ISOLATION AND IDENTIFICATION OF *STAPHYLOCOCCUS AUREUS* IN COW MILK AND MILK PRODUCTS: IT'S PUBLIC HEALTH IMPILCATION AND ANTIBIOTIC RESESITANCE IN SHASHEMENE TOWN OROMIA, ETHIOPIA

MSc Thesis

BY

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This is to certify that the thesis entitled **"ISOLATION AND IDENTIFICATION OF *STAPHYLOCOCCUS AUREUS* FROM COW MILK AND MILK PRODUCTS: IT'S PUBLIC HEALTH IMPILICATION AND ANTIBIOTIC RESITANCE IN SHASHEMENE TOWN"** submitted in partial fulfillment of Masters of Science (MSc) in Veterinary Public Health in the Graduate Program of Faculty of Veterinary Medicine, has been carried out by Hassen Jilo Id. No GPuePuR 0004/14 under our supervision. Therefore we recommended that the student has fulfilled the requirements and hence here by can submit the thesis to the department.

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First, I declare that this thesis is my original work and that all sources of materials used for this thesis have been duly acknowledged. It has been submitted in partial fulfillment of the requirements for an advanced (MSc) degree at Hawassa University, Faculty of Veterinary Medicine. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate. Brief quotation from this thesis are allowable without special permission provided that accurate acknowledge of source is made.

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TABLE OF CONTENTS **PAGE**

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LIST OF ABBREVIATIONS

FDA	Food and Drug Administration
EFSA	European Food Safety Authority
MRSA	Methicillin Resistant *Staphylococcus aureus*
MSA	Mannitol salt agar
MSSA	Methicillin Sensitive *Staphylococcus aureus*
NAP	Nutrient Agar Plate
PAB	Purple agar base
SCC	Somatic Cell Count
SE	Staphylococcal Enterotoxin
SFP	Staphylococcal food poisoning

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figures **Page**

ABSTRACT

A cross sectional study was conducted from May 2023 to April 2024 in Shashemene town Oromia, Ethiopia, aimed to isolate and identify *Staphylococcus aureus* and their resistance to different antimicrobials. A total of 220 samples were collected using a simple random sampling technique for bacteriological isolation and 120 questionaries' were collected from farm owners, milk collection centers (MCCs) and retailer shops. A standard bacteriological and biochemical tests were used to isolate *Staphylococcus aureus*. *Staphylococcus aureus* was isolated from 35.9 % (N=79) of the total samples. The isolation rate was found higher in MCCs (43.5%) than milk retailer shop (34.9%) and farms (27.8%). All (n=79) isolates of *Staphylococcus aureus* were tested for *antimicrobial* susceptibility of 9 selected drugs. The isolates were susceptible to sulphamethoxazole-trimethoprim (95.23%), vancomycine (90.47%) and Gentamycin (80.9%); however, they were highly resistant to Ampicillin (100%), Amoxicillin clavulanic acid (76.19%), Streptomycin (76.19%), Nalidicix acid (71.4%), and Tetracycline (66.66%), and also moderately resistant to Ciprofloxacin (38.07%). Lacks of stringent regulation and monitoring in the dispensing and use of antimicrobials in the area contribute to the occurrence of high antimicrobial resistance to these drugs. Furthermore, an attempt was made by using semi structured questioaire survey that include farmers, consumers, owners and milk collectors at MCC. It revealed raw milk consumption behavior, and inadequate knowledge of milk borne disease. This study showed that *Staphylococcus aureus* is widespread in milk and milk products and developed multiple drug résistance. It is therefore, creation of public awareness about, pasteurization or boiling of milk prior to consumption, rational use of drugs and periodic assessment of the antimicrobial sensitivity of drugs prior to use are paramount important.

Keywords: Antimicrobial resistance, Milk, *Staphylococcus aureus*, Shashemene, Zoonosis

1. INTRODUCTION

1.1. Background

As per multiple researches works Akineden *et al*., (2001); Cabral *et al*., (2004); Katsuda *et al*., (2005) *Staphylococcus aureus* stands as the predominant and economically noteworthy bacteria responsible for inflaming infections in dairy ruminant animals. (Asperger et al., 2003) reported that between 30% and 40% of cases of mastitis are associated with the bacteria. When raw milk and milk products are handled, the environment can contaminate them, giving *Staphylococcus aureus* access to milk (Scherrer *et al*., 2004; Jorgensen *et al*., 2005). *Staphylococcus aureus* comes in two varieties: pathogenic and non-pathogenic (Akineden *et al*., 2001; Cabral *et al*., 2004; Katsuda *et al*., 2005).

Staphylococcus aureus is the most common and commercially significant bacterium causing inflammatory infections in dairy ruminants. The former typically causes disease in its hosts and is coagulase-positive. Severe toxic shock syndrome or abscesses are two possible symptoms of infection. A plant's hygienic state, as well as the cleanliness of its equipment and personnel, can all contribute to the contamination of milk during the milking process. Second only to salmonellosis in terms of frequency, staphylococcal food poisoning (SFP) is one of the most prevalent foodborne infections globally (Aycicek *et al.,* 2006).

According to (Ateba *et al*., 2010), *Staphylococcus* bacteria can cause everything from minor skin infections to more serious illnesses including pneumonia and septicemia. When handling milk, milk products, and storage equipment before or after milking, poor hygiene practices including coughing or sneezing, as well as not washing hands, might lead to the SFP (de Oliveira., 2011), Further spreading of the infection and the generation of toxins by enterotoxigenic strains of Staphylococcus species may result by storing milk at room temperature for an extended period of time prior to consumption. The study conducted by (Tarekgne *et al*., 2015) indicates that the prevalence of *Staphylococcus aureus* is higher in the warmest months of the year, indicating that

temperature has an effect on the pathogen. According to (Argaw, 2018) the incidence of *Staphylococcus* species is decreased by the samples' origin and the acidic content of cheese and yogurt.Even though milk is an extremely valuable food, many microorganisms can grow in raw milk (Helena *et al*., 2010). (Zecconi and Piccinini, 1998) assert that milk and its derivatives are thought to be a source of human *Staphylococcus aureus* infections. Numerous illnesses, ranging widely in severity, are brought on by *Staphylococcus* aureus in both humans and animals. According to (Lowy *et al.* 1998), there could be anything from a minor skin infection to serious pneumonia and sepsis. Prior studies have indicated that variations in cow breeds, locations, management styles, and milking techniques are some of the risk factors linked to *Staphylococcus* aureus infections in animals. *Staphylococcus aureus* antibiotic resistance has also emerged as a critical global public health concern. Additionally, it has been noted that certain *Staphylococcus aureus* strains exhibit resistance to a variety of antimicrobial agents (Sudhanthiramani *et al*., 2015).

Raw milk and its byproducts, such as cream, butter, yogurt, fermented milk (known as "Ergo"), cottage cheese (known as "Ayib"), and cream are frequently consumed in Ethiopia (Gonfa et al., 2001). Nonetheless, a lack of refrigeration facilities, the nation's consumption habits of raw milk and milk products, and unhygienic food production and preparation methods are likely to blame for the high incidence of SFP (Ararsa, *et al*., 2014). Prior research carried out in the Oromia region of Ethiopia revealed a high prevalence of multidrug-resistant *Staphylococcus aureus* in raw milk and milk products as well as a high rate of Staphylococcus species isolation from raw milk and milk products (Gebremedhin *et al*., 2022).

Today's globe faces a variety of difficulties, some of which include rapid population growth, transportation infrastructure, and climate change. Climate change is a predisposing factor that forces various microorganisms to adapt to their surroundings, thereby exposing common precautionary measures to dangers. Since the first medication was created by Alexander Fleming in 1940, the world has been treating both humans and animals with various antibiotics (Tan and Tatsumura. 2015). But over time, commonly used medications have led to drug resistance, sometimes even multi-drug resistance (Kehrenberg *et al*., 2015). *Staphylococcus aureus* is one of the common bacteria that can harm both humans and their animals (Haag *et al.,* 2019).

Drug-resistant *Staphylococcus aureus* in particular the methicillin-resistant *Staphylococcus aureus* (MRSA) has received particular attention, according to Kumar and his colleagues' critical review. But drug-susceptible *Staphylococcus aureus* has been posing a health risk to people and their animals. In developed nations, particularly in America, Europe, and Australia, the true prevalence of drug-resistant and drug-susceptible *Staphylococcus aureus* has been extensively researched (Kumar *et al*., 2021). Consequently, from 20 to 80% of MRSA infections are more than MSSA infections, necessitating the use of alternative medications to treat these infections. In developing countries, the effect of both the MRSA and MSSA could be beyond the studies reported in the developed countries due to the inappropriate and none proportional use of drugs both in the medical and veterinary scenarios.

Due to the inappropriate and disproportionate use of medications in both veterinary and medical settings, the effects of MRSA and MSSA in developing nations may exceed those of studies conducted in developed nations. One of the developing nations is Ethiopia, where pharmaceuticals are sold in open markets alongside other goods for purchase, resulting in the spread of bacteria that are resistant to drugs. Ethiopia is one of the developing countries where drugs are sold in open markets as any shopping goods which lead to a wide distribution of drug-resistant bacteria. The general trend of *Staphylococcus aureus* (both susceptible and resistant) in the last ten years in Ethiopia is not well analyzed, despite a meta-analysis on methicillin-resistant *Staphylococcus aureus* from 2004 to 2015 in Ethiopia indicating a high prevalence of the disease.

Due to *Staphylococcus aureus* ability to produce enterotoxins that can result in life-threatening intoxications, the majority of its effects on public health are linked to animals and the products they produce for food production (Fisher *et al*., 2018). Additionally, it is the cause of a broad spectrum of infections, ranging from minor skin infections to serious illnesses. Serious illnesses in animals caused by virulent *Staphylococcus aureus* strains include suppurative disease, arthritis, omphalitis, urinary tract infections, and clinical and subclinical mastitis (intramammary infections), which causes a large financial loss to the dairy industry in ruminants (Haag *et al*., 2019). It is estimated that up to 90% of dairy farms have *Staphylococcus aureus* caused mastitis infections.

