JIMMA UNIVERSITY
COLLEGE OF AGRICULTURE AND VETERINARYMEDICINE
SCHOOL OF VETERINARY MEDICINE

PREVALENCE, RISKFACTORS AND ANTIMICROBIAL SUSCEPTIBILITY PATTERNSOF *STAPHYLOCOCCUS AUREUS* ISOLATES FROM MASTITIC LACTATING COW'S MILKIN DAIRY FARM AT SHINSHICHO TOWN, KEMBATA TEMBARO ZONE, SOUTHERN ETHIOPIA

MSc THESIS

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Prevalence, Risk factors and Antimicrobial Susceptibility Patternsof *Staphylococcus aureus* isolates from Mastitic lactating Cow's Milkin Dairy Farm at Shinshicho town, KembataTembaro Zone, Southern Ethiopia

A thesis submitted to the School of Veterinary Medicine, Jimma University, in partial fulfillment of the requirements for the Degree of Master of Science in Veterinary Public Health.

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STATEMENT OF AUTHOR

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BIOGRAPHICAL SKETCH

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LIST OF ABBREVIATIONS, SYMBOLS AND ACRONYMS

a_w	Water Activity
BAP	Blood Agar Plate
CI	Confidence Interval
CLSI	Clinical Laboratory Standard Institutes
CMT	California Mastitis Test
CNS	Coagulase Negative Staphylococcus
CSA	Central Statistical Agency
DNA	Deoxyribo Nucleic Acid
E_n	Redox Potential
FBD	Food Borne Diseases
Fc	Fragment crystallizable region
GIS	Geographical Information System
KM	Kilometer
MDR	Multiple Drug Resistant
MHAP	Muller Hinton Agar Plate
MSA	Mannitol Salt Agar
NAP	Nutrient Agar plate
NMC	National Mastitis Council
OF	Oxidative Fermentative
OR	Odds Ratio
PAB	Purple Agar Base
SE	*Staphylococcal* Enterotoxin
SFP	*Staphylococcal* Food Poisoning
SNNPR	Southern Nation Nationalities Peoples Region
TSB	Tryptone Soya Broth
TSST	Toxic Shock Syndrome toxin

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ABSTRACT

Bovine mastitis is the most prevalent infectious disease in dairy herds worldwide. *Staphylococcus aureus* is the major microorganism causing infectious mastitis. A cross-sectional study was conducted from December 2018 to September 2019 in Shinshicho town, Kembata Tembaro Zone, Southern Ethiopia, to determine the prevalence, associated risk factors and antimicrobial susceptibility patternsof *S.aureus* isolates from mastitic lactatingcow's milk indairy farm. A total of 384 lactating dairy cows were screened for mastitis based on clinical examinations and California mastitis test (CMT) result, out of which 160 were positive. The occurrence of mastitis was 41.7 %in cows and 21.1% in quarters. Out of the occurrences of mastitis, 5% and 36.7% were clinical and subclinical respectively. *Staphylococcus aureus* was isolated from 1.8% (n=7) and 14.3% (n=55) of the clinical and subclinical mastitis respectively with the total isolation frequency of 16.1% (n=62). Multivariable logistic regression analysis of the effect of different risk factors on the prevalence of *S.aureus* showed that; adult cows were more likely positive than their younger counterparts (OR=3.64, 95%CI: 1.64-8.11), cows in late lactation stage were more likely to be positive than cows in early lactation stage (OR=4.21, 95%CI: 1.52-11.66), the dairy farms having a large herd size greater than 5 cattle were more likelypositivefor *S.aureus* than farms having a small herd size(OR=3.05, 95%CI: 1.65-5.61), and dairy farms with muddy floor were more likely positive than cemented floor husbandry systems (OR=2.26, 95%CL:1.23-4.17). All the *S.aureus* isolates were subjected to antimicrobial susceptibility tests. The highest rate of susceptibility was to Chloramphenicol (95.2%) followed by Gentamicin (91.9%), Cephalothin (90.3%), Kanamycin (88.7%) and Streptomycin (80.6%). The current study revealed that; *S.aureus* has absolute resistance to Ampicillin, Amoxicillin, Penicillin-G, and Polymyxin. Also, 53.2% of the isolates were found to be multiple drug resistance phenotypes. *Staphylococcus aureus* is one of the pathogens causing mastitis in dairy farms in the study area and might be imposing public health impacts if raw milk is consumed. Larger herd size, aged cow, late lactation stage and muddy or soil floor are risk factors associated with the occurrence of *S.aureus* mastitis. Hence, more attention should be given in large herd, culling old cow, treating in late lactation stage and wise use of antimicrobial.

Keywords: *Antimicrobial susceptibility, Milk, Prevalence, Risk factors, Shinshicho, S.aureus.*

1. INTRODUCTION

1.1. Back ground of the study

Ethiopia has the largest livestock population in Africa comprising about 60.39 million cattle. Out of this total cattle population, the female cattle constitute about 54.68%, from this 11.3% and 20.52% were dairy and milking cows, respectively (CSA, 2017/2018). The sector holds its share from 15 to 17% of gross domestic product, 35 to 49% of agricultural domestic product and 37 to 87% of household incomes (Behnke and Metaferia, 2011). Livestock remains to be a major national resource and form an interconnected chain in the mixed crop-livestock production system (Gebrewold *et al.*, 2000). Moreover, cows represent the largest population of cattle in the country but the annual consumption of milk in Ethiopia is low as compared to the average milk consumption of other countries. Local milk production does not satisfy the country milk requirement due to low input husbandry practice and widespread livestock diseases (Mohammed *et al*., 2004).

Ethiopia holds large potential for dairy development due to its large cattle population and the favorable climate for improving high yielding animal breed (Mohamed *et al*., 2004). Therefore, the contribution of the dairy sector especially the smallholder system in Ethiopia to poverty alleviation and sustainable food production in the country is assumed to be considerable, given the substantial potential for income and employment generation from high-value dairy products (Mohamed *et al*., 2004). In spite of that, qualities and quantity of milk in Ethiopia deteriorate due to various causes, out of which mastitis is a factor contributing to reduced milk production (Fekadu, 1995). However, among the different factors, the sector is constrained by mastitis, which incurs serious economic losses to the dairy industry (Getahun *et al.,* 2007).

Among various etiological agents, *S.aureus* is a major mastitis-causing pathogen that also poses food safety and antimicrobial resistance threats (Kumar *et al*., 2011). It is a versatile pathogen in humans and animals which is responsible for a diverse spectrum of diseases ranging from minor skin infections to life-threatening diseases, such as pneumonia and meningitis (Jangra and Singh, 2010). Besides, *S. aureus* is an important cause of clinical mastitis in dairy cows causing a huge economic loss worldwide (Lundberg *et al*., 2014). The report from Ethiopia also indicated that *S.aureus* is the most predominant cause of mastitis in

dairy cows (Getahun *et al*., 2007). *Staphylococcus aureus* can express a wide array of potential virulence factors, including surface protein that promotes adherence to damaged tissues and /or exotoxins and enzymes that can cause a variety of infection in the skin and soft tissues, including intramammary mastitis (Iwasuki *et al*., 2006).

The bacterial contamination of milk from an infected dairy cow may render it unsuitable for human consumption by causing food-poisoning or provides a vehicle for the spread of zoonotic diseases to humans (Bitew *et al*., 2010). Milk and products derived from the milk of dairy cows can harbor a variety of microorganisms and can be important sources of foodborne pathogens. The presence of foodborne pathogens in milk is due to direct contact with contaminated sources in the dairy farm environment and excretion from the udder of an infected animal (Oliver *et al*., 2005). The prevalence of food-borne pathogens in milk is influenced by numerous factors such as farm size, breed of cows, farm management practices, variation in sampling and types of samples evaluated, differences in detection methodologies used, geographical location and season (Oliver *et al*., 2005). However, in spite of the variation, all of these surveys demonstrated quite clearly that milk can be a major source of food-borne pathogens of human health significance (Oliver *et al*., 2005).

Staphylococcus aureus can be transmitted to humans through contaminated and untreated milk and milk products (Seifu *et al*., 2004). *Staphylococcus aureus* presents on the skin and mucosa of food-producing animal reservoirs that include dairy cow and it is frequently associated with subclinical or clinical mastitis leading to the contamination of dairy products. This bacterium is considered the most commonly occurring major pathogen of the cow's mammary gland (Chaffer *et al*., 1999).

Most of the time many cases of clinical *S.aureus* mastitis is treated with antimicrobial, but the cure rate is very variable due to a breed of cow and bacterial factors such as parity of the cow, durations of the infection and β-lactamase production of the bacteria (Lundberg *et al*., 2014). It has been believed that all bacterial infections were treatable with a vast array of effective and antimicrobial agents. However, the emergence of resistance to multiple antibiotics among *S.aureus* has created challenges for health professionals and researchers (Motamedi *et al*., 2010). It has been reported that shortly after the introduction of penicillin in the 1940s,

resistance developed in *S.aureus*, followed by resistance to methicillin and more recently to glycopeptides, for example, vancomycin (Monroe and Polk, 2000).

Antimicrobial has been widely used in the treatment of infection caused by *S.aureus* in dairy animals, Waters *et al.* (2011) and their use has been linked to the spread of resistant bacteria to humans through the consumption of milk and milk products (Marshall and Levy, 2011). In addition, the extensive misuse of antibiotics in all contexts has created strong selection pressure, which has resulted in the survival and persistence of resistant strains (Shryock *et al*., 2013). This poses a challenge to veterinarians, health professionals and dairy cattle owners due to its negative impact on the response to antimicrobial therapy (Abera *et al*., 2010).

1.2. Statement of Problem

Staphylococcus aureus is the most significant pathogen among *Staphylococci* species related to subclinical intramammary infections in dairy cows leading to severe economic losses in dairy industry worldwide (Godden *et al.*, 2002). Even thought, different classes of antimicrobials can be used against thesepathogens, *S. aureus* mastitis has been found to respond poorly to commonly used antimicrobials treatment (Barkema *et al*., 2006).Furthermore, the emergence of pathogenic microorganism resistance to commonly used antimicrobials is one of the most serious global medical problems concerns of the 21stcentury.

Antimicrobial resistance seems to be increasing and multiple antibiotic-resistant strains have started to emerge (Otter and French, 2010). Infection with antimicrobial-resistant bacteria has been known to be associated with frequent treatment failure and increased severity of the disease (Frinch and Hunter, 2006). One of the most important bacteria in this regard is *S.aureus* (Cosgrove *et al*., 2003). The use of antimicrobial agents in animals and humans results in the emergence and dissemination of resistant bacteria. Resistant bacteria from food animals may be passed through the food chain to humans resulting in resistant infections. The increasing resistance to antimicrobial agents is important in the treatment of human diseases (Anderson *et al*., 2003). The problem with *S.aureus* becomes more complicated when it was found that it quickly developed resistance and was capable of producing many antibiotic-resistant strains (Farzana *et al.,* 2004). The development of resistance has been attributed to

the indiscriminate and extensive use of antibiotics for therapeutic or as growth promoters in food animal production (Normanno, 2005).

In Ethiopia, irrational and misuse behavior of antimicrobials were reported by Beyene *et al*. (2015) which could aggravate the antimicrobial resistance of bacteria issues in the country. The study on *Staphylococcus* species isolated from animal origin food in central and southern Ethiopia by Tessema *et al*. (2016) and South-Eastern Ethiopia, Beyene *et al.* (2016) have reported an alarming level of MDR. However, a good estimate of the magnitude of the Prevalence of *Staphylococcus aureus* and their antimicrobial resistance pattern for animals is hardly available in Ethiopia. *Staphylococcus aureus* isolates from the dairy farm in North-Western Ethiopia, suggesting contagious nature within and between farm observed type strains that are often resistant to commonly used Antimicrobial (Mekonnen *et al.,* 2018).

Recently, there have been some studies conducted on isolation, identification, and drug resistance patterns of *S.aureus* in lactating dairy cow milk in various parts of Ethiopia, such as in Kombolcha town by Asmelash *et al.* (2016), Wolaita Sodo by Biniam *et al.* (2017), Assosa Town by Asmelash *et al*. (2017) and Asella Town by (Befikadu *etal*., 2018). However, Prevalence, associated risk factors and antimicrobial susceptibility test of *S.aureus* isolates from mastitic lactating cow's milk has been insufficiently investigated in the Shinshicho town. Moreover, to date, there was no published document on its magnitude, distribution and associated risk factors in mastitic lactating dairy cow's milk in Kembata Tembaro Zone in general and around Shinshicho town particular.

Since the antimicrobial susceptibility test for *S.aureus* is essential to make decisions regarding the uses of antimicrobial treatment in dairy cows and the prerequisite for establishing control strategies. Besides, the lack of such facts treatment measures fails and designing control programs might be simply assessment works. However, the identification of antimicrobial resistance patterns of *S.aureus* data is needed to get some insight into their overall negative economic implications and public health significance. Therefore, the study was the first of its kind to isolate *S.aureus* from mastitic lactating cow's milk in the Shinicho town and is subsequently, the baseline for which future *S.aureus* isolation on animal-based food products.

1.3. Objectives

- To isolate, identify and determine the prevalence of *Staphylococcus aureus* in mastitic lactating cow's milk in the Shinshicho town.
- To assess different potential risk factors associated with the prevalence of *Staphylococcus aureus* in mastitic lactating dairy cows in the study area.
- To investigate the antimicrobial susceptibility test of *Staphylococcus aureus* isolates to commonly used antimicrobial in the study area.

2. LITERATURE REVIEW

2.1.General description of *Staphylococcus aureus*

The genus *Staphylococcus* comprises of several species and subspecies (Kwok and Chow, 2003). To date, more than 50 species and subspecies of *Staphylococci* have been described according to their potential to produce coagulase. It is broadly grouped into two, namely, coagulase-positive and coagulase-negative *Staphylococcus* (CNS) (Sasaki *et al*., 2010; Becker *et al*., 2014). CNS consists of a group of various *Staphylococcus* species that affect diverse host ranges. Some of them have evolved to cause mastitis in farm animals Pyorala and Taponen (2009) whilst some species colonize post-surgical wounds (cause infections inimmunecompromised people) in humans (Becker *et al*., 2014).

