet J*.; 67(1):12.

Bochniarz M., Wawron W., Szczubial M., 2013. Coagulase-negative staphylococci (CNS) as an aetiological factor of mastitis in cows. *Polish Journal of Veterinary Sciences* 16, 487-492.

Cemil Kürekci. 2016. Prevalence, antimicrobial resistance, and resistant traits of coagulase-negative staphylococci isolated from cheese samples in Turkey. J. Dairy Sci, 99:2675-2679.

Cengiz S, Dinc G, Cengiz M. 2015. Evaluation of Antimicrobial Resistance in Staphylococcus Spp. Isolated from Subclinical Mastitis in Cows. *Pak Vet J* 35: 334-338.

Cepas V, López Y, Muñoz E, Rolo D, Ardanuy C, Martí S. 2019. Relationship between biofilm formation and antimicrobial resistance in gram-negative Bacteria. *Microb Drug Resist.* 25:72–9.

Chaje, cka-Wierzchowska W, Zadernowska A, Nalepa B, Sierpinska M, Łaniewska-Trokenheim L. 2015. Coagulase-negative staphylococci (CoNS) isolated from ready-to-eat food of animal origin–phenotypic and genotypic antibiotic resistance. *Food Microbiol,* 46:222–226.

CSA. 2020. Agricultural Sample Survey 2019/20 [2012 E.C.]. Volume II report on livestock and livestock characteristics (private peasant holdings). Central Statistical Agency (CSA): Addis Ababa, Ethiopia.

De Oliveira A, Pereira VC, Pinheiro L, Riboli DFM, Martins KB, Cunha M. 2016. Antimicrobial resistance profile of planktonic and biofilm cells of Staphylococcus aureus and coagulase-negative staphylococci. *Int J Mol Sci.* 17:1423.

Debaraj Bandyopadhyay, Jaydeep Banerjee, Samiran Bandyopadhyay, Bimalendu Mondal, Pramod K Nanda, Indranil Samanta. 2016. First report on vancomyin-resistant Staphylococcus aureus in bovine and caprine milk. *Microbial drug resistance*.

El-Jakee JK, Aref NE, Gomaa A, El-Hariri MD, Galal HM, Omar SA, et al. 2013. Emerging of coagulase negative staphylococci as a cause of mastitis in dairy animals: An environmental hazard. *Int J Vet Sci Med*. 1:74–8.

Etinosa O. Igbinosa, Abeni Beshiru, Lucy U. Akporehe and Abraham G. Ogofure. 2016. Detection of methicillin-resistant Staphylococci isolated from food producing animals: a public health implication. *Veterinary Sciences*, 3:14.

Fijalkowski K., Struk M., Karakulska J., Paszkowska A., Giedrys-Kalemba S., Masiuk H., Czernomysy-Furowicz D., Nawrotek P. 2014. Comparative Analysis of Superantigen Genes in *Staphylococcus xylosus* and Isolates collected from a Single Mammary Quarter of Cows with Mastitis. *Journal of Microbiology* 52, 366-372.

Fisher EL, Otto M, Cheung GYC. 2018. Basis of virulence in enterotoxin-mediated staphylococcal food poisoning. *Front Microbiol.* 9:436. doi: 10.3389/fmicb.2018.00436.

Fowoyo PT, Ogunbanwo ST. 2017. Antimicrobial resistance in Coagulase-negative Staphylococci from Nigerian traditional fermented foods. *Ann Clin Microbiol Antimicrob* 16:4

Frey Y., Rodriguez J.P., Thomann A., Schwendener S., Perreten V., 2013. Genetic characterization of antimicrobial resistance in coagulase-negative staphylococci from bovine mastitis milk. *Journal of Dairy Science* 96, 2247-2257.

Fry PR, Middleton JR, Dufour S, Perry J, Scholl D, Dohoo I. 2014. Association of coagulase negative staphylococcal species, mammary quarter milk somatic cell count, and persistence of intramammary infection in dairy cattle. *J Dairy Sci.* 97:4876–85.

Ghias W, Sharif M, Yazdani M, Ansari F, Rabbani M. 2016. Isolation and identification of Methicillin and Vancomycin resistance from pus samples of injured skin patients in Lahore, Pakistan. *Biomed Lett*, 2(2):103-112.

Hashemi, M., et al. (2011). "The prevalence of clinical and subclinical mastitis in dairy cows in the central region of Fars province, south of Iran." *Iranian Journal of veterinary research, Shiraz University* **12**: 236-241.