1.2. Statements of the Problem

Staphylococcal food poisoning is one of the most prevalent food-borne diseases worldwide Staphylococcus varies between farm and dairy products due to storage, handling, use of unsanitary utensils, and milking circumstances. (M. Acco *et ai*.,2003).

Milk is widely considered one of the world‘s most valuable complete foods because it is a highly Nutritious substance that contains macro and micronutrients fat, protein, carbohydrates, minerals, vitamins. Protein, fat, and lactose in milk are important sources of energy. Thus, milk is the most popular food for human consumption (Teklemichael *et al*., 2013), In the study area or city there is a huge potential of milk production and hence the society consumes a high amount of unprocessed cow‘s milk and milk product in their everyday diet. Besides, in Ethiopia, raw milk is available in an open-air retailer shop where consumers purchase either for home consumption or consume there in the form of “Ergo”. However, the isolation of *Staphylococcus aureus* from milk and milk product has not been continuous assessed yet based on this reason the current study need to isolate and estimate the prevalence *Staphylococcus aureus* form raw milk, cheese and yoghurt in Shashemene town. But some studies were conducted in Ethiopia to evaluate mostly the hygienic practices and microbiological quality of milk. (Melese *et al*., 2015).

1.3. Objective of the study

1.3.1. General Objective

Identification of *Staphylococcus aureus* from cow milk and milk products; assessment of public awareness about its public health significance in Shashemene town, Oromia, Ethiopia

1.3.2. Specific Objectives

- To isolate and estimate the prevalence *Staphylococcus aureus* form raw milk, cheese and yoghurt in Shashemene town.
- To figure out the antimicrobial susceptible pattern of *Staphylococcus aureus*
- To assess the public health implication of raw milk/milk product contaminated by *Staphylococcus aureus* through questionnaire survey in the study area.

2. LITERATURE REVIEW

2.1. General Characteristics of *Staphylococcus aureus*

Staphylococcus aureus belongs to the family *Micrococcaceae* and is part of the genus *Staphylococcus*, which contains more than 30 species such as *S. epidermidis*, *S. saprophyticus* and *S. haemolyticus*. Among the staphylococcal species, *Staphylococcus aureus* is by far the most virulent and pathogenic for humans. *Staphylococcus aureus* is a 1 μm, Gram-positive cell that in the laboratory may be observed as single cells, in pairs or as grape-like irregular clusters. It is characterized as coagulase- and catalase positive, non-motile, non-spore-forming and as facultative anaerobic. It grows in yellow colonies on nutrient rich media and is referred to as the yellow *staphylococci* (Winn Washington, 2006). *Staphylococcus aureus* was discovered in 1880 by the surgeon Sir Alexander Ogston. He observed grape-like clusters of bacteria when examining a purulent discharge from patients with post-operative wounds during microscopy. He named them staphylé, the Greek expression for a bunch of grapes. In 1884, Rosenbach succeeded in isolating yellow bacterial colonies from abscesses and named them *Staphylococcus aureus* , "aurous" from the Latin word for golden (Rosenbach, 1884).

Staphylococcus aureus has the ability to adapt to different environments and it may colonize the human skin, nails, nares and mucus membranes and may thereby disseminate among recipient host populations via physical contact and aerosols (Lowy, 1998). Colonization with *Staphylococcus aureus* is an important risk factor for subsequent *Staphylococcus aureus* infection (Wertheim *et al.,* 2004). *Staphylococcus aureus* causes a wide range of infections from a variety of skin, wound and deep tissue infections to more life-threatening conditions such as pneumonia, endocarditis, septic arthritis and septicemia. This bacterium is also one of the most common species in nosocomial infections. However, little is known about the virulence factors behind all these conditions. In addition, *Staphylococcus aureus* also cause food poisoning, like milk and milk product, scalded-skin syndrome abdominal pain, head ache, diarrhea and toxic shock syndrome, through production of different toxins (Winn Washington, 2006).

Staphylococcus aureus is the most frequent and significant species of the family, because of its potential pathogenicity both in humans and animals (Quinn *et al.,* 2011). *Staphylococcus aureus* is found as normal flora on the skin, anterior nares, nasopharynx, and mucous membranes of animals and humans. It is also found in environmental sites such as soil, water, and air (Pal, 2001). *Staphylococcus aureus* has also been isolated from different foods of animal origin and was reported as the third most common cause of food-borne illnesses around the world (Hachemi *et al*., 2019).

The public health impact of *Staphylococcus aureus* is interlinked to the animals and their products that are used for food production. Such food from animal origin may be contaminated with one or more preformed *Staphylococcal enterotoxins* (SEs) produced by the organism and cause human diseases (Normanno *et al*., 2007). The prevalence of *Staphylococcus aureus* in milk and dairy products has been reported from many countries as well as different geographical regions of the same country. It was observed that the prevalence rates varied significantly. Increased prevalence of *Staphylococcus aureus* has been documented in milk and milk products produced from developing countries as compared to the developed countries. Such a difference in the prevalence rates was attributed to the variations in the management system of dairy cows in the respective countries (EFSA, 2009).

2.2. Pathogenesis *Staphylococcus aureus*

The nose is regarded as the major site of *Staphylococcus aureus* carriage from where the organisms can spread to other parts of the body have shown that elimination of nasal carriage by using topical mupirocin also eliminates hand carriage. From these observations, it can be concluded that the nose provides an environment in which *Staphylococcus aureus* can propagate and maintain itself for prolonged periods. The proposed pathogenesis for a number of endogenous infections would be that from the nose, the skin become colonized, causing subsequent infection in patients with impaired skin sites, e.g., in hemodialysis and CAPD patients and in patients with intravascular catheters (Kluytmans et al., 1997)

In several studies, the elimination of nasal carriage reduced the incidence of *Staphylococcus aureus* infections. a significant reduction in the rate of surgical-wound infection after intervention

with mupirocin nasal ointment. Nasal treatment with mupirocin led to a reduction by a factor of four in the incidence of *Staphylococcus aureus* bacteremia per patient year in carriers receiving hemodialysis. When the nares were treated topically to eliminate nasal carriage, *Staphylococcus aureus* usually disappeared from other areas of the body. In patients receiving hemodialysis, 87 percent of those who carried *Staphylococcus aureus* in their nares and on their hands carried the same strain at both sites. Treatment with topical mupirocin, which eliminates nasal carriage, also eliminates hand carriage. The proposed mechanism of pathogenesis for a number of endogenous infections is the colonization of the skin from the anterior nares, which causes subsequent infection in patients with areas of impaired skin, such as patients receiving dialysis and patients with intravascular catheters (Eiffv *et a*l., 2001).

The pathogenic process of *Staphylococcus aureus* infection begins with colonization of host skin or mucosal surfaces and involves bacterial attachment to host cells often via components of the extracellular matrix. To persist, the organism produces molecules that decrease the effectiveness of complement mediated and antibody-mediated opsonophagocytosis and block effectors of host immune cell killing, such as reactive oxygen species and antimicrobial peptides. Ultimately, the organism expresses specific factors that damage host cells and degrade components of the extracellular matrix, contributing to persistence and facilitating spread within normally sterile sites of the host (Bradley and Nizet, 2011).

2.2.1. The Occurrence of *S. aureus* in humans, animals, and food of animal origin

Staphylococcus aureus is present as normal flora of humans and animals and is an opportunistic pathogen that is widely distributed throughout the world (Dini *et al.,* 2019). However, it is well recognized as an invasive human pathogen, resulting in significant morbidity and mortality. It has also been frequently isolated from the animals and the food of animal origin (Wang *et al*., 2018). *Staphylococcus aureus* is a well-known bacterium that develops antibiotic resistance. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a strain of *Staphylococcus aureus,* which has been

noted to acquire resistance to different groups of antibiotics and become multi-drug resistant (Chambers *et al*., 2009).

The MRSA strains can be divided into two different groups, the ones which cause hospital infections (healthcare-associated (HA-MRSA) and those which cause infections in the community (community-associated (CA-MRSA), (Chambers and Deleo, 2009). Even though these two groups have similar microbiological characteristics, they differ in the risk factors, genetic structure, virulence determinants, and antibiotic resistance. CA-MRSA strains carry type IV or V *Staphylococcus* chromosomal cassette mec (SCCmec) element and usually possess the Panton-Valentine toxin/leucocidin (PVL) and are not multi-drug resistant. HA-MRSA species carry type I, II or III SCCmec, do not possess PVL and show multidrug resistance. The PVL is a pore-forming toxin encoded on the genes of bacteriophages and is transmitted to the *Staphylococcus aureus*, which is associated with increased virulence to humans (Aung *et al.,* 2019). The CA-MRSA strains are considered more virulent than HA-MRSA strains because they possess specific virulence factors (Wang *et al*., 2018).

