MILK SAFETY ASSESSMENT, ISOLATION AND ANTIMICROBIAL SUSCEPTIBILITY PROFILE OF *Staphylococcus aureus* IN SELECTED DAIRY FARMS OF MUKATURI AND SULULTA TOWN, OROMIA REGIONAL STATE, ETHIOPIA

M.Sc. THESIS

SEMA REGASA

NOVEMBER, 2018
HARAMAYA UNIVERSITY, ETHIOPIA

Milk Safety Assessment, Isolation and Antimicrobial Susceptibility Profile of *Staphylococcus aureus* in Selected Dairy Farms of Mukaturi and SulultaTown, Oromia Regional state, Ethiopia

A Thesis Submitted to the College of Veterinary Medicine
(School of Graduate Studies)
HARAMAYA UNIVERSITY

In Partial Fulfillments of the Requirements for the Degree of
(MASTER OF SCIENCE IN VETERINARY PUBLIC HEALTH)

Sema Regasa

November, 2018
Haramaya University, Ethiopia

SCHOOL OF GRADUATE STUDIES

HARAMAYA UNIVERSITY

We hereby certify that we have read and evaluated this Thesis entitled**: Milk Safety Assessment, Isolation and Antimicrobial Susceptibility Profile of *Staphylococcus aureus* in Selected Dairy Farms of Mukaturi and Sululta Town, Oromia Regional state, Ethiopia**; prepared under our guidance by **Sema Regasa.** We recommend that it be submitted as fulfilling the Thesis requirement.

Shimelis Mengistu (Assoc. Professor.)	_____	_____
Major Advisor	Signature	Date
Ashebr Abraha (Asst. Professor)	_____	_____
Co-Advisor	Signature	Date

As members of the Board of the Examiners of the M.Sc. Thesis Open Defense Examination, We certify that we have read and evaluated the thesis prepared by Sema Regasa and examined the candidate. We recommend that the thesis be accepted as fulfilling the Thesis requirement for the degree of Master of Science in (Veterinary public health).

_____	_____	_____
Chair Person	Signature	Date
_____	_____	_____
Internal Examiner	Signature	Date
_____	_____	_____
External Examiner	Signature	Date

Final approval and acceptance of the thesis is contingent up on the submission of its final copy to the council of Graduate Studies (CGS) through the candidate's department or school graduate committee (DGC or SGC).

DEDICATION

This thesis is dedicated to my family and friends for their never-ending and overwhelming support through the length of my work. Thank you all for believing in me.

STATEMENT OF AUTHOR

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

This Thesis is submitted in partial fulfillment of the requirements for Veterinary medicine M.Sc. Degree at Haramaya University. The Thesis is deposited in the Haramaya University Library and is made available to borrowers under the rules of the library. I solemnly declare that this Thesis has not been submitted to any other institution anywhere for the award of any academic degree, diploma or certificate.

Brief quotations from this Thesis may be made without special permission provided that accurate and complete acknowledgement of the source is made. Request for permission for extended quotations from or reproduction of this Thesis in whole or in part may be granted by the head of School or Department when in his or her judgment the proposed use of the material is in the interest of scholarship. In all other instances, however, permission must be obtained from the author of the Thesis.

Name: Sema Regasa Signature: _____

Submission Date:_____

Collage of veterinary medicine, Haramaya University

BIBLIOGRAPHY SKETCH

The name of mentioned author Sema Regasa was born on 4thMarch, 1992G.C from his mother Ayelech Tesfaye and his father Regasa Beyene at Werra Jarso Woreda, North Shoa Zone, and Oromia National Regional State, Ethiopia. He attended his Elementary; Secondary and Preparatory School education in Werra Jarso Woreda. Grade (1-4) at Gebagubba Elementary School 2001-2004 G.C, grade (5-8) at Modjo Elementary School 2005-2008 G.C and grade (9-12) at Gohatsion Secondary and preparatory School 2009-2012 G.C.

Then he joined Ambo University at 2013 and graduated with BSc Degree in Veterinary laboratory technology at 2015 G.C. After graduation he was employed as GA in Dilla University since September 2015 up to August 2016 G.C. and at 2017 G.C. He has got the chance to pursue his M.Sc. Study in Veterinary Public Health at Haramaya University.

ACKNOWLEDGEMENTS

First, I like to thank the almighty God for giving me the grace, strength and endurance throughout the length of the study. Secondly, I would like to express my deepest gratitude to my academic advisors Dr. Shimelis Mengistu and Dr. Ashebr Abraha for their invaluable inputs towards the completion of this work.

I would also like to thank the National Veterinary Institute Research Development Director directorate (Dr. Esayas Gelaye) and technicians (Mr. Eyob Aseffa and Mr. Dawit Dufera) for standing with me while laboratory work handling. A big thank you goes to my family, friends and colleagues who have encouraged me through the course of the work.

To all those whom I have not mentioned by name and who, in one way or another, contributed to the success of this work, receive my sincere thanks.

ABBREVIATIONS AND ACRONYMS

BAMO	Bacteriological Analytical Manual Online
BAP	Blood Agar Plate
cfu	colony forming unit
CHP	Centre for Health Protection
CLSI	Clinical laboratory Standard Institute
CSA	Central Statistical Authority
FBD	Food Borne Disease
Fc	fragment crystallizable
FSA	Food Standards Agency
GIT	Gastro Intestinal Tract
IgG	Immunoglobulin G
MOARD	Ministry of Agriculture and Rural Development
MDR	Multi- Drug Resistance
MRSA	Methicillin -Resistant *Staphylococcus aureus*
MSA	Mannitol Salt Agar
MWLPO	Mukaturi Woreda Livestock Production Office
PAB	Purple Agar Base
PH	Power of Hydrogen
RAMS	Risk Assessment Microbiology Section
SDAO	Sululta District Agricultural Office
SE	Staphylococcal Enterotoxin
SFP	Staphylococcal Food Poisoning
SSTI	Skin and Soft Tissue Infections
TSA	Tryptic Soy Agar
UK	United Kingdom
WHO	World Health Organization

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

LIST OF TABLES

ABSTRACT

Milk wished-for human consumption has to be free from potentially harmful bacteria. A cross-sectional study was conducted from November 2017 to June 2018 to assess milk handling practice among dairy farms, to estimating the prevalence of S. aureus in raw milk and swab, to assess associated risk factor of S. aureus and S.aureus load in raw milk, and the antimicrobial susceptibility profile of S.aureus isolates in selected dairy farms of Mukaturi and Sululta Town, Oromia Regional State, Ethiopia. The possible risk factors for S.aureus contaminations in milk were evaluated face to face interviewed 77 respondents through a structured questionnaire randomly base on their intimate to the farm. A total of 247 samples (183 raw milk from lactating cow by simple random sampling technique collected from purposively selected dairy farms and depending on number of worker, frequencies of farm visit and material used 64 swab samples) were examined using standard microbiological techniques. The antimicrobial susceptibility profiles of the isolates were also investigated using disc diffusion method. In the study area with regard to milk handling, 72.1% of respondents (milk consumers) used plastic containers for milk handling meanwhile only 37.2% kept milk in refrigeration before consumption and 60.5% of milk users had habit of raw milk consumption. From milking personnel 47.1% store milk at room temperature temporarily between 6-12 hours till transport to collection center with no means of cooling aid. Overall, 16.6% (n= 41) of the samples were positive for S. aureus. The prevalence of S. aureus was 15.3% from udder milk and 25%, 20% and 10% from milkers' hand, milking bucket and drying towel swab respectively. The prevalence of S.aureus in milk were statistically significant variation with respect to age (p=0.000), parity (P= 0.000) and regarding with drainage condition of milking area (P=0.035), farming area (P=0.035) and management system (P=0.035). The isolates were found to be resistant to penicillin G (97.6%), and Amoxicillin (43.9%). According to this study, 12(42.9%) raw milk samples had $\geq 10^4$cfu/ml S.aureus count, which is above the recommended level for human consumption. The study revealed a prevalence of antimicrobial resistant S. aureus from raw milk cow and swabs, poor milk handling practices, raw milk consumption behavior in study area. Proper handling and hygiene decrease milk contamination by S.aureus and make it safe for human consumption.

Key word: *Staphylococcus aureus, Milk, lactating cow, Antimicrobial susceptibility, Ethiopia.*

1. INTRODUCTION

Milk is considered as nature's single most complete food and is definitely one of the most valuable and regularly consumed foods. But at the same time, it is highly vulnerable to bacterial contamination and hence is easily perishable (Girma *et al.,* 2014). Milk and milk products are considered as the sources of illness associated with milk collection and normal processing conditions that may allow the presence of bacteria in the dairy cows and the dairy environment to be introduced directly into milk. Once introduced, the highly nutritive milk medium supports rapid microbial growth (Thaker *et al.*, 2013).