The coagulase-positive *Staphylococci* group, the three major pathogenic ones are *S. aureus*, *S. intermedius* and *S. hyicus* (Devriese, 1990). These three species are associated in many diverse and specialized forms with healthy and diseased farm animals and most of the common pet animals (Devriese, 1990). They are the major pathogen causing mastitis although they have tropism to different body parts of various hosts (Graveland *et al*., 2011). They are versatile pathogens with the ability to transfer between humans and livestock (Zadoks *et al*., 2000). In humans, they cause mild skin infection to more severe diseases such as pneumonia and septicemia (Ateba *et al*., 2010). In animals, their frequent involvement in mastitis results in the contamination of milk and dairy products.

2.1.1. Morphology and characteristics

The *Staphylococcus aureus* bacterium commonly referred to as 'staph' was discovered in 1882 by a Scottish surgeon Sir Alexander Ogston in his effort to identify the etiology of suppuration (formation of pus) in humans (Ogston, 1984). The name "*Staphylococci*" was given to represent its round grape-like clustered morphology and "*aureus*" for its ability to form golden to yellow-pigmented colonies (Bairdparker, 1990). *Staphylococci* are gram-positive *cocci*, approximately 1µm in diameter that tends to occur in irregular clusters Roberts and Greenwood (2003) resemblingbunches of grapes. Most *Staphylococci* are facultative anaerobes and catalase-positive.They are non-motile, oxidase-negative and do not form spores (Quinn *et al*., 2002). *Staphylococcus aureus* produces golden yellow colonies on blood agar; they appear as glistening, smooth, entire, raised, translucent colonies that often have a golden

pigment. The colonies are 2-3mm in diameter after 24hours incubation and most strains show β-haemolysis surrounding the colonies (Bhunia, 2008).

The cell wall of *Staphylococcus aureus* contains three main components: the peptidoglycan comprising repeating units of N-acetyl glucosamine β-1, 4 linked to N-acetyl muramic acid; a Ribitol teichoic acid bound via N-acetyl mannosaminyl-β-1, 4- N-acetyl glucosamine to a muramyl-6-phosphate; and Protein A, which is covalently linked to the peptidoglycan and particularly is characterized by its ability to bind to Fc component of the immunoglobulin in plasma causing auto agglutination. Most of the other species of *Staphylococci* lack protein A in their cell walls (Bhunia, 2008).

2.1.2. Growth and survival characteristics

The growth and survival of *S. aureus* is dependent on several environmental factors such as temperature, water activity (a_w), and pH, the presence of oxygen and composition of the food (Stewart, 2003; Norman *et al*., 2005). These physical growth parameters vary for different *S. aureus* strains presented in Table1. The temperature range for the growth of *S. aureus* is 7–48°c, with an optimum of 37°c. Also, it is resistant to freezing and survives well in food stored below -20°c; however, viability is reduced at temperatures of -10 to 0 ^{o}c. It is readily killed during pasteurization or cooking. Growth of *S. aureus* occurs over the pH range of 4.0–10.0, with an optimum of 6–7 (Stewart, 2003). It is a facultative anaerobe so it can grow under both aerobic and anaerobic conditions. However, growth occurs at a much slower rate under anaerobic conditions (Stewart, 2003).

Table 1.Factors affecting growth and enterotoxin production by *S.aureus*

Organism growth			*Staphylococcal enterotoxin* production	
Factors	Optimum	Rage	Optimum	Rage
Temperature	37°c	7-48°c	37-48°c	10-45°c
pH	6 -7	4-10	7-8	4-9.6
($a_{w)}$	0.98	0.83-0.99∗	0.98	0.85-0.99$^+$
NCl (%)	0	0 – 20	0	0-10
Redox potential (E_n)	>+200mv	< +200mv to > +200mv	>+200mv	<100mvto> +200mv
Atmosphere	Aerobic	Anaerobic aerobic	Aerobic(5-20% dissolved $O_{2)}$	Anaerobic aerobic

*aerobic (anaerobic 0.90-0.99), +aerobic (anaerobic 0.92-0.99), Sources (Hennekinne*et al*., 2012)

2.1.3. Enzyme produced by *Staphylococcus aureus*

Staphylococcus aureus produces various enzymes such as coagulase which clots plasma and coats the bacterial cell, probably to prevent phagocytosis, *Hyaluronidase* (also known as spreading factor) and breaks down *hyaluronic* acid and helps in spreading it (Zakour *et al*., 2011). *Staphylococcus aureus* also produces deoxyribonuclease, which breaks down the DNA, lipase to digest lipids, *Staphylokinase* to dissolve fibrin and aid in the spread, and beta-lactamase for drug resistance (Cencigoga *et al*., 2003). Depending on the strain, *S. aureus* is capable of secreting several exotoxins, which can be categorized into two groups' that include *Staphylococcal* enterotoxins (SE) and toxic shock syndrome toxin (TSST). Many of these toxins are associated with specific diseases and their toxins are dangerous in terms of food safety whereby their biological activity remains unchanged even after thermal processing of food (Dinges, 2000).

2.2. Epidemiology of *Staphylococcus aureus*

2.2.1. Source of contamination and reservoirs

Staphylococci are commonly found in a wide variety of mammals and birds, and contamination of *S. aureus* to food has two main sources: human carriage during food processing and dairy animals in case of mastitis. Human strains are mainly involved in SFP. However, animals are also known to be a potential source of primary contamination. For example, in the case of *Staphylococcal* mastitis of ruminants such as cows, *S.aureus* can be carried over from the udder into the milk (Hennekinne*et al*., 2012).

The natural ecological niches of *S.aureus* are the nasal cavity and the skin of warm-blooded animals. The skin, mucosa membranes, teats and udder of milking animals are the most important reservoir of this pathogen. In the case of an infected udder, *S. aureus* can contaminate milk during milking and it is responsible for approximately 30-40% of all mastitis cases in the world (Medvedova and Valik, 2012). The principal reservoir of *S. aureus* is the human carrier. A high proportion of healthy people have*Staphylococci* in the nasopharynx and on the skin. The organism has been isolated from the head, body, legs, and nose of cows, from the hands and nose of people, and the environment such as the milking equipment, bedding materials and watercourses (Deoliveira *et al.*, 2007).

2.2.2. Risk factors associated with *Staphylococcus aureus*

Staphylococcus aureus is predisposed by several epidemiological risk factors that play a significant role in causing mammary incompetence to protect it from the invasion of infectious agents. From the intrinsic host, risk factors age of the lactating dairy cows and parity have significantly influenced the occurrences of *S.aureus* due to the increased opportunity of infection with time and the prolonged duration of infection, especially in a herd without mastitis control program (Radostits *et al*., 2007). Also the different lactation stages of the cows associated with *S. aureus* mammary infection because of an absence of dry period therapy and birth-related influence (Radostits *et al.,* 2000).

The main source of *S. aureus* infections is the udder of infected cows transferred through milker's hands, utensils, towels, and floors in which the cow was kept (Radostits *et al.*, 2007). Moreover, the risk factors include the host factors, environmental factors and pathogen factors (Shaheen *et al*., 2016). Furthermore, the risk factors which is contributed to the occurrences of *S.aureus* infection in the dairy farm are attribute, the age levels of the animals, numbers of calves (parity), the milking hygienic conditions, lactation periods, the previous mammary gland treatments and the housing systems are greatly associated with *S.aureus* infections in the dairy farms (Mulugeta and Wassie, 2013; Asmelash*et al*., 2016; Biniam*et al.,* 2017).

2.2.3. Means of transmission

Staphylococcus aureus exists in air, dust, sewage, water, milk, food, or on food equipment, environmental surfaces, humans, and animals (Bennett and Monday, 2003). Infected milk is generally considered to be the primary source of the pathogens, and milking liners are the main vector of transmission since they have been frequently shown to be contaminated with similar *S. aureus* strains to those in infected milk (Zadoks *et al.,* 2001). It is also present in the nasal passages and throats and on the hair and skin of 50% or more of healthy individuals. This incidence is even higher for those who associate with or who come in contact with sick individuals and hospital environments. Although food handlers are usually the main source of food contamination in food-poisoning outbreaks, equipment, and environmental surfaces can also be sources of contamination with *S. aureus*. Human intoxication is caused by ingesting enterotoxins produced in food by some strains of *S. aureus*, usually because the food has not

been kept hot enough (60°c, 140°F, or above) or cold enough (7.2°c, 45°F, or below) (Bennett and Monday, 2003).

2.2.4. The distribution of *Staphylococcus aureus* in lactating dairy cow's milk in Ethiopia

Due to changing management conditions and using different diagnostic tests, a wide variation in the prevalence of *S. aureus* has been reported (Rodistits *et al*., 2000).

Table 2.The Prevalence of *S. aureus* isolated from cow milk in a different parts of the country

Study Area	Prevalence (%)	References
Shashemane	28.1%	Abera*et al*., 2012
Addis Ababa	15.1%	Abebe*et al*., 2013
Addis Ababa	15.5%	Abebe*et al*., 2013
Addis Ababa	17.2%	Gizaw,2014
Kobolicha	26.6%	Asmelash*et al*., 2016
Fitsum	32.14%	Fitsum, 2016
WolaitaSodo	15.5%	Biniam *et al.,* 2017
Sabata	19.6%	Ayele*et al*., 2017
Asella town	44.6%	Kemal *et al*., 2017
Asellatown	46.5%	Befikadu*et al*., 2018

2.3. Pathogenesis and clinical forms of *Staphylococcus aureus*

2.3.1. *Staphylococcus aureus* infection in lactating dairy cows

The pathogenesis of *S. aureus* in the mammary gland most likely involves the generally accepted concept of specific colonization. In vitro adhesion of *S. aureus* to the ductular and alveolar mammary gland, epithelial cells indicate that colonization might be an important step in the development of mastitis (Gyles*et al*., 2008). Furthermore, *S. aureus* bacteria can bind to extracellular matrix molecules. It is suggested that *Staphylococci* might use matrix proteins exposed by micro lesions or appearing in blood clots as substrates for adhesion as a step in colonization and the development of mastitis infections. *Staphylococcus aureus* isolated from bovine mastitis can bind to fibronectin, fibrinogen, laminin, and different types of collagen (Barkema*et al*., 2006).

Staphylococcus aureus mastitis in dairy cows may be clinical or subclinical, and in its clinical form, the disease may vary from a severe per-acute form to a very mild form without general signs of infection. Unless the treatment of acute infections is successful, the reaction becomes chronic. This chronic reaction may also occur in sub-clinically (Barkema *et al*., 2006). Treatment failures are particularly high in multiparous with more than one infected quarter

and recurrences are frequent and culling is commonly the only solution (Michelle and Jeffrey, 2011).

2.3.2. *Staphylococcus aureus* and food poisoning

*Staphylococca*l food poisoning (SFP) is one of the most common food-borne diseases and results from the ingestion of *Staphylococcal* enterotoxins (SEs) preformed in food by enterotoxigenic strains of *Staphylococcus aureus*. All of them have super-antigenic activity whereas half of them have been proved to be emetic, representing a potential hazard for consumers (Hennekinne *et al*., 2012). Moreover*, S.aureus* is one of the major pathogens causing food poisoning worldwide. Because of their biological activities and structural relatedness *Staphylococcal enterotoxins* (SE) are emetic toxins and classified as members of the pyrogenic toxin super-antigen family (Balaban and Rasooly, 2001). *Staphylococcus aureus* strains can produce heat-stable enterotoxin which is responsible for *Staphylococcal* food poisoning and ranks as one of the most prevalent worldwide causes of gastroenteritis. The presence of *enterotoxigenic Staphylococcus aureus* in milk and also in milk products leads to potential health hazards to the consumers (Boerema *et al*., 2006).

2.4.Laboratory diagnosis of *Staphylococcus aureus*

Staphylococcus aureus can be diagnosed using macro-morphology whereby colony characteristic is observed and the media used is selective containing 0.1% potassium tellurite then incubated at 37°c for 24-48 hours (Arfatahery *et al*., 2015). Micro-morphologically, *S. aureus* presents as gram-positive *cocci* where samples are swabbed onto a microscopic glass slide. This is then stained with Gram stain or dyes like Crystal violet and basic fuchsine space and viewed under the microscope. *Staphylococcus aureus* is gram-positive and stains blue or purple and appears as small round *cocci* or short chains and most commonly as grape-like clusters (Arfatahery *et al*., 2015).

Based on biochemical test identification, sample placed onto a culture media, that provides sources of nutrition, carbon, energy, and nitrogen for the bacteria to grow and commonly used is Mannitol salt agar (Crossley *et al.*, 2009). It is a selective medium with 7-9% salt or sodium chloride that allows *S. aureus* to grow selectively (Tenover *et al.,* 1994). Other various tests can be used to identify *S. aureus*, including the production of protein a cell-bound clumping

factor, extracellular coagulase, and heat-stable nuclease and catalase test (Marti *et al*., 2010). Free (extracellular) coagulase clots plasma in the absence of calcium. Slide agglutination test involves clumping factor (bound coagulase) in that it is cell-bound and requires only fibrinogen.

2.5. Differentiation of *Staphylococcus* Species

Staphylococcus species must be differentiated from *Streptococcus* species and *Micrococc*us *species* presented in Table3. *Staphylococci* are generally catalase-positive and *streptococci* catalase-negative. *Staphylococcus* species are usually categorized by their colonial appearance, haemolytic pattern, and biochemical profile (Quinn *et al*., 2002). The same as the principal reactions of the coagulase-positive *Staphylococci* are indicated in Table 3. It may be particularly important to distinguish *S.aureus* from *S.intermedius* in certain clinical conditions (Quinn *et al*., 2002).