2.2.2. Reservoirs and sources of infections

Animals with persistently colonized body sites (udder and teat skin, muzzle, and vagina) represent the primary reservoir of *Staphylococcus aureus* and sources of infection for others. Animals with subclinical types of inflammatory infections (IMI) are a reservoir of *Staphylococcus aureus* that serves as a source of infection in the dairy environment and represents the most common cause of raw milk contamination. Other sources of *Staphylococcus aureus* include contaminated milking equipment, bedding, and personnel.*Staphylococcus aureus* infected purchased animals and chronically infected animals are a major source of new *staphylococcus* infections within a farm. Farms and cheese-making plants can serve as a reservoir of *Staphylococcus aurues* and can spread the microorganism into the environment. The tonsils and skin of pigs, chickens, and turkeys often harbor *Staphylococcus aureus* and are also potential sources of *Staphylococcus aureus* contamination (Stewart, 2003): Coagulase Negative *Staphylococci* (CoNS) are found to be the reservoir and a potential source for important resistant elements/genes to *Staphylococcus aureus* (John *et al.,* 2019)

2.2.3. Modes of transmission

Staphylococcus aureus can be transmitted from animal to animal, person to person, as well as from animals to humans and vice-versa. Transmission usually occurs by direct contact, often via the hands, with colonized or infected animals or people and contaminated equipment and surfaces (Pal, 2007). The most common transmission pathways include the transfer from an infected mammary gland to an uninfected gland via fomites, such as milking equipment, or the milker's hands, un-controlled animal traffic between different farms and handling or eating food contaminated with *Staphylococcus aureus*. *Staphylococcus aureus* present in the nose and on the skin is shed into the environment by infected or colonized people and animals, indicating the possibility of airborne transmission as a possible route for infection. Vectors like the housefly (Musca domestica) have also been implicated in the transfer of *Staphylococcus aureus* (Cuny *et al.,* 2010).

2.2.4. Predisposing/risk factors

The ability of a microorganism to cause disease depends on the host's susceptibility, immune response, internal and external microbial environment (normal flora), previous infection, antibiotic administered, predisposing conditions like accident/trauma, and the age (Kandi *et al.*, 2018). *Staphylococcus aureus* carriers could be classified into three different classes: those who always carry a strain (persistent carriers), those who intermittently carry different strains (transient carriers) and those people who never carry *Staphylococcus aureus* (non-colonizers). The hosts, especially asymptomatic hosts, play an important role in the spread of *Staphylococcus aureus* into the environment (Van, 2009).

Staphylococcus aureus shows wide adaptability to several environmental and host factors, thus permitting colonization of susceptible environments such as hospitals. The prevalence of

Staphylococcus aureus strains is high in hospital areas as compared to the other regions. The presence of *Staphylococcus aureus* in hospitals is a major concern for public health. The ST398 is the most represented and widely disseminated strain of LA-MRSA that is reported from countries with intensive farming. Poor hygienic standards of the farms and increased resistance rates of *S. aurous* species in mixed farms are important predisposing factors for transmission (Cuny *et al*., 2010). An essential aspect of LA-MRSA is its remarkable degree of host non-specificity and easy transmissibility between animals and humans. *Staphylococcus aureus* owes its strong pathogenic capabilities to the presence of a large number of virulence factors. Moreover, an important risk factor for *Staphylococcus aureus* infections is its tendency to gain resistance to almost all classes of antimicrobial agents. Of particular concern is the acquired resistance that the *Staphylococcus aureus* develops against the β-lactamase stable β-lactam antibiotics (Lowy, 2003).

2.3. Public health importance of *S. aureus*

Staphylococcus aureus is a major pathogen of public health concern throughout the world. MRSA has become a pathogen of increasing importance in hospitals (nosocomial infection), the community, and also in the livestock. Staphylococcal food poisoning is an intoxication that is caused by the ingestion of food contaminated with pre-formed *staphylococcal enterotoxins* (SEs). There are several different types of SEs that include enterotoxin A, D, E and H, and to a lesser extent B, G and I, of which the enterotoxin A is most commonly associated with *staphylococcal* food poisoning in humans. SEs is resistant to the heat and low pH conditions that easily destroy *Staphylococcus aureus* bacteria. The SEs are also resistant to proteolytic enzymes; hence SEs remain unaffected in the gastrointestinal tract after the ingestion (Argudin *et al*., 2010).

The presence of antibiotic-resistant strains has become an emerging zoonotic issue of public health concern. Although extensive researches have been conducted, many people still suffer from staphylococcal infections associated with livestock (Li *et al.,* 2018). Foods associated with the outbreaks of *staphylococcal* food poisoning include meat and meat products, poultry and poultry products, egg, milk, and dairy products, salads, cream-filled bakery products, and sandwich fillings (FDA, 2012). Common symptoms of staphylococcal food poisoning include nausea, vomiting,

abdominal cramps and diarrhea which are usually rapid in onset. In severe cases, headache, muscle cramping and transient changes in blood pressure and pulse rate may occur. *Staphylococcus aureus* can cause various non-food related health issues such as respiratory infections, skin inflammations, wound sepsis, and toxic shock syndrome (FDA, 2012) This bacterium continues to pose major public health challenges because of the problems with antibiotic resistance (Akpaka *et al*., 2017).

2.4. Economic Impact of *S. aureus*

Bovine mastitis is a significant infectious disease with an impact on the economy of milk production. Besides health disorders of the mammary gland, mastitis can also cause significant losses in the milk yield, alterations in its quality (impaired nutritive value of milk), fertility disorders, and even systemic diseases accounting for increased health care and production costs (Pal, 2018). Although clinical mastitis may cause serious damage to the udder and even systemic disorders leading to the culling of affected animals, the subclinical mastitis is, in general, a more insidious form of the disease because it is invisible to the farmer, leading to delayed diagnosis and also spreads widely among the dairy herds. This results in reduced milk quality and yield, which in turn leads to a reduction in the farmer's income as well as that of the dairy industry. Like dairy cows, mastitis in dairy sheep and goats can cause economic losses as a consequence of a decrease in milk production, undervaluation of milk with high somatic cell counts, mortality and treatment costs. In poultry economic losses may result from decreased weight gain, decreased egg production, lameness, mortality, and condemnation at slaughter (Halasa *et al*., 2009).

2.5. Prevalence of *S. aures* in raw milk in Ethiopia

Due to changing management conditions and using of different diagnostic tests, wide variation in the prevalence of *Staphylococcus aureus* have been reported (Rodistitis, 2000).

Table 1: Summary of Staphylococcus aureus prevalence isolated from raw milk in different parts of Ethiopia.

Study Area	Prevalence (%)	References
Jimma town	13.9	(Getahun and GebreSelassie, 2003)
Debra Zeit	8	(Mokennen et al., 2011)
Abaya, Borana pastoral	6.8	(Worku et al., 2012)
Hawassa	17.9	(Daka et al.,(2012)
Addis Ababa	16.2	(Mekuria et al., 2013)
Dire Dawa	25	(Tesfay *et al*., 2013)
Wolaita sodo	15.1	(Tadesse,2014)
Ambo and Guder town	12.6	(Megersa, 2015)
Bahir dar	15.02	(Bitewa, 2015)
Sebeta	19.6	(Ayele et al., 2017)

2.6. Identification method of *Staphylococcus aureus*

Methods of bacterial identification can be phenotypic techniques based on profiling either an organism's metabolic attributes or some aspect of its chemical composition or genotypic techniques based on profiling an organism's genetic material (primarily its DNA) (Sandle, 2013). Primary isolation and identification of the pathogen is crucial for microbiological diagnosis and

other research purposes. In order to isolate and identify *Staphylococcus aureus* a number of authors have used a variety of selective and/or differential culture media (Thaker *et al*., 2013).

A common medium used for the isolation of pathogenic staphylococci is the mannitol salt agar (MSA). Some organisms cannot tolerate high osmotic pressure. Media containing higher than normal salt concentrations that inhibit the growth of these non-tolerant organisms other than the salt tolerant *staphylococc*i (Pal, 2007), Mannitol salt agar contains a high salt concentration so only salt tolerant staphylococci will grow on high salt concentration of this medium that inhibits the growth of most other organisms.

Additionally, MSA contains the sugar mannitol. *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. 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. (Jay, 2000; Quinn et al. 2002).

2.6.1. Cultural and gram's stains test

Most media contain an indicator to distinguish *Staphylococcus aureus* and or inhibitory substances to aid the isolation of *Staphylococcus aureus* from other organisms of which most utilize an indicator system comprising a carbohydrate, most commonly mannitol and a pH indicator, traditionally phenol red, to highlight potential *Staphylococcus aureus* (Perry *et al*., 2003). Among the most common media used for isolation of *a Staphylococcus aureus* are mannitol salt agar (MSA) and Staphylococcus-110 agar, which are selective and differential media and take advantage of the tolerance of *Staphylococcus aureus* to 7.5 % NaCl as well as its metabolic activities, and mannitol fermentation in MSA or gelatinase activity in *Staphylococcus*-110 agar media. Baird–Parker agar also a moderately selective and differential medium, used to detect

tellurite tolerance and lecithinase activity of *Staphylococcus aureus* and *Aureus* chrom select agar base ,MRSA chrom select agar base and Vogel johnson agar are all used to isolate *Staphylococcus aureus* (Martınez *et al*., 2013). Different Identification methods like Gram-staning, catalase test, coagulase test, DNAase production, Methyl red –Vogusproskeur , motility test , serotyping, bio typing , Phage typing and antibiotics sensitivity test are used for *Staphylococcus aureus*.

Gram staining: The Gram stain was first used in 1884 by Hans Christian Gram most widely used staining procedure in bacteriology, is a complex and differential staining procedure. Through a series of staining and decolourization steps, organisms in the Domain Bacteria are differentiated according to cell wall composition as gram negative and gram positives. *Staphylococcus aureus* is gram positive cocci arranged in grape like clusters (Quinn *et al*., 2011).

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

Oxidase test: A piece of filter paper was moistened in a petridish with 1 percent aqueous solution of tetramethyl –p-phenylenediamianedihydrochloride. The test colony was streaked firmly across the filter paper with a glass rod. The disappearance of dark purple color along the streak on the filterpaper was considered as *Staphylococcus*. Oxidase test usually used as differentiation for Staphylococci (oxidase negative) from Micrococci (oxidase positive) (Quinn *et al*., 2002).