Milk safety problem is pretty common (Girma *et al.,* 2014). This is especially true in developing countries like Ethiopia where production and consumption of raw milk and various dairy products often takes place under unsatisfactory hygiene conditions (Wubete, 2004). The safety of raw milk and raw milk products with respect to staphylococcal poisoning is of great concern around the world. Milk can be contaminated by *Staphylococcus aureus* when there is infection of the mammary gland. In addition, it can be contaminated during or after milking by poor hygienic practices, such as improper washing of hands when handling milk storage equipment and coughing or sneezing. In this case, human activity is responsible for the contamination, as these bacteria colonize the nasal pathways in human beings. Improper cleaning of storage and preparation areas and unclean utensils are also responsible for contamination of raw foods (Singh and Prakash, 2010).

Staphylococcus aureus is a gram positive organism that serves as an opportunistic pathogen and frequent colonizer of the epithelium causing severe diseases in human and animals. Due to its zoonotic potential, control of *S. aureus* is not only of great economic importance in the dairy industry but also a significant public health concern (Kümmel *et al.,* 2016).In livestock *S. aureus* is an important cause of mastitis, skin and soft tissue infections (SSTI) and to lesser extent infections of the locomotors system (Girma *et al.,* 2014).

In human, *Staphylococcus aureus* is a leading cause of gastroenteritis resulting from the consumption of contaminated food. Staphylococcal food poisoning is due to the absorption of Staphylococcal enterotoxins preformed in the food. Pathogenic staphylococci are commonly associated with skin lesions, infected lacerations; boil the presence, with an incidence ranging

10-20% of the pustules that are common in farm workers. The most common symptoms are nausea, vomiting, retching, diarrhea, abdominal cramping, and prostration (CHP, 2011; Thaker *et al.*, 2013). In severe cases, patients may present with headache, muscle cramping, severe fluid and electrolytes loss with weakness and low blood pressure or shock. Patients usually recover within two days, but can take longer in severe cases that may require hospitalization. Death following a case of staphylococcal food poisoning is very rare and may occur among the elderly, infants, and severely debilitated persons (CHP, 2011).

Higher prevalence of antimicrobial resistant *S. aureus* was isolated in the dairy farms of highly condensed cows with poor milking hygiene and poor environmental hygiene (Amanu *et al.,* 2016). Now days *S. aureus* strains have developed resistant to the penicillin drugs and resistant to all β-lactam drugs. There is no effective long-term decolonization therapy for *S. aureus* carrier (Mekonnen, 2015).It has been reported that, *Staphylococcus aureus* isolates showed multiple resistant to various antimicrobial agents. The indiscriminate use of those antimicrobial agents might account, at least in part, for such a high resistance (Fikru, 2014).

In Ethiopia, the number of intensive and semi intensive dairy farms have been increasing from time to time due to urbanization, increased human population and income growth. However, the management practices of these dairy farms remained traditional (Teshome, 2016). Moreover, in traditional practice the status of cleanliness of the milker, udder of the cow, milking environment and the milking equipment could be the chief source of initial milk contamination and there is lack of standard hygienic condition followed by producers during milk production. Hygienic control of milk and milk products in Ethiopia is not usually conducted on regular bases (Melese and Addisu, 2015).

The hygienic conditions are different according to the production systems, 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 wash the udder before milk, milking equipment and hands (Teshome *et al.,* 2014). Reports indicated that, 98% of the yearly milk is produced by survival 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 (Yilma *et al.,* 2007).

Many foods support growth of *S. aureus* and toxin production; however, milk, dairy products and meats are common vehicles and are probably the most frequently implicated which play an important role in staphylococcus food poison (Smith, 2007). Utensil and milker hand is recognized as the most likely source of contamination of dairy products with *S. aureus* (Girma *et al.,* 2014).

The distribution and higher prevalence of *Staphylococcus aureus* according to the study conducted at different parts of Ethiopia indicates lack of proper personal, environmental hygiene and sanitation; and animal husbandry practices. Fikru, (2014) reported 17.2% from farm and abattoir samples at central Ethiopia, Lencho, (2015) reported 13.9% from farm samples (milk; udder, hand and utensil swab) at Ambo and Guder town and Ayele *et al.,* (2017) reported 19.6% at farm level and 80% at milk collection center at Sebeta. Milk, equipment and hand of human might contain resistant *Staphylococcus aureus* posing a potential risk to consumers. A study on the prevalence of *Staphylococcus aureus* and risk factor contributing for contamination of milk in dairy farm is limited in the study area. Thus, there is a need for study on the status of *S. aureus* and milk handling practice in the study area so as to forward the possible management options for *Staphylococcus aureus*.

Therefore, the objectives of this study were:-

- ✓ To assess milk handling practice among selected dairy farms
- ✓ To estimate the prevalence of *S aureus* in milk of dairy cow and swabs from different contact surfaces
- ✓ To assess associated risk factors for *S aureus* in dairy cow and *S. aureus* load in raw milk sample
- ✓ To determine the antimicrobial susceptibility profile of *S aureus* isolates in the study area

2. LITERATURE REVIEW

2.1. Overview of Milk Safety

2.1.1. Handling practice affecting hygiene of milk

Milk is a well-known medium that favours the growth of several microorganisms. Even if milk produced from mammary gland of healthy mammals is sterile fluid, contamination of microbes starts from udder of milking animal, poor milking practice, milking environment (contaminated air, excreta of animals), poor handling practices (lack of treatment like cooling with refrigerator, appropriate heating) and lack of cold chain transportation and storage system until it is ready for consumption (Teshome, 2016).

The equipment used for milking, processing and storage determine the quality of milk and milk products. The left-over of milk and other dirt particles within the container may result in the contamination of milk (Teshome *et al*., 2014). Diseased lactating animals may show increased shedding of pathogens directly into raw milk or through their feces which may contaminate the production and milking environment. Infected animals with no signs of disease (asymptomatic carriers) may also harbor and shed pathogens, often intermittently, into milk. On the other hand, intensive housing practices may increase the risk of udders contamination as a result of high stocking density, concentration of waste, stress, soiled bedding and feces contamination. Contaminated or poorly prepared feed and poor nutritional practices increase fecal shedding of pathogens that affect scouring area (RAMS, 2009).

Contaminated water used for stock drinking, udder washing and cleaning increases risk of environmental contamination. Poor milking practices, such as milking of dirty, chapped or cracked teats, inadequate maintenance of milking equipment and poor personnel hygiene can lead to contamination of raw milk. Inappropriate temperature control of raw milk after milking and inappropriate temperature control of milk during delivery can lead to growth of pathogens. Packaging and poor hygiene may contribute to cross-contamination of raw milk (Khan *et al.,* 2008).

2.1.2. Milk safety and its public health significance

Milk safety, from milk hygiene practices points of view can be endangered starting from farm to the mouth of consumers. More importantly, consumers are concerned about the safety of dairy products and the conditions under which they are produced. Raw milk may contain microorganisms pathogenic for man and the contamination can generally occur from three main sources; within the udder, outside the udder, and from the surface of equipment used for milk handling and storage. Pathogenic bacteria may present in raw milk as a direct consequence of udder disease. Total number of organism in milk as disease causative agent in relation to its proper evaluation for consumption is important. It is therefore critically important to ensure high quality raw milk through the production of milk from healthy animals and handling under good hygienic condition (Girma *et al.,* 2014).

Milk and milk products pose a health risk to consumers if it is contaminated by any pathogens and subjected to temperature abuse where these organisms can multiply to high counts and may produce toxins (Melese and Addisu, 2015).

2.1.3. Milk-borne pathogenic microorganisms

Various bacteria may have access to milk and milk products from different sources and cause different types of milk-borne illnesses. Sometimes milk and milk products may carry microorganisms or their toxic metabolites (poisons/toxins). Some of these microorganisms are pathogenic and cause illness to humans while others cause spoilage in milk rendering it unsuitable (unsafe) for human consumption. Bacteria are the leading and common microbial milk safety hazards. Raw milk is inherently dangerous and may contain a whole host of pathogens, including: *Staphylococcus aureus, Campylobacter jejuni, Salmonella species, E. coli, Listeria monocytogenes.* Illnesses caused by these bacteria can be especially problematic for infants, young children, the elderly, pregnant women and the immune compromised (Girma *et al.,* 2014).

Staphylococcal food poisoning is caused by the Staphylococcal enterotoxins (SEs) in the contaminated food. The onset of symptoms depends on susceptibility to the SEs, the amount of contaminated food eaten, the amount of toxin/pathogen in the food ingested and the general

health of the patients (CHP, 2011). With regard to the dose of microbes (cfu/ml), the guideline for the microbial standard level of milk in reference to *Staphylococcus aureus* set various levels of safety, such as good (<20), acceptable (20 to <100), unsatisfactory(100 to $<10^4$)and hazardous ($\geq 10^4$) (Gilbert *et al*., 2000).

