

Thesis Ref. No. \_\_\_\_\_

IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY PROFILES OF  
*STAPHYLOCOCCUS* SPECIES ISOLATED FROM RAW MILK, SWABS OF  
UDDERS, MILKING UTENSILS AND MILKERS HANDS IN SMALL HOLDER AND  
DAIRY FARMS IN AMBO AND GUDER TOWN

MSc Thesis



By

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Veterinary Microbiology, Immunology and Veterinary Public Health

October, 2015  
Bishoftu, Ethiopia

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A Thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Science in Veterinary Microbiology

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October, 2015  
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Title: Identification and Antimicrobial Susceptibility Profiles of *Staphylococcus* Species Isolated from Raw Milk, Swabs of Udders, Milking Utensils and Milkers Hands in Small Holder and Dairy Farms in Ambo and Guder Town

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## **DEDICATION**

This thesis work is dedicated to my friend Tsedalu Eshetu, who has been a constant source of support and encouragement during the challenges of graduate school and life. I am truly thankful for having you in my life. This work is also dedicated to my family, Megersa Marami, Zenebu Bekele and Tsedalu Beyena, who have always loved me unconditionally and whose good examples have taught me to work hard for the things that I aspire to achieve.

## STATEMENT OF AUTHOR

First, I declare that this thesis is my actual work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an advanced (MSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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## LISTS OF ABBREVIATIONS

$\chi^2$	Chi-Square
BaCl <sub>2</sub>	Barium Chloride
BHI	Brain Heart Infusion
CFU	Colony Forming Unit
CLSI	Clinical & Laboratory Standards Institute
CNS	Coagulase Negative <i>Staphylococcus</i>
CPS	Coagulase Positive <i>Staphylococcus</i>
FBD	Food Borne Disease
H <sub>2</sub> SO <sub>4</sub>	Sulfuric Acid
IgG	Immunoglobulin G
Kb	Kilo Base
Mb	Mega Base
MIC	Minimum Inhibitor Concentration
mRNA	Messenger Ribonucleic Acid
MRSA	Methacillin Resistant <i>Staphylococci aureus</i>
MSA	Mannitol Salt Agar
PBP	Penicillin-Binding Protein
PCR	Polymerase Chain Reaction
rRNA	Ribosomal Ribonucleic Acid
SET	<i>Staphylococcus</i> Enterotoxin
SFD	Staphylococcal Food Borne Disease
SU	Standard Units
SXT	Sulphametoazole-Trimethoprim
TSST	Toxic Shock Syndrome Toxin
UK	United Kingdom
VRSA	Vancomycin-Resistant <i>Staphylococci aureus</i>

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## ABSTRACT

*A cross sectional study was conducted in Guder and Ambo town from December to June 2015, to estimate the prevalence, determine the associated risk factors and antibiotic susceptibility profiles of isolated Staphylococcus species from raw milk, udder swabs, milkers' hand and swabs of utensils. A total of 339 samples were collected from small holder and dairy farms. These samples contained 135 raw milk, 25 swabs from milkers' hands, 135 swabs of udder and 44 swab samples from milking utensils. Primary and secondary biochemical tests were used to identify the Staphylococcus species; risk factors were assessed through interview and personal observation and Antibiotic disk diffusion method using Kirby Bauer technique was applied for Antimicrobial susceptibility test. Staphylococcus species identified from all samples were categorized into Coagulase Negative Staphylococcus, Staphylococcus aureus, Staphylococcus intermedius and Staphylococcus hyicus. The prevalence of Coagulase Negative Staphylococcus, Staphylococcus aureus, Staphylococcus hyicus and Staphylococcus intermedius found from milk in the study area were 40%, 12.6%, 16.2% and 5.9%, respectively. The prevalence of Coagulase Negative Staphylococcus was 43.7% from swab of udder, 36.6% from swab of milking utensils and 40% from swab of milkers hands. In addition, the prevalence of S. aureus was 15.5% from swab of udder, 9% from swab of milking utensils and 20% from swabs of milkers hands. Using chi- square test association between risk factors and isolation of Staphylococcus species from milk was analyzed. The result indicated that the prevalence of Staphylococcus species in milk had statistically significant association based on parity ( $P= 0.036$ , usage of towels ( $P=0.006$ ) and previous treatment ( $P= 0.022$ ). Out of 108 coagulase positive Staphylococcus species identified, 50 isolates were subjected to 12 antimicrobial susceptibility disks (Erythromycin, Pencillin, Steptomycin, Tetracycline, Sulphamethoxazole-Trimethoprim, Vancomycin, Ciprofloxacin, Nitrofuranton, Amoxacylin, Nalidixic Acid, Doxycycline and Ampicillin) (Oxoid, Basing Stoke, UK). The result of antimicrobial susceptibility tests indicated that 100% isolated Staphylococcus aureus were resistant to Ampicillin and Penicillin, 90% to Amoxicillin, 45% to Nalidixic Acid, 40% to Tetracycline and 20% to Doxycycline. Out of tested S. intermedius isolates 100% were resistant to Ampicillin, 70% to Nalidixic Acid and Pencillin, 50% to Vancomycin and 10% to Amoxacillin. Among 20 S. hyicus isolates 90%*

*were resistant to Ampicillin, 85% to Penicillin, 60% to Amoxicillin, 15% to Streptomycin and Tetracycline. On the contrary, all tested Staphylococcus species isolates were susceptible to Ciprofloxacin. The current study revealed that the prevalence of Staphylococcus species in raw milk was high and isolates of Staphylococcus species were resistant to a number of drugs. Therefore, raw milk should be pasteurized and handled hygienically. In addition, further research should be done on the factor favor multidrug resistant Staphylococcus species and other risk factors responsible for milk contamination in the study area.*

**Keywords:** *Ambo, Antimicrobial Susceptibility Profiles, Dairy Farms, Guder, Identification, Isolation, Raw Milk, Small holder, Staphylococcus*

## 1. INTRODUCTION

Milk 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 (Roberts and Greenwood, 2003) in the dairy farm environment, contaminated milking equipment, the hands of the milkers' (Cullor, 2004) and excretion from the udder of an infected animal (Oliver *et al.*, 2005). The contamination of milk with pathogenic bacteria has been known to occur mainly due to unhygienic way of handling and processing (Singh and Prakash, 2010).

*Staphylococci* are normal inhabitants of the skin and mucous membranes of animals and humans. They are also wide spread in nature and have been isolated sporadically from a wide range of environmental sources such as air, water, soil and plant surfaces, meat, poultry and dairy products (Singh and Prakash, 2010). Pathogenic strains are usually coagulase-positive and have been found to cause disease in their hosts throughout the world (Larsen *et al.*, 2000). They are capable of causing mild to life threatening diseases, which also includes food borne illnesses. Several species in this genus are having capability to produce a wide range of heat stable enterotoxins (Fagundes *et al.*, 2010).

Milk is considered vehicles of *S. aureus* for infection in humans (Zecconi and Hahn, 2000). Milk has been known to be contaminated by *S. aureus* when there is infection of the mammary gland or by bad hygiene habits, such as coughing or sneezing and not washing hands when handling milk storage equipment, during or after milking, and in this case, human activity is responsible for the contamination, as these bacteria colonizes the nasal pathways in human beings (De Oliveira *et al.*, 2011).

Humans and animals have been known as the primary reservoirs (Argaw, 2015). The presence of *S. aureus* in food has been known to cause food poisoning by releasing enterotoxins into the food and also cause Toxic Shock Syndrome by release of super antigens into the blood stream (Todar, 2005). Pathogenicity of *S. aureus* is due to the

membrane active substances such as cytolytic toxins, consisting of four haemolysins and a leukocidin. This genus may have alpha, beta, gamma and delta haemolysin and the pathogenic members of species *aureus* display beta haemolysis (Presscott *et al.*, 2002).

In Ethiopia, there is no standard hygienic condition followed by producers during milk production. The hygienic condition has been known to vary according to the production system, adapted practices, level of awareness, and availability of resources. In most of the cases under smallholder condition, the common hygienic measures taken during milk production especially during milking are limited to letting the calf to suckle for few minutes and/or washing the udder before milking. The quality of the water used for cleaning purpose (to wash the udder, milk equipment, hands), however, is not secured (Yilma, 2003).

Studies revealed the prevalence of *S. aureus* in dairy products in different areas causing mastitis especially in bovine (Adiss, 2009). In Ethiopia 98% of the annual milk is produced by subsistence farmers who live in rural areas under unsatisfactory hygiene conditions, where cooling and other facilities needed for dairy industry are not sufficiently owned by the farmers. This makes these types of foods potential carriers of pathogenic microorganisms, such as enterotoxin producing *Staphylococcus* species (Yilma, *et al.*, 2007).

Now days most *S. aureus* strains have developed resistant to the penicillin drugs and many are resistant to all  $\beta$ - lactam drugs. All strains of *S. aureus* have a huge arsenal of virulence factors, and now many are adding multidrug resistance as well. The development of new drugs is not as to keep up with developing resistance except a few newer drugs that currently are fairly effective against *S. aureus* strains (ceftaroline, ceftobiprole, dalbavancin, iclaprim, tigecycline) (Reygaert, 2013).

Studies isolating *Staphylococcus aureus* from bovine mastitic milk found different parts of Ethiopia showed high levels of resistance to ampicillin, penicillin, polymixin B, and streptomycin (Mekonnen *et al.*, 2005; Getahun *et al.*, 2008; Abera *et al.*, 2010; Girma *et*

*al.*, 2012; Belayneh *et al.*, 2013). Considering the large portion of the Ethiopian population that lives in close proximity to their livestock, there is potential for transmission of resistant *Staphylococcus aureus* from livestock to humans through the consumption of milk (Desta, 2013).

The production of good quality milk is a challenge that can be overcome provided that basic care is taken at the source of production. Education of handlers and principally of consumers on the dangers of consuming bad quality raw and pasteurized milk is needed (De Oliveira *et al.*, 2011). The prevalence of pathogenic *Staphylococcus* species found in fresh bovine milk around the area, Ambo is not known. Additionally, study on the risk factors contributing for contaminating the fresh milk is not done in the study area. Study on antibiotic susceptible profiles of pathogenic *Staphylococcus* species in the study area is also important to manage *Staphylococcus* species. Therefore, the aims of the present study were:

- To estimate the prevalence of *Staphylococcus* species in raw milk, swabs of utensils, milkers hands and udder from smallholder and dairy farms in Guder and Ambo town;
- To determine risk factors associated with *Staphylococcus* species isolation and
- To evaluate the antibiotic susceptibility profile of the isolates.