***Mannitol salt Agar (Mannitol fermentation)*:** The colonies that were confirmed by gram's staining reaction, haemolysis on the blood agar, colony characterization ,catalase positive and oxidase negative were selected and streaked on Mannitol salt agar plate and incubated at 37^0C and examined after 24-48 h for growth. The presence of growth and change of PH in the medium (red to yellow) was regarded as presumptive identification of *Staphylococcus aurous* or coagulase positive Staphylococcus aurous. Phenol red pH indicator detected the acidic metabolic product of mannitol. Fermentation of mannitol by *Staphylococcus aureus* causes yellow discolouration of the medium within 24 hrs. Of incubation (Quinn *et al*., 2002)

Blood agar: consists of a culture medium widely used in clinical microbiology laboratories, being easy to prepare and low cost this (M. Drancourtet al.,2007) culture medium consists of 5% blood from some mammal, usually that of the sheep. In addition, it allows the identification and isolation of several bacteria, therefore they are nonspecific, however, the distinction of species and genera is possible, according to the hemolytic standards of each strain. In (E. Yehet al., 2009) these senses, colonies of the genus Staphylococcus on blood agar, are checked with a white or yellow tint, creamy, opaque, and convex.

Some species, such as *Staphylococcus aureus*, form a transparent halo close to the places found in the culture medium, as they are capable of providing a β-hemolysis (total hemolysis) However, the difference in hemolytic patterns cannot be used as the sole diagnostic criterion, and there must be the use of staining methods, biochemical tests, and selective means to contribute to the correct identification of any and all microorganisms. (T.V.M.D.et al.,2013).

Coagulse test: This test is used to detect the presence of coagulase enzyme in the isolated pathogen that differentiates pathogenic *Staphylococcus aureus* from non-pathogenic strains which is standard test for routine identification of Staphylococcus *aureus* (Bannerman *et al*., 2003). The reaction was considered positive, if any degree of clotting from a loose clot to a solid clot that is immovable when the tube is inverted (tilted) was visible within the tube and no degree of clotting would be taken as negative. Purple agar Base (1% maltose fermentation) Purple agar base (PAB) with the addition of 1 percent maltose was used to differentiate the pathogenic staphylococci, particularly the coagulase-positive isolates. The suspected culture was inoculated on PAB media plate with 1% of maltose and incubated at 37^0_C for 24-48 hours. The identification was based on the fact that *Staphylococcus aureus* rapidly ferment maltose within 24 hrs and the acid metabolic products cause the pH indicator (bromocresol purple) to change the medium and colonies to yellow. The rapid fermentation (24hrs) was considered as *Staphylococcus aurous* isolates (Quinn *et al*., 2002).

Deoxyribonuclease test: Deoxyribonuclease test is one of the biochemical test used to identify this bacteria *and* determine the ability of an organism to hydrolyze DNA and pathogenic *Staphylococcus aureus* are DNAse positive is (Jahan *et al*., 2015).

Serotyping: Serotyping is based on differences in the structure of antigenic determinants (such as lipopolysaccharides and membrane proteins) located on the surface of bacterial cells (Mehndiratta and Bhalla, 2012).

Bio typing: Biotyping is one of the methods developed for differentiating bacterial strains. biotyping of *Staphylococcus aureus* is based on the results of three reactions production of staphylokinase and β-hemolysin enzymes, coagulation of bovine plasma and observation of growth properties on an agar containing crystal violet (Brzozowski *et al*., 2017)

Phage typing: Phage typing is one of the most useful phenotypic methods in epidemiological studies. Phages are inoculated on agar surface on which the tested *Staphylococcus aureus* isolate is located. Areas where lysis of bacterial cells has occurred are called 'phage plaques (Stark, 2013). Currently, this method is less frequently used, but it can provide valuable information about the prevalence of epidemic *Staphylococcus aureus* strains (Gali *et al*., 2013).

2.7. Treatment

The objective of treatment in human patients is to replace fluids, salt, and minerals that are lost by vomiting or diarrhoea (Sandel and McKillip, 2004). Some strains of Staphylococcus have acquired genes making them resistant to multiple antimicrobial agents. These organisms are uniformly resistant to penicillins and cephalosporins. Penicillinase resistant penicillins such as oxacillin and flucloxacillin are used for serious infections. First or second generation cephalosporins such as cephalothin, cephalexin and cefuroxime are usually safe in patients who are hypersensitive to penicillins. 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 (Kloos and Bannerman, 1994; Rho and Schaffner, 2007).

2.8. Control and Prevention

Treatment options for *Staphylococcus aureus* infections can be difficult and infected animals should be identified for their likelihood of cure. Identifying and eliminating cows through strategic treatment or culling is important for controlling disease. Using herd records to isolate cows with high SCCs or recurrent clinical mastitis is necessary to target infected cattle for testing. Herds with greater than 50% of positive milk cultures would indicate a significant problem. It is more common for herds to have less than 30% of milk samples that are positive for *Staphylocsoccu aureus*. Cows that have an SCC of greater than 400,000, but test negative for *Staphylococcus aureus* should be retested within 2- 4 weeks due to sporadic shedding of the bacterium. Frequent samples provide a better idea of the infection rate. (R Mellenberger and Kirk, 2001),

Prevention is achieved by way of a good, long-term*a Staphylococcus aureus* management program and is more successful than antimicrobial therapy. Mastitis vaccination programs are currently not very efficacious against *Staphylococcus aureus* infections. *Staphylococcus aureus* infections are caused by contamination during the milking process in many cases, which is why excellent pre- and post-milking teat sanitation, milking hygiene including using single-use towels, and maintaining clean milking equipment are necessary for reducing transmission of this and many other contagious pathogens (R.Mellenberger and Kirk, 2001).

3. MATERIALS AND METHODS

3.1. Study Area

The study was conducted in Shashemene town West Arsi Zone. West Arsi zone is located in southern Ethiopia, the zone capital city is found at 250 km distance from Addis Ababa. The zone extends from 7^0 6' 40" to 7^0 23' 20" latitude and 38^0 28' 0" to 38^0 48'0" longitude. Most parts of the zone have elevations of ranging from 1500 to over 2300 m. Shashemene town is the administrative center of the zone. The mean annual temperature of the zone is found between 20-25°C in the highland and 10-16°C in the lowland area. However, there is a slight variation of temperature from month to months. October to May is the hottest months while June-September is the coldest. On average, the zone gets annual mean rainfall 13.7 mm per year. The farming system of the area is mixed crop livestock farming. The livestock population of the zone is 3,352768 head of cattle, (MoLF, 2018).

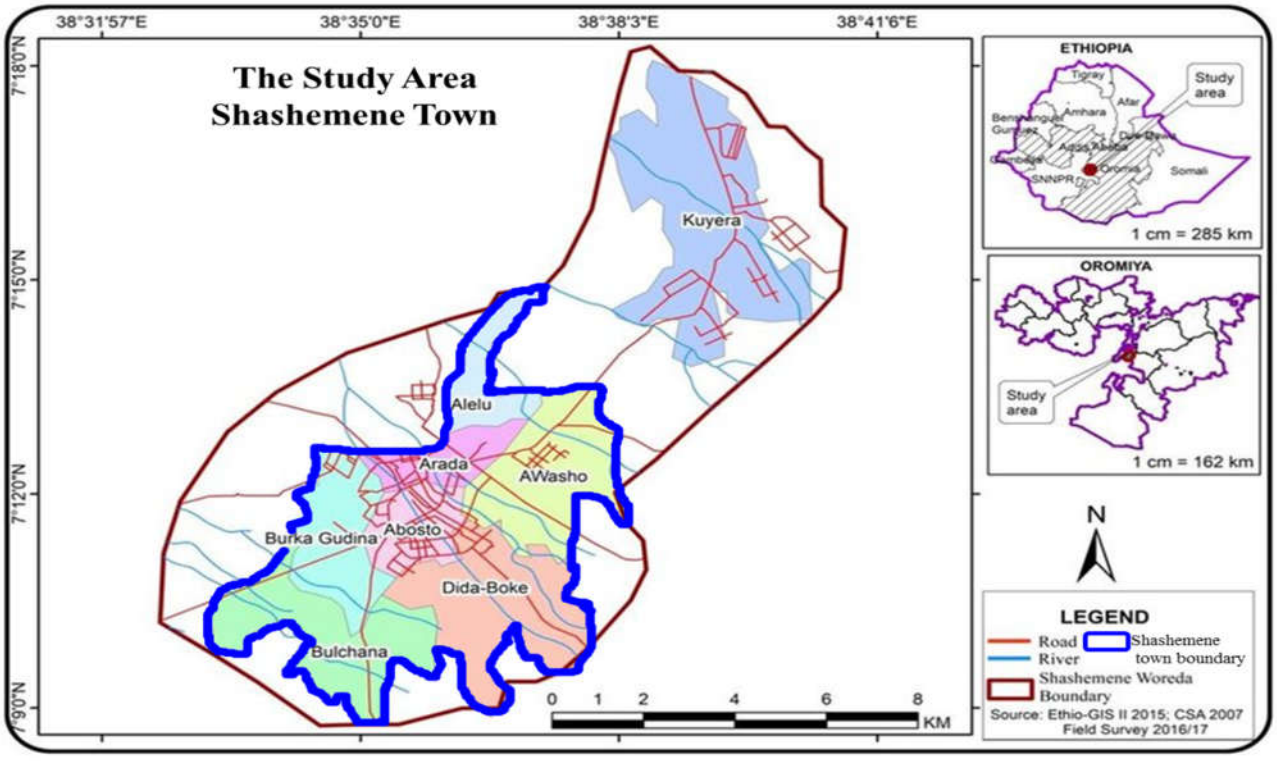

Figure 1:- Map of study area

3.2. Study design and sampling method

A cross-sectional study was carried out from May 2023 to April 2024 at Shashemene town. Milk samples were collected from dairy farms, milk collection centers and milk seller shops. Additionally, its public health significance was assessed by preparing question to the milk consumer, producer, and seller. Before collecting samples general information about the dairy farms, obtained from Agricultural Offices and also for retailer shops, and potential milk producing obtained from Kebeles were in the study area. Based on the information obtained those Kebeles having more dairy farms, retailer shops and MMCs were selected purposively for this study. Simple random sampling technique was employed to select dairy farms and retailer shops in each Kebele. Similarly, farm, MCC and milk retailer shop was selected by simple random sampling to take appropriate raw milk samples. Besides, milk seller and owners were interviewed about the public health significance of *Staphylococcus aureus*. Following general milk material and personal hygiene, milk sample was collected from the farm MCC, and milk retailer shop. The milk storage, transportation how to be collected, management hygiene and personal hygiene history was recorded for each case. The screening of positive samples by bacteriological test and further microbiological examination was analyzed in the laboratory.