2.2. *Staphylococcus aureus* and Its General Characteristics

Staphylococcus aureus is one organism of particular interest in food safety. This facultative anaerobic gram positive bacterium is a major cause of food borne intoxications and outbreaks throughout the world because of its ubiquity and its ability to persist and grow under various conditions (Quinn *et al*., 2005).

The organism has a diameter of 0.5 –1.5 μm and characterized by individual cocci, which divide in more than one plane to form grape-like clusters. It is, non-motile, non-spore forming, and cannot produce endospores but is highly resistant to drying, especially, when associated with organic matter such as blood, pus, and other tissue fluids. They are quite resistant to desiccation and high osmotic conditions. These properties facilitate their survival in the environment and growth in food products. But, Staphylococcal cells are destroyed by heat but if they have already produced enterotoxin in a food, the toxins can survive approved doses of irradiation and some thermal processes, including pasteurization (Melese and Addisu, 2015).
The organisms are able to grow in a wide range of temperatures (7°C to 48°C with an optimum of 30°C to 37°C), pH (4.2 to 9.3, with an optimum of 7.0 to 7.5); and sodium chloride concentrations(up to 15%NaCl). The organism produces catalase and coagulase. The Staphylococcal cell wall is resistant to lysozyme and sensitive to lysostaphin, which specifically cleaves the pentaglycin bridges of Staphylococcus spp. These characteristics enable the bacteria to survive in a wide variety of foods, especially those require manipulation during processing, and including fermented food products like cheeses (Le Loir *et al.,* 2003; CHP, 2011).

2.3. Pathogenesis of *Staphylococcus aureus*

The allocation of Staphylococci as normal flora of domestic animals is maybe the most important epidemiologic factor in Staphylococcal disease. A large number of commonly accepted virulence factors are associated with *S. aureus* but, it is yet to be elucidated which of these are important for infection of the bovine udder. Virulence factors may be divided in three functional categories: Factors that mediate adhesion of bacteria to host cells; those that produce tissue damage; and those that protect the bacteria against the host's immune system and antibiotics and coagulase-negative Staphylococci are normally less virulent and express fewer virulence factors. *S. epidermidis* readily colonizes implanted devices (Baron, 1996).

Staphylococcus aureus bacteria produce various enzymes and toxins that destroy cell membranes and can directly damage milk-producing tissue. Initially, the bacteria damage the tissues lining the teats and gland cisterns within the quarter, which eventually leads to formation of scar tissue. The bacteria then move up into the duct system and establish deep seated pockets of infection in the milk secreting cells. This is followed by the formation of abscesses that wall-off the bacteria to prevent spread but allow the bacteria to avoid detection by the immune system. The abscesses prevent antibiotics from reaching the bacteria and are the primary reason why the response to treatment is poor (Mullarky *et al.*, 2010).

Capsulated *S. aureus* strains are more resistant to phagocytises than non capsulated strains, and allow the bacteria to remain in the infected hosts. *Staphylococcus aureus* produces many virulence factors helps that bacteria in disease development (Todar, 2008).

Table 1. Structural component involved in the pathogenesis of *S. aureus* and their respective recognized functions

Structural component	**Biological activities**
Capsule	Preventing its recognition by receptors on phagocytic cells. Facilitates the adherence of bacteria to host cell.
Protein A	Inhibiting phagocytic engulfment. Surface component which binds Fc (fragment crystallizable) portion of IgG and inhibits opsonization.
Cytoplasmic membrane	Site of biosynthetic and respiratory enzymes.
Peptidoglycan	Osmotic stability. Stimulates production of endogenous pyrogen (endotoxin-like activity) and inhibits phagocytosis.

(Source: Baron 1996; Quinn *et al*., 2002; Todar, 2008)

Table 2. Enzyme involved in pathogenesis of *S. aureus* and their respective recognized functions

Enzyme	**Biological activity**
Coagulase	Conversion of prothrombin to thrombin, which converts fibrinogen to fibrin (clot)
Catalase	Converts Hydrogen peroxide to Oxygen and water
Hyaluronidase	hydrolyses hyaluronic acids
Fibrinolysin/staphylokinase	Degrade fibrin clots by converting plasminogen to the fibrinolytic enzyme plasmin.
Lipases	Break fats down into their fatty acid and glycerol components. Degrades protective fatty acid on skin.
Penicillinase	Cleaves the ß-lactam ring of the penicillin molecule.

(Source: Baron 1996; Todar, 2008)

Table 3. Toxin involved in pathogenesis of *S. aureus* and their respective recognized functions

Toxin	**Biological activity**
Alpha (α),Beta (β) and Leukocidin toxin	Action on erythrocyte in vitro/ haemolysin and membrane-damaging
Toxic shock syndrome toxin-1	toxin mediated effect on T-cell and microphage
Enterotoxins (A-E,G-I)	stimulate release of inflammatory mediators in mast cells
Exfoliative toxin	Cleavage of desmosomes in the stratum granulosum of the epidermis

(Source: Baron 1996; Tondar, 2008)

2.4. Diseases Caused by *Staphylococcus aureus*

Staphylococci are human and animal pathogen, known for their ability to become resistant to antibiotics. *Staphylococcus aureus* is a bacterium present on skin and in mucous membranes in 20-30% of healthy people, and is also carried by a wide range of animals. *S. aureus* is also found on food and can cause food poisoning via the production of enterotoxins in food, where there is a lack of appropriate temperature/time control. In human it cause typically local skin and wound infections but can occasionally cause more severe infections in the body (FSA,2017) and causes superficial skin lesions (boils, styes) and localized abscesses in other sites and deep-seated infections, such as osteomyelitis and endocarditic and more serious skin infections (furunculosis) (Baron, 1996).

Staphylococcus aureus is a pyogenic pathogen known for its capacity to induce abscess formation at sites of both local and metastatic infections. This classic pathologic response to *S. aureus* defines the framework within which the infection can progress. *S. aureus* food poisoning is a self-limiting disease, which usually lasts 24-48 hours and the main symptoms include vomiting and diarrhea. Rarely, *S. aureus* food poisoning can develop into systemic disease, causing symptoms such as fever and hypotension (FSA, 2017).

In livestock *S. aureus* is an important cause of mastitis, skin and soft tissue infections (SSTI) and to lesser extent infections of the locomotors system. In cattle mastitis caused by *S. aureus* can be expressed by wide spectrum of clinical signs, from mild cases without clinical signs to extreme cases with lethal exit. Mastitis caused by *S. aureus* in cattle may vary from the prevalent subclinical form of infection to a severe gangrenous form. Per acute form of infection is often seen as a gangrenous mastitis with lethal exit. Chronic and sub-acute cases are the most common forms and from the herd health point of view they are the most important. In many occasions their clinical feature is characterized by elevation of somatic cells only (Benić *et al*., 2011) and often found in older cows as a low grade, sub clinical mastitis or from small ulcers on the teats (Girma *et al.,* 2014).

2.5. Distribution of *Staphylococcus aureus*

Staphylococcal food poisoning is one of the common causes of the food borne illnesses in many parts of the world. About a quarter of people among the world population carry one or other strain at any one time, and, if they develop an infection, their own colonizing strains are likely to be responsible for such an infection. The broad distribution of Staphylococcus as normal flora of domestic animals is perhaps the most important epidemiologic factor in Staphylococcal disease (CPH, 2011).

The natural ecological niches of *S. aureus* are the nasal cavity and the skin of warm blooded animals. *S. aureus* dissemination on different farms may be due to their closeness and to the community status of the milk refrigerating tank *S. aureus* can be isolated from different body parts of an animal as well as from the environment. Furthermore *S. aureus* can found on the milkers' hands as well as on the nasal mucous membrane of the humans working at the dairy farms, in bedding and the drinkers. The different studies conducted in different part of Ethiopia showed variable prevalence of *S. aureus.* A study conducted by Anagaw *et al*. (2013) showed that 17.6% methicillin resistance *Staphylococcus aureus* were found from urine, eye discharge, genital swab, body fluid, pus, wound swab and discharge samples in northwest of Ethiopia. In line with that, it was suggested that inadequate hygiene condition of dairy, environment, poor milking procedure, poor animal health service and lack of proper attention to health of the mammary gland cause for the contamination of milk and milk products in the

dairy farms (Mekibib *et al.,* 2010). Using routine bacteriological investigation methods, Mekonnen *et al*. (2011) reported that 24% Ethiopian cottage cheese (Ayib) samples were found to harbor Staphylococcus species. In other study, the most prevalent mastitis causing pathogen was Staphylococcus, of which the predominant (47.1%) isolate was hemolytic and coagulase positive *S. aureus* (Mekibib *et al*., 2010). Milk, meat, equipment, hand and nose of human's contain Staphylococci species posing a potential risk to consumers (Fikru, 2014). A study conducted at Asella by Fufa *et al.,* (2016) showed that (11.9%), (11.1%), (33.3%), (11.1%), and (33.3%) *Staphylococcus aureus* were isolated from udder milk, tank milk, tank swab, polled hand and nasal swab sample respectively.