Table 3.The differentiations of gram- positive *cocci*

Organism	Appearance in stained smears	Coagulase production	Catalase production	Oxidase production	O-F Test [a]	Bacitrain disc (0.04unit)
Staphylococcus species	Irregular clusters	±	+	-	F	Resistant
Micrococcus species	Packets of four	-	+	+	O	Susceptible
Streptococcus and Enterococcus species.	Chain	-	-	-	F	Resistant

a, oxidation fermentation tests O=oxidative, F= fermentative; Source (Quinn *et al*., 2002)

Colonial characteristics: - *Staphylococcal* colonies are usually white, opaque and up to 4mm in diameter. The colonies of bovine and humans strains of *S.aureus* are golden yellow. Colonies of same coagulase-negative *staphylococci* are also pigmented (Quinn *et al*., 2002).

Haemolysis in sheep or oxblood agar: - Four *Staphylococcal* haemolysins are recognized, alpha, beta, gamma, and delta. Individual haemolysins differ antigenically, biochemically and their effects on the red blood cells of different animal species (Quinn *et al*., 2002).Strains vary in their haemolysin-producing ability and animal strain of *S.aureus* and *S.intermedius* usually produces both alpha and beta-haemolysin. On ruminant blood agar, the alpha-haemolysin causes a narrow zone of complete haemolysin immediately around the colony, and beta

haemolys in produces a wide zone of partial or incomplete haemolysis. This is referred to as double haemolysis (Quinn *et al*., 2002).These haemolysins act as a toxin in vivo. CN *Staphylococci* exhibit variation in their ability to produce haemolysis which usually develops slowly. Isolates of *S.hyicus* are non-haemolytic (Quinn *et al*., 2002).

Slide and tube coagulase tests**: -** In these tests, a suspension of *Staphylococci* is mixed with rabbit plasma either slide or in a small tube**.** The fibrinogen in rabbit plasma is converted to fibrin by coagulase: The slide test detects the presence of a bound coagulase or clumping factor on the bacteria surface. A positive reaction is indicated by the clumping of bacteria within 1 to 2 minutes (Quinn *et al*., 2002). The tube test detects free coagulase or *Staphylo coagulase* which is secreted by the bacteria into the plasma. It is the definitive test for coagulase production and a positive reaction is indicated by clot formation in the tube following incubation at 37°c for 24 hours (Quinn *et al*., 2002).

Biochemical tests for differentiating *S.aureus* and *S.intermedius*:-Purple agar, containing bromocresol purple as a P^{H} indicator and 1% maltose, is used to differentiate *S.aureus* from *S.intermedus* (Quinn *et al*., 1994). *Staphylococcus aureus* utilizes maltose and the acid produced to change the medium and colonies from purple to yellow. *Staphylococcus intermedius*, poor maltose fermenters, do not change the color of the medium (Quinn *et al*., 2002). Differentiation of *S.intermedius* and *S.psudintermedius* can be carried out by biochemical methods including detection of mannitol fermentation udder anaerobic condition.

Table 4.Distinguishing features of coagulase-positive *Staphylococci*

Species	Colony color	Haemolysis on sheep blood agar	Coagulase production		Acetoin production	Maltose utilization [a]
			Tube test	Slide test		
S.aureus	Golden yellow [b]	+	+	+	+	+
S.intermedius	White	+	+	V	-	±
S.hyicus	White	-	V	-	-	-
S.aureus sub spp.anaerobius[c]	White	+	+	-	-	Na
S.delphini	White	+	+	-	-	Na
S.schleiferi sub spp. Coagulans	White	+	+	-	+	Na

a= 1%maltose in purple agar base, b=bovine and human strains only, c=anaerobic, Na =not available, += over 90% strains positive, - = over 90% strains negative, ±= poor utilization, v= variable reactions. Source :-(Quinn*et al.,* 2002)

2.6. Isolation and identification of *Staphylococcus aureus*

The isolation and identification of *Staphylococcus* species are conducted based on colony morphology, hemolytic properties, Gram-stain, catalase production, coagulase production, and biochemical profile or sugar fermentation (Quinn *et al*., 2002; Aycicek*et al*., 2005). Samples were inoculated aseptically on the surface of the blood agar plates by spreading with a sterile loop in such a way that bacteria are ultimately deposited singly because when the bacteria are at a sufficient distance from each other, the whole progeny of each accumulates locally during growth to form a discrete mass or colony which is readily visible to the naked eye. Each colony was presumed to be a pure culture, consisting exclusively of the descendants of a single cell (Leloir *et al*., 2003; Shah, 2003).

On blood agar medium, *Staphylococcal* colonies appear opaque to golden yellow, glistening, smooth and in circular form. Blood agar is the medium of choice for isolation of the organism from samples, and on 24 hours incubation *Staphylococci* give good growth of creamy, often deeply pigmented colonies that is surrounded by the narrow zones of clear haemolysis, a broader zone of incomplete haemolysis or none depending on the species (Quinn *et al*., 2002; Bendahou *et al*., 2008). Some species of *Staphylococcus* synthesize the enzyme hemolysin. Hemolysin is an exoenzyme that lyses red blood cells. If a colony of bacterial cells is producing hemolysin and secreting it into the medium, there will be around, clear zone surrounding the colony because the red blood cells in that area have been lysed. The presence or absence of hemolytic properties, therefore, cannot be used as a definitive identification of *Staphylococcus* species as some species and strains of *Staphylococcus* species may not cause hemolysis (Quinn *et al*., 2002; Salandra*et al*., 2008).

Preparation and examination of Gram-stained smears from typical colonies show Gram-positive spherical bacterium *(coccus*), which on microscopic examination appears in pairs, short chains, or bunched, grape-like clusters (Aycicek*et al*., 2005). Catalase test is important to distinguish *Streptococci* (catalase-negative) from *Staphylococci*, which are catalase-positive. The catalase test determines if the organism produces the enzyme catalase that breaks down hydrogen peroxide (H_2O_2) to water and oxygen (Shah, 2003). When mixed with 3% H_2O_2, catalase-positive organisms will generate bubbles of oxygen, which are visible to the naked eye while catalase-negative organisms do not. This enzyme allows organisms to

breakdown harmful metabolites of aerobic respiration and may be seen in aerobic and facultative anaerobic organisms. It is preferable to test colonies for catalase production from media without blood since erythrocytes possess catalase activities (Quinn *et al*., 2002).

Pathogenic organisms require mechanisms to help them overcome host defense mechanisms. One mechanism involves coating the bacterial cells in a body substance, such as fibrin, to fool the immune system. The coating of a natural body substance will not trigger an immune response and this is accomplished through the production of coagulase. The coagulase is *exoenzymes* that cause fibrin of blood plasma to be deposited on bacterial cells resulting in clot formation. Pathogenic *Staphylococci* produce coagulase, while non-pathogenic strains are coagulase-negative (Shah, 2003; Morrison, 2008).

The common medium used for the isolation of pathogenic *Staphylococci* is the Mannitol salt agar (MSA). Some organisms cannot tolerate high osmotic pressure. The media contains higher than normal salt concentrations that inhibit the growth of these non-tolerant organisms other than the salt-tolerant *Staphylococci* (Baird and Lee, 1995). The MSA contains the sugar Mannitol and *Staphylococcal* organisms can utilize Mannitol as a fermentable carbohydrate (food source) and will produce acid end products from this metabolism. Since this process is invisible an indicator is added to the media to detect changes in pH and Phenol red is the indicator used in MSA. It is red at a neutral pH but turns yellow if conditions in the media become acidic. Pathogenic *Staphylococci* not only grow on the medium, but they also produce acid from it. This acid production turns the pH indicator from red to yellow. Non-pathogenic *Staphylococci* can grow on the medium but produce no acid from it and the medium remains pink (Jay, 2000; Quinn *et al*., 2002).

2.7. Antimicrobial resistance

2.7.1. Antimicrobial resistance problem and causes

Antimicrobial resistance is an ever-increasing global public health threat. Now a day, it is well documented that clinically important bacteria are not only characterized by a single drug resistance but also by multiple antibiotic resistance (Levy and Marshall, 2004). The WHO(2014) annual report indicated that resistance to common antibiotics has reached alarming levels in many parts of the world indicating that many of the available treatment

options for common infections in some settings are becoming ineffective (WHO, 2014). The ever-increasing antimicrobial resistance problem is not only associated with potential treatment failure but also brings a fundamental alteration in the microbial ecosystems of humans, animals, and the environment. Also, it increases the infection rates and facilitates the emergence of more virulent bacterial pathogens that could lead to more severe infections (Barza *et al*., 2002).

One of the biggest issues in antimicrobial resistance is the use of antibiotics in animals. There is a continuous debate on the association between antimicrobial use in the production of food animals and the emergence of resistant organisms in humans. In veterinary medicine, antibiotics are given for the treatment of contagious and infectious animal diseases including clinical and subclinical mastitis at the dairy farm level (Aarestrup, 2005).

However, besides human and veterinary use, antibiotics are widely used in animal husbandry and other agricultural practices and this has significantly exaggerated the antibiotic resistance problems globally according to Aarestrup (2005) of these practices, the most serious is the continuous administration of sub-therapeutic doses of antibiotics as growth promoters for food animals. This practice favors the emergence and propagation of a large number of resistance genes (Marshall and Levy, 2011). Commensal bacteria could constitute a risk by being a reservoir of resistance genes. Resistant commensal bacteria of food animals may pass on their resistance to zoonotic bacteria and reach the intestine tract of humans (vandenbogaard and Stobberingh, 2000).

2.7.2. Antimicrobial resistance of *Staphylococcus aureus*

Staphylococcus aureus is notorious for its ability to become resistant to antibiotics. The development of multidrug resistance of *S. aureus* is a global problem. *Staphylococcus aureus* develops drug resistance more readily because of its ability to produce an exopolysaccharide barrier and their location in the micro abscess that limits access to the antimicrobial (Jeljaszewicz *et al*., 2000). However, all the resistance traits *S.aureus* has acquired since the introduction of antimicrobial chemotherapy in the 1930s, methicillin resistance is clinically the most important, since a single genetic element confers resistance to the most commonly

prescribed class of antimicrobials the beta-lactam antibiotics, which include penicillin, cephalosporin, and carbapenems (Grundmann *et al*., 2006).

There is a growing concern over the transmission of resistant bacteria via the food chain (Singer *et al.*, 2003). This poses a challenge to veterinarians, health professionals, and dairy cattle producers due to its negative impact on the response to antimicrobial therapy (Abera *et al*., 2010; Asmelash *et al*., 2016; Kemal *et al*., 2017). Studies carried out in different countries reported increased antimicrobial resistance among *Staphylococcus aureus* isolates (Thaker *et al*., 2013).

2.8.Public Health Significance

Staphylococcal food poisoning is one of the major concerns in public health programs worldwide (Leloir *et al*., 2003).*Staphylococcal* infections are frequent but are usually contained by immune mechanisms to the site of entry. The highest incidence of the disease usually occurs in people with poor personal hygiene, overcrowding, and children (Rho and Schaffner, 2007). In the developing countries, the surveillance system of FBD hardly exists and it is, therefore, difficult to estimate the real magnitude of the problem. Even in countries where surveillance services are very efficient, the precise incidence of food poisoning is not known, as outbreaks are often not reported to public health authorities (Boschipinto, 2008). Hence, the incidence of FBD caused by *Staphylococci* is thought to be much higher than reported since many cases remain undeclared (Argaw and Addis, 2015).

Foodborne diseases are a serious and growing problem in the world (Baron, 2007). *Staphylococcus aureus* is a significant cause of FBD, causing an estimated 241,000 illnesses per year in the United States (Kadariya *et al.,* 2014). Globally, an estimated 2 million people died from diarrheal diseases in 2005, approximately 70% of diarrheal diseases are foodborne. It is estimated that up to 30% of the population suffer from foodborne illnesses each year in some industrialized countries (WHO, 2015). Among foodborne disease, *Staphylococcal* food poisoning is of major concern in global public health programs. *Staphylococcal* organisms alone have found to cause hospitalization rates as high as 14%. Although not considered especially lethal, death can ensue if large amounts of SE are ingested, fatality rates range from

0.03% in the general population to as high as 4.4% for highly sensitive persons such as immune-compromised persons, elderly persons, and children (Kerouanton *et al*.,2007).

2.9. Prevention, Control, and Treatment

The prevention is certainly possible to maintain low herd prevalence with less than two, cows infected (in one or more quarters) with *Staphylococcus aureus*. Since the majority of herds are most important a reservoir of the pathogens is infected cows. These can be achieved through carrying out treatment; culling, drying off infected quarters and ensuring new heifers/cows are uninfected. However, there are other potential sources of *S.aureus* associated with the environment and these may be of special significance in particular herds in which the condition is difficult to control with the traditional methods. Sources of *S.aureus* outside the mammary gland are also the reason why, it is virtually impossible to eradicate *S.aureus* mastitis from a commercial dairy unit (Green and Bradley, 2004).

The control measures include education of those who prepare food at home and other food handlers, so that they will take proper personal hygiene measures, prohibiting individuals with abscesses or other skin lesions from handling food, refrigeration at 4°c or lower of all foods to prevent bacterial multiplication and the formation of toxins. Foods must be kept at room temperature for as little time as possible. The veterinary milk inspection service should supervise dairy installations, the correct operation of refrigeration units and their use immediately after milking, and refrigerated transport of the milk to pasteurization plants (Acha and Szyfres, 2003).

However, control is both important and difficult as *Staphylococcus aureus* can persist for months in dust, curtains and human carriage is often permanent. Reservoirs and routes of spread differ, so different measures are appropriate in different circumstances. Prevention is much concerned with the destruction of the bacteria and with the inhibition of growth (Baron, 2007). The effective methods for preventing SFP are aimed at eliminating food contamination through high standards of personal hygiene to prevent food contamination by food handlers. This is through public education concerning hand washing, wearing gloves during food preparation and storing foods at the proper temperature to inhibit growth or destroy the

pathogen and minimize toxin production as heating food after the toxin is formed will not be an effective control measure (Baron, 2007).