## 2. LITERATURE REVIEW

### 2.1. General Characteristics of *Staphylococcus*

#### 2.1.1. *The genus Staphylococcus*

Taxonomically, the genus *Staphylococcus* is categorized under the bacterial family Staphylococcaceae (SU, 2014). To date, more than 50 species and subspecies of *Staphylococci* have been described according to their potential to produce coagulase. Their classification thus distinguishes between coagulase-producing strains, designated as Coagulase-Positive *Staphylococci* (CPS) and noncoagulase-producing strains, called Coagulase-Negative *Staphylococci* (CNS). Among CNS, some species are known to play an important role in the fermentation of meat and milk-based products and are therefore considered as food grade (Hennekinne *et al.*, 2012).

#### 2.1.2. *Morphology and characteristics*

*Staphylococci* are Gram-positive cocci, approximately 1 µm in diameter, that tend to occur in irregular clusters (Roberts and Greenwood, 2003) resembling bunches of grapes. The name derives from the Greek words staphyle and kokkos for a 'bunch of grapes' and a 'berry' respectively. Most *Staphylococci* are facultative anaerobes and catalase-positive. They are non-motile, oxidase-negative and do not form spores. Two species, *S. aureus* subsp. *anaerobius* and *S. saccharolyticus* are anaerobic and catalase-negative (Quinn *et al.*, 2002).

The *S. aureus* subsp. *aureus* (referred to as *S. aureus*) and *S. intermedius*, and the coagulase-variable *S. hyicus* are important pathogens of domestic animals. Coagulase production correlates with pathogenicity. Although Coagulase Negative *Staphylococci* are usually of low virulence, some occasionally cause disease in animals and man (Quinn *et al.*, 2002).

*S. aureus* is a spherical gram-positive bacterium (coccus) that on microscopic examination appears in pairs, short chains, or bunched, grape-like clusters (Bennett and Monday, 2003). They are nonmotile and produce golden yellow colonies (Bhunja, 2008). 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 24hr incubation and most strains show  $\beta$ -haemolysis surrounding the colonies (SU, 2014).

The cell wall of *S. aureus* contains three main components: the peptidoglycan comprising repeating units of N-acetyl glucosamine  $\beta$ -1, 4 linked to N-acetyl muramic acid; a ribitol teichoic acid bound via N-acetyl mannosaminy- $\beta$ -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 autoagglutination. Most of the other species of *Staphylococci* lack protein A in their cell wall (Bhunja, 2008).

*Staphylococcus intermedius* is similar to *S. aureus* in much respect. Colonies are grey white, smooth, none pigmented, glistening, beta hemolytic on blood agar. This species possesses two different teichoic acid antigens in its cell wall, Poly (C) and Poly (P). *Staphylococcus intermedius* produces coagulase and hemolysins (alpha, beta, and delta) (Carter and Wise, 2004).

*S. hyicus* colonies are creamy white, glistening, nonpigmented, nonhemolytic, convex, circular on bood agar. It produces a heat stable nuclease. Coagulase and fibrinolysin are produced by some strains. Protein A and enterotoxins are produced by some strains, but latter are not identical to those of *S. aureus* it does not produce alpha, beta or delta hemolysins. *Staphylococcus hyicus*, which is closely related to *Staphylococcus epidermidis* antigenically, occurs less frequently on the skin and in the milk of cattle. This species is recovered from infection in other animals including cattle (Carter and Wise, 2004).

### 2.1.3. *Molecular Structure*

The genomes of 18 different members of the genus *Staphylococcus* have been completely sequenced. This number includes 14 strains of *S. aureus*, 2 strains of *S. epidermidis*, 1 strain of *S. haemolyticus*, and 1 of *S. saprophyticus*. In all cases, the genome is circular and ranges from 2.49 Mb (*S. epidermidis*) to 2.9Mb pairs (*S. aureus* strains). The sequenced strains also exhibit a high amount of diversity within this genus, with some species and strains having acquired resistance to the antibiotics methicillin and/or vancomycin and some containing extrachromosomal elements called plasmids that most often contain additional genes that can contribute to pathogenesis. Among the sequenced isolates, the number of plasmids present varies from 0 to 3 and the size of these elements varies as well. Genetic and sequence data available indicate that in addition to the normal complement of housekeeping genes, the chromosome contains many accessory genetic elements that are not necessary for growth under laboratory conditions (Gillaspy and Iandolo, 2009).

The genotypic standards for assigning an organism to the genus *Staphylococcus* include determination of guanine plus cytosine (G+C) content of 30-39 mol% and phylogenetic trees constructed by comparison of 16S rRNA or 23R rRNA sequences. The phenotypic criteria are based on the ultrastructure and chemical composition of the cell wall, typical form Gram positive bacteria and catalase reaction positive for all species, except for *S. aureus subsp anaerobius* and *S. saccharolyticus*, which are strictly anaerobic (Souza *et al.*, 2012).

#### 2.1.4. Virulence of factors and toxin produced by *Staphylococcus* species

Table 1: Virulence factors involved in the pathogenesis of *Staphylococcus* species and respective Putative functions.

Cellular products	Biological activities	References
Adhesions	Binds to a variety of extracellular matrix protein of the host (fibronectin, fibrinogen and collagen)	(Hirsh <i>et al.</i> , 2004)
Catalase	Converts Hydrogen peroxide to Oxygen and water	(Carter and Wise, 2004;
Coagulase	Conversion of prothrombin to thrombin, which converts fibrinogen to fibrin (clot)	Plata <i>et al.</i> , 2009)
Enterotoxin/pyrogenic super antigen	11 enterotoxin A-M (no F nor J) and Toxic Shock Syndrome Toxin (TSST-1) Resistant to heat and digestive enzyme	(Hirsh <i>et al.</i> , 2004)
Hemolysin ( $\alpha\beta\delta\gamma$ )	Action on erythrocyte in vitro	
Heat stable Nuclease	Cleaves both Deoxyribonucleic acid and Ribonucleic acid.	
Lipase	Degrades protective fatty acid on skin	(Carter and Wise, 2004)
Staphylokinase	Degrade fibrin clots by converting plasminogen to the fibrinolytic enzyme plasmin.	
Leukocidin	Kills granulocytes and macrophage	
Exfoliative toxins A and B (Exfoliatin)	Cleavage of desmosomes in the stratum granulosum of the epidermis.	
Hyaluronidase	Degrades hyaluronic acid, the ground substance of connective tissue	
Lysostaphin	It is lytic enzyme produced by <i>S.simulans</i>	
Protein A	Surface component which binds Fc portion of IgG and inhibits opsonization.	(Quinn <i>et al.</i> , 2002)
Aureolysin	Inactivate antimicrobial peptides	(Costa <i>et al.</i> ,
<i>Staphyloxanthin</i>	Resistance to neutrophil reactive oxidant-based phagocytosis	2013)

## 2.2. Epidemiology of *Staphylococcus*

### 2.2.1. Source of contamination and reservoirs

The broad distribution of *Staphylococci* as normal flora of domestic animals is perhaps the most important epidemiologic factor in staphylococcal disease. Ready availability of virulent organisms, combined with factors related to host resistance and health management, encourage the development of most infections. Opportunities for occurrence of staphylococcal mastitis are provided by the widespread use of the milking machine, although hand-milked, suckled, and dry cows are also affected. (Medvedova and Valik, 2012).

Coagulase positive species *S. aureus* and *S. intermedius* inhabit the distal nasal passages, external nares, and skin, especially near mucocutaneous borders such as the perineum, external genitalia, and bovine udder. They also occur as transients in the gastrointestinal tract. Coagulase negative *Staphylococci*, especially *S. epidermidis*, are predominant among the resident skin flora but also the upper respiratory tract (Medvedova and Valik 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 contaminant. In the case of an infected udder, *S. aureus* can contaminate milk during milking in a density ranging from 10<sup>1</sup>-10<sup>8</sup> CFU/ml, mostly about 10<sup>4</sup> CFU/ml. It is responsible for approximately 30-40% of all mastitis cases in the world (Medvedova and Valik 2012).

In primary production and the dairy environment, except for milk producing animals, human beings and operational environment belong among the main sources of product contamination (Medvedova and Valik 2012). The principal reservoir of *S. aureus* is the human carrier. A high proportion of healthy people (30% to 35%) have *Staphylococci* in the nasopharynx and on the skin. A carrier with a respiratory disease can contaminate foods by sneezing, coughing, or expectorating. Similarly, he may contaminate food he

handles if he has a staphylococcal skin lesion. However, even if not sick himself, the carrier may contaminate food by handling different food ingredients, equipment, utensils, or the finished product. The proportion of toxigenic strains isolated from various sources (humans, animals, and food) is very high (Pedro and Boris, 2001).

### 2.2.2. *Vehicles of transmission*

*Staphylococci* exist in air, dust, sewage, water, milk, food, or on food equipment, environmental surfaces, humans, and animals. Humans and animals are the primary reservoirs. *Staphylococci* are 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).

### 2.2.3. Distribution of *Staphylococcus* species in raw milk in Ethiopia

Table 2: The prevalence of *Staphylococcus* species isolated from raw milk, and milk storage in the different areas of Ethiopia.

Type of samples	Study Area	Species of <i>Staphylococcus</i>	Prevalence (%)	References	
Raw cow milk	Addis Ababa	<i>S. aureus</i>	16.2	(Mekuria <i>et al.</i> , 2013)	
		<i>S. aureus</i>	8	(Mokennen <i>et al.</i> , 2011)	
	Dire Dawa	<i>S. intermedius</i>	6		
		<i>S. hycius</i>	6		
		CNS	13		
		<i>S. aureus</i>	25	(Tesfay <i>et al.</i> , 2013)	
		<i>S. aureus</i>	13.9	(Getahun and Gebre-Selassie, 2003)	
	Abaya, Borana pastoral area	Bishoftu	<i>S. aureus</i>	44	(Desissa <i>et al.</i> , 2013)
		<i>S. aureus</i>	6.8	(Worku <i>et al.</i> , 2012)	
		<i>S. epidermidis</i>	3.33		
Milk storage container	Abaya, Borana pastoral area	<i>S. intermedius</i>	2.33		
		<i>S. aureus</i>	7.29		
		<i>S. epidermidis</i>	3.03		
		<i>S. intermedius</i>	2.81		

### 2.3. *Staphylococci* Infection in Cattle

Staphylococcal mastitis occurs in goats, sows, and ewes; in cattle it is most often chronic and subclinical, but can be acute or even gangrenous, especially in postparturient cows (Songer and Post, 2005).