3.3. Sample size determination

The sample size was determined using the recommended formula (Thrusfield, 2005) with 95% confidence interval, 5% absolute precision and expected prevalence of *Staphylococcus aureus 16.9%* from previous report which is close and has similar feature with the current study area (Tadessa *et al*., 2014)**.**

$$n = \frac{Z^2 \times Pexp\ (1 - Pexp)}{}$$

Where, n = sample size, z = statistic for a level of confidence=1.96

d = precision, p = expected prevalence = 16.9%

Accordingly 216 sample where calculated but to increase precision 220 lactating dairy cows milk and milk products were sampled.

3.4. Sample size and milk sample collection

A total of 220 milk samples were collected from Farm, MCC and milk retailer shop. Among total samples from MCCs, 14 yoghurt, 21 cheese and 50 raw milk was collected, 15 yoghurt. 17 cheeses and 40 raw milk collected, from milk farm and 13 yogurt samples, 20 cheese samples and 30 raw milk samples were collected from retailer shop respectively. A 25 ml volume of Milk samples were collected according to the procedure recommended by (Quinn et al., 1999). into sterile universal bottles. Samples were identified by farm, MCC, milk retailer shop and samples date. Milk sampling and screening was performed for each retailer and farm of the sample taken. Collected samples were transported using ice-box to Hawasa University, faculty of Veterinary Medicine, Microbiology Laboratory. As soon as arrival to the laboratory it was stored at 4°C for a maximum of 24 hours until cultured on standard bacteriological media was prepared (Quinn *et al*., 2002).

3.5. Bacterial isolation and identification

The refrigerated samples were thawed by allowing equilibrating at room temperature for not less than 30 minutes before inoculation of the culture media. A loop-full of each milk sample was streaked on nutrient ager. Bacteriological examination was done according to the (Quinn *et al*., 2002). On Nutrient Agar plates streake with the milk sample was incubated aerobically at 37^0C and examined for growth after 24 - 48hr. *Staphylococcus species* colonies will usually white, opaque and up to 4mm in diameter.

The colony of bovine strain of *Staphylococcus aureus* wills golden yellow. *Staphylococcus aureus* and *Staphylococcus intermedius* usually produce both alpha (α) and beta (β) haemolysin while Coliform bacteria appear on blood agar as grey to brown colonies ranging in size from 3 to 5 mm in diameter. Primary isolation and identification of *Staphylococcus* species was made after sub-culturing of those colony which shows the same growth on both blood agar and nutrient agar to examine clear colony morphology (size, shape and colour) of the colonies, presence or absence of haemolysis or type of haemolysis on blood agar, gram's staining reaction, 10 µL 3% KOH test, catalase test, oxidative fermentative test and oxidase test.

Known genera of staphylococcus was sub-cultured on selective and differential media on Mannitol salt agar and those grown on Mannitol salt agar and shown yellow pigmentation was sub cultured on nutrient agar for coagulase test. The slide coagulase test was conducted by 0.5 ml of rabbit plasma placed on clean slide and an equal 0.5ml of an overnight broth culture of the *Staphylococcus* mixed to seen the clumping or clot formation (Quinn *et al*., 2002).

3.6. Antimicrobial susceptibility testing

The Clinical and Laboratory Standards Institute's (CLSI, 2021) criteria were followed when testing for antibiotic susceptibility of a *Staphylococcus aureus* isolates. In order to conduct susceptibility testing, the isolates' direct colony suspension was adjusted to match a 0.5 McFarland standard turbidity level. The Clinical and Laboratory Standards Institute's recommendation was followed in determining the isolated strains' susceptibility to antimicrobial agents using the disk diffusion method on Mueller-Hinton agar. All subclasses of antibiotics as well as those frequently used to treat bovine mastitis were chosen for the susceptibility test. So, in all, nine antimicrobials were employed in this investigation (CLSI, 2021). The discs that contained the antibiotics were: Amoxicillin-clavulinic acid (30µg), Ampicillin (10µg), Vancomicin (30µg), Gentamicin (10µg), Streptomcin (10µg), Ciprofloxacin (5µg), Sulfamethoxazoletrimethoprim (25µg), Nalidixic acid (NA 30 µg), and tetracycline (30 µg), Finally, the diameters of the zone of inhibition around the disks were measured to the nearest millimeter using caliper, and the isolates were classified as susceptible, intermediate and resistant according to the interpretative standards of Clinical and Laboratory Standards Institute (Quinn *et al*., 2002; CLSI, 2021). Moreover, isolates showing resistance to two or more antimicrobial subclass were considered as multidrug resistant (Intrakamhaeng and Komutarin, 2012).

3.7. Questionnaire survey

The study's target population, which consists of dairy farm owners, consumers, and milk collectors at MCC, had their knowledge, attitudes, and practices regarding the handling and consumption of milk in the study area evaluated using a pre-tested structured questionnaire. Before being administered, the questions were written in English and then translated into Amharic and afan Oromo. After that, the responses were entered into the original form and translated into English.

The questioner used Arsham (2002) as a guide when determining the sample size

.$N=0.25/SE^2$

Where, N-sample size SE-standard error

SE is at a maximum (5%) when p = 0.05 and the calculated sample size was a minimum of 100. A total of 120 respondents were interviewed

3.8. Questionnaire survey -Based Investigation

The questionnaire survey sample was taken from a total of 120 sample questionnaire prepared purposively to interview the consumer and person from which sample was collected (120) was interviewed. Therefore, totally 120 individuals were interviewed. Verbal consent was obtained from the respondents and the objective of the survey was explained to the respondents before start of the interview. Identification of respondents for questionnaire survey was based on random selection of volunteers who use the milk from shop, shop seller, worker, merchant, civil servant and farmers from Shashemene town. The participant was asked with structured and Semi structured questionnaire which covers demographic data (including age, sex, educational level and location). The potential risk factors of bacteria such as the habit of raw milk consumption, age, occupation, educational levels, presence and usage of management like boiling of milk and knowledge of *staphylococcus* food borne disease and its zoonotic importance and other necessary information was collected through face-to-face interviews.

3.9. Data Management and Analysis

All the data obtained from both questioner survey and findings in the laboratory was entered, cleaned and coded into Microsoft Excel 2010 spread sheet computer program. Then it was analyzed by using SPSS version 12.0 for windows software, and Chi-square ($\chi 2$) test were applied to compare the infection status with regard to the hypothesized risk factors like sample type, origin, habit of raw milk consumption, age, sex, occupation and presence and usage of milk pasteurization.

4. RESULTS

4.1. Overall Prevalence of *Staphylococcus aureus*

Among 220 samples examined, 35.9 % (N=79) were positive for *staphylococcus aureus*. The study has showed relatively a higher prevalence rate of *staphylococcus aureus* at MCCs (43.5%) than milk retailer shop (34.9%) and farms (27.8%). There was no statistically a significant difference (P>0.05) in isolation of *staphylococcus aureus* from farms, milk retailer shop and MCCs (Table 2).

Table 2:- Overall isolation rate of *Staphylococcus aureus* and its association with source of sample

Sources	**No examined (%)**	**No Positive (%)**	**X^2-value**	**P-value**
Farm	72 (32.7)	20 (27.8)	4.24	0.12
MCC	85(38.6)	37 (43.5)		
SHOP	63(28.6)	22 (34.9)		
Total	220(100.0)	79 (35.9)		

4.2. Isolation rate of *S. aureus* in milk and milk products from farm, MCC and shop

It was observed that out of the total 72 samples collected from farm, 20 (27.78%) were found to be positive for *Staphylococcus aureus*. (Table 3). Likewise, from a total of 63 samples collected from milk retailer shops, 22(34.9%) and 85 samples collected from mccs 37(43.53%) were found positive for. Which cheese 7(33.3%), raw milk 23(46.0%) and yogurt 7(50.0%). In all cases, there were no statistical difference (p>0.05) among cheese, raw milk and yoghurt (Table 3).

Table 3:- Association of S. aureus isolation rate with sample type in farm, MCC and shop

Sample type	No examined (%)	No Positive (%)	X^2-value	P-value
Milk sample from farm				
Cheese	17 (23.6)	5(29.41)	0.3	0.98
Raw milk	40(55.6)	11(27.5)		
Yogurt	15(20.8)	4(26.67)		
Total	**72(100.0)**	**20(27.78)**		
Milk sample from Shop				
Cheese	20(31.7)	6(30.0)	0.65	0.72
Raw milk	30(47.6)	12(40.0)		
Yogurt	13(20.6)	4(30.7)		
Total	**63(100.0)**	**22(34.9)**		
Milk sample from MCC				
Cheese	21(24.7)	7(33.33)	1.25	0.53
Raw milk	50(58.8)	23(46.0)		
Yogurt	14(16.5)	7 (50.0)		
Total	**85(100.0)**	**37(43.53)**		

4.2. Antimicrobial susceptibility test

From the total of 79 isolates from dairy farm, MCCs and milks seller shop of *Staphylococcus aureus* 21 pure sample were tested for antimicrobial susceptibility by 9 selected antibiotics. Antibiotics of veterinary and human health relevance were considered in this study has demonstrated, the existence of alarming levels of resistance of *Staphylococcus aureus* to commonly used antimicrobial agents. The isolates were highly resistant to **Ampicillin (100%), Amoxicillin clavulanic acid (76.19%), Streptomycin (76.19%), Nadilic-acid (71.4%), Tetracycline (66.66%)** respectively and moderately resistant to **Ciprofloxacin (38.09%).**

However, they were also susceptible to **Sulphamethoxazole-trimethoprim (95.23%), Gentamycine (80.95%)** and **Vancomycine (90.47%)** (Table 4).