Moreover, persons with lesions containing purulent exudates should not be permitted to handle food until proper medical advice is sought. In general, measures such as serving hot meal immediately after cooking, reheating cooked foods thoroughly, rapid refrigeration of cooked foods, proper washing of hands before and after food preparation, avoiding food service worker with skin infections in food establishments and using clean utensils and equipment will certainly reduce the incidence of food poisoning outbreaks due to *Staphylococcus aureus* (Baron, 2007).

The objective of treatment in human patients is to replace fluids, salt, and minerals that are lost by vomiting or diarrhea. Some strains of *Staphylococcus* have acquired genes making them resistant to multiple antimicrobial agents. These organisms are uniformly resistant to penicillin and cephalosporin. Penicillinase resistant penicillin such as oxacillin and flucloxacillin are used for serious infections. First or second-generation cephalosporin such as cephalothin, cephalexin, and cefuroxime are usually safe in patients who are hypersensitive to penicillin. Vancomycin is usually effective for methicillin-resistant *Staphylococci*. Erythromycin and its newer relatives are used in milder infections. The infections can also be treated with combination therapy using sulfa drugs and minocycline or rifampin (Rho and Schaffner, 2007).

3. MATERIALS AND METHODS

3.1. Description of the study area

The study was conducted in Shinshicho Town, which is found in KembataTembaro Zone Southern Nation Nationalities and Peoples Regional State in SouthernEthiopia. The townis locatedat a distance from Addis Ababa 367 km and 293km through Shashamane and Hosanna respectively. The land coverage of the town is 1543 hectares and it is situated at a longitude of about 37°46′East and latitude of 7°12′North with an altitude of 1875meters above sea level (Fig.1) (Shinshicho town financial and economic development office, 2018).

The rainfall over much of the area is typically bimodal with the major rainy season usually extending from June to September and short rain season occurs from January to May. The minimum and maximum mean annual rainfall range from 1200 to 1500mm. The average minimum and maximum daily temperatures are 18°c and 31°c respectively. The Shinshicho town has two administrative Kebeles, namely Ketela and Metoma. The total human population of the town is estimated to be 55,563 of which 25,025 males and 30,538 were females (Shinshicho town financial and economic development office, 2018).

The agricultural production system of the study area is depending on a mixed farming system. The dairy farms in the town commonly kept improved hybrid and cow are managed under smallholder dairy farms in the semi-intensive management system. They are often provided with a supplementary diet in addition to the natural pasture and agricultural by-products. The livestock population of the town is estimated to be 11739 cattle, 3035 sheep, 3870 goats, 39100 poultry and 1450 equine (Shinshicho Town Administrative Agricultural Office, 2018).

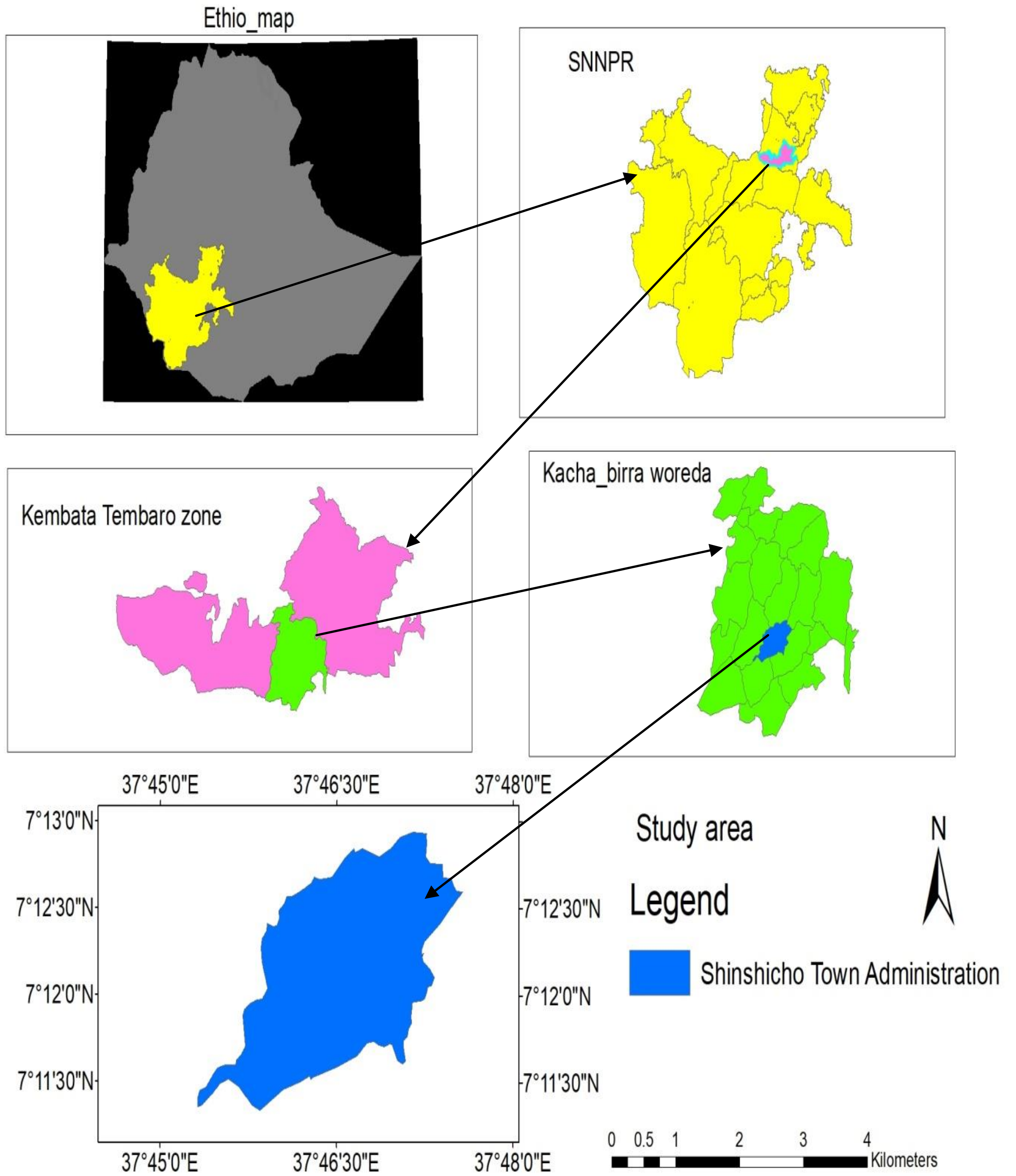

Figure 1.Location map of the study area. Source: - (Ethiopia GIS Data Base 1994)

3.2. Study Design

The cross-sectional study design was conducted from December 2018 to September2019in Shinshicho townto determine the prevalence, associated risk factors and antimicrobial susceptibility patterns of *S.aureus* isolates from mastitic lactating cow'smilk indairy farm at Shinshicho town.

3.3. Study animals

The study animals were lactating dairy cows in the Shinshicho town. The breeds of animals were (Holstein-Friesian, Jersey and Zebu cross) in the selected dairy farms which were kept under a semi-intensive management system. Age of the study animals were determined based on cattle birth records and their teeth (Appendix II). Their age categorized as described by Torell *et al*. (2003); Asmelash *et al*. (2016) as young (<5years), Adult (5 to 8years) and old (>8years). Besides, parity was categorized as few (1and 2 calves), moderate (3and 4 calves) and many (>4 calves). The lactation stage was grouped as early (1 and 2 months), medium (3 to 6months), and late (>6months). The floor system of the dairy house was classified as cemented and soil or mud types (Asmelash *et al.*, 2016). The hygienic condition of milking arranged as good and poor depending upon washing and drying the udder of lactating cows before milking. The previous treatment of mammary gland infection was assessed as yes and no respectively to (Abera *et al*., 2010).

3.4. Sample size determination and sampling procedures

The sample size of the study was determined based on the sample size determination method as described by Thrusfield (2007) with a 95% confidence interval and 5% desired absolute precision. Since there was no previous study conducted in this area, the expected prevalence of 50% was assumed and this was corresponding to a required maximum sample size by the formula given as follows.

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

Where: -n=required sample size;

Pexp =expected prevalence;

d^2 = desired absolute precision

Therefore, the calculated sample size was 384 of lactating dairy cows.

According to Mekonnen (2018), the sampling frame of the study area indicated that the farms were small-holder dairy farms having an average of two to three lactating cows each. Besides, 250 smallholder dairy farms with the total dairy cows of (199 Holstein-Friesian, 255 Jersey, and 220 Zebu-cross lactating dairy cows) were registered in the Shinshicho administrative town of agricultural office. Thus, 146 smallholder dairy farms having a minimum of two and a maximum of five lactating cows were purposively selected based on the availability of lactating dairy cows and the permission of the owners. Besides, the selected dairy farms were clustered based on their breed and numbers of lactating cows. Then focused on the sampling frame in the clusters, each breed of lactating cows was sampled by simple random sampling techniques by using a lottery method after assigning identification numbers on the piece of paper corresponding to each breed of lactating dairy cows. Hence, 152 Holstein-Friesian, 117 Jersey, and 115 Zebu-cross cows were sampled.

3.5. Questionnaire survey of risk factors

A semi-structured questionnaire was developed and all data based on the study objective was recorded. Data regarding the different potential risk factors were assessed for each of 384 lactating cows based on observation and by interviewing the farm owners. The cow's level factors such as breed, age, parity, lactation stage, pregnancy and nutritional status difference were recorded. The farm-level factors such as herd size, floor types, milking hygiene, previous mastitis infection, dry cow therapy and uses of the towel were recorded. Udder and milk abnormalities (injuries, swelling, milk clots, abnormal secretion, etc.) were also recorded (Appendix I).

3.6. Clinical examination and California mastitis test (CMT)

Each selected lactating cow was screened for mastitis based on clinical examinations and the California mastitis test. Clinical examination of the udder was based on the method previously indicated by (Radostits *et al*., 2007). The clinical findings considered include abnormalities of the secretion, abnormalities of the udder and teat and systemic reaction. The California Mastitis test was performed according to previously established procedures by (NMC, 2004). It is used to determine the prevalence of subclinical mastitis and also as a screening test for the selection of samples to be cultured for the cows under study. The 2mlof milk sample from each quarter was placed into a plastic paddle that has four shallow cups marked A, B, C and

D. An equal amount of CMT reagent was added to the milk. The paddle was rotated to mix the contents. Positive samples were showed gel formation within a few seconds. The CMT result was scored as negative if there was no gel formation and record as zero (0) or positive if there was gel formation ranging from trace (T), weakly positive (+1), Distinct positive (+2) and strongly positive (+3) as per the recommendation is given by(NMC,2004). Hence, the cow was considered mastitis positive if one or more quarters were CMT positive with or without isolation of the microorganisms (Quinn *et al*., 1994).

3.7. Sample collection and transportation

During the study, a total of 324 milk samples were collected from 39 clinical and 285 subclinical mastitis positive quarters. The milk samples were taken from mastitis positive cows not treated early with either intramammary or systematic antimicrobial agents and collected according to the earlier protocol (Quinn *et al*., 2004). To prevent udder contamination quarters were washed with tap water and dried. The teat ends were then cleaned with cotton soaked with 75% ethyl alcohol. Then, after discarding the first three-stream of milk, approximately 8-10ml milk was collected aseptically into a sterile test tube with a tight-fitting cup, per labeled test tube, by holding it in inclined positions, so that the pathogen that going to be recovered come from the mammary gland. To reduce contamination of teat ends during sample collection, the near teats were sampled first and then followed by the far ones, after milking out and discarding the first three drops (Quinn *et al.,* 1999). Finally, milk samples were held in an icebox for transportation to Wolaita Sodo Regional Veterinary laboratory for bacteriological examination to isolate *Staphylococcus aureus*. The samples were immediate culture or kept at 4°c overnight until culture on standard bacteriological media. Prepared bacteria isolation and identification were carried out based on standard bacteriological techniques previously established (Quinn *et al*., 2004).

3.8. Bacteriological culture

The milk samples were bacteriological examined according to the procedures employed by (Quinn *et al*., 2004). The milk samples, taken from infected quarters, were centrifuged to increase the bacterial load and inoculated separately on to sterile blood agar plates (BAP) enriched with 5% heparinized sheep blood and incubated at 37°c for 24-48 hours under aerobic culture conditions (Appendix IV and V).

The plates were examined for the presence of *Staphylococcus* colonies. The identification of bacteria on primary culture was as *Staphylococcus* was done according to the following procedures (Quinn *et al*., 2004).The plates were examined for growth, colony morphology features such as circular, golden, yellow and white in color and beta haemolysis on blood agar within 24-48hours (Quinn *et al*., 2002). Presumed *Staphylococcal* colonies were then sub-cultured on nutrient agar plates (NAP) and incubated at 37°c for 24-48 hours to get a pure culture (clone of cells derived from a single cell). The pure identified colonies in the nutrient agar were preserved and maintained at 4°c for further need (Quinn *et al.,* 2002). The slants were preserved and maintained for further identification.

3.8.1. Gram staining

Grams staining method was used for further identification based on cellular morphology and staining effect. All suspected cultures of *Staphylococcus* species colonies were picked from NAP and smeared on a labeled clean glass slide (Appendix IV). The smeared slides were stained using grams stain. Once stained, the smear was examined under a light microscope by using the oil immersion objective. The slides were examined for the presence of bacterial cells as well as the Grams reaction (color), morphology (chains, pairs, clusters). The Grams stained smears from typical colonies that showed Gram-positive *cocci* occurring in bunched, grapelike irregular clusters were taken as presumptive *Staphylococcus* species (Quinn *et al.,* 2002). Final identification of the *S.aureus* assignment was done based on biochemical tests such as catalase test, oxidase, Mannitol sugar fermentation, coagulase test, and one percent maltose fermentation.

3.9. Biochemical tests

3.9.1. Catalase test

Suspected pure colonies isolated were picked up by a bacteriological loop from nutrient agar plates and mixed with a drop of 3% hydrogen peroxide on a clean glass slide (Appendix IV). If the suspensions form bubble within a few seconds were believed as a catalase-positive organism and further tests were performed. The catalase test usually used to differentiate for *Staphylococci* (catalase-positive) and*Streptococci* (catalase test- negative).Those positive *Cocci* were considered as *Staphylococci* and *Micrococc*i (Quinn *et al*., 2002).