Mastitis in cattle caused by *Staphylococci* is of interest from a public health perspective. In modern milking systems, *S. aureus* is a common pathogen in cows' udder. The agent is transmitted by means of milking machines or the milker's hands, and enters through the

milk duct or superficial lesions on the teat. Mastitis caused by *S. aureus* in cattle may vary from the prevalent subclinical form of infection to a severe gangrenous form. Both forms are economically important because of the losses they cause in milk production (Pedro and Boris, 2001).

## **2.4. Staphylococcal Food-Borne Disease**

SFD is one of the most common FBD and is of major concern in public health programs worldwide. A typical FBD caused by *S. aureus* has a rapid onset following ingestion of contaminated food (usually 3– 5 hours). This is due to the production of one or more toxins by the bacteria during growth at permissive temperatures. However, the incubation period of SFD depends on amount of toxin ingested. Very small dose of SEs can cause SFD (Kadariya *et al.*, 2014)

The onset of SFD is abrupt. Symptoms include hypersalivation, nausea, vomiting, and abdominal cramping with or without diarrhea. If significant fluid is lost, physical examination may reveal signs of dehydration and hypotension. Abdominal cramps, nausea, and vomiting are the most common. Although SFD is generally self-limiting and resolves within 24–48 hours of onset, it can be severe, especially in infants, elderly, and immunocompromised patients (Kadariya *et al.*, 2014).

### *2.4.1. Staphylococcal enterotoxins*

Staphylococcal enterotoxins are short, single-chain polypeptides of approx. 600 to 800 amino acids, forming two unequal domains, A and B. Domain A is larger, and is proposed to be involved in binding to T-cell receptor sites. It contains both the carboxyl and amino termini. Domain B contains a cystein loop, which is suggested to stabilize a necessary conformation for the emetic (vomiting) property. While many efforts have been made to identify the amino acids and domains involved in emesis, the picture is not complete. There are no specific cells or receptors in the intestines linked to SFP, but in the review by

it is suggested that SETs stimulate neurons leading to the vagus nerve, which transmits the signal to the vomiting centre in the brain (Argudin *et al.*, 2010)

SETs are heat-resistant, and may retain biological activity after 28 min at 121 °C. However, heat stability has been reported to vary between different food matrices and is also affected by the purity of the toxin, initial amount, PH, salt concentration and detection method (Balaban & Rasooly, 2000). The ability of SETs to resist proteolytic enzymes such as pepsin and trypsin, helps them to keep activity in the digestive tract after ingestion (Rosengren, 2012).

The *S. aureus* enterotoxins (SEs) are potent gastrointestinal exotoxins synthesized by *S. aureus* throughout the logarithmic phase of growth or during the transition from the exponential to the stationary phase. They are active in high nanogram to low microgram quantities, and are resistant to conditions (heat treatment, low pH) that easily destroy the bacteria that produce them, and to proteolytic enzymes, hence retaining their activity in the digestive tract after ingestion (Argudin *et al.*, 2010).

*S. aureus* can express several different types of protein toxins which are probably responsible for symptoms during infections. Some will lyse erythrocytes, causing hemolysis, but it is unlikely that hemolysis is a relevant determinant of virulence in vivo. Leukocidin causes membrane damage to leukocytes, but is not hemolytic (Todar, 2005).

Systemic release of alpha toxin causes septic shock, while enterotoxins and TSST-1 are superantigens that may cause toxic shock. Staphylococcal enterotoxins cause emesis (vomiting) when ingested and the bacterium is a leading cause of food poisoning (intoxication) (Todar, 2005).

## **2.5. Public Health and Economic Importance**

Staphylococcal Food Poisoning (SFP) is one of the most common Food borne diseases (FBD) and is of major concern in public health programs globally (Hennekinne *et al.*, 2012). Staphylococcal infections are frequent but are usually contained by immune

mechanisms to the site of entry. The highest incidence of disease usually occurs in people with poor personal hygiene, overcrowding and in children (Rho and Schaffner, 2007). In developing countries, the surveillance system of FBD hardly exists and it is therefore, difficult to estimate the real magnitude of the problem (Boschi-Pinto, 2008). 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. Hence, the incidence of FBD caused by *Staphylococci* is thought to be much higher than reported since many cases remain undeclared (Argaw, 2015)

Food borne diseases are a serious and growing problem in the world (Baron, 2007). *S. 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, 2011).

Among FBD, SFP is of major concern in global public health programmes. 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 immunocompromised persons, elderly persons and children (Kerouanton, 2007).

## **2.6. Antibiotic Resistance**

Antimicrobial resistance is genetically based; resistance is mediated by the acquisition of extrachromosomal genetic elements containing resistance genes. Exemplary are plasmids, transposable genetic elements, and genomic islands, which are transferred between bacteria via horizontal gene transfer (Batabyal *et al.*, 2012).

There has been a marked increase in the number of species that have acquired resistance to antibiotics (Table 1) (Chakraborty *et al.*, 2012).

Table 3: Some representative antibiotics, their modes of action and mechanisms of resistance

Category	Some members	Mode of action	Major mechanisms of resistance
$\beta$ -Lactams	Penicillin,	Inhibition of cell wall synthesis	Cleavage by $\beta$ -Lactamases,
Aminoglycosides	Streptomycin, Gentamycin	Inhibition of protein synthesis	Enzymatic modification, efflux, ribosomal mutations, 16s rRNA methylene
Quinolones	Ciprofloxacin, Oxofloxacin, Norfloxacin	Inhibition of DNA replication	Efflux, modification, targets modification
Glycopeptides	Vancomycin	Inhibition of cell wall synthesis	Altered cell walls, efflux

Source (Chakraborty *et al.*, 2012)

### 2.6.1. Methicillin-Resistant *S. aureus* (MRSA)

The introduction of methicillin in 1961 was rapidly followed by reports of methicillin resistance in *S. aureus*. Today, MRSA strains are found worldwide, and most are multidrug resistant. Methicillin-resistant *Staphylococcus aureus* (MRSA) are a type of *Staphylococcus* or "staph" bacteria that are resistant to many antibiotics (Batabyal *et al.*, 2012).

High level resistance to methicillin and all other  $\beta$ -lactams in *Staphylococcus aureus* (methicillin-resistant *S. aureus* strains (MRSA)) is due to acquisition of a gene, *mecA*, responsible for synthesis of a new PBP, PBP 2a, with reduced affinity for  $\beta$ -lactams. In the absence of *mecA*, low level resistance could be due to overproduction of PBP 4 or due to modification of PBP 2 (Perichon and Courvalin, 2009).

PBPs are membrane-bound enzymes that catalyze the transpeptidation reaction, which is necessary for cross-linkage of peptidoglycan chains. PBP2a substitutes for the other PBPs and, because of its low affinity for all  $\beta$ -lactam antibiotics, enables *Staphylococci* to survive exposure to high concentrations of these agents. Thus, resistance to methicillin confers resistance to all  $\beta$ -lactam agents, including cephalosporins. Expression of resistance in some MRSA strains is regulated by homologues of the regulatory genes for *blaZ*. These genes, *mecI* and *mecR1*, regulate the *mecA* response to  $\beta$ -lactam antibiotics in a fashion similar to the regulation of the *blaZ* response to penicillin by *blaR1* and *blaI*. *mecA* is carried on a mobile genetic element and is part of a genomic island designated staphylococcal cassette chromosome *mec* (SCCmec). To date, four different SCCmec elements varying in size from 21 to 67 kb have been characterized. Such islands may also contain additional genes for antimicrobial resistance and insertion sequences, as well as genes whose function is uncertain (Ryu *et al.*, 2014).

#### 2.6.2. Vancomycin resistance

Vancomycin has been the most reliable therapeutic agent against infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA). However, in 1996 the first MRSA to acquire resistance to vancomycin, was isolated from a Japanese patient (Hiramatsu, 2001).

The glycopeptide vancomycin was first released in 1958. Vancomycin is an inhibitor of cell wall synthesis in *S. aureus* and other gram-positive organisms. While beta-lactam antibiotics inhibit cell wall synthesis by binding to the transpeptidase active site of penicillin binding proteins, vancomycin acts by a completely different mechanism so it has been the treatment of choice for serious infections caused by MRSA (Howden *et al.*,

2010), but increase in vancomycin use has led to the emergence of two types of glycopeptide-resistant *S. aureus* (Souza *et al.*, 2012).

Briefly, the resistance gene is located on a transposon called *Tn1546*. This transposon contains a set of genes (including *vanA*) which encode enzymes that replace the C terminal D-Ala-D-Ala residues of the peptidoglycan precursor with D-Ala-D-Lac. Vancomycin binds to normal precursors by forming hydrogen bonds between its peptide portion and the D-Ala-D-Ala dipeptide. The structural change in the precursor leads to loss of vital hydrogen bonds and extreme reduction in affinity of vancomycin to these cell wall precursors (Askari *et al.*, 2012).

## **2.7. Isolation of *Staphylococcus* Species**

Microscopic morphology helps to differentiate bacilli, streptococci, and yeasts from *Staphylococci*, which form irregular or grape-like clusters of cocci. *Staphylococci* may be further differentiated from streptococci on the basis of the catalase test, with the former being positive. Additional features are needed to differentiate *Staphylococci* further from micrococci. Usually *Staphylococci* are lysed by lysostaphin but not by lysozyme, and they can grow in the presence of 0.4 µg/mL of erythromycin. Micrococci are not lysed by lysostaphin, may be lysed by lysozyme, and will not grow in the presence of erythromycin. In a deep stab culture, micrococci will grow at the surface, whereas most *Staphylococci* grow throughout the agar. *Staphylococci* will grow and produce acid from glucose and mannitol anaerobically, whereas micrococci do not. Staphylococcal cells contain teichoic acids in the cell wall and do not contain aliphatic hydrocarbons in the cell membrane, whereas the reverse is true with micrococci. Further, the G-C content (mole percentage) of *Staphylococci* is 30–40% and 66–75% for *Micrococci* (Quinn *et al.*, 1999).