Table 4:- Antimicrobial susceptibility profile of *S. aureus*

Antimicrobials	Unit	Resistant (%)	Intermediate (%)	Susceptible (%)
Amoxicillin clavulanic	30 µg	16(76.19%)	_	**5(23.8%)**
Ampicillin	10µg	21(100)	_	_
Ciprofloxacin	5µg	8(38.09%)	8(38.09%)	**5(23.8%)**
Gentamycin	10µg	2(9.52%)	2(9.52%)	**17(80.95)**
Nalidixic acid	30µg	15(71.4)	5(23.8%)	**1(4.76%)**
Streptomycin	10µg	16(76.19%)	3(14.29%)	**2(9.52%)**
Sulphamethoxzole trimethoprim	25µg	0(0%)	1(4.76%)	**20(95.23%)**
Tetracycline	30µg	14(66.66%)	6(28.57%)	**1(4.76%)**
Vancomycine	30 µg	_	2(9.52%)	**19(90.47%)**

4.3. Results of questioner survey

In addition to the laboratory survey, issues of public health implication arising from *Staphylococcus aureus* and possible sources of milk contamination with *Staphylococcus aureus* were assessed using structured questionnaire survey on 40 farm owners, 40 milk collectors and 40 consumers milk seller shop owners, specifically design for each type of respondent accordingly and a total of 120 respondents were participated. In the current study risk factors for public health such as milk consumption, form of milk consumption, illness of consuming milk, clinical signs and symptom of milk borne disease, awareness on milk born disease and frequency of milk consumption were assessed. Among the total of 40 interviewer dairy farmers, 95% of them consume milk, among these 50% were raw milk consumer. The consumption of raw milk is

relatively higher in an Illiterate (55 %) than those dairy farmers who can join an Elementary school (27.5%), while among the dairy farmers that join high school were only (15.5 %,) consume raw milk. Additionally only 12.5% of the dairy farmers were aware of the occurrence of foodborne diseases due to raw milk consumption, 87.5% of them have no aware of *Staphylococcal* food poisoning associated with consumption of raw milk and milk products. In relation with the cleaning habit of barn, out of 40 farmers interviewed, 45 % and 37.5% of them practiced cleaning of barn two times and three times per day respectively while 17.5% of them clean once per day. In another way, only 20% (n = 8) of farmers wash udder and teat with tap water, while 75% use well water and 5% wash udder with river water before milking however none of the dairy farmers did disinfect their hands before milking using antiseptic solutions but 100% wash hand with detergent. On the other hand, 87.5% of them practiced washing of dairy equipment with detergents before milking and 48.7 %(n=19) of the farmers used plastic containers for milking and only 40 %(n=16) of them use refrigerator (Table 5).

Table 5 :-Summary of questionnaire survey data from farm

Frequency distributions of the variables			
Variables	**Category**	**Frequency**	**Percent (%)**
Education	Illiterate	22	55.0
	Elementary	11	27.5
	High school	6	15.0
Milk Consumption	Yes	38	95.0
	No	2	5.0
Milk Type	Raw	20	50.0
	Ergo	9	22.5
	Ayib	6	15.0
	Boiled	5	12.5
Milk illness	Yes	5	12.5
	No	35	87.5

Time	Rarely	3	7.5
	Common diet	1	2.5
	Frequently	20	50.0
	As required	16	40.0
Duration	1-4 hrs.	29	72.5
	4 hrs.	11	27.5
Temperature	4^0C	16	40.0
	Room T^o	24	60.0
Know about Staph intoxication	Yes	5	12.5
	No	35	87.5
Floor type	Concrete	17	42.5
	Natural	15	37.5
	straw bed	3	7.5
	sand &stone	5	12.5
Milking area	Concrete	17	42.5
	Natural	15	37.5
	straw bed	3	7.5
	sand &stone	5	12.5
Cleaning Time/day	Two times	18	45.0
	Three times	15	37.5
	One time	7	17.5
Cleaning udder	Before milking	27	67.5
	Before & after milking	13	32.5
Milking Equipment	Aluminum can	15	38.5
	Plastic bag	19	48.7
	Clay pot	6	12.8
Source of water for cleaning	River water	2	5.0
	Well water	30	75.0
	Tap water	8	20.0
Habit of washing milk containers	Yes	35	87.5
	No	5	12.5

Hand washing before milking With water	Yes	40	100.0
	No	-	-
Hand washing before milking with inspecting	Yes	-	-
	No	40	100.0

Among 40 retailer shops, 17.5% (n= 7) of the interviewed consumers drink boiled milk while 57.5% (n=23) consume raw milk and 22.5 %(n=9) consume raw milk products like yogurt only 2.5% consume soft cheese. With respect to awareness of milk borne diseases 87.5% (n= 35) retailer shops had no aware of milk borne disease associated with drinking raw milk while only 5% of the respondents had knowledge about staphylococcal food poisoning. Among milk consumers, 50% (n= 20), 25% (n= 10), 20.5% (n= 8), and5 % (n=2) of them purchased milk from MCC, farms, cafe, and from others respectively. Likewise 55% of them (n= 22) used plastic containers while the rest 35% (n= 14) used metallic container and 10% used other container to transport milk to their homes. In contrary 35% (n=14) of them kept milk in a refrigerator while 65% (n= 26) of them kept milk at room temperature (Table 6).

Table 6:- Summary of questionnaire survey data from retailer shops

Frequency Distribution of Variables		
Variable	**Frequency**	**Percent**
Sex		
Male	26	65.0
Female	14	35.0
Age		
Adult (18-33)	25	62.5
Old (>33)	15	37.5
Education		
Illiterate	10	25.0
Elementary	15	37.5
High school	10	25.5
Higher edu.	5	12.5
Form of milk consumed		
Raw milk	23	57.5
Yogurt	9	22.5
Cheese	1	2.5
Boiled	7	17.5
Awareness about milk borne illness		
Yes	5	12.5
No	35	87.5
Knowledge about Staph intoxication		
Yes	2	5.0
No	38	95.0
Illness after consuming milk and its products		
Yes	35	87.5
No	5	12.5
Clinical signs observed		
Diarrhea	16	44.4

Vomiting	9	25.0
Both	9	25.0
Stomach cramp	2	5.6
Where do you purchase milk		
Farm	10	25.0
MCC	20	50.0
Café	8	20.5
Others	2	5.0
Containers used where you purchase milk		
Plastic	22	55.0
Metallic	14	35.0
Others	4	10.0
Time stays prior to consumption at home		
<1 hr.	13	32.5
1-2 hrs.	16	40.0
>2 hrs.	11	27.5
Temperature you keep milk at home		
Refrigerator	14	35.0
Room T^o	26	65.0
Total	**40**	**100.0**

At the mccs, milk from dairy farmers was checked either with organoleptic test and boiling for its freshness. Those milk samples passed the tests were collected from the farmers. Similarly 92.5 % (n=37) of the interviewer from MCCs purchased raw milk from farms. Among them, 27.5% (n= 11) used metallic container while 67.5% (n= 27) used plastic containers and 5 %(n=2) used other material for milk transportation. On the other hand, the respondents of milk collectors indicated that they used different methods of quality assessments like boiling (10%) and visualizing and smelling (60%) before purchasing milk. With respect to milk storage 65% of MCCs was found to be kept milk in a room temperature until consumption. Likewise 2% of them had no aware of the occurrence of milk borne diseases associated with drinking raw milk and 95% of the respondents had no Knowledge about milk borne illness of *Staphylococcal* food poisoning (Table 7).

Table 7:- Summary of questionnaire survey data from MCC

Frequency Distribution of Variables		
Variables	**Frequency**	**Percent**
Age		
Adult (18-32)	21	52.5
Old (>33)	19	47.5
Education level		
Illiterate	16	40.0
Elementary	12	30.0
High school	7	17.5
Higher edu.	5	12.5
Quality Assessment		
Lactometer	7	17.5
Alcohol test	5	12.5
Organoleptic	24	60.0
Boiling	4	10
Time of milk stay		
<1 hr.	19	47.5
1-2 hrs.	11	27.5
>2 hrs	10	25.0
Temperature of Milk storage		
< $4^{o}C$	14	35.0
Room T^{O}	26	65.0
Knowledge about milk borne illness		
Yes	2	5.0
No	38	95.0
Awareness about milk born disease		
Yes	11	27.5
No	29	72.5
Illness after consuming milk and milk product		

Yes	29	72.5
No	11	27.5
Clinical signs observed		
Diarrhea	11	27.5
Vomiting	13	32.5
Both	8	20.0
Stomach cramp	6	15.0
Others	2	5.0
Source of collecting milk		
Farm	37	92.5
Others	3	7.5
Containers used for milk collection		
Plastic	27	67.5
Metallic	11	27.5
Others	2	5.0

5. DISCUSSION

The consumption of raw milk and its derivatives is common in Ethiopia, which is not safe from consumer‘s health point of view as it may lead to transmission of various diseases. It may be contaminated at the site of production and during processing, the cow itself, from air/ dust, unclean milk containers and the milk handlers. Pathogens and other organisms can gain access to milk as a result of the milk handlers‘ activities such as coughing, sneezing, scratching and from body surfaces in contact with milk, particularly the fingers (Getahun and Gebre-Selassie, 2003).