3.9.2. Oxidase test

A piece of filter paper was moistened in a Petri dish with a one percent aqueous solution of tetramethyl –p-phenylene diamine-hydrochloride. The test colony was streaked firmly across the filter paper with a glass rod. The disappearance of the dark purple color along the streak on the filter paper was considered as *Staphylococcus*. Oxidase test usually used as differentiation for *Staphylococci* (oxidase-negative) from *Micrococci* (oxidase positive) (Quinn *et al*., 2002).

3.9.3. Mannitol salt Agar plate (Mannitol fermentation)

The colonies that were confirmed by Gram‘s staining reaction, complete β-hemolysis on the blood agar, colony characterization, catalase-positive and oxidase negative were selected and streaked on Mannitol salt agar (MSA) plate and incubated at 37°c and examined after 24-48 hours for growth and change in the color of the medium (Appendix IV and V). The presence of growth and change of pH in the medium (red to yellow color) were regarded as confirmative identification of the salt-tolerant *Staphylococci*. The phenol red pH indicator detected the acidic metabolic product of Mannitol. The fermentation of Mannitol by *S.aureus* causes yellow discoloration of the medium (Quinn *et al*., 2002). Colonies that develop weak or delayed yellow color after 24 hours of incubation were taken as *S.intermedius* and colonies that failed to produce any change on the medium were considered as *S.hyicus* and Coagulase negative *Staphylococcus* species (Quinn *et al*., 2002).

3.9.4. Anaerobic utilization of glucose

Oxidation fermentation basal medium was prepared and 0.5% glucose was added into the medium. The media were distributed into the individual test tubes (appendix IV). Suspected *Staphylococcus* colonies were inoculated into the inside tube medium using a bacteriological sterile straight wire loop. Following this, using sterile paraffin the medium inside the tube was covered at least 25mm thick. The inoculated tubes were incubated for 5-14 days at 37°c acid and were produced anaerobically if indicator bromothymol blue changes to yellow throughout the tube indicating the presence of *Staphylococcus* species which has been known fermentative. The development of acid in the medium indicates glucose fermentation (Forbes *et al*., 2007).

3.9.5. Coagulase test

Coagulase test was used to differentiate pathogenic *Staphylococci* species from nonpathogenic species. Tube coagulase test was performed in sterile tubes by adding 0.5 ml of selected isolates presumptive identified *Staphylococcus aureus* from Mannitol salt agar were subcultured NAP and grown on tryptone soya broth (TSB) at 37°c for 24 hours and mixed with 0.5 ml of citrated rabbit plasma (Quinn *et al*., 2002). After mixing by gentle rotation, the tubes were incubated at 37°c along with a negative control tube containing a mixture of 0.5 ml of sterile TSB and 0.5 ml of citrated rabbit plasma (Appendix IV). Clotting was evaluated at 30 minutes intervals for the first 4 hours of the test and then after 24 hours incubation. The reaction was considered positive if any degree of clotting from a loose clot to a solid clot that is immovable when the tube is inverted(tilted) were visible within the tube and no degree of clotting was taken as negative (Quinn *et al*., 2002).

3.9.6. Fermentation of one percent of maltose using Purple agar Base

Purple agar base (PAB) with the addition of one percent maltose was used to differentiate the pathogenic *Staphylococci*, particularly the coagulase-positive isolates. The suspected culture was inoculated on a PAB media plate with one percent of maltose and incubated at 37°c for 24-48 hours (appendix V). The isolation was based on the fact that *Staphylococcus aureus* rapidly ferments maltose within 24hours and the acid metabolic products cause the pH indicator (bromocresol purple) to change the medium and colonies to yellow. The rapid fermentation (24hours) was considered as *Staphylococcus aureus* isolates (Quinn *et al.*, 2002).

3.10. Antimicrobial susceptibility testing

All 62 *S.aureus* isolates were subjected to antimicrobial susceptibility testing by Kirby-Bauer disc diffusion method on Mueller Hinton agar following the procedures described by (Quinn *et al*., 2011; CLSI, 2016). Approximately, 3-5 well-isolated colonies from a pure culture of nutrient agar were transferred into a test tube of 5ml tryptone soya broth (TSB) and incubated at 37°c for 6hours. The turbidity of the broth incubated was adjusted by using sterile saline or adding more isolated colonies to obtain turbidity visually comparable with that of 0.5McFarland standards. The Muller-Hinton Agar plates (MHAP) were prepared and a

sterile cotton swab was dipped into the suspension and swabbed on the surface of the Muller-Hinton agar plate.

Antimicrobial of animal and human health significance were taken into consideration. The following antibiotic disc with their corresponding concentration Chloramphenicol (30µg), Gentamicin (10µg), Kanamycin (30µg), Streptomycin (10µg), Cephalothin (30µg), Polymyxin-B (300units), Ampicillin (10µg), Amoxicillin (20µg), Penicillin-G (10units) and Tetracycline(30µg) (Hi media, India) were positioned on to the plates, using sterile forceps by a distance of 24mm (center to center) and pressed gently to ensure complete contact with the agar surface. The plates were allowed for 30 minutes for the diffusion of the active substance of the agents, plates were inverted and incubated at 35-37°c for 16-18hours under aerobic conditions (CLSI, 2016). The inhibition zone was then recorded as the diameter of the zone surrounding the individual disc in which bacterial growth was absent. The inhibition zone diameter of each antibiotic disc was measured to the nearest whole millimeters using a ruler, which is held on the back of the inverted Petri plates the values obtained from the CLSI (2016) were used to interpret the results obtained. *Staphylococcus aureus* isolates were then classified as susceptible, intermediate and resistant to a particular antibiotic (Table5). Multiple antibiotic-resistant (MAR) phenotypes were recorded for isolates showing resistance to three and more antibiotics (Rota *et al.,* 1996; Huber *et al*.,2011).

Table5.Interpretations of antimicrobial susceptibility test (guideline for reading)

Antimicrobial agent	Disk content	Susceptibility (millimeter)	Intermediate (millimeter)	Resistance (Millimeter)
Gentamicin	10µg	≥15	13-14	≤12
Cephalothin	30µg	≥18	15-17	≤14
Kanamycin	30µg	≥18	14-17	≤13
Streptomycin	10µg	≥15	12-14	≤11
Tetracycline	30µg	≥19	15-18	≤14
Penicillin-G	10units	≥29	-	≤28
Polymyxin-B	300units	≥12	-	≤11
Chloramphenicol	30µg	≥18	13-17	≤12
Ampicillin	10µg	≥29	-	≤28
Amoxicillin	20µg	≥20	-	≤19

Source: - (CLSI, 2016).

3.11. Data Management and Statistical Analysis

The data were recorded in the Microsoft Excel spreadsheet 2010 and coded before statistical analysis. All the statistical analysis was performed using Stata 14 statistical software (Stata Corp, 4905 Lake way Drive, College Station, Texas 77845 USA). The prevalence of *S.aureus* was calculated as the proportion of *S.aureus* positive cows (clinical and subclinical) against the total number of animals examined. A cow was denoted as positive for mastitis if at least a single teat with clinical mastitis or CMT positive result was detected. Accordingly, descriptive statistics such as percentages and frequency distribution was used to describe/present bacterial isolates and antimicrobial susceptibility which was expressed as a percent of resistant, intermediate and susceptible. In addition, the proportion of bacteria resistant to at least one of the ten antimicrobial agents and resistant three or more were calculated. The association between the dependent variable, *S.aureus* (0=negative and 1=positive) and categorical independent variables was assessed using multivariable logistic regression analysis. The independent variables evaluated were herd size, age, lactation stage, floor type, previous mastitis and use of a towel. All independent variables with p-value< 0.25 in the initial univariable analysis were checked for multicollinearity using Goodman and Kruskal gamma statistics and those variables whose gamma value ranged between −0.6 and +0.6 were considered in a multivariable logistic regression analysis (Dohoo*et al*., 2009).The final model was built in a backward elimination procedure in reference to log-likelihood ratio Values were considered significant at p<0.05 was taken significant in all analysis.

3.12. Ethical considerations

Permission to conduct this study was obtained from the Research Ethical review board of the College of Agriculture and Veterinary Medicine. Before conducting this study, all the farmers/owners of dairy farmers were informed about the purpose of the study were well aware of the importance and benefit of the study in terms of immediate (provided treatment) and future values. Besides, the research was highly participatory in the sense that dairy owners were provided their cows as research grounds. Furthermore, while collecting samples from lactating dairy cows safe handling procedures were followed. For notification, formal letters were written and sent to smallholder dairy farm owners and as well as a laboratory by the agricultural Office of Shinshicho administrative town.

4. RESULTS

4.1. Prevalence of *Staphylococcus aureus*

A total of 384 lactating dairy cows with either clinical or subclinical (CMT positive) mastitis were examined for the occurrences of *S. aureus*. The overall occurrence of mastitis in the study area (farm) was 41.7% in cows and 21.1% in quarters (Tables 6 and 7). Out of which, 5% (19/384) and 36.7 % (141/384) were found to be clinical and subclinical mastitis, respectively (Table6). Likewise, the quarter level prevalence of mastitis was 21.1% (324/1536), from which7.2% (111/1536) and 13.9% (213/1536) were found in the front and hindquarters, respectively (Table7). Out of the total 1536 quarters examined 19(1.2%) quarters which belong to 4% (15) lactating cows were found to be blind teat. The overall prevalence of *S.aureus* in this study was recorded to be 16.1% (62/384). *Staphylococcus aureus* was isolated from 1.8% (7/384) and 14.3% (55/384) of the clinical and subclinical cases, respectively. There was a statistically significant difference ($P<0.05$) in the prevalence of *S.aureus* between clinical and subclinical mastitis (Table6). Besides, the quarter's level prevalence of *S.aureus* was 4 % and it was varied significantly ($p<0.05$) between the front and hindquarters (Table 7).

Table 6.Prevalence of *Staphylococcus aureus* in types of mastitis

Forms of mastitis	Numberof examined cows	Number of positive cows(%)	Number of *S.aureus*isolates (%)	X^2	P-value
Clinical	384	19(5%)	7(1.8%)		
Subclinical	384	141 (36.7%)	55(14.3%)	103.57	0.00
Total	384	160 (41.7%)	62(16.1%)		

Table7.Prevalence of *Staphylococcus aureus* in quarter's level

Types of mastitis	No.of Examined quarters	Positive front quarters	Positive hind quarters	No. of quarters positive (%)	No. of *S.aureus* isolates	X^2	P-Value
Clinical	1536	14	25	39 (2.5%)	7 (0.5%)		
Subclinical	1536	97	188	285 (18.6%)	55 (3.5%)	143.23	0.00
Total	1536	111	213	324 (21.1%)	62 (4%)		

4.2. Risk factors associated with the prevalence of *S. aureus* isolates

Table 8.Univariable logistic regression analysis of associated risk factors with prevalence of *S. aureus* isolates

Risk factors	Category	No. of cows examined	No. of cows mastitis positive	No. of Cows *S. aureus* positive	Prevalence of *S. aureus* (%)	OR(95%CI)	P-Values
Herd size	≤ 5 herds size	236	98	25	10.6	Ref	
	>5herds size	148	62	37	25	2.81(1.61-4.91)	0.00
Breed	Holstein Friesian	152	74	30	19.7	Ref	
	Jersey	117	53	19	16.2	0.79(0.42-1.49)	0.46
	Zebu-cross	115	33	13	11.3	0.52(0.26-1.05)	0.07
Age in years	Young(<5)	120	36	10	8.3	Ref	
	Adult(5 to 8)	152	66	32	21.0	2.93(1.38-6.25)	0.01
	Old(>8years)	112	58	20	17.9	2.39(1.06-5.37)	0.03
Parity in calves	Few(1 and 2)	125	45	13	10.4	Ref	
	Moderate(3and 4)	186	76	29	15.6	1.59(0.79-3.20)	0.19
	Many(>4)	73	39	20	27.4	3.25(1.50-7.04)	0.00
L/S in months	Early(1 and 2)	98	26	8	8.2	Ref	
	Medium(3 to 6)	229	103	42	18.3	2.53(1,14-5.61)	0.02
	Late(>6)	57	31	12	21.0	3.00(1.14-7.87)	0.03
Pregnancy status	No pregnant	281	125	43	15.3	Ref	
	Pregnant	103	35	19	18.4	1.25(0.69-2.27)	0.46
Floor types	Concreted	270	104	33	12.2	Ref	
	Soil(mud)	114	56	29	25.4	2.45(1.40-4.28)	0.00
PMT	Yes	92	37	11	11.9	Ref	
	No	292	123	51	17.5	1.56(0.77-3.14)	0.21
Milking hygiene	Good	198	85	21	10.6	Ref	
	Poor	186	75	41	22.0	2.38(1.35-4.22)	0.00
Previous infection	Present	141	79	41	29.0	Ref	
	Absent	243	81	21	8.6	0.23(0.13-0.41)	0.00
Uses of towel	Yes	173	77	46	26.6	Ref	
	No	211	83	16	7.6	0.23(0.12-0.42)	0.00
Dry cow therapy	Yes	228	85	51	22.4	Ref	
	No	156	75	11	7.0	0.26(0.13-0.52)	0.00

Ref=Reference categories, OR=odd ratio, CI=Confidence interval, L/S=lactation stages, PMT=previous mastitis treatment.

Multivariable logistic regression analysis of the effect of different risk factors on the prevalence of *S.aureus* isolates was presented in Tables 9 and 10. Hence, multivariable analysis results showedthat; the prevalence of *S.aureus* isolates in adult cows were more likely occurred than their younger counterparts (OR=3.64, 95%CI: 1.64-8.11), and cows in late lactation stage were more likely to be positive than cows in early lactation stage (OR=4.21, 95%CI: 1.52-11.66) (Table 9). Besides, the dairy farms havinga large herd size greater than 5 animals were more likely positive for *S.aureus* than small herd size less than or equal 5 cattle (OR=3.05, 95%CI: 1.65-5.61), and soil or muddy husbandry system were more likely positive than cemented floor husbandry system (OR=2.26, 95%CI:1.23-4.17) (Table 10).