## **2.8. Identification of *Staphylococcus* Species**

Various tests can be used to identify *Staphylococcus*, including production of protein A, cell-bound clumping factor, extracellular coagulase and heat-stable nuclease. A combination of hemolysis and coagulase activities seems to represent an optimal criterion

for the identification of *S. aureus* in cultures from milk samples (Boerlin *et al.*, 2003). In addition, molecular methods have been developed more recently. Comparison of the performance of tests in various studies can be difficult because different strains of *S. aureus* and different species of CNS have been included and may behave in different ways (Brown *et al.*, 2005).

#### 2.8.1. *Tube coagulase test*

Free (extracellular) coagulase clots plasma in the absence of calcium. The tube coagulase test with rabbit plasma and examination of tubes after incubation for 4 hrs and 24 hrs is the standard test for routine identification of *S. aureus*. Tests negative at 4 hrs should be re-examined at 24 hrs because a small proportion of strains require longer than 4 hr for clot formation. Some other species of *Staphylococci*, including *Staphylococcus schleiferi* and *Staphylococcus intermedius*, may also give positive results in tube coagulase tests. In addition, rare strains of *S. aureus* are negative in coagulase tests. For routine testing more rapid tests are now widely used, particularly latex agglutination tests (Quinn *et al.*, 2002; Roberts and Greenwood, 2003).

#### 2.8.2. *Slide coagulase test*

Clumping factor (bound coagulase) differs from free coagulase in that it is cell-bound and requires only fibrinogen. The slide agglutination test for clumping factor is very rapid but up to 15% of *S. aureus* strains are negative, so isolates negative in slide tests should be confirmed with a tube agglutination test. Some less common species of *Staphylococci*, including *S. schleiferi* and *Staphylococcus lugdunensis*, may give positive results in the slide coagulase test. The test is unsuitable for isolates that are not easily emulsified and clumping factor can be obscured by large amounts of capsule (Brown *et al.*, 2005)

#### 2.8.3. *Latex agglutination tests*

Early versions of commercial latex agglutination tests for *S. aureus* detected protein A and/or clumping factor. These tests had problems with some MRSA which produce little

or no clumping factor and protein A. Later formulations of latex tests include protein A and/or clumping factor but also detect various surface antigens, which improved the sensitivity of the tests but at some expense to specificity due to cross-reaction with CNS. In addition, any test including clumping factor may give false-positive results with *S. lugdunensis* and *S. schleiferi* (Brown *et al.*, 2005).

#### 2.8.4. *DNase and heat-stable nuclease tests*

Deoxyribonuclease plates can be used to screen isolates but, as various amounts of DNase are produced by CNS, positives should be confirmed with an additional test. Heat-stable nuclease tests can be used to identify *S. aureus*, although some rare coagulase-negative species can be positive. The metachromatic agar diffusion method for heat-stable nuclease has been particularly used in direct tests on blood cultures but this method is medium dependent. A latex agglutination test based on heat-stable nuclease has also been described (Quinn *et al.*, 1999; Roberts and Greenwood, 2003).

#### 2.8.5. *Commercial biochemical tests*

There are many commercial kits and automated instruments which include identification of *S. aureus*. While performance of these tests may be good, they are slower, technically more time consuming or more expensive than tests such as coagulase and latex agglutination. Hence they are less likely to be used for specific identification of *S. aureus*. In contrast, the Staphychrom II test is a 2 hrs chromogenic test based on prothrombin and protease inhibitors (Brown *et al.*, 2005).

### **2.9. Prevention, Control and Treatment**

There is no significant growth of *Staphylococci* at temperature below 4.4<sup>0</sup> C and the organisms are destroyed when kept at 77<sup>0</sup> C for 20 minutes. Storing foods at temperature less than 4.4<sup>0</sup> C or greater than 60<sup>0</sup> C effectively prevents replication of staphylococcal organisms and significant toxin production (Adiss, 2009).

Control is both important and difficult as *Staphylococci* 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).

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 in relation to hand washing, wearing gloves during food preparation and storing foods at proper temperature to inhibit growth or destroy the pathogen and minimize toxin production as heating food after toxin is formed will not be an effective control measure. 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 equipments will certainly reduce the incidence of food poisoning outbreaks due to *Staphylococcus* (Baron, 2007).

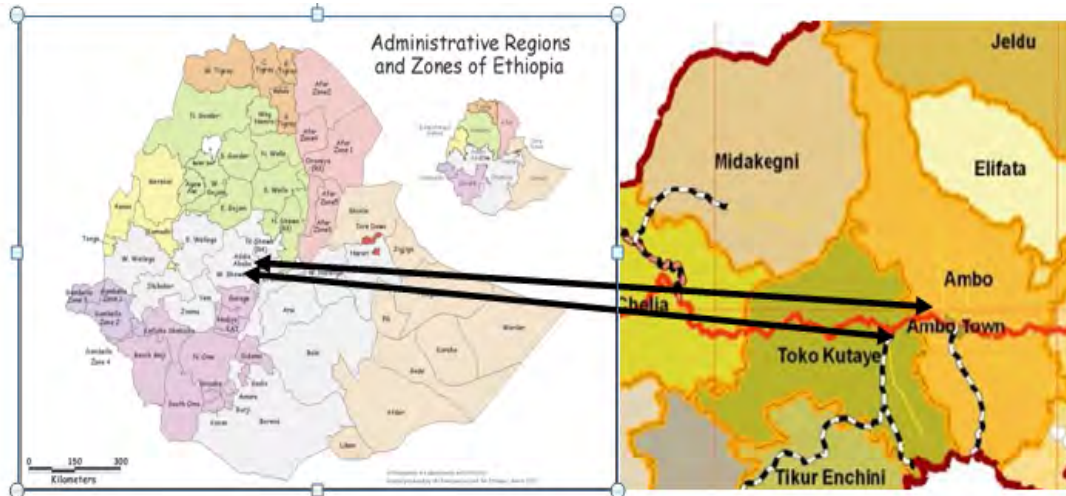
The objective of treatment in human patients is to replace fluids, salt, and minerals that are lost by vomiting or diarrhoea. Some strains of *Staphylococcus* have acquired genes making them resistant to multiple antimicrobial agents. These organisms are uniformly resistant to penicillins and cephalosporins. 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 (Adiss, 2009).

### 3. MATERIAL AND METHODS

#### 3.1. Study Area

This study was conducted in Ambo and Guder Towns. Ambo town is situated at 8°56'30" - 8°59'30" N latitude and 37° 47'30" -37°55'15" E longitude in central Oromia, 114 km west of Addis Ababa. The altitude of the area ranges from 2000 - 2400 m mean above sea level, characterized by warm temperate weather which is locally called 'Bada-dare' (mid altitude). The temperature ranges from 15°C-29°C with average annual temperature of 22°C. It receives a mean annual rain fall ranging from 800-1000 mm with an average of 900 mm. The highest rainfall concentration occurs from June to September and the mean monthly relative humidity varies from 64.6% in August to 35.8% in December. The soil types encountered are Red soil (36.25%), Black soil (34.37%) and Brown soil (29.38%). Livestock are major agricultural resource in this area. Ambo town has three 'Kebeles'. Ambo has a livestock population of 272,168 (137,448 cattle; 40,985 sheep; 20,968 goats; 11,901 donkeys; 8,679 horses; 337 mules and 51,850 poultry). The total human population of Ambo is estimated to be 216,312 with a total of 83,679 (50.08 %) and 29,409 (57.69%) female and 81, 038 (49.92 %) and 22,186 (42.31%) male in rural and urban, respectively (CSA, 2007; Etefa and Dibaba, 2011).

Guder is a town located in the Western Shewa Zone of the Oromia Region of Ethiopia. Located 12km west of Ambo, this town has a latitude and longitude of 8°58'N 37°46'E 8.967°N 37.767°E, with an elevation of 2101 meters above sea level. The total human population of Guder Town is estimated to be 14,742 according to CSA, (2007) with a total of 7,266 males and 7,476 females.



Source: (Etefa and Dibaba, 2011)

Figure 1: Map of the study area.

### 3.2. Study Population

The study population was lactating cows in selected three ‘Kebeles’ of Ambo and two Kebeles of Guder town which are kept under intensive, semi intensive and extensive management system categorized under small holder and dairy farms. The number of small holder and dairy farms incorporated were 5 and 18 respectively. The range of lactating cows categorized under small holder and dairy farms were 1-3 and >3 respectively. Milkers who served in small holder and dairy farms at selected area were included.

### 3.3. Study Design and Sample Type

A cross sectional study was conducted in Guder and Ambo town from December 2014 to June 2015. Types of sample included were milk, swabs of milkers’ hands, udder and milking utensils.

### 3.4. Sample Size Determination and Sampling Procedure

The sample size was calculated using the formula described by Thrusfield (2005) at 5% precision and expected prevalence of 8.25% which was reported by Mokennen *et al.*

(2011) from Bishoftu, which has similar features with the current study area. Accordingly, the calculated minimum number of cows in the study was 116 but to increase precision 135 lactating cows were sampled. In addition, 25 milkers hand swabs and 44 swabs of milking equipment or utensils were sampled. The milker population serves in the smallholder and dairy farms are few in numbers; as the result all of them were included. Therefore, a total of 339 samples were considered for the present study.

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

Where n= sample size  
P<sub>exp</sub>= expected prevalence  
d = desired absolute precision

All dairy farms found in both Ambo and Guder towns were included; but lactating cows among farms were selected randomly. Small holder dairy farms were selected using purposive sampling.

Table 4: Number and type of samples considered for the study.

Type of samples	Total
Raw milk	135
Swabs of udder	135
Swabs of milking utensils	44
Swabs of milkers' hands	25
Total	339

### 3.5. Sample Holding and Transport

Sterilized screw capped test tubes were used for collecting milk samples from the individual cows. Totally, 10 ml of pooled milk sample (equal volume from each quarter) was collected. Sterilized peptone water and sterile wooden sticks swabs were utilized during swab samples handling. Using Ice box, the collected samples were transported to

Ambo University, Veterinary Microbiology laboratory within 24 hrs. The samples were kept at 4°C overnight before culturing (see Appendix I).