Hegde R, Isloor S, Prabhu KN, et al. 2013. Incidence of subclinical mastitis and prevalence of major mastitis pathogens in organized farms and unorganized sectors. *Indian J Microbiol*. 53(3):315-320. doi:10.1007/s12088-012-0336-1

Ibadin EE, Enabulele IO, Muinah F. 2017. Prevalence of mecA gene among staphylococci from clinical samples of a tertiary hospital in Benin city,*Nigeria. Afr Heal2th Sci.* 17:1000–10.

Kadariya J., Smith T.C., Thapaliya D., 2014. And *Staphylococcu*s food-borne disease: an ongoing challenge in public health. *BioMed Research International* 2014, 827965-827965.

Kaliwal BB, Sadashiv SO, Kurjogi MM, Sanakal RD. 2011. Prevalence and antimicrobial susceptibility of Coagulase-negative Staphylococci isolated from bovine mastitis. *Vet World* 4:158.

Kasozi, K.I., Tingiira J. B., Vudriko P. 2014. High Prevalence of Subclinical Mastitis and Multidrug Resistant Staphylococcus aureus are a Threat to Dairy Cattle Production

in Kiboga District (Uganda). *Open Journal of Veterinary Medicine*, 4: 35-43. http://dx.doi.org/10.4236/ojvm.2014.44005

Klibi A, Maaroufi A, Torres C, Jouini A. 2018. Detection and characterization of methicillin-resistant and susceptible coagulase-negative staphylococci in milk from cows with clinical mastitis in Tunisia. *Int J Antimicrob Agents*. 52:930–5.

Kudinha T, Simango C. 2012. Prevalence of coagulase-negative staphylococci in bovine mastitis in Zimbabwe. *J S Afr Vet Assoc*. 73:62–5.

Lee GY, Yang SJ. 2021. Profiles of coagulase-positive and -negative staphylococci in retail pork: prevalence, antimicrobial resistance, enterotoxigenicity, and virulence factors. *Anim Biosci.:*734-742.

Li H, Andersen PS, Stegger M, .2019. Antimicrobial resistance and virulence gene profiles of methicillin-resistant and -susceptible from food products in Denmark. *Front Microbiol.*10:2681.

Mahato S, Mistry HU, Chakraborty S, Sharma P, Saravanan R, Bhandari V. 2017. Identification of variable traits among the methicillin resistant and sensitive coagulase negative staphylococci in milk samples from mastitic cows in india. *Front Microbiol*. 8:1446.

Markey B, Leonard F, Archambault M, Cullinane A, Maguire D. 2013. Clinical veterinary microbiology e-book. United States: *Mosby Ltd.*

May L, Klein EY, Rothman RE, Laxminaraya R. 2014. Trends in antibiotic resistance in coagulase-negative staphylococci in the United States, 1999 to 2012. *Antimicrob Agents Chemother*. 58:1404–9.

Morgenstern M, Erichsen C, Hackl S, Mily J, Militz M, Friederichs J *et al*. 2016. Antibiotic resistance of commensal and coagulase-negative staphylococci in an international cohort of surgeons: a prospective point-prevalence study. *Plos One*. 11(2):e0148437.

Moser A, Stephan R, Ziegler D, Johler S. 2013. Species distribution and resistance profiles of coagulase-negative staphylococci isolated from bovine mastitis in Switzerland. *Schweiz Arch Tierheilkd* 155: 333-338.

Mpatswenumugabo, J. P., Bebora, L. C., Gitao G. C., Mobegi V. A., Iraguha, B., Kamana O., and Shumbusho B. 2017, Prevalence of Subclinical Mastitis and Distribution of Pathogens in Dairy Farms of Rubavu and Nyabihu Districts, Rwanda. *Journal of Veterinary Medicine*, Volume 2017, Article ID 8456713, 8 pages https://doi.org/10.1155/2017/8456713.

Mungube EO, Tenhagen BA, Regassa F, Kyule MN, Shiferaw Y, Kassa T, Baumann MP. 2005. Reduced milk production in udder quarters with subclinical mastitis and associated economic losses in crossbred dairy cows in Ethiopia. *Trop Anim Health Prod.* 37(6):503-12. doi: 10.1007/s11250-005-7049-y. PMID: 16248222.

Mureithi D K and Njuguna M N 2016 Prevalence of subclinical mastitis and associated risk factors in dairy farms in urban and peri-urban areas of Thika Sub County, Kenya. *Livestock Research for Rural Development* 28 (2) 2016

Neeraj Shrivastava, Varsha Sharma, Anju Nayak, Shrivastava AB, Sarkhe BC, Shukla PC *et al*. 2017. Prevalence and Characterization of Methicllin-Resistant *(*MRSA) mastitis in dairy cattle in Jabalpur, Madhya Pradesh. *Journal of Animal Research* 7(1):77-84.