In this study, from 220 lactating cow‘s milk samples subjected to bacteriological examination, (79/220), 35.9% were found to be positive for *Staphylococcus aureus*. This finding is nearly in agreement with the findings observed in Jimma, 35.7% (Tolosa *et al*, 2016), Shinshicho, 38.78% (Abraham *et.al*, 2023); However, the result of the present study showed a slight lower contamination rate compared to other works, 48.8% (Daka *et al,* .2011) in Awasa and 42.1% (Abera *et a*l, .2010) in Adama .The result is also slightly higher than (Ayele *et al*,. 2017) with 19.6 prevalence of *Staphylococcus aureus* in raw milk in Sebete while wide variation in the prevalence of *Staphylococcus aureus* has also been reported. This variation is largely attributed to the changing management conditions and using of different diagnostic tests (Rodistitis, 2000).

In the present study, 46.8% (37/79) of the milk samples from the MCCs were found to be contaminated with. *Staphylococcus aureus* The results showed a higher contamination rate at MCCs than dairy farms and milk retailer shop. This might be attributed to cross contamination of milk while bulking and poor handling during transportation from farm to collection centers and at milk collection centers ((Getahun and Gebre-Selassie, 2003; Addis *et al*, .2010). The contamination of *Staphylococcus aureus* at MCC centers was higher than the previous work (Desissa *et al.,* 2012; Wubete, 2004) where *Staphylococcus aureus* was isolated at recovery rate of 75% and 72%, respectively from bulk tank milk. But, this finding was similar when compared with the report by (Addis *et al*., 2010) from which they recovered 46% of *Staphylococcus aureus* from milk at MCCs. The isolation of *Staphylococcus aureus* from room temperature and milk containers was 65% and 67.5%, respectively. These clearly indicated that milk storage and milk containers could be the potential sources of contamination of milk with *Staphylococcus aureus* and at milk collection the

fact that staphylococci are ubiquitous organisms and also 55% and 48.7% among in the milk containers the number of isolation rate of *Staphylococcus aureus* in milk retailer shop and from dairy farm were observed respectively this number show the contamination of *Staphylococcus aureus* was seen in the milk storage container especially with plastic container.

The antimicrobial susceptibility tests carried out in this study indicated the occurrence of resistance of *Staphylococcus aureus* to some of the commonly used antimicrobials. This study presents the sensitivity of the *Staphylococcus aureus* isolates towards Sulphamethoxazol trimethoprim (95.23%),Vancomycine (90.49%). Gentamycine (80.95%), However, the isolates were found to be highly resistant to Ampicillin (100%), and amoxicillin-clavulanic acid (76.19 %), Streptomycin (76.19%), Nadili-acid(71.8%) and tetracycline(66.66%). The high resistance pattern of the isolates to Ampicillin was relatively similar to the findings reported by; 95% (Endrias Zewdu Gebremedhin *et al.*, 2022), and highly similar to 100% (Balcha *et al.*, 2022), 100% (Abraham *et al*., 2023).

This is probably due to resistance to β-lactams and frequent use of the antibiotics. Absolute resistance to Ampicillin must be of a great concern; since this antibiotic represents, the main antibiotic group recommended for *Staphylococca*l mastitis treatment and regular use of antibiotics for the treatment of cows may result in the spread of resistant strains. Antibiotic resistance is carried on plasmids and transposons which can pass from one *Staphylococcal* species to another (Hulya *et al.,* 2006). The prevalence of antibiotic resistance usually varies between isolates from the different sampled stations and even between isolates from different herds on the same farm (Waage *et al*., 2002). Moreover, the present study showed resistance pattern of *Staphylococcus aureus* to streptomycin (76.19%), tetracycline (66.66%).

The findings are nearly similar with the report of (Tadesse, 2014) with a resistant pattern of streptomycin (66.7%) and tetracycline (69.2%) in and around wolaita sodo, south of Ethiopia. This is due to the fact that these drugs specifically tetracycline and streptomycin are commonly used in the treatment of infections in the study area. Lacks of stringent regulation and monitoring in the dispensing and use of antimicrobials in the country have also might contribute to the occurrence of high antimicrobial resistance to these drugs. Antibiotic therapy is an important tool in the treatment

of *Staphylococcus aureus* related infections. However, the misuse or intensive use of antibiotics can lead to the development of resistance among different bacterial strains (White *et al*., 2001).

The hygienic condition or quality of milk has serous implication on public health safety. The questionnaire result mainly gave broad understanding of the milking and hygienic practice in the study area. Maintaining the hygienic conditions of dairy house, milking area, milking equipment is important for production of good quality milk (Magnusson *et al*., 2007; Yilma, 2007). Especially cleaning the udder of cow before milking is important since it could have direct contact with the ground, urine dung, and feed refusals while resting. Similarly not washing udder before milking can important possible contaminants into the milk. The current study revealed that, 32.5% of the farmers didn‘t wash udder and teat of the cows before milking and all of them didn‘t wash and use antiseptics their hands but 87.5% washed equipment with tap water before milking. This is slightly lower than he report of (Bejano, 2014) 75.8% of farmers did not clean udder before milking. These practices combined with the observation of the occurrence of *Staphylococcus aureus* on hands of milkers‘ indicated that farmers lack knowledge on the importance of good milking practices in minimizing microbial contamination of milk raising the public health issues. Yet pre-milking udder preparation and employing good milk handling practices play an important role in minimizing contamination at the farm with *Staphylococcus aureus* (Michelle and Jeffrey, 2011).

In the present study, the use of plastic containers for milking, storing, collecting and transporting at farm (48.7%), milk collection centers (67.5%) and milk retailer shop (55%) was observed respectively. Plastic containers have inherent characteristics that make them unsuitable for milk handling. Plastic containers scratch easily and provide hiding places for bacteria during cleaning and sanitization and poor conductor heat and hence will hinder effective sanitization by heat leading to bacterial contamination of the milk (Omoe *et al*, .2005; GK, 2011). In this study among the milk retailer shop, 87.5% of them didn‘t have awareness about milk borne diseases associated with consumption of raw milk. This result is close to a study done by (Bejano, 2014) around Assossa district, 100% of them have no awareness about milk borne disease among farmers.

In a traditional practice it is less expensive to take milk from the bulk tank than buying pasteurized retail milk (Stephen *et al*., 2009). Though the results showed relatively a lower percentage of raw milk consumption, still these individuals are at a greater risk of contracting food born intoxication than those who do not consume raw milk. Cross-contamination with pathogenic microorganism can gain access to milk either in contamination or direct excretion from the udder into milk (ElZiney and Al-Turki, 2007).Thus consumption of raw milk with no treatment may pose public to *Staphylococcus* food born poisoning as a result of contamination.

Similarly numerous epidemiological reports have implicated non-heat treated milk and raw milk products as the major factors responsible for illness caused by food borne pathogens (El-Ziney and Al-Turki, 2007). Similar to the milk retailer shop, 50% of farm‘s drink raw milk and raw milk products like yogurt (22.5%) and 87.5% of them has no awareness of milk borne diseases. This is in line with (Ayele *et al*., 2017) who reported 95% of consumers have no awareness of staphy food with milk born disease. Consumers are the last group of the food chain and therefore they are at risk of any malpractice occurring in the chain. Also 60% of the farm’s kept milk at room temperature. Lack of refrigeration facilities at farm and household level in developing countries of tropical regions, with high ambient temperature implies that raw milk will easily be spoiled during storage and transportation (Gilmour, 1999; Godefay and Molla, 2000).

6. CONCLUSION AND RECOMMENDATIONS

In conclusion, the present study revealed the occurrence of contamination of milk with *Staphylococcus aureus* along the milk value chain at farm, milk collection centers and milk seller shops. The high prevalence of *Staphylococcus aureus* at MCCs might indicate cross contamination of milk while bulking and poor handling during transportation from farm to collection centers and at milk collection centers. The study further showed that *Staphylococcus aureus* has developed multiple drug resistance to commonly used antimicrobial like ampicillin, amoxicillin and Nalidixic acid. The study also revealed poor milk handling practices, raw milk consumption behavior, inadequate knowledge of milk borne disease and occurrence of antimicrobials resistant *Staphylococcus aureus*. In general, the study has revealed the possibility of the public health risk posed by *Staphylococcus aureus* in Shashemene town. Therefore, creation of public awareness about good milk handling practices, milk borne diseases and their prevention is important.

In light of the present study, the following recommendations are forwarded;

- Raw milk meant for human consumption should be pasteurized or heat treated.
- Milk needs to be kept cold from the time it is produced until it is consumed.
- Public education is necessary to raise awareness about the need for improved control measures and the ensuing decrease in *Staphylococcal* food poisoning.
- Future studies should consider investigation and designing of cost effective preventive and control options that would enable to reduce milk contamination by S. aureus and there by the associated public health risks.
- Furthermore, prior to use, it is advised to monitor, use medications judiciously, and periodically determine the drugs' antimicrobial sensitivity.