### **3.6. Isolation and Identification of *Staphylococcus* Species**

The samples were directly inoculated on blood agar plate containing 7% sterile sheep blood. It was incubated at 37 ° C for 24- 48 hrs. Then the colonies characterization were seen and recorded. The colonies grown on blood agar plates were taken and smeared. Gram's staining was employed for identifying cell morphology, color and shape. Suspected colonies were being sub cultured on nutrient agar plates and further biochemical tests were done.

#### *3.6.1. Gram staining*

All suspected cultures of *Staphylococcus* species colonies were picked and smeared on labeled clean glass slide. The smeared slides were stained using gram stain. Once stained, the smear was examined using the oil immersion (1000 magnification) lens. The slides were evaluated for the presence of bacterial cells as well as the Gram reaction (color); morphologies (e.g. cocci or bacilli) and arrangements (e.g. chains, pairs, cluster) As Quinn, *et al.* (2002). The suspected colonies checked by gram stain reaction were further tested (see appendix III).

#### *3.6.2. Catalase test*

Suspected pure colonies isolated were received from nutrient agar plate and mixed with a drop of 3% Hydrogen Peroxide on a clean glass slide. If the suspensions forms bubble it were believed as Catalase positive bacteria and further tests were performed (see appendix III).

### 3.6.3. *Oxidase test*

The isolated colonies were picked and smeared onto Kovac's reagent dampened filter paper. The color of the paper was observed and waited till 30 s. Development of a deep blue to purple color in 10 to 30 s is a positive reaction (Isenberg, 2007).

### 3.6.4. *Anaerobic utilization of glucose*

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

### 3.6.5. *Growth of Staphylococcus on Mannitol Salt Agar plate*

The colonies that were identified by gram staining, oxidase test and catalase test were subcultured on MSA plates and incubated at 37°C examined after 24-48hrs for growth and change in the color of the medium (see appendix III; Figure 3). The presence of growth and change of PH in the media (red to yellow color) were regarded as confirmative identification of the salt tolerant *Staphylococci*. Phenol red PH indicator detected the acidic metabolic product of mannitol. Fermentation of mannitol by *S. aureus* causes yellow discoloration of the medium (Quinn *et al.*, 2005). Colonies that develop weak or delayed yellow color after 24 hrs of incubation were taken as *S. intermedius* and colonies that failed to produce any change on the medium were considered as *S. hyicus* and CNS.

### 3.6.6. *Tube coagulase test*

Colonies that were grown on the MSA plate, subcultured on Brain Heart Infusion medium (BHI) incubated at 37°C for 24 hrs. The 0.5 ml of rabbit plasma and a drop of young colonies taken from BHI were mixed and incubated for 4-24hrs at 37°C. The clotting of suspension was evaluated at 30 minutes intervals for the first 4 hr of the test and then after 24 hr incubation (see appendix III; Fig. 2). The reaction was considered as positive, if any degree of clotting was visible within the tube when tilted as (Bennett and Lancette, 2001).

### 3.6.7. *Fermentation of 1% maltose using purple agar base*

Purple agar base (PAB) with the addition of 1% maltose was used to differentiate the pathogenic *Staphylococci*. The suspected culture was inoculated on PAB media with 1% maltose and incubated at 37°C for 24-48 hours. *S. aureus* rapidly ferment maltose and acid metabolic products cause the PH indicator (bromocresol purple) to change the medium and colonies to yellow. *S. intermedius* gives weak or delayed reaction and *S. hyicus* did not ferment maltose but attacks the peptone in the medium producing an alkaline reaction (a deeper purple) around the colonies (see appendix II; Figure 3) (Quinn *et al.*, 2002)

## 3.7. **Antimicrobial Susceptibility Test**

The isolate of *S. aureus*, *S. hyicus*, and *S. intermedius* (totally n=50) were randomly selected and subjected to 12 Antimicrobial susceptibility disks. The lists of Antimicrobial disks (Oxoid, Basing Stoke, UK) utilized were Erythromycin, Pencillin, Steptomycin, Tetracycline, Sulphamethoxazole-Trimethoprim, Vancomycin, Ciprofloxacin, Nitrofuranton, Amoxacylin, Nalidixic Acid, Doxycycline and Ampicillin. The method applied for antimicrobial testing was antibiotic disk diffusion method, using Kirby Bauer technique. About 2-3 pure colonies of the isolates was taken from the Nutrient agar and suspended in Tryptose soya broth and then, incubated at 37°C for 1-2hrs. The suspension was then checked for the development of slight turbidity, against 0.5 MacFarland solutions (which is prepared by mixing 1% BaCl<sub>2</sub> and 1% H<sub>2</sub>SO<sub>4</sub>, with a different proportion, and

by taking the concentration, which is estimated to contain equivalent to about,  $10^4$  to  $10^6$  organisms/mille liter). It was inoculated, by dipping a sterile cotton swab into it, and wiping on the Muller Hinton agar, according to the standard procedure (CLSI, 2014) and then the antimicrobial discs were firmly placed on it and the plates were incubated at  $37^\circ\text{C}$  for 24 hrs (see appendix IV).

After the final incubation time, the zone of inhibition around each disc was measured, with the help of a caliper and the results were interpreted as sensitive, intermediate and resistant using a standard zone of interpretative chart (CLSI, 2014).

### **3.8. Permission and Ethical Consideration**

Ethical approval was taken from the College of Veterinary Medicine and Agriculture committee for sample collection.

### **3.9. Data Collection**

During sample collection data regard to the animal identifiers, the farm condition and management were collected through interview and personal observation. Information likes presence of site, breed, type of farm, age, parity, lactation stage, blind teat, teat lesion, udder tick infestation, floor type, previous udder treatment, usage of towels for cleaning and drying udder before milking and udder washing before milking were gathered and considered as risk factors (Table 13).

### **3.10. Data Management and Analysis**

Ms- excel spread sheet program was used for raw data storage and the analysis was carried out by STAT Version 11 (Stata Corp., 2009). Descriptive statistics was conducted to summarize the raw data. The results were presented using table and graphs. Chi- Square test was used to check the presence of association between risk factors and isolation of *Staphylococcus* species. The significance level was adjusted at  $\alpha= 0.05$ .

## 4. RESULTS

### 4.1. Isolation and Identification of *Staphylococcus* Species

Out of 339 samples collected from small holder and dairy farms, 247 (72.9%) *Staphylococcus* isolates were identified. The prevalence of *Staphylococcus* species in milk was 74.8% (101/135) (Table 5).

Table 5: Isolate of *Staphylococcus* species derived from milk, swabs of udder, milking utensils and milkers' hands.

Sample type	Positive isolates	Total samples	Prevalence (%)
Milk	101	135	74.8
Swabs of milking utensils	30	44	68.2
Udder swabs	98	135	72.6
Milkers' hands swabs	18	25	72
Total	247	339	72.9

$\chi^2 = 0.7621$  P- value = 0.859

Each of samples was examined with primary and secondary biochemical tests and species of *Staphylococcus* distributed in all samples were categorized into four. They are *S. aureus* with a total of 47 isolates (13.9%); the second, third and fourth were represented respectively by *S. intermedius* with 21 isolates (6.2%), *S. hyicus* with 40 isolates (11.7%) and Coagulase Negative *Staphylococci* (CNS) with 139 isolates (41%) isolates (Table 6). There no statistically associated between species of *Staphylococcus* identified and type of samples.

In the present study, the prevalence of *S. aureus*, *S.intermedius*, *S.hyicus* and CNS in fresh raw milk was 12.6%, 5.9%, 16.2 % and 40% respectively. The percentage of *S. aureus* found to be contaminated the udders, milking utensils and milkers' hands were 15.5%,

9%, and 20% respectively. The number of *S. intermedius* isolate identified from swabs of udder and milking utensils were 9 and 4 respectively. No isolate of *S. intermedius* were found from swabs of milkers' hands. In addition, 9, 6, and 3 isolates of *S. hyicus* were identified from swabs of udder, milking utensils and swabs of milkers' hands respectively. The percentage of CNS identified was high when compared to other Coagulase Positive *Staphylococcus* species that was found from milk (40%), udder swabs (43.7%), swabs of utensils (36.6%), and swabs of milkers' hand (40%) (Table 6).

Table 6: Proportion of *Staphylococcus* species isolated from various samples.

Sample Type	Identified <i>Staphylococcus</i> Species								Total isolates
	CNS		<i>S. aureus</i>		<i>S. intermedius</i>		<i>S. hyicus</i>		
	Isolate	%	Isolate	%	Isolate	%	Isolate	%	
Milk	54	40	17	12.6	8	5.9	22	16.2	101
Udder Swabs	59	43.7	21	15.5	9	6.6	9	6.6	98
Swabs Of Milking Utensils	16	36.6	4	9	4	9	6	13.6	30
Swabs of Milkers' Hands	10	40	5	20	0	0	3	12	18
Total isolates	139	41	47	13	21	6.2	40	11.7	247

$$\chi^2 = 10.567 \quad P\text{-value} = 0.566$$

#### 4.2. Prevalence of *Staphylococcus* in Small Holder and Dairy Farms

As it is indicated on Table 7 slight difference was observed on the prevalence of staphylococcus species isolation between small holder and dairy farms but it was not significant variation ( $P > 0.05$ ).

Table 7: Percentage of *Staphylococcus* species identified from milk, swabs of udders, milkers' hands and milking utensils from small holder and dairy farms.