Nibret M., Yilikal, A. and Kelay. B. 2011. "A cross sectional study on the prevalence of subclinical mastitis and associated risk factors in and around Gondar, Northern Ethiopia," *International JournalofAnimal andVeterinaryAdvances* 3(6): 455–459.

Nonnemann B, Lyhs U, Svennesen L, Kristensen KA, Klaas IC, Pedersen K. 2019. Bovine mastitis bacteria resolved by MALDI-TOF mass spectrometry. *J Dairy Sci.* 102:2515–24.

Nunes RSC, Del Aguila EM, Paschoalin VMF. 2015. Safety evaluation of the coagulase-negative staphylococci microbiota of salami: superantigenic toxin production and antimicrobial resistance. *BioMed Res. Int*, 483548.

Oguttu JW, Qekwana DN, Odoi A. 2017. An exploratory descriptive study of antimicrobial resistance patterns of Staphylococcus Spp. Isolated from Horses Presented at a Veterinary Teaching Hospital. *BMC Vet Res*. 13:269.

Osman K, Alvarez-Ordóñez A, Ruiz L, Badr J, ElHofy F, Al-Maary KS. 2017. Antimicrobial resistance and virulence characterization of Staphylococcus aureus and coagulase-negative staphylococci from imported beef meat. *Ann Clin Microbiol Antimicrob*. 16:35.

Osman Kamelia, Badr Jihan, Khalid S Ali-Maary, Ihab MIMoussa, Ashgan M Hessain, Zenab MS Amin Girah *et al*. 2016. Prevalence of the antibiotic resistance genes in coagulse-positive and negative Staphylococcus in chicken meat retailed to customers. *Frontiers in Microbiology.* 7:1846.

Otto M. 2012. Coagulase-negative staphylococci as reservoirs of genes facilitating MRSA infection. Bioessays, 35:4-11.

Pyzik E, Marek A,Stepien-Pysniak D, Urban-Chmiel R, Jarosz LS, Jagiello-Podebska I. 2019.Detection of antibiotic resistance and classical enterotoxin genes in coagulase-negative staphylococci isolated from poultry in Poland. *J Vet Res.* 63:183–90.

Qolbaini E N., Artika M., Safari D Detection of Subclinical Mastitis in Dairy Cows using California Mastitis Test and Udder Pathogen. *Curr. Biochem*. 1 (2): 66 - 70

Romero J., Benavides E., and Meza C. 2018. Assessing Financial Impacts of Subclinical Mastitis on Colombian Dairy Farms. Front. *Vet. Sci*. 5:273. doi: 10.3389/fvets.2018.00273

Saidi, R., Khelef, D., Kaidi, R., 2013. Subclinical mastitis in cattle in Algeria: Frequency of occurrence and bacteriological isolates. *Journal of the South African Veterinary Association* 84(1), 1- 5 http:// dx.doi.org/10.4102/jsava. v84i1.929

Sayeed A., Rahman A., Bari S., Islam A., Rahman M., Hoque A.2020. Prevalence of sub-clinical mastitis and associated risk factors at cow level in dairy farms in Southwestern part of Bangladesh. Printed 1-14.

Schmidt T., Kock M.M., Ehlers M.M., 2015. Diversity and antimicrobial susceptibility profiling of staphylococci isolated from bovine mastitis cases and close human contacts. *Journal of Dairy Scien*ce 98, 6256-6269.

Sharma A. and Sindhu N. 2007. Occurrence of clinical and subclinical mastitis in buffaloes in the State of Haryana (India). *Ital. J. Anim. Sci.* 6, (Suppl. 2): 965-967.

Shittu, A.; Abdullahi, J.; Jibril, A.; Mohammed, A.A., and Fasina, F.O. 2012. Sub-clinical mastitis and associated risk factors on lactating cows in the Savannah Region of Nigeria. *BMC Vet. Res.* 8(1): 134.

Shrestha LB, Bhattarai NR, Khanal B. 2017. Antibiotic resistance and biofilm formation among coagulase-negative staphylococci isolated from clinical samples at a tertiary care hospital of eastern Nepal. *Antimicrob Resist Infect Control*. 6:89.

Silva NCC, Guimarães FF, Manzi MDP, Gómez-Sanz E, Gómez P, Araújo JP. 2014. Characterization of methicillin-resistant coagulase-negative staphylococci in milk from cows with mastitis in Brazil. *Antonie van Leeuwenhoek Int J Gen Mol Microbiol*. 106:227–33.

Srednik ME, Tremblay YDN, Labrie J, Archambault M, Jacques M, Cirelli AF. 2017. Biofilm formation and antimicrobial resistance genes of coagulasenegative staphylococci isolated from cows with mastitis in Argentina. *FEMS Microbiol Lett*. 364:fnx001.