2.6. Sources and Reservoirs of *Staphylococcus aureus*

The Staphylococcus is ubiquitous in nature with humans and animals as the primary reservoirs (Mekonnen, 2015). They are present in the nasal passages and throat, in the hair, and on the skin of probably 50% or more of healthy individuals. Man's respiratory passages, skin and superficial wounds are common sources of *S. aureus*. Staphylococci can be isolated from animals, with the bovine being the most important because of the involvement of Staphylococci in mastitis and contamination might generally occur from three main sources: within the udder, exterior to the udder and from the surface of milk handling and storage equipments, but the surrounding air, feed, soil, feces and grass are also possible sources of contamination (Solomon *et al.,* 2013).

Many foods can support growth of staphylococci and toxin production. While *S. aureus* is allowed to grow in foods, it can produce a toxin that causes illness. Although cooking destroys the bacteria, however the toxin produced is heat stable and may not be destroyed. In most of the time the contaminated foodstuff reaches a temperature that allows *S. aureus* growth because of a failure in the refrigeration process, or because a growth permissive temperature is required during the processing, for instance, cheese making (CHP, 2011).

2.7. Mode of Transmission of *Staphylococcus aureus*

Staphylococci are most often transmitted by direct or indirect contact with a person who has a discharging wound (septic and non-septic lesions), a clinical infection of the respiratory or urinary tract, or one who is colonized with the organism. Milk can act as a vehicle for transmitting the *S. aureus* from animal to human to cause the severe food borne intoxication. It has been recorded that *S. aureus* causes the subclinical mastitis and contaminate the udder and milk; acting as the main source of contaminants. Contaminated milking equipment's and the milker's hands, hands of healthcare personnel and food preparers may be the source of infection (De Oliveira *et al.,* 2011; Fikru, 2014).

Contaminated surfaces and medical equipments are also possible sources of Staphylococci and Staphylococcal food poisoning is the result of the consumption of a heat stable preformed (produced in the food) protein enterotoxin that are produced by certain strains of Staphylococci species. Milk, milk products and meat, especially handled foods, are common vehicles that are frequently implicated in Staphylococcal food poisoning (Smith, 2007). Poor personal hygiene of the food handlers and improper storage of cooked food were identified as the contributing factors (CHP, 2011).

2.8. Public Health and Economic Importance of *Staphylococcus aureus*

Staphylococcal food poisoning is one of the most common food borne diseases in both humans and animals globally, resulting from the ingestion of Staphylococcal enterotoxin preformed in food by strains of coagulase-positive staphylococci, mainly *S. aureus* (Mekonnen, 2015). *Staphylococcus aureus* is a significant cause of FBD, causing an estimated 241,000 illnesses per year in the United States (Kadariya *et al.,* 2014). Globally, an estimated 2 million people died from diarrheal diseases in 2005; approximately 70% of diarrheal diseases are food borne. It is estimated that up to 30% of the population suffer from food borne illnesses each year in some industrialized countries (WHO, 2011).

The presence of milk bacteria resistant to antimicrobial agents is known as an important public-health issue and is mainly related to the treatment of mastitis (Zdolec *et al.,* 2016).

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 and is economically important because of the losses they cause in milk production (Lencho, 2015).

Apart from the food borne intoxication, antibiotic resistance of *S. aureus* against the common antibiotics is the greatest public health issues everywhere. *S. aureus* shows very high resistance against Penicillin, vancomycin, and methicillin compared to other antibiotics (Kitara *et al.,* 2011). Milk from the local vendors with a high percentage of *S. aureus* and its antibiotic resistance may lead to very serious public health issues (Sudhanthiramani *et al*., 2015).

2.9. Antimicrobial Resistance of *Staphylococcus aureus*

Antimicrobial resistant Staphylococcus is major public health concern since the bacteria can be easily circulating in the environment. Methicillin-resistant *Staphylococcus aureus* is any strain of *Staphylococcus aureus* that has developed, through the process of natural selection, resistance to beta-lactam antibiotics, which include the penicillin (methicillin, dicloxacillin, nafcillin, and oxacillin) (Deurenberg and Stobberingh, 2008). It is also called multi-drug resistant *Staphylococcus aureus*. The misuse and extended use of antibiotics in humans, veterinary medicine, and agriculture and in stock farming as growth promoters are some selective pressures that encourage the bacteria to develop antimicrobial drug resistance at an increasing rate (Andremont, 2015). One of the main reasons for the increase environmental multi-drug resistant is the indiscriminate use of antimicrobials during animal husbandry (Fikru, 2014).

2.10. Isolation and Identification of *Staphylococcus aureus*

The isolation and identification of Staphylococcus species is conducted on the basis of colony morphology, hemolytic properties, Gram-stain, catalase production, coagulase production and biochemical profile or sugar fermentation (Quinn *et al.,* 2002). The organism is isolated by streaking material from the clinical specimen or from a blood culture onto the solid media

such as blood agar, tryptic soy agar or heart infusion agar. Then the media were examined for the presence of Staphylococcus colonies based on their morphological aspects (MARD, 2005). Gram staining is differentiates bacteria into two groups; gram positive and gram negative. Hence after the gram staining, the gram positive cells appear as purple and gram negative cells appear as pink and occurring in bunched grapelike irregular clusters presumptive Staphylococcus species (Quinn *et al.,* 2002).

The catalase test is important in distinguishing Streptococci which are catalase-negative from Staphylococci which are catalase positive. The enzyme catalase is present in most cytochrome containing aerobic and facultative anaerobic bacteria. Catalase has one of the highest turnover numbers of all enzymes such that one molecule of catalase can convert millions of molecules of hydrogen peroxide to water and oxygen in a second. Organisms which produce the enzyme break down the hydrogen peroxide, and the resulting O_2 production produces bubbles in the reagent drop, indicating a positive test. Organisms lacking the cytochrome system also lack the catalase enzyme and are unable to break down hydrogen peroxide, into O_2 and water and are catalase negative (Baron, 1996).

Mannitol salt agar (MSA) also distinguishes bacteria based on the ability to ferment the sugar mannitol, the only carbohydrate in the medium. 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*., 2002).

The coagulase tests used were both slide coagulase and tube coagulase tests. The slide coagulation 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 coagulation test. Staphylococci, including *S. schleiferi* and *S.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).

2.11. Detection of *Staphylococcus aureus* in Food Borne Intoxication

Immunological methods the most specific and sensitive tests for the enterotoxins are based on the reactions with specific antibodies. The first tests developed were based on the reaction of the enterotoxin with the specific antibodies in gels to give a precipitin reaction. These were the only laboratory methods available until radio immuno assay was applied, and later the enzyme-linked immunosorbent assay and the reversed passive latex agglutination method were developed. The gel-diffusion methods have been used primarily for the detection of enterotoxin production by staphylococcal strains, although the reversed passive latex agglutination method is used for testing strains for low production of enterotoxin. The radio immuno assay method was used for testing for enterotoxin in foods until the enzyme-linked immunosorbent assay and reversed passive latex agglutination were available (Bergdoll and Lee Wong, 2006).

The detection of enterotoxin in foods requires methods that are sensitive to less than1 ng/g of food. The quantity of enterotoxin present in foods involved in food-poisoning outbreaks may vary from less than 1ng/g to greater than 50ng/g. Although little difficulty is usually encountered in detecting the enterotoxin in foods involved in food poisoning outbreaks, outbreaks do occur in which the amount of enterotoxin is less than 1ng/g such as the case of the 2% chocolate milk. In such instances, the enterotoxin can be detected only by the most sensitive methods. Another situation in which it is essential to use a very sensitive method is in determining the safety of a food for consumption, where it is necessary to use the most sensitive methods available in order to show that no enterotoxin is present. The most important methods used to detect enterotoxins in foods are enzyme-linked immunosorbent assay method, the reversed passive latex agglutination method and screening methods (Bergdoll and Lee Wong, 2006).

2.12. Control of Staphylococcus aureus

Effective methods for preventing Staphylococcal food poison (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. For most patients supportive therapy such as resting and fluid replacement using oral rehydration fluids can be sufficient. Anti-spasmodic and anti-emetics may be considered to help control symptoms of vomiting. For patients who are highly susceptible to severe fluid and electrolytes loss, they may require hospital care and intravenous fluid replacement. Antibiotics are not useful in treating Staphylococcal food poisoning as the toxin is not affected by antibiotics (CHP 2011).