Table 9.Multivariable logistic regression analysis of cow's level associated risk factors with prevalence of *S. aureus* isolates

Risk factors	Category	No. of cows examined	No. of Cows *S. aureus* positive	Prevalence of *S.aureus* (%)	OR (95%CI)	P-values
Age in years	Young(<5)	120	10	8.3	Ref	
	Adult(5 to 8)	152	32	21.0	3.64(1.64-8.11)	0.00
	Old(>8)	112	20	17.9	2.52(1.11 ,5.72)	0.03
Lactation stages in months	Early(1and2)	98	8	8.2	Ref	
	Medium(3-6)	229	42	18.3	2.89(1.28-6.53)	0.01
	Late(>6)	57	12	21.0	4.21(1.52-11.66)	0.01

Ref=Reference categories. OR= Odd ratio, CI=Confidence interval.

Table10. Multivariable logistic regression analysis of farm level associated risk factors with *S.aureus* isolate

Risk factors	Category	No. of Examined farms	Mastitis positive farms	No. of *S.aureus* positive farms	Prevalence of *S.aureus* (%)	OR (95%CI)	P-values
Herd size	≤5 herd size	83	54	18	21.7	Ref	
	>5 herd size	63	52	24	38.0	3.05(1.65-5.61)	0.00
Floor type	Concreted	102	70	25	24.5	Ref	
	Soil(muddy)	44	36	17	38.6	2.26(1.23-4.17)	0.01
Previous mastitis infection	Absent	94	62	23	24.5	Ref	
	Present	52	44	19	36.5	0.30(0.16-0.55)	0.00
Uses of towel	Yes	86	61	20	23.3	Ref	
	No	60	45	22	36.7	0.25 (0.13-0.47)	0.00

Ref=Reference categories. OR= Odd ratio, CI=Confidence interval

4.3. Antimicrobial Susceptibility testing

All 62 *S.aureus* isolates were subjected to antimicrobial susceptibility tests, the results of which were presented in Table11. Of the entire antimicrobial used in this study, the isolates were the highest rate of Susceptibility towards Chloramphenicol 59 (95.2%) followed by Gentamicin 57(91.9%), Cephalothin 56(90.3%), Kanamycin55 (88.7%), and Streptomycin 50 (80.6 %). Whereas, the highest rate of resistance among the isolates were against Penicillin-G 62(100%), Polymyxin 62(100%), Amoxicillin 62(100%), Ampicillin 62(100%) and Tetracycline 50 (80.6%) (Table11).

Table 11Distribution of inhibition Zone diameter values of different antimicrobial for *S.aureus* isolates (n=62)

AMA	Inhibition Zone diameter by millimeters																												
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	≥29
AMX			33	6	7	4	5	3	1		2	1																	
AMP		50	6		4					2																			
CEP												1			2	1	2	4	6	17	5	8	6	8	2				
CHR													2	1					5	4	13	5	16	9	7				
GEN										1		1	3		4	5	7	16	6	5	3	6	5						
KAN								1		1		2	1		1		1	7	10	19	6	13							
PG		62																											
PLY		62																											
STM				2			1	2		1	2	1	2	1	3	4	22	11	1	9									
TTC			15	11	18	6									2	1			2	3	4								

The black color shaded = intermediate zone diameter and break points; Gray color shaded = resistant zone diameters and Green color shaded = Susceptibility zone diameters. AMA= antimicrobial agents, AMX=Amoxicillin AMP=Ampicillin, CEP = Cephalothin, CHR= Chloramphenicol, GEN=Gentamicin, KAN = Kanamycin PG=Penicillin, PLY=Polymyxin, STM=Streptomycin and TTC=Tetracycline

4.4. Multiple Drug resistance phenotypes of *S. aureus* isolates

Multiple Drug resistances (MDR) phenotype was determined for the isolates as presented in Table 12. The predominant multiple antimicrobial resistance phenotypes for the isolated in the study area (farms) were AMP-AMX-PG-PLY-TTC and AMP-AMX-PG-PLY in 14.5% and 9.7% of the isolates, respectively. Likewise, MAR phenotype AMP-PG-PLY, AMX-PG-PLY-TTC, and AMP-AMX-PG-PLY-STM-TTC were obtained in 4.8% of the isolates. The MAR phenotypes AMX-PG-PLY, AMP-AMX-KAN-PG-PLY and AMP-AMX- CEP-PG-PLY-STM-TTC were obtained 3.2% of the isolates. The MAR phenotype AMP-AMX-CHR-PG-PLY-TTC, AMP-AMX-KANA-PG-PLY-STM-TTC, and AMP-AMX-CEP-GEN-KAN-PG-PLY-TTC were obtained 1.6% of the isolates (Table12). Out of the total *S.aureus* isolates recovered from the study area, 53.2% of the isolates developed multiple antimicrobial resistance phenotypes. Among all MDR phenotype of *S.aureus* isolates, 22.6% of them were resistant to three and four antibiotics, 24.2% of them were resistant to five and six antibiotics and 6.4% of the isolates were resistant to seven and eight antibiotics. The Percentages of the phenotype were calculated by dividing the number of particular MDR phenotype by the total number of isolates identified in the study area (Table12).

Table 12.The predominant MDR phenotype for *S.aureus* isolates (n=62)

MDR pattern	Phenotype	Number observed	Percentage (%)
Three	AMP-PG-PLY	3	4.8
	AMX-PG-PLY	2	3.2
Four	AMP-AMX-PG-PLY	6	9.7
	AMX-PG-PLY-TTC	3	4.8
Five	AMP-AMX-PG-PLY-TTC	9	14.5
	AMP-AMX-KAN-PG-PLY	2	3.2
Six	AMP-AMX-PG-PLY-STM-TTC	3	4.8
	AMP-AMX-CHR-PG-PLY-TTC	1	1.6
Seven	AMP-AMX-CEP-PG-PLY-STM-TTC	2	3.2
	AMP-AMX-KANA-PG-PLY-STM-TTC	1	1.6
Eight	AMP-AMX-CEP-GEN-KAN-PG-PLY-TTC	1	1.6

MDR=Multiple Drug resistances, AMP=Ampicillin, AMX=Amoxicillin, PG=Penicillin, PLY=Polymyxin, CEP=Cephalothin, CHR= Chloramphenicol, GEN=Gentamicin, KAN=Kanamycin, TTC=Tetracycline and STM=Streptomycin.

5. DISCUSIONS

The present study showed that; an overall occurrence of mastitis 41.7% in cows and 21.1% in quarters. This finding was analogous with the previous report by Biniam *et al*. (2017) who recorded mastitis 40.9% in cows and 21% in quarters in Wolaita Sodo, Southern Ethiopia. This report was also in agreement with the assertion by Radostits *et al*. (2000) that, in most countries and irrespective of the cause, the prevalence of mastitis is about 50% in cows and 10-25% in quarters. The infection rate in cows was comparable with the findings of Asmelash *et al*. (2017) who found a prevalence of 39.32% in and around Assosa town, Ethiopia. The current finding of the study was higher than that of Mulugeta and Wassie (2013); Bitew *et al*. (2010) reported an overall prevalence of mastitis 29.5% around Wolaita Sodo and 28.8% at Bahir Dar, respectively. In contrast, the occurrence of mastitis was low when compared with the findings of Asmelash *et al*. (2016) and Befikadu *et al*. (2018) who reported a prevalence of 56% in and around Kombolcha and 59.9% in Asella town, Ethiopia respectively. This variability in the prevalence of *S.aureus* caused mastitis between different reports could be attributed to differences in farm management systems or differences in study methods and laboratory instruments employed by the investigators.

The occurrence of subclinical mastitis recorded in the current study was 36.7% and that of clinical mastitis 5%. The result was inlined with 36.18% subclinical mastitis prevalence reported by Biniam *et al.* (2017) in and around Wolaita Sodo, Southern Ethiopia. In this study, the rate of subclinical mastitis (36.7%) was higher than that of the clinical mastitis (5%) as was reported by Abera *et al*. (2010) (36.7%versus10%) in Adama town and Biniam *et al*. (2017) (36.18% versus 4.66%) in and around Wolaita Sodo. In most reports including the present study, the prevalence of subclinical mastitis was higher than clinical mastitis. This could be attributed to little attention given to subclinical mastitis, as the infected animal shows no obvious symptoms and secrets normal milk and farmers, especially smallholders, are not well informed about invisible loss from subclinical mastitis. In Ethiopia, the subclinical type of mastitis received little attention and efforts have been concentrated on the treatment of clinical cases (Almaw *et al*., 2008).

The occurrence of mastitis in front and hindquarter were 7.2% and 13.9 % respectively. The hindquarter was more affected than the front quarter and the current result was related to the

finding of Biniam *et al*. (2017) who reported 7.33% and 13.66% in front and hindquarters respectively. This is because the hindquarters are highly predisposed to contamination with dirt. In addition to this, a large amount of milk is produced from hindquarters and as a result, the pressure on the teat canal forces the canals to be opened widely which allows the entrance of bacteria. The observation of blind quarters in this study might be an indication of a serious mastitis problem on the farms and the absence of culling that should have served to remove a source of mammary pathogens for the cows.

In this study, the prevalence of *Staphylococcus aureus* was found, significantly associated with types of mastitis and frequently isolated in subclinical mastitis than the clinical cases which was in agreement with the finding of Befikadu *et al*. (2018) in Asella town and Biniam *et al*. (2017) in Wolaita Sodo and other similar studies that proved *Staphylococcus aureus* is the principal causative agent of subclinical mastitis Abera *et al*. (2010) in Adama town Ethiopia, but, disagreed with *Staphylococcus aureus* isolated in clinical more than subclinical mastitis Asmelash *et al*. (2016) in and around Kombolcha, Ethiopia. Despite the fact, poor treatment successes and limited dry cow therapy may cause this disagreement.

The overall prevalence of *Staphylococcus aureus* in the study population and study area was found to be 16.1% which was inlined with the prevalence (15.5%) reported from dairy farms of Addis Ababa city of Ethiopia by Abebe *et al*. (2013) and (15.1%) from Wolaita Sodo dairy farms by Biniam *et al*. (2017), 17.2% reported from Egypt by Osman (2010), but present result was lower when it was compared that of Abera *et al*. (2010); Fitsum (2016); Kemal *et al*. (2017); Befikadu *et al*. (2018) who reported 42.1%,32.14%,44.62% and 46.5% of *S.aureus* isolates at Adama town, Wolaita Sodo and Asella town, respectively. The possible explanation for the variability in the prevalence of *S.aureus* in mastitis cows among different reports might be that *S.aureus* is a contagious pathogen transmitted from one cow, infected to another healthy individual by contact with animals during unhygienic milking procedures (Rowe, 1999). Therefore, difference in farm management practices breeds of targeted cows, level of production and variations in the study methods and materials employed by the investigators, environmental inconsistency and variation in awareness between dairy producers, how the disease transmits, may contribute this variation.

The highest prevalence of *S. aureus* (38%) was observed in dairy farms having larger herd size greater than 5 cattle categories followed by herd size less than 5 animals. An increase in prevalence with increased herd size was observed with a highly significant association with the prevalence of *S.aureus* isolates (p=0.00). The significantly higher risk was observed in herd size greater than 5 cattle (OR=3.05, 95%CI: 1.65, 5.61),than corresponding herds. This result was agreed with the finding of Kemal *et al*. (2017) from Asella town, Ethiopia. This due to *S.aureus* have adapted to survive in the udder; known by their contagious nature and are shed in the milk which serves as a source of infection for other health cows during the milking process. It is generally observed that large herds are characterized by increased stocking density and increased risk of exposure to infection (Radostits *et al*., 2007).

The current result revealed that the prevalence of *Staphylococcus aureus* isolates was significantly differenced with the age categories. A significant association of age with the prevalence of *S.aureus* isolates was reported by other authors (Asmelash *et al*., 2016; Kemal *et al*., 2017) in and around Kombolcha and in Asella town Ethiopia respectively. In the present study, age categories of adult (5 to 8 years) of lactating dairy 3.64times were more likely infected with *S.aureus* isolates as compared to young and old age categories of cows in this study area. The increased prevalence of *S.aureus* within the increased age of animals in this study can be related to increased susceptibility of pathogenic organisms in udder relaxed sphincter muscle of teat (Erskine *et al*., 2002).

Multivariable logistic regression revealed that the prevalence of *S.aureus* associated with mastitis was significantly varied among the stage of lactation, which was highest in the late stage of lactation followed by mid and early stages of lactation in that order. Hence, late lactation stages 4.21 times were more likely infected as compared to early and medium lactation stages. This result was found to be consistent with the previous reports of Nibret *et al*. (2012); Legesse *et al*. (2015) in Addis Ababa and Hawassa towns, respectively. Nevertheless, the reports from the Southern part of Ethiopia indicated a higher prevalence in the early stage of lactation which disagrees with this study result (Dego and Tareke, 2003; Biffa *et al*., 2005). *Staphylococcus aureus* is a contagious pathogen and infectious increase as exposure to this infectious agent increases, however, the difference might be attributed to difference in management practice and lack of dry cow therapy.

The present study was revealed that; thedairy farms with muddy floor were 2.26 times more likely infected with*S.aureus* than cemented husbandry systems. Houses with soil floor increased the risk of *S.aureus* infection. The association between soil floor and high prevalence of *Staphylococcus aureus* revealed in this finding was agreed with the result of Kemal *et al*. (2017) who reported from Aselle town, Arisi Zone, Eastern Ethiopia, but as opposed to this, reports from Addis Ababa and Asella town indicated that a high proportion of *S.aureus* was isolated from cemented floor husbandry systems (Legesse *et al*., 2015; Befikadu *et al*., 2018). This is due to association with poor sanitation and cows which were maintained in dirty and muddy common barns with bedding materials that favor the proliferation and transmission of mastitis pathogens. The main sources of infection are udder of infected cows transferred through milker's hand, towels and environment (Radostits *et al*., 2007).In this study multivariable logistic regression analysis results showed that; the presence of previous mastitis infection and no used of the towel in the dairy farms were moderate effect as a comparison between the absent of the previous infection in the farm and separate used of towel. This result was agreed with the previous study of Asmelash *et al*. (2017) in and around Assosa town, Ethiopia.