Type of farms			Sample type				Total isolates
			Milk	Udder swabs	Swabs of utensils	Swabs of milker hands	
Small holder farms	Results (%)	CNS	36.4	45.5	20	40	37.5
		<i>S.aureus</i>	18.2	18.2	0	20	15.6
		<i>S. intermedius</i>	9.1	0	20	0	6.3
		<i>S.hyicus</i>	18.2	9.1	20	20	15.6
		Total isolates	81.8	72.3	60	80	75
Dairy farms	Results (%)	CNS	40.3	43.5	38.5	40	41.4
		<i>S.aureus</i>	12.1	15.3	10.3	20	13.6
		<i>S. intermedius</i>	5.6	7.25	7.7	0	6.2
		<i>S.hyicus</i>	16.1	6.45	12.8	10	11.4
		Total isolates	74.2	72.5	69.4	70	72.6

$\chi^2 = 10.567$  P- value = 0.57

#### 4.3. Association between Risk Factors and *Staphylococcus* Isolation

The Chi square test showed that three variables were found to be significantly associated with the isolation of *Staphylococcus* as it is indicated on Table 8. The prevalence of *Staphylococcus* species in milk were statistically significant variation with respect to parity (P= 0.036), usage of towels (P=0.006) and previous treatment (P= 0.022)

Table 8: The association between risk factors and isolation of *Staphylococcus* species in milk

Risk factors		Positive isolates	Total sample	Prevalence	95% CI	$\chi^2$	P- value
Site	Kebele 01	21	29	72.4	69.3 - 75.5	17.38	0.136
	Kebele 02	33	40	82.5	79.7- 85.3		
	Kebele 03	20	29	69.0	65.9-72.0		
	Guder	27	37	73.0	70.2-75.7		
Breed	Local	38	46	82.6	80.0-85.2	3.87	0.424
	Cross	63	89	70.8	69.0-72.5		
Type of Farm	Small holder	9	11	81.8	76.5-87.2	0.79	0.939
	Dairy farms	92	124	74.2	72.7-75.7		
Age (years)	<3	53	71	74.6	72.6-76.7	10.42	0.237
	3-6	42	58	72.4	70.2-74.6		
	>6	6	6	100.0	92.0-108.0		
Parity	1-2	63	86	73.3	71.4-75.1	16.51	0.036
	3-4	30	40	75.0	72.3-77.7		
	>4	8	9	88.9	82.7-95.0		
Lactation Stage (months)	<3	35	42	83.3	80.6-86.1	4.57	0.802
	3-6	44	61	72.1	70.0-74.3		
	>6	22	32	68.8	65.9-71.6		
Blind Teat	No	88	115	76.5	74.9-78.1	2.57	0.632
	Yes	13	20	65.0	61.5-68.5		
Teat Lesion	No	91	118	77.1	75.5-78.7	4.61	0.329
	Yes	10	17	58.8	55.2-62.5		
Tick Infestation	No	53	71	74.6	72.6-76.7	1.09	0.396
	Yes	48	64	75.0	72.9-77.1		
Floor Type	Muddy	31	41	75.6	72.9-78.3	2.02	0.732
	Concrete	70	94	74.5	72.7-76.2		
Previous Treatment	No	87	109	79.8	78.1-81.5	11.41	0.022
	Yes	14	26	53.8	51.0-56.7		
Usage of Towels	No	63	83	75.9	74.0-77.8	11.30	0.006
	Yes	38	52	73.1	70.8-75.4		
Udder Washing	No	52	69	75.4	73.3-77.4	4.77	0.311
	Yes	49	66	74.2	72.2-76.3		

#### 4.4. Antimicrobial Susceptibility Profiles of *Staphylococcus* Species

Out of 108 *Staphylococcus* species identified, 50 isolates were subjected to antibiotic susceptibility tests (Table 9). Ampicillin, Penicillin and Amoxacylin were drugs to which

a large proportion of *S. aureus* isolates were resistant. As it is indicated in Table 9, most isolates (90% to 100%) were resistant to these three drugs. *S. aureus* isolates were also slightly resistant to Nalidixic Acid (45%), Tetracycline (40%), and Doxycycline (20%).

*S. intermedius* isolates were found to be highly resistant to most antimicrobials. From the total of 10 *S. intermedius* isolates 100% were resistant to Ampicillin, 70% to Nalidixic Acid, 70% to Penicillin, 50% to Vancomycin and 10% to Amoxacylin. The resistance level of 20 *S. hyicus* isolates were 90% to Ampicillin, 85% to Penicillin, 60% to Amoxacylin, 15% to Streptomycin, 15% to Tetracycline, 10% to Nitrofurantion, and 5% to Nalidixic Acid. All 50 tested species of *Staphylococcus* were susceptible 100% to Ciprofloxacin.

#### **4.5. Multidrug resistance of *Staphylococcus* Species**

The present study indicated that 94% multidrug resistant Coagulase Positive *Staphylococcus* species isolated from milk of lactating cows, from swabs of udder, milking utensils and milkers hands. All tested isolates of *S. aureus* were resistant to Penicillin and Ampicillin. Two isolates of *S.aureus* were resistant to six drugs (Tetracycline, Penicillin, Amoxacylin, Nalidixic Acid, Doxycycline and Ampicillin). On the contrary, all 20 tested isolates of *S.aureus* were susceptible to Ciprofloxacin, Streptomycin and Sulphametoxazole-Trimethoprim. A total of 20 tested isolates of *S.hyicus* were susceptible to Doxycycline and Ciprofloxacin. One isolate of *S.hyicus* was found to be resistant to Tetracycline, Penicillin, Amoxacillin, Streptomycin and Ampicillin. All isolates of *S.intermedius* tested were susceptible to Ciprofloxacin and Sulphametoxazole-Trimethoprim, but all of 10 tested isolates were resistant to Ampicillin.

Table 9: Antimicrobial susceptibility profiles of 20, 10, and 20 isolates of *S. aureus*, *S. intermedius* and *S. hyicus*, respectively.

Antibiotics	Species	Susceptible		Intermediate		Resistant	
		No	%	No	%	No	%
Amoxicillin	<i>S. aureus</i>	0	0	2	10	18	90
	<i>S. intermedius</i>	9	90	0	0	1	10
	<i>S. hyicus</i>	5	25	3	15	12	60
Ampicillin	<i>S. aureus</i>	0	0	0	0	20	100
	<i>S. intermedius</i>	0	0	0	0	10	100
	<i>S. hyicus</i>	2	10	0	0	18	90
Ciprofloxacin	<i>S. aureus</i>	20	100	0	0	0	0
	<i>S. intermedius</i>	10	100	0	0	0	0
	<i>S. hyicus</i>	20	100	0	0	0	0
Doxycycline	<i>S. aureus</i>	13	65	3	15	4	20
	<i>S. intermedius</i>	8	80	2	20	0	0
	<i>S. hyicus</i>	20	100	0	0	0	0
Erythromycin	<i>S. aureus</i>	16	80	4	20	0	0
	<i>S. intermedius</i>	7	70	3	30	0	0
	<i>S. hyicus</i>	15	75	5	25	0	0
Nalidixic Acid	<i>S. aureus</i>	7	35	4	20	9	45
	<i>S. intermedius</i>	3	30	0	0	7	70
	<i>S. hyicus</i>	6	30	13	65	1	5
Nitrofurantion	<i>S. aureus</i>	13	65	7	35	0	0
	<i>S. intermedius</i>	7	70	3	30	0	0
	<i>S. hyicus</i>	16	80	2	10	2	10
Penicillin	<i>S. aureus</i>	0	0	0	0	20	100
	<i>S. intermedius</i>	3	30	0	0	7	70
	<i>S. hyicus</i>	3	15	0	0	17	85
Streptomycin	<i>S. aureus</i>	20	100	0	0	0	0
	<i>S. intermedius</i>	7	70	3	30	0	0
	<i>S. hyicus</i>	17	85	0	0	3	15
Sulphamethoxazole -trimethoprim	<i>S. aureus</i>	20	100	0	0	0	0
	<i>S. intermedius</i>	10	100	0	0	0	0
	<i>S. hyicus</i>	18	90	2	10	0	0
Tetracycline	<i>S. aureus</i>	12	60	0	0	8	40
	<i>S. intermedius</i>	10	100	0	0	0	0
	<i>S. hyicus</i>	17	85	0	0	3	15
Vancomycin	<i>S. aureus</i>	20	100	0	0	0	0
	<i>S. intermedius</i>	1	10	4	40	5	50
	<i>S. hyicus</i>	17	85	3	15	0	0

## 5. DISCUSSION

### 5.1. Overall Prevalence of *Staphylococcus* Species

Cow's milk may be contaminated from different sources and at different processes. Milk that is contaminated by animals, airborne dust or droplets at the site of production and during processing presents a health hazard. It may be contaminated from the cow itself, from air/ dust, unclean milk containers and the milk handlers. Milk can be contaminated by microorganisms directly from the milk handlers who have direct or indirect contact with the milk especially if these persons are in the process of shedding pathogenic organisms. 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).

The present study showed that 247 *Staphylococci* isolates were detected out of 339 sample collected; 101 (40.8%) originated from raw fresh cows' milk, 30 (12.4%) milking utensils, 98 (39.7%) swabs of udder and 18 (7.3%) swabs of milkers' hands. Gizaw, (2014) reported a prevalence of 55.1% *Staphylococcus* from milk that agrees with the present study and 44.9% of prevalence from container and human swabs which disagrees with the current study. The variation in prevalence of *Staphylococcus* reported from milking utensils may be usage of disinfectant when cleaning utensils, size of the sample and study methods designed.

The prevalence of *S. aureus* in milk was 13.9%. This finding is similar with the study done by Mekuria *et al.* (2013) who reported 15.5 % and 17.2% by reported by Gizaw, (2014) who isolated *S. aureus* strains from human and animal sources in and around Addis Ababa, Ethiopia. However, the present findings were lower than that of Bendahou *et al.* (2008) who reported 40% *S. aureus* isolates from milk and milk products at North Morocco. This finding is also in contrast with findings of Jahan *et al.* (2015) who reported 25.5% *S. aureus* from raw milk. In the current study, milk samples were collected directly

from cows' udder before contacting milking utensils that might decrease the prevalence of *S aureus*.

The study revealed that 6.2% *S. intermedius* and 11.7% *S. hycius* isolates were identified from all samples (out of 339). This result agrees with the study done by Gizaw, (2014), who reported 7.4% of *S. intermedius* and 8.2% *S. hycius* from milk and human origin samples. The current finding is also similar with study of Bendahou *et al.* (2008) who reported 2% *S. intermedius* and 4 % *S. hycius* in dairy cows. This finding also shares with Mokennen *et al.* (2011) study, who reported the prevalence of 4% *S. hycius*.

The current study showed that 41% CNS were isolated from milk, swabs sample of udder, milking utensils and milkers' hands. This finding is closely comparable with the findings of Bendahou *et al.* (2008) who reported 54% CNS prevalence from milk and milk products in North Morocco.