In general, measures such as serving hot meal immediately after cooking, reheating cooked foods thoroughly, rapid refrigeration of cooked foods, good personal hygiene/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 can certainly reduce the incidence of food poisoning outbreaks due to Staphylococcus (Baron, 2007) and also, storing foods at temperature less than 4.4^0C or greater than 60^0C effectively prevents replication of Staphylococcal organisms and significant toxin production (Ash, 2008). On other hand keeping kitchens and food-serving areas clean and sanitized and if food is to be stored longer than two hours, keeping hot foods hot (over 140°F) and cold foods cold (40°F or under) reduce food poisoning outbreaks due to Staphylococcus (Baron, 2007).

3. MATERIALS AND METHODS

3.1. Description of the Study Areas

The study was conducted in Mukaturi and Sululta town, Oromia Regional State, Ethiopia. The study area was selected based on their category of livestock production area, according to the record of dairy development and credit activities.

Mukaturi: Mukaturi is a capital town of Wuchale district located 78 km north-west of Addis Ababa. Agro-ecologically, the district is categorized into three: Dega, Woina-Dega and Kolla constituting 87%, 11% and 2% of the total area of the woreda, respectively. The district is bordered by Yaya Gulale and Debre Libanos in the west and Sululta in the south, Jida in the East and fitche/selale in the north. The district has geographical location of $9^0$18'-$9^0$46'N and $38^0$42'-$39^0$07'E latitudes and longitudes, respectively. The agro-climatic zones of the district are temperate (*"Beda"*) ranging from 2300-3300, accounting for about 77.2%, sub-topical (*"Beda-Dare"*) ranging from 1500-2300 m, accounting for about 20.8% and tropical (*"Gamoji"*) ranges from 500-1500m, accounting for about 2% of the district area. The average annual rain fall and temperature of the study area are about 1000 mm and 25 ^{0}C, respectively. The economy of the district is based on agriculture, which is made of traditional crop farming system and animal husbandry. Agriculture is being the main stay of the district economy. Nowadays, the sector is showing a significant improvement because of better extension services and provision of adequate agricultural inputs. The number of cattle used for milk purpose at North Shoa Zone during 2016 was 236, 808 (CSA, 2017).The district has a total of 94,141 cattle populations of which 26,142 were cows, 12,193 heifers, 12,628 female calves and the remaining 43,178 were male cattle (MWLPO, 2017).

Sululta: Sululta town is one of the towns of Oromia Special Zone surrounding Finfine of Oromia National Regional State. Sululta town is 26 km from Addis Ababa to north and east. Geographically, district is demarcated by Wuchale and Yaya Gulalle district in North, Addis Ababa city and Welmera district in South, Jida and Bereh district in the East and Mulo district in the West direction. The study area is located at 9° 11^0N latitude and 38^0 45° E longitude. The average altitude in the town is 2765m above mean sea level. Agro-ecologically, the district is categorized into three: Dega, Woina-Dega and Kolla constituting 71%, 25.4% and

3.6% of the total area of the district, respectively. The altitude of the district ranges from 2851 to 3700 meters above sea level. The high annual rain fall is 1447mm with mean of 1140mm and minimum of 834mm. In the area the months with high rain fall are July to September with low temperature, whereas the temperature is high in the month between Decembers to march. The farming system of the district is rain-fed and mixed agriculture. Livestock husbandry and crop production are the predominant economic activities and the major source of livelihood in the district. The main farming of the study area is livestock rearing followed by crop production. The livestock feed resource is hay, crop residue and grazing land. The total cattle population in the district is estimated at 224,600 and 15% are cross-breed (SDAO, 2012).

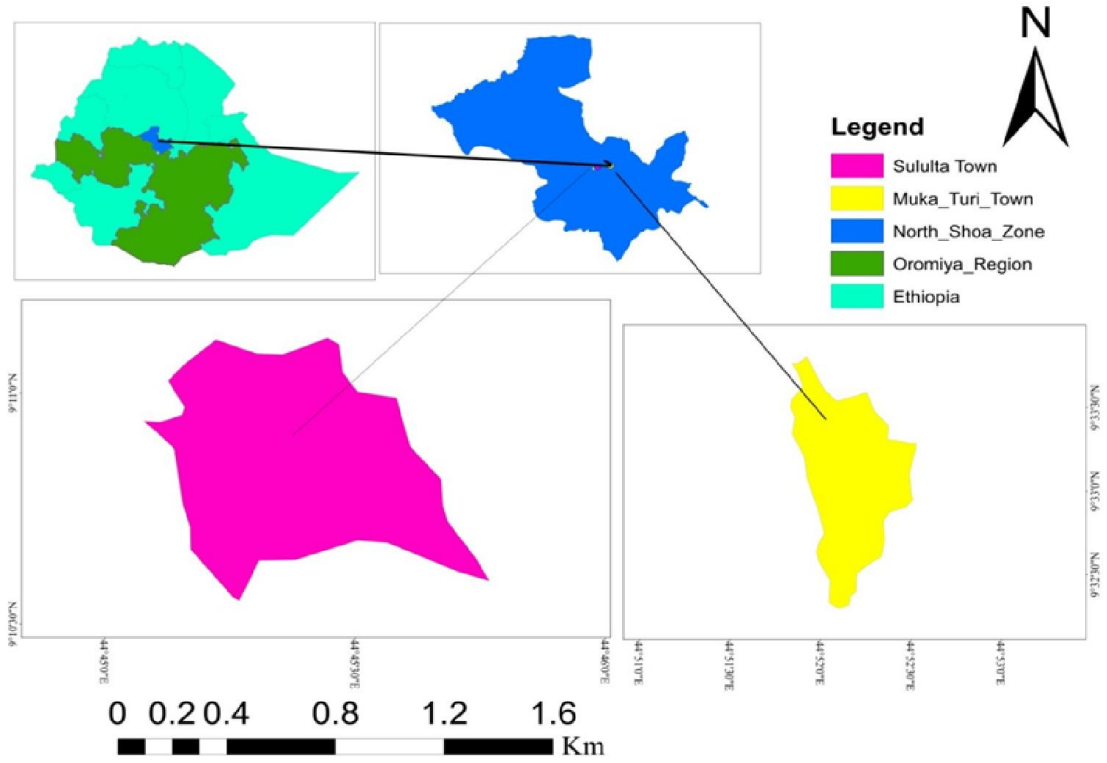


Figure 1: Map of the study area.

3.2. Study Population and Materials

The study population was apparently health cross breed lactating cows in selected dairy farm which were kept under intensive and semi-intensive management system. Age of the study dairy cows were determined from information of cattle birth records kept, transferred with the cattle as they move from one operation to another or from owner and categorized according to Abera *et al.,* (2013) as young (≥ 3 - 5 years), adults (> 6 - ≥ 9 years), and old (> 9 years). Parity was also categorized as few (with 1 - 2 calves), moderate (3 - 4 calves) and many (> 4 calves). Lactation stage was classified as early (< 3 months), medium (3 - 6 months) and late (> 6 months). Animal's body condition score was categorized as poor, moderate and good based on vertebrae at middle of the back, fat deposit behind shoulder and in brisket area, rear view of the hook bone (cross-section), side view of the line between hook and pin bones and cavity between tail head and pin bone (Sharad *et al*., 2016). Drainage conditions of the milking areas were categorized as poor and good from view of accumulated dirty sewage and muddy or properly cleaned area. Milker's who served in dairy farms at selected area were part of the study. In addition to animals, milker's hand, milking bucket and drying towels were parts of the study.

3.3. Study Design and Sample Type

A cross-sectional study was conducted from November, 2017 to June, 2018 to estimate the prevalence of *Staphylococcus aureus* from udder milk and swabs of different contact surfaces as well as to assess milk handling practices and *S.aureus* load in raw milk sample. In addition, antimicrobial susceptibility profile of isolated *Staphylococcus aureus* was performed using standard laboratory methods. Types of samples included were raw milk from cow udder, and swab from milker's hand, milking bucket and drying towel.

3.4. Questionnaire Survey

Structured questionnaire was used face to face interviewed respondents randomly based on their intimate to the farm to collect information on possible risk factors for *Staphylococcus aureus* contaminations in milk. Risk factors considered in the current study was cleaning conditions of the barn/milking environment, hygiene of milking cows' udder and milk

handlers, hygiene of milking equipment with special emphasis to hygiene of milking and milk handling practices, utensils used for milking, milk storage and uses of milk (for selling or domestic purposes). Furthermore, milk consumption behaviors and their awareness on the risk of zoonotic diseases that are associated with the consumption of raw milk was also assessed (Annex 8.1).