In vitro antimicrobial susceptibility tests of *S. aureus* isolates revealed that the highest rate of susceptibility among the isolates was recorded against Chloramphenicol, Gentamicin, Cephalothin, Kanamycin and Streptomycin with frequencies of 95.2%, 91.9%, 90.3%, 88.7%, and 80.6%, respectively. This result was followed the finding of Abera *et al.* (2010) from Adama town who reported susceptibility of *S.aureus* to Chloramphenicol (100%), Gentamicin (91.7%), Kanamycin (88.9%) and Streptomycin (86.1%), Thaker *et al*. (2013)in India who reported the isolates sensitive to Cephalothin(100%) and Gentamicin(90%), Kemal *et al*. (2017) in Asella who reported the isolates susceptible to Chloramphenicol (97.5%) and Gentamicin(95.3%) and Girmu (2016) from Afar Ethiopia who reported the *Staphylococcus aureus* susceptible to Chloramphenicol (100%), Streptomycin (94%) and Gentamicin (90%). These antimicrobial agents was no longer used for the treatment of animal diseases in many countries Pace *et al*. (2006), which might be contributed to the current findings recorded. Deneeling *et al*. (2007) who reported that the tested livestock-associated MRSA isolates were highly susceptible to most classes of antimicrobial drugs, except β-lactams and tetracycline, the latter of which has been attributed to its high usage in animal husbandry.

On the other hand, *S. aureus* isolates showed the highest resistance among others to a Penicillin-G (100%), Polymyxin-B (100%), Amoxicillin (100%), Ampicillin (100%), and Tetracycline (80.6%). The current investigation was in harmony with the report of Eyasu (2018) who reported the resistance of *Staphylococcus aureus* to penicillin (100%), Ampicillin (100%), Amoxicillin-clavulanic (82.7%) and Tetracycline (60.49%) in Arsi Negelle, Ethiopia. Moreover, the present report was comparable with the result of Lencho (2015) in Ambo and Gudar town who recorded 100% to both penicillin and Ampicillin followed by 90% to Amoxicillin. This finding was similarly done by Asmelash *et al*. (2016) Kombolcha reported *S.aureus* resistance to penicillin (100%), amoxicillin (100%) and tetracycline (77.4%) and also similarly reported *S.aureus* resistance to penicillin (100%) and amoxicillin (100%) by Jahan *et al*. (2015) in Bangladesh. The variability in susceptibility result could partly arise on how frequent a drug was in use dairy cow's treatments in the study area.

The beta-lactams, tetracycline, sulfonamides and some aminoglycosides have become the first line of antimicrobial agents used for the treatment of bovine mastitis in Ethiopia. *S.aureus*is frequently resistant to other antibiotic agents in clinical use, including β-lactams, fluoroquinolones, aminoglycosides, rifampin, and mupirocin (Carbon, 2000). The resistance of *S. aureus* to penicillin-G may be attributed to the production of beta-lactamase enzyme that inactivates penicillin and closely related antibiotics. Resistance to penicillin-G is used as a marker to assess the susceptibility of *S. aureus* isolates against other β -lactam antibiotics (Waage *et al*., 2002; Pace *et al*., 2006). This correlates with the present findings. In the study area, the beta-lactamase was the drug of choice for therapy of intramammary infections, such that frequent and often inadequate use of these medications has probably contributed to the emergence of resistant bacteria in the herds. A similar finding was reported by Kemal *et al*. (2017) that the emergence of antimicrobial-resistant strain is nearly always a result of repeated therapeutic and/or indiscriminate use of them. Probably around 50% of mastitis caused by *S.aureus* strains produce beta-lactamase and there is evidence that these strains are more difficult to cure with all antibiotics (Green and Bradely, 2004).

The current study has demonstrated the existence of alarming levels of resistance of *Staphylococcus aureus* to commonly used antimicrobial (penicillin-G, amoxicillin including tetracycline) in the study farms. The finding under reports from previous studies in

other countries Gentilini (2000) and Edward *et al.* (2002) in Argentina, suggesting a possible development of resistance from prolonged and indiscriminate usage of the same antimicrobial. Therefore, the important to implement a systematic application of an in-vitro antimicrobial susceptibility test before use of antimicrobial in both therapeutic and prophylactic of *Staphylococcal* mastitis infections.Antibiotic-resistant *S.aureus* isolates pose a severe challenge to both veterinary and health professions and dairy cattle producers because they hurt therapy (Brouillette and Malouin, 2005). The antimicrobial resistance level in this study was relatively high. Furthermore, 53.2% of *S.aureus* isolates were resistant to three or more antimicrobial classes including most of the commonly used antimicrobial in the study area. This result was inlined with the findings of Abebe *et al*. (2013) who reported from Addis Ababa, 50.6% and Kashoma *et al*. (2015) who reported 43.2% from Tanzania. This result was slightly higher than the finding of Mohammed (2015) who reported 26.09% of multiple drug resistance of *S.aureus* isolated from cow milk in Tanzania.

The variation of multiple drug resistance frequencies was due to prolonged usages of common antimicrobial for treatment and prophylactic purposes in the study area. Multiple antibiotic-resistant *S. aureus* strains have been isolated from milk obtained from dairy animal samples in many parts of the world (Waage *et al*., 2002; Shitandi and Sternesjo, 2004; Pesavento *et al*., 2007; Ateba *et al*., 2010). The prevalence of antibiotic resistance between *S. aureus* usually varies between isolates from the different sampled stations and even between isolates from different herds and/or flocks of the same farm (Waage *et al*., 2002). Besides, *S. aureus* has developed multidrug resistance in many regions of the world and the usage of antibiotics correlates with the emergence and maintenance of antibiotic-resistant traits within pathogenic strains (Shitandi and Sternesjo, 2004).

6. CONCLUSION AND RECOMMENDATIONS

In this study, the prevalence of mastitis was 41.7 % (36.7% subclinical and 5% clinical mastitis) in dairy cows and 21% at quarter's level. The present study showed that; the overall prevalence of *S. aureus* in the study animals was 16.1%, which was 1.8% and 14.3% in clinical and subclinical cases respectively. Among the various potential risk factors were assessed: herd size, age, lactation stages, floor type, previous mastitis infection and uses of the towel were found to be significantly associated with the prevalence of *Staphylococcus aureus* isolates in lactating dairy cows. The antimicrobial susceptibility status of all isolates from lactating dairy cows in the study area was also tested to some commonly used antimicrobial agents, thus, resistance was observed that the isolates have zero susceptibility to Penicillin-G, Polymyxin, Amoxicillin, and Ampicillin, but highly sensitive to Chloramphenicol (95.2%) following, Gentamicin (91.9%), Cephalothin(90.3%), Kanamycin (88.7%) and Streptomycin(80.6%). The isolates showed also, 53.2% multidrug resistance to a great extent to commonly used antimicrobial. *Staphylococcus aureus* is one of the pathogens causing mastitis in dairy farms in the study area and might be imposing public health impacts if raw milk is consumed. Larger herd size, aged cow, late lactation stage, and muddy or soil floor are risk factors associated with the occurrence of *S. aureus* mastitis.

Based on the above concluding remarks the following recommendations are forwarded.

- Establishment of standardized monitoring systems in smallholder dairy farms should be implemented to minimize mastitis infection in the study area.
- Early diagnosis and screening of cows for mastitis should be implemented.
- Improvement of floor type from soil to concrete should be implementing in the study area.
- More attention should be given in larger herd, culling old cow and treating in late lactation stage and wise use of antimicrobial.
- Use of Gentamicin, Cephalothin, Kanamycin, and Streptomycin should be encouraged to treat mastitis infected cow in the study area
- Regular antimicrobial susceptibility testing should be employed helps to select effective antimicrobial that ultimately reduces the development of resistance towards commonly used antimicrobial.
- Further advanced molecular characterization study should be conducted for a factor favors multidrug resistance development.

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8. APPENDIXES

Appendix I. Questionnaire format

Owners Name: ___________________Kebele _______ Dairy farm name________

Block/village_____________Date of data Collection___________________

1. History of the cow:

Breed_____ Age_______ Calving date________ parity________pregnancy status pregnant_____non pregnant______

Body condition Good________ Medium______ emaciated_____________

Tick infestation: present ________________absent______________

Teat lesion: present _______________absent_________________

Edema of udder and teat present__________________ absent __________

Blindness of teat canal: Blind_______not blind______

Herd size less than1-5cattle______above 5cattle _________

Gross milks quality;

Watery___________blood tinged ______clots/flakes_______ normal______________

Sample collected from: RH____________RF____________LF__________LH_____

CMT score: RH________RF___________LF_________LH_____________

2. Milking hygiene

Do you wash udder before milking? yes_________ no______

Do you dry after washing? yes____________ no____________

Separate towels used for each cow? yes_______________ no_______________

Do you wash your hand before and in between milking? yes___________ no__________

Do you wash your hand in between milking? yes_____________ no_________

When do you milk cows with mastitis? first________ last_________ any time ________

3. Flooring systems

Floor type: concrete_______stone______soil________slopy_____leveled__________

Roof: metal sheet___________ grass______________

Wall: concrete____________mud______________________others__________

Manure removal: daily______weekly______monthly______

Other (specify) __________General hygiene: Good____________ poor___________

4. Farm situation

Can you differentiate healthy udder from diseased udder? Yes_______ No_______

is there any previous mastitis problem in the farm? Yes_________ no__________

Do you treat mastitis cases yes____________ no___________ What Person treating mastitis? Veterinary professional__________ myself___________ is there any alternative measures Yes_______No_________What are those alternatives___________________

5. Drug usage

Do you name drugs used for mastitis treatment __________________________

Is there problem of cure after therapy? Yes ________________ no __________________

Do you have knowledge about dry cow therapy? Yes___________ no____________

Do you practice dry cow therapy? yes__________ no_____________

Appendix II. Format used for recording data in the flied

Appendix.Table1. Farm visit data collection format

No	Date/sample collection	Sample code	Subclinical mastitis				Clinical mastitis				CMT Score
			RF	RH	LF	LH	RF	RH	LF	LH	

RF=Right front, RH=Right hind, LF=Left front, LH=Left hind quarters

Appendix table 2.Interpretation for the California Mastitis Test

CMT score	Interpretation	Visible reaction
0	Negative	milk fluid is normal
T	Trace	slight precipitation
1	Weak positive	distinct precipitation
2	Distinct positive	mixture thickens with gel formation
3	Strong positive	strong gel that is cohesive with a convex surface

Source: (NMC, 2004)

Appendix table 3.Record sheet for Laboratory for isolation of *S.aureus*

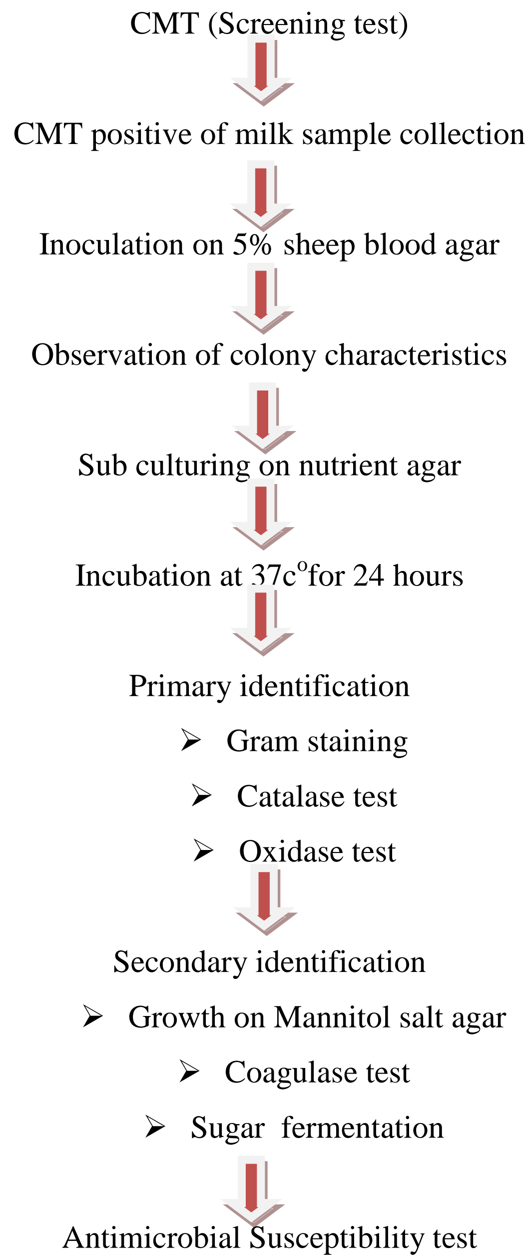
Samples code	Types of sample	Colony characterization on BAP	β-hemolysin	Grams stain	Catalase reaction	MSA	Manito's fermentation/PAB

Appendix table 4. Handy guide to determining the age of cattle by the teeth

At birth to 1 month	Two or more of the temporary incisor teeth present. Within first month, entire 8 temporary incisors appear.
2 years	As a long-yearling, the central pair of temporary incisor teeth or pinchers is replaced by the permanent pinchers. At 2 years, the central permanent incisors attain full development
2-1/2 years	Permanent first intermediates, one on each side of the pinchers, are cut. Usually these are fully developed at 3 years
3-1/2 years	The second intermediates or laterals are cut. They are on a level with the first intermediates and begin to wear at 4 years
4-1/2 years	The corner teeth are replaced. At 5 years the animal usually has the full complement of incisors with the corners fully developed
5 to 6 years	The permanent pinchers are leveled, both pairs of intermediates are partially leveled, and the corner incisors show wear.
7 to 10 years	At 7 or 8 years the pinchers show noticeable wear; at 8 or 9 years the middle pairs show noticeable wear; and at 10 years, the corner teeth show noticeable wear
12 years	After the animal passed the 6th year, the arch gradually loses its rounded contour and becomes nearly straight by the 12th year. In the meantime, the teeth gradually become triangular in shape, distinctly separated, and show progressive wearing to stubs. These conditions become more marked with increasing age.