Out of 44 swabs of milking utensils of a dairy farm, 4 (9%) isolates were found to be *S. aureus*. The study conducted by Pramar *et al.*, (2014), reported high prevalence 18.8% of *S. aureus* which were isolated from swabs of milking equipments of a dairy farm. The dissimilarity of prevalence of *Staphylococcus* isolation probable the hygienic status of equipment sampled of present the study was better than the previous study. A total of 25 swabs from milkers' hands were collected and 20% (5/25) were found to be *S. aureus*. This finding is higher when it is compared with a study conducted by Pramar *et al.* (2014), who reported 2.55% prevalence of *S. aureus* from swabs of milkers hands. This variation might be due to small number of sample in the current study.

When prevalence of *Staphylococcus* species in small holder and dairy farms is compared there was slightly higher prevalence of the *Staphylococcus* species in small holder than in dairy farms even though, statistically not significant association. The probable reason for this could be use of towel for cleaning and drying of cows' udder before milking in dairy farms is common which might have lowered the prevalence of *Staphylococcus* species.

## 5.2. Risk factors Associated with Prevalence of *Staphylococcus* Species

Based on observation and interview data collected during the study period in the small and dairy farms it was found that use of hygienic towels for cleaning and drying animals' udders before milking minimized the prevalence of *Staphylococcus* species. High prevalence of *Staphylococcus* species was observed in small holder and dairy farms that do not use hygienic towels for cleaning and drying animals udder than those that use towels for cleaning and drying animal's udder (P= 0.006).

History of previous treatment was considered as a risk factor. Accordingly, prevalence of *Staphylococcus* species has showed significant variation among small holder and dairy farm that had history of previous treatment and those that do not have history of previous treatment (p = 0.022). These finding is similar with the study done by (Mekuria *et al.*, 2013).

It was also found that prevalence of *Staphylococcus* species increases as parity number increases (P =0.036). This could be due to the fact that as the parity number increases there is high degree of contamination of the udder and milk through milking process.

## 5.3. Antimicrobial Susceptibility Profiles of *Staphylococcus* Species

The prevalence and degree of antimicrobial resistance in Veterinary Medicine are increasing worldwide. The dissemination of antimicrobial resistant *Staphylococci* is presenting a challenge to both human and animal health professionals (Al-Thani and Al-Ali, 2012). The antimicrobial susceptibility tests carried out in current study indicated that 100% of *Staphylococcus aureus* were resistant to Ampicillin and Penicillin followed by 90% to Amoxicillin. Comparison of the study done by Begum *et al.* (2007) at Bangladesh revealed that 82.86% of *S. aureus* isolates were resistant to Penicillin-G and 37.14 % to Amoxicillin. Similarly, the study done by Jahan, *et al.* (2015) reported 100% resistance of *S. aureus* to both Penicillin-G and Amoxicillin.

The present study also revealed that *S. aureus* isolates have shown 45% resistance to Nalidixic acid, 40% to Tetracycline and 20% to Doxacycline. This is similar with the findings of Sori *et al.* (2011) who reported 46% of antimicrobial resistance pattern of *S. aureus* to Amoxicillin-clavulanic acid, 87.2% to Penicillin, 92% to Nalidixic acid and 46% to Amoxicillin, but it disagrees with the observation made by Sori *et al.* (2011) in the case of Tetracycline (0%) in dairy farms in Jimma town, Ethiopia. The probable explanation for this could be; *S. aureus* strains have the capacity to change their resistance behavior to the exposed antimicrobials (Quinn *et al.*, 1999). But, Al-Thani and Al-Ali, (2012) study indicated the high resistance of *Staphylococcus aureus* to tetracycline (78.9%) agrees the current study. This is not surprising because penicillin G and tetracycline are the most commonly used antimicrobials for the treatment of infection or mastitis in veterinary practice in Ethiopia. Moreover, penicillin resistance is plasmatic and, it spread out very quickly to several other strains (Mekuria *et al.*, 2013). Similarly, Daka *et al.* (2012) reported 67.9% resistance pattern of strains of *S. aureus* isolates from milk to Penicillin G, 70.9% to Ampicillin, 30.9% to Amoxicillin-Clavulanic acid, and 0% to Ciprofloxacin.

Daka *et al.* (2012) also reported that *S. aureus* isolates were resistant to Erythromycin (32.1%), Trimethoprim-Sulfamethoxazole (7.7%) and Vancomycin (38.5%). On the contrary, the current study indicated that *S. aureus* isolates were susceptible to Erythromycin (100%) Trimethoprim-Sulfamethoxazole(100%) and Vancomycin (100%).

The isolated *S. intermedius* were resistant to Ampicillin (100%), Nalidixic acid (70%) and Penicillin (70%) and susceptible to Ciprofloxacin (100%), Tetracycline (100%) and sulphamethoxazole-trimethoprim (100%). These findings are correlated to Intorre *et al.*, (2007). In this study it was reported that *S. intermedius* was susceptible to Ciprofloxacin (100%).

The present study revealed that 94% multidrug resistant Coagulase Positive (90% of *S. aureus*, 100% of *S. hiycus*, 90% of *S. intermedius*) *Staphylococcus* species isolated from milk of lactating cows, from swabs of udder, milking utensils and milkers hands. This is comparable with findings of Sharma *et al.* (2011) who reported higher prevalence of

multidrug resistant *S. aureus* from raw milk of dairy cattle in India, and found out that 70% of the isolates were resistant to amoxicillin-sulbactam, erythromycin and vancomycin. Pereira *et al.* (2009) showed that 70 to 73% of *S. aureus* strains isolated from various foods were resistant to  $\beta$ -lactam such as Pencillin and Ampicillin.

## 6. CONCLUSION AND RECOMMENDATIONS

The present study showed high prevalence of *Staphylococcus* species in raw milk in Guder and Ambo town. The result of swab samples from the udder of the cow, milkers' hands and storage container showed higher prevalence of *Staphylococcus* species. This indicated possible contamination of milk with *Staphylococcus* species from milkers' hands, milking utensils and udder of the cow. The presence of pathogenic *S. aureus* poses a public health hazard and rise concerns about the safety of these food product. The results obtained in this study also showed that the *Staphylococcus* species isolated from raw milk, swab samples from milking utensils and milker's hands were resistant to common antibiotics that could be a threat to mastitis control and public health. Based on the findings of the present study the following recommendations are forwarded.

- Antiseptics and disinfectant should be encouraged after washing hand and cleaning milking utensils.
- The site of the udder placement enables to harbour the microorganisms from the floor and environment, thus cleaning the udder with antiseptics and drying using clean and separate towels should be preferred.
- Raw milk intended for human consumption must be subjected to pasteurization or heat treatment at least equivalent to pasteurization temperature.
- Further research should be done on the factor favour multidrug resistant *Staphylococcus* species and other risk factors responsible for milk contamination in the study area.

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## 8. APPENDEXS

Table 10: Record sheet used during sample collection and processing

Serial Number	Sample type	Sample code	Name of the farm	Site	Date of collection	Date of culturing on BAP	Growth on BAP	Hemolysis on BAP	Pigmentation on BAP	Pigmentation on NAP	Catalase test	Oxidase test	Gram stain	Growth on MSA	Coagulase test	Growth on PAP	Glucose Fermentation	Voges-Proskauer test	Results	
1.																				

### Appendix I: Procedures for Collecting Milk Samples

1. Label tubes prior to sampling (date, farm, and cow identity).
2. Brush loose dirt, bedding, and hair from the udder and teats.
3. Thoroughly wash and dry grossly dirty teats and udders before proceeding with sample collection. Udders should be washed as a last resort.
4. Discard several streams of milk from the teat (strict foremilk) and observe milk and mammary quarters for signs of clinical mastitis. Record all observations of clinical signs.
5. Dip all quarters in an effective premilking teat disinfectant and allow at least 30 seconds contact time.
6. Dry teats thoroughly with an individual towel.
7. Beginning with teats on the far side of the udder, scrub teat ends vigorously (10 to 15 seconds) with cotton balls or gauze pledgets moist (not dripping wet) with 70% alcohol.
8. Teat ends should be scrubbed until no more dirt appears on the swab or is visible on the teat end.

9. A single cotton ball or alcohol swab should not be used on more than one teat. Take care not to touch clean teat ends. Avoid clean teats coming into contact with dirty tail switches, feet, and legs. In herds where cows are not cooperative, begin by scrubbing the nearest teat until clean, obtain the sample, and move to the next teat.
10. Begin sample collection from the closest teat and move to teats on the far side of the udder. Remove the cap from the tube or vial but do not set the cap down or touch the inner surface of the cap. Always keep the open end of the cap facing downward. Maintain the tube or vial at approximately a 45 degree angle while taking the sample. Do not allow the lip of the sample tube to touch the teat end. Collect one to three streams of milk and immediately replace and tightly secure the cap. Do not overfill tubes, especially if samples are to be frozen.
11. To collect a composite sample (milk from all four quarters in the same tube), begin sample collection with the nearest teats and progress to the teats on the far side of the udder. One to 2 ml of milk should be collected from each quarter of the udder.
12. Store samples immediately on ice or in some form of refrigeration. Samples to be cultured at a later date (more than 48 hours) should be frozen immediately.

Source: (NMC, 2004)

Appendix II: Media ingredients and its preparation as manufacturer instructions used in the present study.

Blood Agar (Oxoid England)

Typical formula (g/l):

., Lab-Lemco <sup>®</sup> powder	10.0
Peptone	10.0
Sodium chloride	5.0
Agar	15.0
Final PH 7.3 + 0.2 at 25 °C	

Instructions:

Suspend 40g in 1L of demineralized (distilled) water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121 OC for 15 minutes. Cool to 45-50 °C and add 7% sterile defibrinated blood.

Nutrient Agar (Oxoid, England)

Compositions:

Typical formula (g/l)

Lab-Lemco“powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Agar	15.0
Final PH 7.4 + 0.2 at 25 °C	

Instructions:

Suspend 28g in 1L of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121 OC for 15 minutes.

Mannitol Salt Agar (Oxoid, England)

Ingredients	Units
„Lab-Lemco“powder	1.0
Peptone	10.0
Mannitol	10.0
Sodium chloride	75.0
Phenol Red	0.025
Agar	15.0
Final PH 7.5 + 0.2 at 25°C	

Instructions:

Suspend 111g in 1L distilled water and bring to the boil to dissolve completely. Sterilize by autoclaving at 121OC for 15 minutes. Mix well before pouring into sterile Petri dishes.