Suja KRS, Sheela P, Jyothis S, Radhakrishnan EK. 2017. Virulence factors associated with Coagulase Negative Staphylococci isolated from human infections. 3 Biotech. 7:1–10.

Sumon, S. M. R., et al. (2017). "Subclinical mastitis in dairy cows: somatic cell counts and associated bacteria in Mymensingh, Bangladesh." *Journal of the Bangladesh Agricultural University* **15**: 266.

Tafa, F., Terefe, Y., Tamerat, N., and Zewdu, E. 2015. Isolation, identifications and antimicrobial susceptibility pattern of coagulase positive Staphylococcus from subclinical mastitic dairy cattle in and around Haramaya University. *Ethiopian Veterinary Journal, 19(2), 41. https://doi.org/10.4314/evj.v19i2.8*

Tremblay YDN, Caron V, Blondeau A, Messier S, Jacques M. 2014. Biofilm formation by coagulase-negative staphylococci: impact on the efficacy of antimicrobials and disinfectants commonly used on dairy farms. *VetMicrobiol.* 172:511–8.

Vitali LA, Petrelli D, Lamikanra A, Prenna M, Akinkunmi EO. 2014. Diversity of antibiotic resistance genes and staphylococcal cassette chromosome mec elements in faecal isolates of coagulase-negative staphylococci from Nigeria. *BMC Microbiol*, 14:106.

Xu J, Tan X, Zhang X, Xia X, Sun H.2015. The diversities of staphylococcal species, virulence and antibiotic resistance genes in the subclinical mastitis milk from a single Chinese cow herd. *Microb Pathog*. 88:29–38.

Yimam TM, Kasse GE, and Yitie MT 2020. Study on Prevalence of Bovine Subclinical Mastitis and Associated Risk Factors in Smallholder Dairy Farms of Mecha District, West Gojam, Ethiopia. *Epidemol Int J* 4(5): 000161

Youssif N H., Hafiz N M., Halawa M A. Saa M F. 2020, Influence of Some Hygienic Measures on the Prevalence of Subclinical Mastitis in a Dairy Farm. *Int. J. Dairy Sci.,* 15: 38-47.

Yu W, Kim HK, Rauch S, Schneewind O, Missiakas D. 2017. Pathogenic conversion of *coagulase-negative staphylococci. Microbes Infect*. 19:101–9.

Zeryehun, T., & Abera, G. 2017. Prevalence and Bacterial Isolates of Mastitis in Dairy Farms in Selected Districts of Eastern Hararghe Zone, Eastern Ethiopia. *Journal of Veterinary Medicine*, 2017, 1–7. https://doi.org/10.1155/2017/6498618

Zmantar T, Kouidhi B, Miladi H, Bakhrouf A. 2011. Detection of macrolide and disinfectant resistance genes in clinical and *Coagulase-negative Staphylococci*. *BMC Res*. 4:453.

8. APPENDICES

Annex 1. Procedures and interpretation of laboratory tests which used for identification of staphylococci

1. Gram Stain Procedure

1. Pure colonies are picked by bacteriological loop and placed clean slide
2. Make the thin bacterial colonies smear and allow it to dry on the air
3. Fixed the dried smear by passing though flame 1 or 2 but not over to
4. Flood the smear by grams crystal violet leat stand for one minute
5. Pour of the stain and wash with gentle water
6. Flood with grams iodine solution and allow it to remain 60 second
7. Pour off the iodine solution and gentle wash with water
8. Decoralized with grams iodine solution 95/0/o acetol alcohol for 12-20 second
9. Counter stain with gram sefranine or carbon fuschin for 60 sec
10. Wash off the red sefranine solution with water
11. Slide my shake to remove water and to make dry it
12. Examine finished slide under microscope oil emersion

Interpretation: the bluish color gram positive and pinkish red negative

2. Coagulase Test Procedure

1. To test tube are taken and label test and negative control
2. Each tube filled with 0.5ml of rabbit plasma
3. To the tube labeled and test o.5ml overnight broth culture bacteria is added
4. The tube labeled negative control only o.5ml of sterilized tube are added
5. All the tube are mixed gentle, incubated 37c and observed at 4 hours
6. It the test is remain negative until 4hrs of 37c the tube is kept at room temperature for overnight incubated
7. Avoid the tube shaking during reading doubt or false result my occur due to down of clot

Results: positive is the soiled clot remain overnight but not clot for negative result

3. California Mastitis Test (CMT)

1. Put ½ CMT solution on the paddle cup with equal amount of fresh milk always use so much CMT solution as milk
2. Brief rotary motion of paddle brings out the reaction
3. Degree of thickening shows the the reduction in lactose, casein and rennet coagulation properties and increase in corpuscular protein, chlorides.