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8. ANNEX

Appendix I. Questionnaire format for Farm owner/worker questioner

Name _____Date_____

Address /kebele _____ Age_____ sex_____

Educational Level? A. Illiterate B. Grade < 5 C. Grade 6-12 D. Grade >12

1. Do you consume the milk at home?

A. Yes B. No

2. If Yes, by what form?

A. Raw (fresh whole milk) B. Ergo (naturally fermented milk)

C. Ayib(Ethiopian cottage cheese) D. boiled

3. Did one of your family members become ill after consuming raw milk?

A. Yes B. No

4. If yes, which of the following signs did he or she showed?

A. Diarrhea only B. Vomiting only

C. both diarrhea and vomiting D. abdominal pain and cramp

5. Do you know any milk borne diseases?

A. Yes B. No

6. If, yes to question number 5 can you name them----------

7. How often do you and your family consume the milk and its products?

A. rarely B. As common diet

C. Frequently D. As required

8. Is there any time gap between taking & using the milk?

A. yes B. No

9. For how long do you keep milk at home?

A. 1-4hrs B .4-10hrs C.10-16hrs D. more than 16hrs

10. At what temperature do you keep milk? a. <4 oc b. Room temperature

11. What is your level of education(who consume raw milk)?

a. Elementary /Read and write b. college graduate c. high school d. no education

12. Do you know about staphylococcal food born intoxication?

a. yes b. no

13. What kind of floor did you use for housing dairy cows?

A. concrete ground B. natural floor

C. straw bedded floor D. stone and sand E. Made from wood

14. What about for milking areas?

A. concrete ground B. natural floor

C. straw bedded floor D. stone and sand E. Made from wood

15. How often did you clean your cows‘ house?

A. Three times a day B. two times a day C. one times a day D. weekly E. monthly

16. How often did you clean your milking cows‘ udder?

A. No cleaning B. Only before milking

C. Only after milking, D. Before and after milking.

17. What kind of milking equipment did you use?

A. Aluminum cans B. Plastic beaker

C. clay pot D. other traditional

18. What is the source water do you use to clean your equipment, teat and udder?

A. River B. well water C. tap water D. stagnant water

19. Do you wash the milk container with soap, detergent?

A. yes B. No

20. Do you wash your hand before milking? A. yes B. No

Questionnaire format for consumer

Name_____ Date_____

Address_____ Age_____ idecational level_____

1. In what form do you consume milk? a. Boiled milk b. Yoghurt c. Cheese d. raw milk

2. Do you have awareness about milk born disease? a. Yes b. No

3. Do you know about Staphylococcus food born disease? a. Yes b. No

4. Do you experience illness after consuming milk and milk product? a. Yes b. No

5. If yes, what kind of clinical sign did you experience? a. Diarrhea b. Vomiting c. Stomach cramp d. Other _____

6. Where do you purchase milk? a. Farm b. MCC c. Hotel /café d. Other _____

7. What kind of container do you use to collect milk? a. Plastic b. Metallic c. Other _____

8. How long the milk stay at home prior consumption? a. <1hr b. 1-2hr c. >2hr

9. At what temperature do you keep milk? a. < 4 oc /refrigerator b. Room temperature

Milk collection center

Questionnaire format for MCCs

Name_____ Date_____

Address_____. Age_____ Sex_____

Idecational level_____

What kind of quality assessment does you before collecting milk?

a. Lactometer reading b. Alcohol test c. Organoleptic test(smell, test and visualize) d. Other _____

2. How long the milk stay at Milk collection Centre?

a. <1hr b. 1-2hr c. >2hr

3. At what temperature milk kept at the center?

a. <4^{o}c b. Room temperature

4. Do you have awareness about milk born disease?

a. Yes b. No

5. Do you know about Staphylococcus food born disease?

a. Yes b. No

6. Do you experience illness after consuming milk and milk product?

a. Yes b. No

7. If yes, what kind of clinical sign did you experience?

a. Diarrhea b. vomiting c. Stomach cramp d. Other _____

8. From where do you collect milk?

a. Farm b. Other source _____

9. What kind of container do you use to collect milk?

a. Plastic b. Metallic c. Other _____

Table 8:- The resistance of each antimicrobial was determined depending on the following measure of zone inhibition diameter**.**

Antimicrobial agents	**Unit µg**	**Diameter of zone of inhibition to nearest Mm**		
		Resistant≤	**Intermediate**	**Susceptible≥**
Amoxicillin- clavulanic acid	30	19	-	20
Ampicillin	10	28	-	29
Streptomycin	10	11	12-14	15
Tetracycline	30	14	15-18	19
Trimethoprim-sulphamethoxazole	25	10	11-15	16
Gentamicin	10	9	13-14	15
Vancomycin	30	12	10-11	12
Nalixidic acid	30	13	14-18	19
Ciprofloxacin	5	15	16-20	21

Source (CLSI, 2012)

Gram's staining procedures

1. Make a thin bacterial colony smear and allow it to dry on the air
2. Fix the dried smear by passing through the Bunsen flame two to three times taking care not to overheat the smear
3. Flood the fixed smear with Gram's crystal violet (primary stain). Let stand for 60 seconds.
4. Pour off the stain and gently wash with tape water.
5. Flood with Gram's iodine (mordant) solution. Allow it to remain for 60 seconds.
6. Pour off the iodine solution and gently wash with tape water.
7. Decolorize with Gram's decolourizer 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.
8. Counter stain with Gram's safranin solution or carbolfuschin (counter stain) for 60 seconds.
9. 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.
10. Examine the finished slide under a microscope (oil immersion objective).
11. Interpretation: Bluish purple color indicate Gram positive and pinkish colour indicate Gram negative bacteria

Figure 2:- Appendix Figure gram stain of S.aureus

Catalase test procedure

1. Pick a colony from an 18-24 hours culture and place it on a clean glass slide.

2. Put one drop of 3% H_2O_2 over the organism on the slide.

3. Observe for immediate bubbling (gas liberation) and record the result.

4. Interpretation: A positive result is the rapid evolution of O_2 as evidenced by bubbling and a negative result is no bubbles or only a few scattered bubbles.

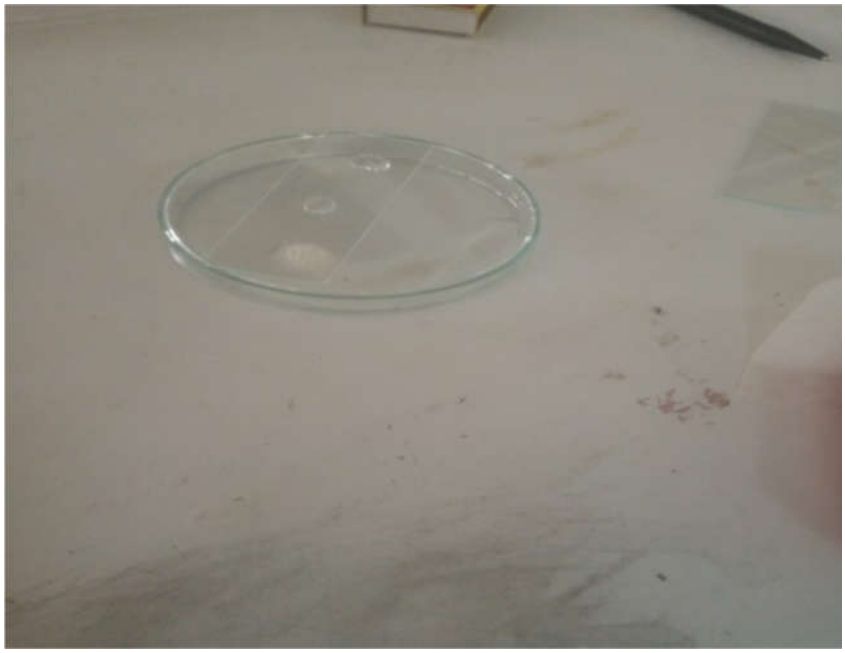

Figure 3:- Appendex S.aureus Slide catalese test

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Coagulase test procedure

1. Three test tubes are taken and labeled ―test‖, ―negative control‖ and ―positive control‖.

2. 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.

3. 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.

4. All the tubes are mixed gently, incubated at 37^{o}C and observed up to four hours. If the test remains negative until four hours at 37^{o}C, the tube is kept at room temperature for overnight incubation.

5. 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.

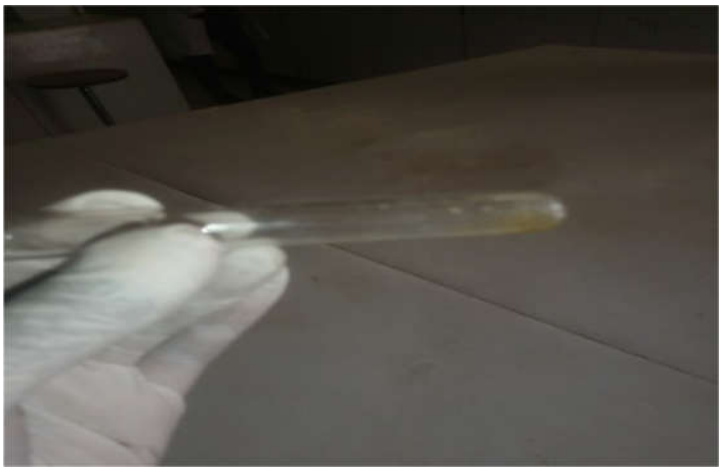

Figure 4:- Appendix figure Positive coagulase reaction

Procedure for the disk diffusion methods

1. At least 4-5 well isolated colonies of the same morphological type are selected from nonselective agar plate and just the top of the colonies are touched.

2. Then a suspension was made in a saline or broth without pre incubation.

3. The turbidity of both suspensions is adjusted by comparison with a 0.5 McFarland turbidity standard.

4. The standard and the test suspension are placed in similar 4-6 ml, thin glass tube or vial.

5. 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.

6. Inoculation of bacterial suspension

7. 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.

8. The swab is streaked in three directions and continuously brushed over the MullerHinton or by rotating the plate for complete cover of the agar surface.

9. 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.

10. 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).

11. This is equivalent to 6 discs per standard 90 mm Petri plate.

12. After incubation, the diameter of the zones of inhibition are measured to the nearest mm using a ruler or calliper.

13. The diameters are read from the back of the plate, when the test is on the comparatively clear Muller-Hinton medium.

14. The diameter of the zones should be read across the centre of the discs.

15. An interpretation of the size of the zones of inhibition is made with reference.

Figure 5:- Appendix Antibiotic sensitivity test on muller hinton agar

Figure 6:- Appendix S.aureus growt on rmanitol salt agar

Figure 7:- Appendex S.aureus bacterial culture

Figure 8:- Appedex.S.aureus examine under microscope