## Purple Agar Base (Difco, France)

### Compositions:

#### Typical formula (g/l):

Proteose peptone	10.0
Beef extract	1.0
Sodium chloride	5.0
Bromcresol Purple	0.02
Agar	15
Final PH 6.8 + 0.2 at 25 °C	

#### Instructions:

Suspend 31g of the powder in 1L of purified water. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to dissolve the powder. Autoclave at 121°C for 15 minutes. When preparing 0.5-1% carbohydrate fermentation, dissolve 5-10g of the desired carbohydrate in the basal medium prior to sterilization by autoclaving.

## Brain Heart Infusion

Ingredients	Units
Pancreatic digest casein	14.5g
Agar	5g
Brain Heart Solids from infusion	8g
Peptic digest of Animal Tissue	5g
Sodium chloride	5g
Dextrose	2g
Sodium Phosphate Dibasic	2.5g
PH 7.4±0.2 at 25 OC	
Distilled water 1liter	

#### Instruction

Dissolve 52g in 1000 ml distilled water stir and dissolve completely and sterilized by autoclaving for 15 minutes at 121OC .Cool to room temperature before

Table 11: Primary and secondary biochemical tests used in the study to identify *Staphylococcus* species.

No.	Hemolysis	Pigmentation	Gram stain	Catalase test	Oxidase test	Fermentation on MSA	Tube coagulase test	VP test	Fermentation(1 % maltose using Purple agar)	Results
1	+	+/-	+	+	-	+	+	+	+	<i>S. aureus</i>
2	+	-	+	+	-	+	+	-	+/-	<i>S. intermedius</i>
3	-	-	+	+	-	-	+	-	-	<i>S. hyicus</i>
4	-/+	-/+	+	+	-	+/-	-	+/-	±	CNS

Table 12: The resistance of each antimicrobial was determined depending on the following measure of zone inhibition diameter.

Antimicrobial agents	Disc content in µg	Diameter of zone of inhibition to nearest mm		
		Resistant ≤	Intermediate	Susceptible ≥
Amoxicillin	20	19	-	20
Ampicillin	10	28	-	29
Ciprofloxacin	5	15	16-20	21
Doxycycline	30	12	13-15	16
Erythromycin	15	13	14-22	23
Nalixidic acid	30	13	14-18	19
Nitrofuranton	300	14	15-16	17
Penicillin	10	28	-	29
Streptomycin	10	11	12-14	15
Tetracycline	30	14	15-18	19
Trimethoprim-sulphamethoxazole	25	10	11-15	16
Vancomycin	30	9	10-11	12

Source (CLSI, 2014)

### Appendix III: Biochemical tests and procedures.

#### 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 tap 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 tap water.
7. 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 tap water.
8. Counterstain with Gram's safranin solution or carbolfuchsin (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 be shaken to remove most of the water and air-dried.
10. Examine the finished slide under a microscope (oil immersion objective).
11. Interpretation: Bluish purple colour indicates Gram positive and pinkish colour indicates Gram negative bacteria

#### 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.

#### Procedure for oxidation and fermentation test

1. 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 sterilised tube.
2. 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
3. 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
4. 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.
5. Examine tubes daily for colour change.

#### Interpretation:

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

#### Coagulase test procedure

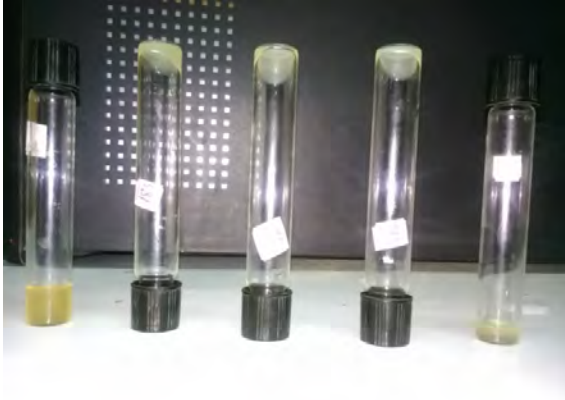
1. Three test tubes are taken and labeled “test”, “negative control” and “positive control”.
2. Each tube is filled with 0.5 ml of 1 in 10 diluted rabbit plasma. To the tube labelled test, 0.1 ml of overnight broth culture of test bacteria is added.
3. To the tube labelled positive control, 0.1 ml of overnight broth culture of known *S. aureus* is added and to the tube labelled negative control, only 0.1 ml of sterile broth is added.
4. 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.
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.

#### Appendix IV: Procedure for the disk diffusion methods

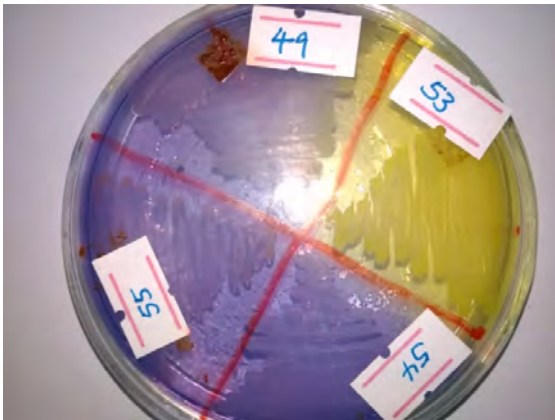
1. At least 4-5 well isolated colonies of the same morphological type are selected from non-selective agar plate and just the top of the colonies are touched.
2. Then a suspension was made in a saline or broth without pre incubation.
3. The turbidity of both suspensions is adjusted by comparison with 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 Muller-Hinton 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.



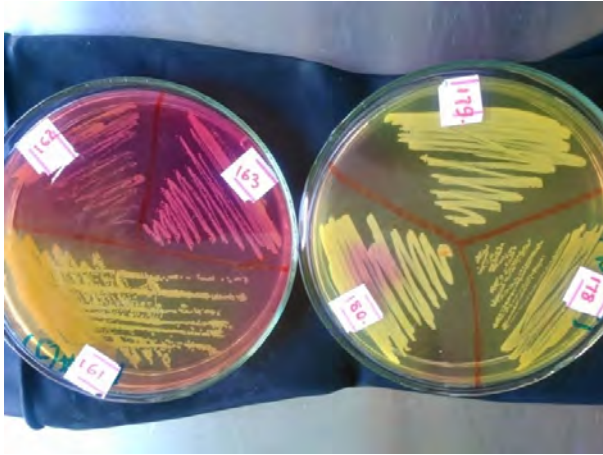
**Figure 2:** Tube coagulase test.

The second, third and fourth test tubes produced heavy clot and stayed there in the test tube while the test tube stood upside down which represents coagulase positive *Staphylococcus* species. But the first and fifth test tube not produced clotting. They are categorized into CNS.

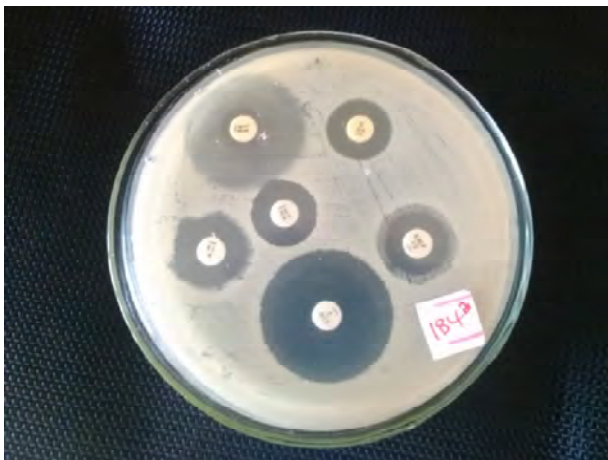


**Figure 3:** *Staphylococcus Species* Colonies grown on purple agar plate

These colonies changed the color of the medium into yellow. Colonies changed purple color into yellow was *S. aureus*.



**Figure 4:** *Staphylococcus* species Colonies grown on Mannitol Salt Agar plate. The yellow colonies fermented the Mannitol and the red ones are non ferment.



**Figure 5:** Plate indicated antibiotic diffusion methods.

Table 13: Data collection format

No		Date
1.	Code _____	
2.	Owner' name _____	
3.	Addresses _____	
4.	ID No. _____	
5.	Type of farms	<ol style="list-style-type: none"> <li>1. Small</li> <li>2. Large</li> </ol>
6.	Breed	<ol style="list-style-type: none"> <li>1. Local</li> <li>2. Cross</li> <li>3. Exotic</li> </ol>
7.	Parity	<ol style="list-style-type: none"> <li>1. 1-3</li> <li>2. 4-7</li> <li>3. &gt;7</li> </ol>
8.	Lactation stage (Months)	<ol style="list-style-type: none"> <li>1. Early (&lt;2)</li> <li>2. Medium (3-6),</li> <li>3. Late (&gt; 6)</li> </ol>
9.	Age (years)	<ol style="list-style-type: none"> <li>1. 3-5 (Young)</li> <li>2. 6-9 (Adults)</li> <li>3. &gt;9 (Old)</li> </ol>
10.	Animal Body condition score	<ol style="list-style-type: none"> <li>1. Good</li> <li>2. Medium</li> <li>3. Poor</li> </ol>
11.	Presence of blind teats	<ol style="list-style-type: none"> <li>1. Yes</li> <li>2. No</li> </ol>
12.	Occurrence of teats lesion	<ol style="list-style-type: none"> <li>1. Yes</li> <li>2. No</li> </ol>
13.	Availability of ticks on the udder and teats	<ol style="list-style-type: none"> <li>1. Yes</li> <li>2. No</li> </ol>
14.	Udder washing before milking	<ol style="list-style-type: none"> <li>1. Yes</li> <li>2. No</li> </ol>
15.	Usage of antiseptic and disinfectant for cleaning during milking	<ol style="list-style-type: none"> <li>1. Yes</li> <li>2. No</li> </ol>
16.	Type of floor in animal house	<ol style="list-style-type: none"> <li>1. Muddy</li> <li>2. Floor (Concrete)</li> </ol>
17.	Type of management system used	<ol style="list-style-type: none"> <li>1. Intensive</li> <li>2. Semi Intensive</li> <li>3. Extensive</li> </ol>
18.	Previous udder treatment	<ol style="list-style-type: none"> <li>1. Yes</li> <li>2. No</li> </ol>
19.	Presence of drain of excretion and water in the house	<ol style="list-style-type: none"> <li>1. Yes</li> <li>2. No</li> </ol>