Catalase and spontaneoses free fatty acid when using the bulky milk slight to medium thickening indicate chronic mastitis in the herd.

Test should be not used colostrum milk

Premilk is prefable to striping 2-3 discarded

Score	**interpretation**	**visible reaction**
O	negative	milk fluid normal
T	traces slight	precipitation
1	weak distinct	precipitation but not gel formation
2	distinct positive	mixture of thickness is gel formation
3	strong positive	viscosity is greatly increased, strong gel cohesive surface

4. Catalase Test

1 Pick a colony from an18-24hrs culture and place it clean slide
2 Put on one drop of H2O2 over the organisms on the slide
3 Observe forimidate bubbling (gas forming) and record the result

Results: Positive result is rapped evidenced by bubbling and negative result is no bubbling

5. Mannitol Salt Agar

Composition (g/Litre):

Lab-Lempo powder	1.0
Peptone	10.0
Mannitol	10.0
Sodium chloride	75.0
Phenol red	0.025
Agar	15.0

Final PH 7.5 + 0.2 at 25 0C

Procedures:

1. The colonies that were confirmed by gram staining reaction hemolysis on blood agar.
2. Catalase positive was selected and streaked on mannitol salt agar Plate,
3. Incubate at 37^0c and examined after 24-48hrs for growth

The presence of growth and fermentation of mannitol by S. Aureas causes yellow discoloration of the medium.

Instructions:

Suspend 111g in 1L distilled water and bring to the boil to dissolve completely, sterilize by autoclaving at 121 0C for 15 minutes. Mix well before pouring in to sterile petridishes.

6. Nutrient agar

Composition (g/Litre):

- Lab-Lemco powder……..1.0
- Yeast extract ……2.0
- Peptone…….5.0
- Sodium chloride……....5.0
- Agar……15.0

pH: 7.4 ± 0.2

Preparation: Dissolve 28g of the components or the dehydrated complete medium in 1000ml of distilled water, by heating if necessary. Sterilize for 15 min in the autoclave set at 121 °C. Transfer about 15 ml of the melted medium to sterile small Petri dishes and proceed.

7. Purple Agar Base (PAB)

1. The suspected culture was inoculated on PAB media plate with 1% of maltose.
2. Incubated at 370C for 24-48 hours to differentiate S. aureus from other CPS isolates.
3. The identification and confirmation of was based on the fact that:-

a. S. aureus rapidly ferment maltose and the acid metabolic products cause the PH indicator (bromocresol purple) to change the medium and colonies to yellow.

b. S. intermedius gives a weak or delayed reaction

c. S.hicus did not ferment maltose but attacks the peptone in the medium producing an alkaline reaction (a deeper purple) around the colonies.

6. Procedure of Milk Sample

1. Label tube prior to sampling (date, farm and cows identity)
2. Brush loose the dirty, beading and teat thoroughly wash and dry it grossly before sample collection
3. Discard several streams of the milk from teat and observe any clinical sign of mastitis
4. Dip all quarter an infective dis infective and not allow for 30 sec contact time
5. Dry the teat with individual towels
6. Begging with teat on the far side of the udder scrub the teat with cotton for 10-15 sec not well moisted with 70 /0 of alcohol
7. Teat ends should be scrubs until it well clean
8. The single cottons ball or alcohol swab is should not be used more than one teat can not to clean end of teat
9. Being sample collection from closest teat and to move on the far side of the inner surface of the teat all was the open tube down ward facing.
10. Maintain the tube approximately 45% degree while taking milk do not over fill tube specially when the frozen the sample.
11. To collect a composite sample collection start from near to far one to two ml should be collected in every quarter or teat.
12. Store sample immediately on ice or frozen not exceeds 48hrs before culturing.

Annex 2. Plating and biochemical tests record sheet format used for *staphylococci* isolation

Sample _____

Code of sample	CMT results		Colony characteristics on different media				Biochemical tests					Staphylococci results		
	Positive	Negative	On blood agar		On mannitol salt agar		Gram reaction	Catalase test	Coagulase test	Purple base agar	Rabbit plasma	Negative	CPS	CNS
			Pigmentation	Hemolysis	Growth only	Growth and fermentation								
1														
2														
-														
-														
-														
-														
-														
379														

Annex 3. Profiles of Staphylococcal species isolated from bovine subclinical mastitis cases.