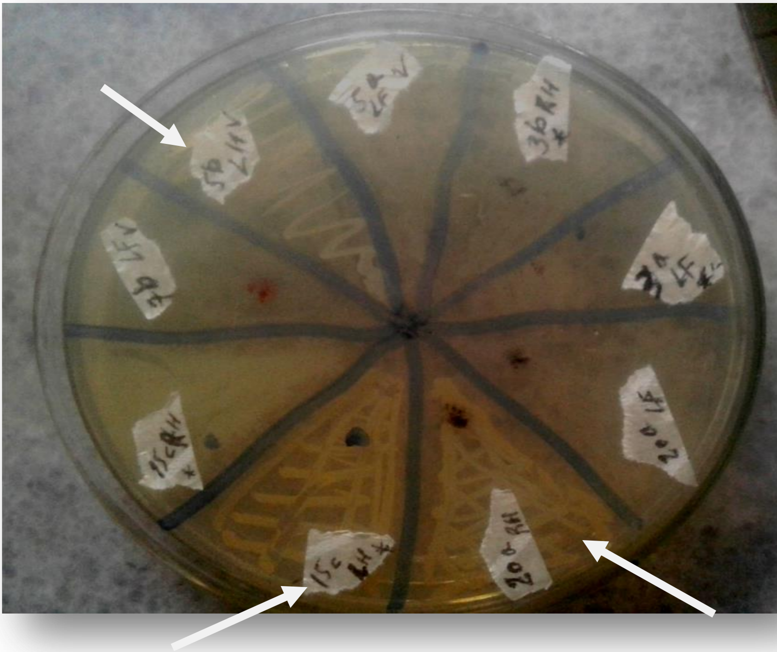
Source: (Torell*et al*., 2003).

Appendix III. Flow chart for isolation and identification of *S.aureus* from milk



Appendix IV. Primary and secondary identification tests.

Appendix figure 1.Growth on Blood Agar.



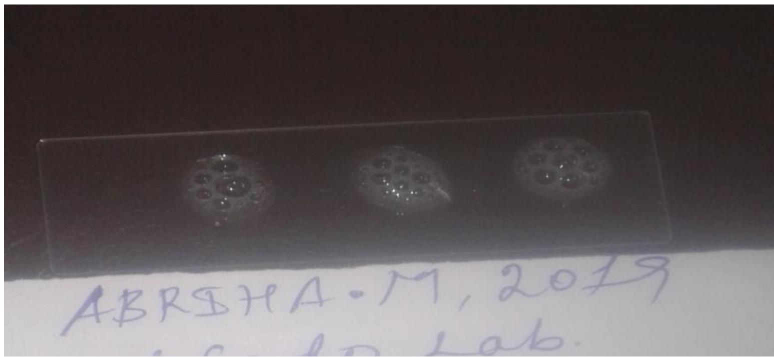
Appendix figure 2. Growth of *S.aureus* on nutrient agar

Gram’s staining procedures (carter, 1984)

- Make a thin bacterial colony smear and allow it to dry on the air
- Fix the dried smear by passing through the Bunsen flame two to three times taking care not to overheat the smear
- Flood the fixed smear with Gram's crystal violet (primary stain). Let stand for 60 seconds.
- Pour off the stain and gently wash with tape water.
- Flood with Gram's iodine (mordant) solution. Allow it to remain for 60 seconds.
- Pour off the iodine solution and gently wash with tape water.
- Decolorize with Gram's decolorizer solution 95% acetone alcohol) for 15-20 seconds until the blue dye no longer flows from the smear and gently wash the smear with tape water.
- Counter stain with Gram's safranin solution or carbon fuschin (counter stain) for 60 seconds.
- Wash off the red safranin solution with water. Blot with bibulous paper to remove the excess water. Alternatively, the slide may shake to remove most of the water and air-dried.
- Examine the finished slide under a microscope (oil immersion objective).
- Interpretation: Bluish purple color indicate Gram positive and pinkish color indicate Gram negative bacteria

Catalase test (Quinn *et al*., 1999)

Principle: the breakdown of hydrogen peroxide into oxygen and water is mediated by the enzyme Catalase. Procedure: a loopful of the bacterial growth is taken from the top of the colonies avoiding the blood agar medium. The bacterial cells are placed on a clean microscope slide and a drop of 3% H_2O_2 is added. An effervescence of oxygen gas, within a few seconds, indicates a positive reaction.



Appendix figure 3. Slide Catalase test positive**.**

Coagulase test procedure

- Three test tubes are taken and labeled “test”, “negative control” and “positive control”.
- Each tube is filled with 0.5 ml of 1 in 10 diluted rabbit plasma. To the tube labeled test, 0.1 ml of overnight broth culture of test bacteria is added.
- To the tube labeled positive control, 0.1 ml of overnight broth culture of known S. aureus is added and to the tube labeled negative control, only 0.1 ml of sterile broth is added.
- All the tubes are mixed gently, incubated at 37°C and observed up to four hours. If the test remains negative until four hours at 37°C, the tube is kept at room temperature for overnight incubation.
- Avoid shaking or agitating the tube during reading. Doubtful or false negative results may occur due to break down of the clot.

Result:-

Positive result is indicated from a loose clot suspended to a solid clot that is immovable, which remains in place even after inverting the tube. No degree of clotting is observed in negative result.

Mannitol salt agar

The colonies that were confirmed by staining reaction, Catalase test, and coagulase test were streaked on Mannitol salt agar plate and incubated at 37 ^{O}C and examined after 24-48 h for growth. The presence of growth and change of pH in the media (red to yellow color) regarded as presumptive identification for *S. aureus (*Quinn *et al., 2000)*

Appendix figure 5.Growth on Mannitol salt agar

Appendix figure 4.Tube coagulase test

Appendix table 5.Differential test used for identification of *S.aureus* from others species.

S/N	*Staphylococcus* species	Hemolysis	Coagulase	Pigment production	Fermentation of sugars	
					MAS	PBA
1	*S.aureus*	+	+	+	+	+
2	*S.intermedius*	+	+	-	±	±
3	*S.hyicus*	-	+	-	-	-
4	CNS	-	-	-	-	-

+ = 90% or more strains are positive, + = 90% or more strains are weakly positive, - =90% or more strains are negative. Source: (Quinn, *et al.,* 1999)

Appendix table6.Differentiation of *Staphylococcus* and *Micrococcus species.*

Tests	*S.aureus*	CNS	*Micrococcus*
Catalase	+	+	-
Hemolysis	+	-	-
Coagulase	+	-	-
Mannitol(A)	+	-	-
Maltose(A)	+	V	-
Glucose(A)	+	+	-

+= Positive reaction, - =negative reaction, v=variable reaction, A=acid production

Procedure for oxidation and fermentation test

- Prepare O-F base medium and when it is cooled at 50°c, add 10 ml of sterile glucose in to 100 ml of O-F base, for a final concentration of 10% glucose and dispense into a sterilized tube. Heat two tubes of medium in boiling water for 10 minutes to drive off the oxygen, cool and inoculate by inserting a straight wire vertically
- Incubate one tube aerobically and the second tube anaerobically or seal the surface with a layer of sterile liquid paraffin oil to create an aerobic condition
- Incubate both tubes at 37°C for 24-48 hours or more, up to 7 days with the caps loose. Longer incubation may be required for slowly growing species.
- Examine tubes daily for color change.

Interpretation:-

Fermentation: Yellow color in both tubes with or without gas. Oxidation: Yellow color only in tube without oil. No oxidation/fermentation: any change in the color of the tubes. The carbohydrates have not been fermented or oxidized.

Procedure for the disk diffusion methods

- At least 4-5 well isolated colonies of the same morphological type are selected from non-selective agar plate and just the top of the colonies are touched.
- Then a suspension was made in a saline or broth without pre incubation.
- The turbidity of both suspensions is adjusted by comparison with a 0.5 McFarland turbidity standard.
- The standard and the test suspension are placed in similar 4-6 ml, thin glass tube or vial
- The turbidity of the test suspension is adjusted with broth or saline and compared with the turbidity standard, against a white back ground with contrasting black lines, until the turbidity of the test suspension equates to that of the turbidity standard.
- Inoculation of bacterial suspension
- A sterile, nontoxic swab on an applicator stick is dipped in to the standardized suspension of bacteria and excess fluid is expressed and rotating the swab firmly against the inside of the tube above the fluid level.
- The swab is streaked in three directions and continuously brushed over the Muller Hinton or by rotating the plate for complete cover of the agar surface.
- The inoculated plates are allowed to stand for 3-5 minutes but no longer than 15 minutes and the discs are placed on the agar surface using sterile forceps or an antibiotic dispenser.
- Each disc is gently pressed with the point of a sterile forceps to ensure complete contact with the agar surface. The disc should be placed no closer together than 24 mm (centre to centre).
- This is equivalent to 6 discs per standard 90 mm Petri plate.
- After incubation, the diameters of the zones of inhibition are measured to the nearest mm using a ruler or caliper.

- The diameters are read from the back of the plate, when the test is on the comparatively clear Muller-Hinton medium.
- The diameter of the zones should be read across the centre of the discs.
- An interpretation of the size of the zones of inhibition is made with reference.

Appendix table 7.Interpretations of antimicrobial susceptibility test (guideline for reading)

Antimicrobial agent	Disk content	Susceptibility (millimeter)	Intermediate (millimeter)	Resistance (Millimeter)	expire Date
Gentamicin	10µg	≥15	13-14	≤12	2020
Cephalothin	30µg	≥18	15-17	≤14	2020
Kanamycin	30µg	≥18	14-17	≤13	2019
Streptomycin	10µg	≥15	12-14	≤11	2020
Tetracycline	30µg	≥19	15-18	≤14	2019
Penicillin-G	10units	≥29	-	≤28	2020
Polymyxin B	300units	≥12	-	≤11	2019
Chloramphenicol	30µg	≥18	13-17	≤12	2019
Ampicillin	10µg	≥29	-	≤28	2020
Amoxicillin	20µg	≥20	-	≤19	2020

Source (NCCL, 2011and 2016)

Appendix figure 6. Antibiotic sensitivity test

Appendix V. Medias used for bacteriological examination

1. Blood Agar Base (Infusion) Code: (Hi media, India, M073):-

A Blood Agar Base that has been specifically formulated to give improved hemolytic reactions with sheep blood.

Directions:

Suspend 40.0 grams in 1000ml distilled water Heat to boiling to dissolve the medium completely sterilized by autoclaving at 15 Ibs pressure (121^0c) for 15 minutes cool to 45-50°c and aseptically defibrinated blood mix well and pour in to sterile Petri plates.

Standard Formula:

Ingredients	Grams/liter
Meat infusion	10.00
Tryptose	10.00
Sodium chloride	5.00
Agar	15.00
Final pH(25°c) 7.3±0.2	

2. Nutrient Agar (ISO-6579 ISO -10273 ISO19250):-

Uses: - for the cultivation of non-fastidious microorganisms in water, feces and clinical-samples

Preparation:-

Suspender 23 gram of the medium in one liter of distilled water. Mix well and dissolve by heating with frequent agitation boil for one minute until complete dissolution. Dispense in to appropriate container and sterilize in autoclave at 121°c for 15minute.

Formulas in gram per liter of distil water:-

Ingredients	Grams/liter
Gelatin peptone	5.00
Beef extract	3.0
Bacteriological Agar	15.00
Agar	15.00
Final pH 6.8±0.2 at (25c^o)	

3. Grams staining reagent
Crystal violet
Grams Iodine
Decolorized (Alcohol)
Carbon fuchsin (Safranin)

4. Mannitol salt Agar Code: (Himedia, India, MH118):-
Used for selective isolation of pathogenic staphylococci from pharmaceutical products in accordance with the microbial limit testing by harmonized methodology of USP/EP/BP/JP/IP/mdium13.

Directions:-Suspend 111.02 grams in 1000ml purified/distilled water. Heat to boiling to dissolve the media completely Sterilized by autoclaving at 15lbs pressure (121°c) for 15 minutes or as per validated cycles.

Standard and formula: _

Ingredients	Grams/liter
Peptic digest of animal tissues	5.00
Pancreatic digest of casein	5.00
Beef extract	1.00
Sodium chloride	75.00
D-mannitol	10.00
Phenol red	0.025
Agar	15.00
pH after sterilization 7.4±0.2	

5. Purple agar base (Himedia, India)

Standard and formula:-

Ingredients	Grams/liter
Proteose peptone	10.0
Beef extract	1.0
Sodium chloride	5.0
Bromocresol purple	0.02
Agar	15.
Final pH after sterilization 6.8 ±0.2 at 25^{0}c	

Instructions for use:

Suspend 31g of the powder in 1 liter of purified water. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Autoclave at 121^{0}c for 15 minutes. When preparing 0.5-1% carbohydrate fermentation, agar dissolves 5-10g of the desired carbohydrate in the basal medium prior to sterilization by autoclaving.

6. Tryptone soya broth (Himedia, India)

Standard and formula:-

Ingredients	Grams/liter
Pancreatic digest of casein	17.0
Papaic digest of soybean meal	3.0
Sodium chloride	5.0
Di-basic potassium phosphate	3.5
Glucose	2.5
Final pH after sterilization 7.3 ±0.2 at 25^0C	

Instructions for use:

Dissolve 30g in 1 liter of distilled water and distribute into final containers. Sterilize by autoclaving at 121^0C for 15 minutes.

7. Muller Hinton Agar (Oxoid, England):_

Composition (g/l): beef extracts 2.00gm, acid hydrolysate of casein 17.5gm, starch1.50gm, agar 17.0gm.

Direction: suspend 38 g of the powder in 1 liter of distilled water. Mix thoroughly, heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Autoclave at 121^oc for 15 minutes.