3.5. Sampling and Sample Size Determination

The sample size for this study was determined by the following formula given by Thrusfield (2007), accordingly, the sample size 'n' was calculated as:

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

Where 1.96 = the value of Z at 95% confidence interval,

d =desired absolute precision,

n=required sample size, and

P_{exp}=expected prevalence

Therefore, by using the above formula and taking in to account 95% confidence interval, desired absolute precision of 5% and an expected prevalence of 13.9% which was reported by Lencho (2015) from Ambo and Guder Town, which has similar features with the current study area. Based on the above formula totally 183 milk samples were collected by simple random sampling techniques from lactating cows in purposively selected dairy farms. Eight dairy farms were purposively selected based on the availability of one or more lactating animals and willingness of the dairy farm owners to be part of the study. Then 183 lactating cows were selected using simple random sampling techniques after assigning of identification tags for each lactating animals. Depending on number of workers, frequency of visiting the farm and materials they used in the farm, 24 swab samples from milker's hand, 30 swab samples from milking buckets and 10 swab samples from drying towel were collected. Overall, 247 samples were used to isolate and identify *S. aureus*.

3.6. Sample Collection and Transport

According to Tsegalem *et al.,* (2016) twenty five milliliter volume of raw milk sample was collected aseptically from each 183 apparently healthy lactating cows using sterile universal bottles with screw cap followed the procedure of Annex 8.2. The swab samples from milker's hand, milking buckets and drying towel were taken before milking by wipe zigzag over above contact surfaces using moisten sterile swabs in saline solution and thereafter kept in sample bottles containing sterile physiological saline solution to prevent desiccation. All samples were immediately transported using ice box to bacteriology laboratory at National Veterinary Institute, Debre Zeit and the samples were kept at 4ºC for isolation of the target bacteria within 24 hours of collection.

3.7. Laboratory Analysis

3.7.1. Isolation and Identification of *Staphylococcus aureus*

The bacteriological medium used was prepared according to the manufacturer's recommendations and milk samples were subjected to bacterial culture according to the procedures described by Quinn *et al.* (2002). Briefly, a loop full of milk samples and swabs were cultured on blood agar base enriched with 7% sheep blood. The inoculated plates were then incubated aerobically at 37^{o}C for 24 to 48 hours. Then the plates were examined for the presence of Staphylococcus colonies. Colonies characterization was based on their morphological aspects. Thus, colony with morphological features such as circular, golden yellow and white in color and B-hemolysis on blood agar within 24-48 hours were preliminarily considered as *Staphylococcus* colonies. This suspected colonies transferred to nutrient agar plate and incubated at 37^{0}c for 24hr to get pure colonies.

Then primary identification of suspected colonies was performed based on Grams reaction; cellular morphology by followed the procedure (Annex 8.4A) and catalase test (Annex 8.4B). The gram stained smears from typical colonies that showed gram-positive cocci occurring in bunched/grapelike clusters were taken as presumptive *Staphylococcus* species and subjected to catalase test. Catalase positive colonies of Staphylococci were then selected and sub-cultured on mannitol salt agar and incubated aerobically at 37^{0}C for 24-48 hours. The colonies of

Staphylococci which produced a yellow pigment on the media (Annex 8.4C) were subjected to coagulase tests (Annex 8.4D) and cultured on purple base agar (with 1% maltose). Finally, *Staphylococcus aureus* was determined and identified as coagulase-positive; rapidly ferment maltose and change the medium and colonies appear to be yellow in colour (Quinn *et al.,* 2002).

3.7.2. Enumeration of *Staphylococcus aureus* from raw milk samples

Parallel to inoculation on blood agar, according to Aberra, (2010) serial dilutions of milk samples were prepared up to 10^{-6}in normal saline water and from each critical dilution best for countable range one ml sample suspension was aseptically transferred to Baird Parker agar plates followed the procedure mentioned on annex 8.4. The inoculums' was spread over surface of agar plate, using sterile bent glass streaking rod and retain plates in upright position for about ten minutes or until excessive moisture is getting absorbed from the surface of the agar. Then plates were inverted and incubated for 24-48 hrs at 35-37°C. Finally, the plate containing colonies with typical appearance of circular, smooth, convex, moist, and gray to jet-black, frequently with light-colored (off-white) margin, surrounded by opaque zone and frequently with an outer clear zone in the medium was taken as *Staphylococcus aureus.* According to BAMO, (2001) plates that contained 20-200 colonies were selected for *Staphylococcus aureus* count and total *S.aureus* colonies from two consecutive plates of each sample were converted into colony forming units per milliliter (cfu/ml) using a formula given by PHE (2016).

$$N = \frac{\sum C}{V\,(n1 + 0.1\,n2)\,d}$$

Where; N= number of bacterial colonies counted,

C= sum of colonies identified on two consecutive dilution steps, where at least one contained 20 colonies and less than 200 colonies.

n1= is the number of plates counted at the first dilution

n2= is the number of plates counted at the second dilution

V= volume of inoculums on each dish/plate, in milliliter and

d= dilution rate corresponding to the first dilution selected (the initial suspension is a dilution).

3.7.3. Antimicrobial susceptibility test

The antimicrobial susceptibility profile of *Staphylococcus aureus* isolates was performed using disc diffusion method. Briefly, a suspension of *S. aureus* isolates was prepared in 5ml a sterile saline solution (0.85% NaCl) to match with 0.5MacFarland turbidity standard. The suspensions were then swabbed over the entire surface of Mueller Hinton agar (Oxoid) with a sterile cotton swab (Hudzicki, 2009) and kept on the bench until excessive moisture is absorbed in to the media. Then disks containing single concentrations of each antimicrobial agent (Oxoid, Basing Stoke, and UK) were placed onto the inoculated surface and incubation overnight at 37°C. Clear zones of bacterial growth inhibition were measured in mm using a straight line ruler followed the procedure mentioned on annex 8.4. The diameters of growth inhibition zone were interpreting and recorded as susceptible, intermediate, and resistant according to the recommendation given by CLSI (2017) (Annex 8.1.2). Based on their accessibility and habitually uses in humans and veterinary for the susceptibility testing, the following antimicrobial drugs and concentrations were used: Amoxicillin (AMX) (25µg), Ampicillin (AM) (10µg), Pencillin (10µg), Tetracycline (TE) (30µg) and Erythromycin (ER) (15µg).

3.8. Data Management and Analysis

Microsoft Excel 2007 was used for data management and computation of descriptive statistics were applied to compute prevalence of *Staphylococcus aureus,* percentages of antimicrobial susceptibility profile and proportions of questionnaire data by using SPSS version 20 software. Chi- square test (x^2) was used to check the presence of association between risk factors and prevalence of *Staphylococcus aureus.* The significance level was set at $P< 0.05$.

4. RESULTS

4.1. Respondents Knowledge and Practices on Milk Handling and Consumption

The study involved 77 respondents who had milk consumer and who had milking personnel intimate to the farm at Mukaturi and Sululta town. From total respondents 43 were milk consumer and 34 were milking personnel. With regard to school education category, of milk consumers that interviewed 58.1% had educated while 52.9% had no formal educated from milking personnel. With regard to age category, 60.5% and 47.1% of milk consumer and milking personnel respectively falls within the age group of 21-30 years. The majority of the respondents are male (Table 4)

Table 4. Socio demography of respondents

Categorize	Milk consumer			Milking personnel		
	Mukaturi (N=24)	Sululta (N=19)	Total (N=43)	Mukaturi (N=16)	Sululta (N=18)	Total (N=34)
Gender						
Male	18(75%)	8(42.1%)	26(60.5%)	10(62.5%)	16(88.9%)	26(76.5%)
Female	6(25%)	11(57.9%)	17(39.5%)	6(37.5%)	2(11.1%)	8(23.5%)
Age						
18-20	3(12.5%)	2(10.5%)	5(11.6%)	3(18.8%)	8(44.4%)	11(32.4%)
21-30	15(62.5%	11(57.9%)	26(60.5%)	9(56.2%)	7(38.9%)	16(47.1%)
31-40	5(20.8%)	3(15.8%)	8(18.6%)	4(25%)	3(16.7%)	7(20.6%)
41-50	1(4.2%)	3(15.8%)	4(9.3%)	0(0%)	0(0%)	0(0%)
School						
Educated	13(54.2%)	12(63.2%)	25(58.1%)	6(37.5%)	10(55.6%)	16(47.1%)
No formal educated	11(45.8%)	7(36.8%)	18(41.9%)	10(62.5%)	8(44.4%)	18(52.9%)

4.1.1. Hygienic practices of consumer on milk use and milk handling activity

In this study from 43 milk consumer, 37.2%, 48.8% and 14.0% were buy milk from farm, collection center and others area respectively. Of consumer 72.1% had used plastic container to buy or transport milk and 27.9%, 23.3% and 48.8% had kept on milk at home under room temperature before consumption for <2 hours, 2-6 hours and 6-12 hours respectively. While 37.2% preserving the milk at below 4^0c in refrigerator, the rest put at room temperature with no cooling. Additionally, of the consumer 60.5% consume raw milk and 32.6%, 4.7% and 62.8% young children, adult and infant age respectively had show more GIT disturbance associated with drinking of raw milk. From respondent information the occurrence of GIT disturbance associated with drinking of raw milk observed in infant than children and adult age. Of the consumer 25.6% had no any health risk associated with raw milk consumption or aware of milk borne disease associated with drinking raw milk (Table 5).