Tests	Character	Staphylococcus species isolates	
		Staphylococcus Aureus	Coagulase Negative Staphylococcus
Growth on blood agar	Pigmentation	+	_
	Hemolysis	+	_
Growth on mannitol salt agar	Growth only	_	+
	Growth and fermentation	+	_
Gram reaction		+	+
Cell morphology and arrangement		Coccci in cluster	Cocci in cluster
Catalase test		+	+
Coagulase test		+	_
Parple base agar		+	_

Annex 4. Questionnaire survey and Observation format for knowledge, attitude and hygienic practice of participants in each selected dairy farms.

HARAMAYA UNIVERSITY

POSTGRADUATE PROGRAM DIRECTORATE

College of Veterinary Medicine

Department of Veterinary Public Health

Dear respondents'

The purpose of this questionnaire were to together information about to estimate the prevalence of staphylococci and subclinical mastitis from lactating cows and to assessment of hygienic practice in dairy farms at Haramaya district for partial fulfillment of the requirement for the degree of Master of Science in Veterinary Public Health.

General Directions:

1. You are kindly requested to give genuine response.
2. The study is entirely academic and all responses are confidential.
3. Feel free to respond.

Your participation in this study was purely voluntary and you are free to accept and refuse your consent to participate. The researcher promises to treat all information you provide as strictly confidential and will not disclose individualized information to any one unrelated to this study.

Thank you in advance!!

Format 1: Questionnaire for dairy farm workers and consumers interview.

This questioner is for the purpose assessments on hygienic handling practice of cow milk, therefore I would like to Acknowledge you that your good cooperation by providing true information.

Section I: Socio demographic Information of Respondents

1. Region_____
2. District/woreda _____
3. Village/PA _____
4. Name of Respondent _____
5. Age_____
6. Sex: A. Male B. Female
7. What is your Occupation?

A. Farmer B, Employed. C. Housewife D, Private Business E, Student F. other……

8. What is your Education Level?

A. Illiterate B. Elementary C, Secondary School D, College E. Degree (University level) F, Other

Section II: Interview on Milkers and Consumers about milk hygiene and handling practices at dairy farms.

1) Do you wash your hands before milking? (A)Yes…… (B)No……
2) If yes what technique do you use?

a) Normal water B) Warm water C) Normal water and soap D) Warm water and soap

3) Do you wash your cow's udder before milking? (A) Yes….. (B) No……..
4) If yes, when do you wash it? (A) Cleaned before milking (B) cleaned after milking (C) cleaned before and after milking
5) If you wash the udder or teat what do you use for drying? (A) Individual towel B) Common towel (C). Just with hands (D). No drying at all
6) Do you wash your Hands between milking of another cow? A) Yes B) No

7) What is the source of the water used for washing the udder and milk utensils? (A) River/ stream (B). Tap / piped (C). Hand dug well (D). Others
8) Which container do you use for milking? A. Plastic, B. Traditional, C. Aluminum/St
9) How do you wash your milking bucket?

a) Immediately after milking, B Just before the next milking, C. Others _____

10) What do you use to clean your milking bucket? A) Detergent B) Cold or hot water C) Other_____
11) Source of milk for you sale? A, Household, B, Collection from different people who have lactating cows
12) How do you sell your milk?

a) A, Separately, B, Mixing with each other

13) How many times per day do you milk your cow? A. Once, B. Twice, C. Thrice
14) At what time do you milk your cow? A, morning B, evening, C, afternoon
15) When you sale your milk? A, morning B, evening, C, afternoon
16) How do you store you evening milk? _____
17) Do you sell the evening milk the next day? A. Yes, B. No
18) If yes, how do you sell it?

A. Mixed with the fresh one, B. Separately, C. Other_____

19) What type of equipment are you using for transporting milk?

A) Traditional, B. Plastic containers, C. Aluminum/Steel

20) By what you transport your milk to the market? A, on foot B, vehicle c, others_____
21) How long do you carry the milk to reach the final selling site (town)?

A) A, 1- 3KM, B, 4-5KM, C, >6KM

22) What is the main cause of spoilage of the milk? _____
23) How do you prevent your milk from spoilage?
24) How do you drink milk? A) Raw milk B) after boiling
25) If you boil/pasteurize the milk, for how much boil it?_____
26) Do you have knowledge about the disease which transmits from animal to human?

A) Yes B) No

27) If yes, how can get? A) By training B) formal C) informal

Appendices Table 3. **Dairy Farms Observation Format**

No	Observation Type	Observation Result
1	Is any disinfectant Used in the farm?	Used-------- Not Used----------
2	Smoking or eating or chewing while milking	Smoking------- Chewing-------- Eating----------
3	Apron (any protective clothes)	Worn--------- Not worn---------
4	Do you washed hand before milking?	Washed---------- Not washed----------
5	How usage of towels while milking?	Common-------- Not common-------
6	Udder hygiene	Good------ Poor-------
7	House hygiene	Good------ Poor-------

Annex 5. Photos up on filed and laboratory based investigation

Figure 1. Identified subclinical mastitis by CMT

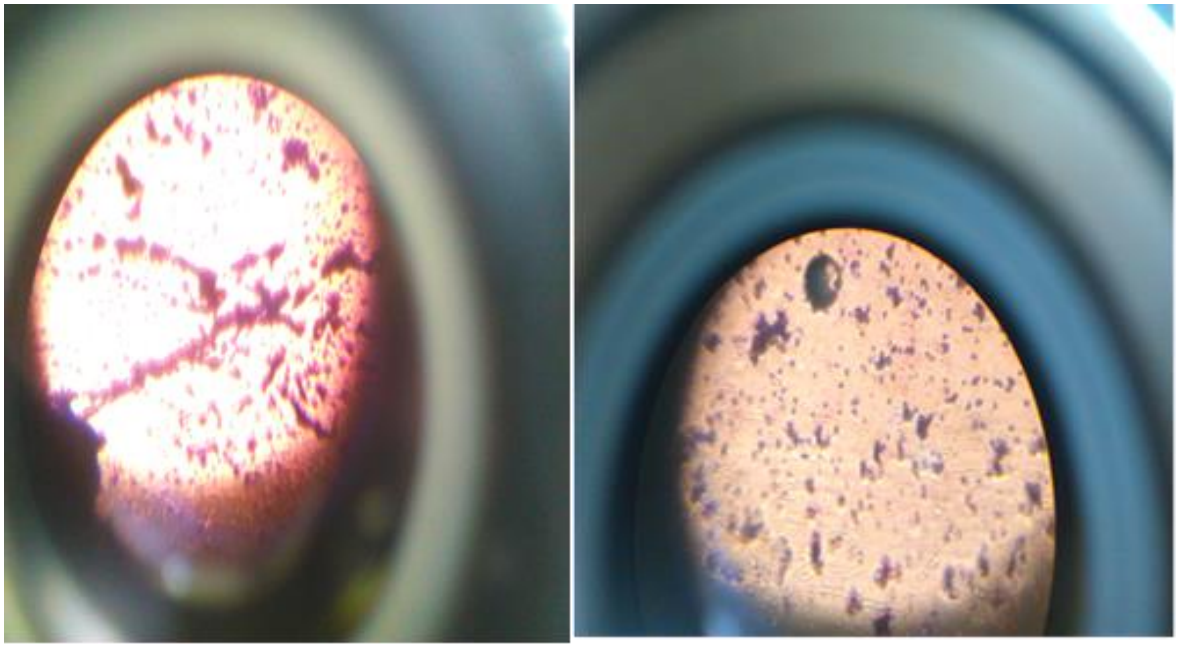

Figure 2. Gram stain of staphylococci under microscope

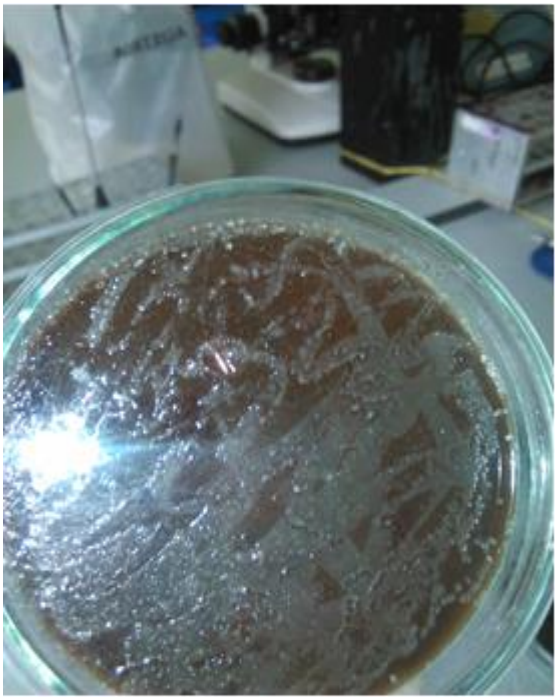

Figure 3. Growth Staph. on blood agar.