4.1.2. Hygienic practices of milking personnel during milking and milk handling practice

In the present study from 34 milking personnel, 73.5% respondents wash hand before milking and 85.3% wash between milking. On the other hand, 76.5% washed and dried udder and before milking. However, 64.7% did not wash and dried udder after milking and did not use drying towel separately for udder. Additionally, 76.5% uses plastic for milking and 29.4% uses for milk storage. From milking personnel 55.9% had practiced cleaning of barn once a day and the rests cleaning twice a day. The 47.1% milking personnel had stored milk at home under room temperature before sell or transport to collection center for 6-12 hours and the 35.3% had stored milk at home under room temperature before sell or transport to collection center for <2 hours and while the rests store between 2-6 hours. Additionally, 58.8% practiced washing of milking equipment and storage container with detergents before milking (Table 6)

Table 5. Milk consuming/use and milk handling activity by consumer in the study area

Variables	Mukaturi(N=24)	Sululta(N=19)	Overall(N=43)
Milk buy from			
Direct from farm	7(29.2%)	9(47.4%)	16(37.2%)
from collection	13(54.2%)	8(42.1%)	21(48.8%)
Other	4(16.7%)	2(10.5%)	6(14.0%)
Kinds of containers used			
Plastic	18(75.0%)	13(68.4%)	31(72.1%)
Stainless steel	6(25.0%)	6(31.6%)	12(27.9%)
Milk stay at home prior consumption under room temperature.			
<2 hours	4(16.7%)	8(42.1%)	12(27.9%)
between 2-6 hours	2(8.3%)	8(42.1%)	10(23.3%)
between 6-12 hours	18(75.0%)	3(15.8%)	21(48.8%)
Where you put the milk at home			
with no cooling	17(70.8%)	10(52.6%)	27(62.8%)
in refrigerator	7(29.2%)	9(47.4%)	16(37.2%)
Habit of milk consumption			
Raw	14(58.3%)	12(63.2%)	26(60.5%)
Boiling	10(41.7%)	7(36.8%)	17(39.5%)
Do you mix fresh and left over milk for consumption			
Yes	6(25.0%)	8(42.1%)	14(32.6%)
No	18(75.0%)	11(57.9%)	29(67.4%)
Do you know any health risk associated with raw milk consumption			
Yes	20(83.3%)	12(63.2%)	32(74.4%)
No	4(16.7%)	7(36.8%)	11(25.6%)
Do you know any GIT disturbance associated with drinking of raw milk			
Yes	21(87.5%)	11(57.9%)	32(74.4%)
No	3(12.5%)	8(42.1%)	11(25.6%)
Which age show more GIT disturbance associated with drinking of raw milk			
young children	9(37.5%)	5(26.3%)	14(32.6%)
Adult	1(4.2%)	1(5.3%)	2(4.7%)
Infant	14(58.3%)	13(68.4%)	27(62.8%)
Did you suffer from milk borne infection	8(33.3%)	6(31.6%)	14(32.6%)
Yes	16(66.7%)	13(68.4%)	29(67.4%)
No			

Table 6. Hygienic practices during milking and milk handling practice by milking personnel in the study area

Variables		Mukaturi(N=16)	Sululta(N=18)	Overall(N=34)
Hand washing before milking				
	yes	10(62.5%)	15(83.3%)	25(73.5%)
	no	6(37.5%)	3(16.7%)	9(26.5%)
Hand washing between milking				
	yes	11(68.8%)	18(100%)	29(85.3%)
	no	5(31.2%)	0(0%)	5(14.7%)
Udder washing and drying before milking		11(68.8%)	15(83.3%)	26(76.5%)
	yes	5(31.2%)	3(16.7%)	8(23.5%)
	no			
Udder washing and drying after milking		6(37.5%)	6(33.3%)	12(35.3%)
	yes	10(62.5%)	12(66.7%)	22(64.7%)
	no			
Use drying towel separately for udder				
	yes	5(31.2%)	7(38.9%)	12(35.3%)
	no	11(68.8%)	11(61.1%)	22(64.7%)
Antiseptic use during milking				
	yes	8(50%)	7(38.9%)	15(44.1%)
	no	8(50%)	11(61.1%)	19(55.9%)
Milking utensil used				
	plastic	11(68.8%)	15(83.3%)	26(76.5%)
	stainless steel	5(31.2%)	3(16.7%)	8(23.5%)
Milk storage containers				
	plastic	0(0%)	10(55.6%)	10(29.4%)
	stainless steel	16(100%)	8(44.4%)	24(70.6%)
Detergent use for milk container				
	yes	10(62.5%)	10(55.6%)	20(58.8%)
	no	6(37.5%)	8(44.4%)	14(41.2%)
Barn cleaning				
	Once a day	10(62.5%)	9(50%)	19(55.9%)
	Twice a day	6(37.5%)	9(50%)	15(44.1%)
Milk stored at home under room temperature before sold				
	<2 hour	0(0%)	12(66.7%)	12(35.3%)
	Between 2-6 hour	6(37.5%)	0(0%)	6(17.6%)
	Between 6-12 hour	10(62.5%)	6(33.3%)	16(47.1%)

4.2. Overall Prevalence of *Staphylococcus aureus*

The current study revealed an overall prevalence of 16.6% *Staphylococcus aureus*. Based on sample types the prevalence of *S. aureus* in milk, milker's hands, milking bucket, and drying towel were 15.3, 25, 20, and 10%, respectively (Table 7).

Table 7. Prevalence of *Staphylococcus aureus* isolate from raw milk and swab samples

Sample type	Total samples examined	Number of positive samples	Prevalence %
Milk sample	183	28	15.3
Hand swab	24	6	25
Bucket swab	30	6	20
Towel swab	10	1	10
Total	247	41	16.6

4.3. Risk Factors Associated with Prevalence of *Staphylococcus aureus*

In the present study, the prevalence of *S. aureus* was found higher in, animals of old age, giving many births (>4 calves), and poor body condition than their counter categories. With regard to environmental variables, the prevalence was higher in Mukaturi than Sululta having semi-intensive management system than intensive and poor milking environment than good one. The prevalence of *Staphylococcus aureus* in milk showed statistically significant variation ($p<0.05$) with respect to age, parity, management system, drainage condition of milking area and farming area (Table 8).

Table 8. Prevalence of *Staphylococcus aureus* in milk and associated risk factors

Risk factors	Total samples examined	Number of samples positive	Prevalence	X^2	P-value
Farming area					
Mukaturi	78	17	21.8%	4.424	0.035
Sululta	105	11	10.5%		
Age					
Young (≥3-5)	59	6	10.2%	16.201	0.000
Adult (>6- ≥9)	101	12	11.9%		
Old (>9)	23	10	43.5%		
Management system					
Intensive	105	11	10.5%	4.424	0.035
Semi intensive	78	17	21.8%		
Parity level					
Few (1-2calve)	56	3	5.4%	17.895	0.000
Mid (3-4 calve)	107	16	15%		
Many (>4calve)	20	9	45%		
Lactation stage					
Early (<3 moth)	60	8	13.3%	1.131	0.568
Mid (3-6 moth)	101	15	14.9%		
Late (>6 moth)	22	5	22.7%		
Body condition scour					
Poor	7	3	42.9%	4.286	0.117
Modern	146	21	14.4%		
Good	30	4	13.3%		
Drainage condition of milking area					
Poor	104	21	20.2%	4.448	0.035
Good	79	7	8.9%		

4.4. *Staphylococcus aureus* Load in Raw Milk Samples from Cow Udder

In the present study from 28 *Staphylococcus aureus* positive raw milk samples, 12 (42.9%) raw milk samples had *S.aureus* count, which is above the recommended level for human consumption. From 12 *Staphylococcus aureus* positive raw milk samples, nine samples had levels of *Staphylococcus aureus* corresponding to 10^4cfu/ml and three samples had levels of 10^5cfu/ml (Table 9). The remaining 16 samples (57.1%) had less than 20 cfu/ml which below the recommended level.