Figure 4. Colony of Staph.on monnitol agar

Figure 5. Transferring Staph. From mannitol to nutrient ager

Figure 6. Growth of Staphylococci on nutrient agar media

Figure 7. Staphylococci identified by catalase test

Figure 8. Tube coagulase test conducted by rabbit plasma

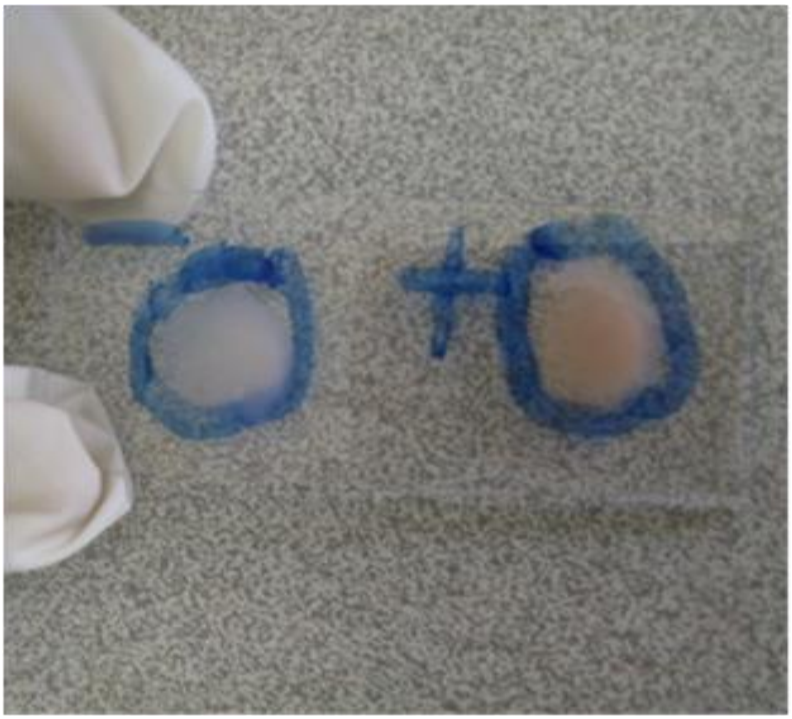

Figure 9. Slide coagulase test conducted by rabbit plasma

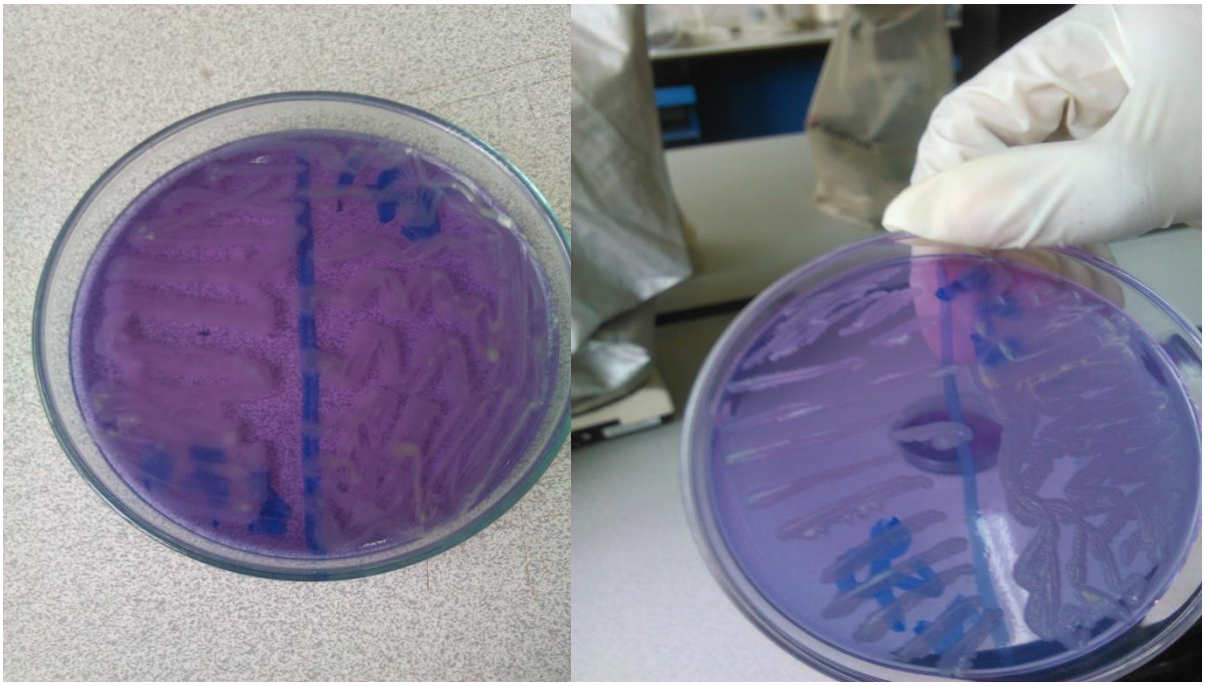

Figure 10. Identified colony of staphylococci on purple base agar