Table 9. *Staphylococcus aureus* load from raw milk collected directly from udder

Contaminated raw milk samples	*S. aureus* (cfu/ml)	*S. aureus* log10 cfu/ml
MSD119	1.15×10^5	5.062411
MSD176	5.64×10^4	4.750999
MSD221	7.09×10^4	4.850702
MSD271	5×10^4	4.69897
MSD314	5.27×10^4	4.722035
MIDC24	5.82×10^4	4.764787
MIDC59	5.18×10^4	4.714482
SGF9	7.64×10^4	4.882887
SGtF3	1.06×10^5	5.026793
SGtF4	7.36×10^4	4.867092
SWF6	1.04×10^5	5.015512
SYF2	8×10^4	4.90309

MSD: Mukaturi Selale Dairy, MIDC: Mukaturi International Dairy Cow, SGF: Sululta Gize Farm, SGtF: Sululta Getinet Farm, SWF: Sululta Wende Farm, SYF: Sululta Yilma Farm

4.5. Antimicrobial Susceptibility Profiles of S. *aureus* Isolates

The present study demonstrated the existence of susceptibility levels of *S. aureus* to commonly used antimicrobial agents in the study area. Thus, 75.6%, 56.1% and 51.2% of the *S. aureus* were found to be susceptible to Ampicillin, Amoxacillin and Tetracycline respectively. On other hand 51.2% of *S.aureus* was intermediate for Erythromycin. The resistance profile of Penicillin and Amoxacillin was also 97.6% and 43.9% respectively (Table 10).

Table 10.Antimicrobial susceptibility profiles of (n=41) isolates of *S. aureus*.

Anti microbial disk	Intermediate (I)		Resistence (R)		Sensitive **(S)**	
	No	%	No	%	No	%
AMP10µg	0	0	10	24.4	31	75.6
ER15µg	21	51.2	11	26.8	9	22
AMX25µg	0	0	18	43.9	23	56.1
P10µg	0	0	40	97.6	1	2.4
TE30µg	10	24.4	10	24.4	21	51.2

Amoxicillin (AMX) (25µg); Ampicillin (AMP) (10µg); Pencillin (10µg); Tetracycline (TE) (30µg) and Erythromycin(ER) (15µg), µg = micro gram

Based on analysis of multidrug resistance patterns of *S. aureus* isolates, 2.4% exhibited resistance to Ampicillin, Amoxacillin, Penicillin, and Tetracycline. Whereas, 2.4% isolate is susceptible to all antibiotics used. On the other hand, 21.95% isolates were showed resistance to the combination of Amoxacillin and Penicillin. However, 9.76% isolates were showed resistance to the combination of Erythromycin, Amoxacillin, and Penicillin (Table 11).

Table 11.Multi-drug resistance (MDR) profile of *S.aureus* isolates

Resistant to drug combination	Antimicrobial	Resistant isolates	
		Number	%
One drug	P	10	24.39
Two drug	AMX,P	9	21.95
	P,TE	2	4.88
	ER,P	5	12.20
Three drug	ER,P,TE	1	2.44
	AMP,AMX,P	1	2.44
	AMX,P,TE	3	7.32
	ER,AMX,P	4	9.76
	AMP,P,TE	3	7.32
	AMP,ER,P	1	2.44
Four drug	AMP, AMX, P, TE	1	2.44
None	Resistance to none (susceptible to all)	1	2.44
Total		41	100.00

5. DISCUSSION

5.1. Hygienic Practices during Milking and Milk Handling Practices

A total of 77 respondents comprising of those who had milk consumers and who had milking personnel at Mukaturi and Sululta town intimate to the farm were interviewed. From total respondents 40 were from Mukaturi town and 37 were from Sululta town. Whereas, of total respondents 43 were milk consumer and 34 were milking personnel. The majority (52.9%) of milking personnel has no formal education in study area. This indicates that more intervention is needed to aware milking personnel or farmers in order to improve their hygienic milk handling and husbandry practices.

In the study area, 37.2%, 48.8%, and 14.0% respondents buy milk from farm, collection center and others sources, respectively. With regard to milk handling, 72.1% of respondents (milk consumers) used plastic containers for milk handling meanwhile only 37.2% kept milk in refrigeration before consumption. The study also revealed that 60.5% of milk users had habit of raw milk consumption. The present finding is in agreement with study from Sebeta showed that large proportion (66%) of consumers use plastic container and only 10% kept milk in a refrigerator, while 90% of them kept milk at room temperature (Ayele *et al*., 2017). Disagreeably, study in Debre-Zeit also reported that 31.8% of dairy producers and 36% consumers had the habit of drinking raw milk (Fanta, 2010). The variation in milk consumption habits could be due to the strong traditionally habit of the people in the current study area for utilizing raw milk and milk products were greatly at risk of obtaining these pathogen and limit of awareness on milk borne disease.

Of consumer 25.6% had no any health risk associated with raw milk consumption or aware of milk borne disease associated with drinking raw milk. A study in Sebeta also reported that 54% had no aware of milk borne disease associated with drinking raw milk (Ayele *et al.,* 2017). From respondent information the occurrence of GIT disturbance associated with drinking of raw milk observed in infant than children and adult age. Consequently, 32.6%, 4.7% and 62.8% young children, adult and infant age respectively had showed more GIT disturbance associated with drinking of raw milk in present study. Newborns and children seem to be more exposed to milk contaminants than adults, since they consume larger

quantities of milk more and are susceptible and have weak immune system. Cleaning the udder of cows before milking is important since it could have direct contact with the ground, urine, dung and feed refusals while resting (Melese and Tesfaye, 2015**).** About 76.5% of respondents wash and dry udder before milking and about 35.3% of respondents use drying towel separately for udder after washing in present study. Contradictory to this study, Melese and Tesfaye (2015) in and around Jigjiga city of Somali Region reported that about 92% of respondents did not use udder washing before milking and all the interviewees did not use towel to dry udder after washing.

Utensil used for milking and storage determine the safety of milk and milk products. In this study, apart from one all dairy cow milking personnel in selected dairy farms practice hand milking and 76.5% uses plastic bucket for milking and 29.4% uses for milk storage. Similar to this finding, the works by Melese and Tesfaye (2015) in and around Jigjiga city of Somali Region reported that all respondents practice hand milking and above 60% of the interviewed households used plastic jars as milking utensil and transport utensil. The use of plastic and traditional containers can be a potential source for the contamination of milk by bacteria, because this allows the multiplication of bacteria on milk contact surfaces during the interval between milking. There may be difficulty of removing all milk residues from traditional containers that are porous by nature with the common cleaning systems. In this study, about 55.9% of the respondents clean the barn once per day. In agreement with the report by Melese and Tesfaye (2015) in and around Jigjiga city of Somali Region about 75% of the respondents clean the barn once per day. As a result of current study, from the total of milking personnel (47.1%) store milk at room temperature temporarily between 6-12 hours till transport to collection center with no means of cooling aid. This would certainly support the growth and multiplication of *S. aureus* as it is able to survive and multiply in a variety of food substrates, at appropriate temperatures. The overall effect of these poor milk-handling practices could lead to contamination of the dairy product as realized in the study.

Milk may be contaminated by animals, milking personnel, dust or air droplets at the site of production and during milking processing may be presents a health hazard. It may be contaminated from the cow itself, from air/ dust, unclean milk containers, unseparate drying

towel 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. The bacteria can gain access to milk as a result of the milk handlers' while, coughing, sneezing and from body surfaces in contact with milk (Thaker *et al.,* 2013**).**

5.2. Overall Prevalence of *Staphylococcus aureus*

The present study showed that 16.6% *Staphylococcus aureus* isolates were detected out of 247 sample collected, 15.3% originated from raw cows' milk, 25% swabs of milkers' hands, 20% swab of milking bucket and 10% swab of drying towel. In the present study 16.6% is slightly higher when it is compared with a study conducted by Lencho (2015) who reported 13.9% at Ambo and Guder town. This variation might be due to small number of sample in the current study. Whereas, this finding is in line with Abebe *et al*., (2013) who reported 15.5% at Addis Ababa, Fikru, (2014) who reported 17.2% at Addis Ababa, Ayele *et al.,* (2017) who reported 19.6% at Sebeta, Abunna *et al., (*2013) who reported 21.1% at Addis Ababa and Tessema and Tsegaye (2017) who reported 21.2% at Alage ATVET College Dairy Farm, Ethiopia.

However, the result of the present study showed a slight lower prevalence rate compared to other works, Wubete, (2004) who reported 27% at Addis Ababa, Bedada and Hiko, (2011) who reported 39.1%, *S. aureus* isolates at Asella and Sudhanthiramani *et al.,* (2015), who reported 39.1%, *S. aureus* isolates at region of Tirupathi, India. This is due to in the current study, milk samples were collected directly from cows' udder before contacting milking utensils that might decrease the prevalence of *S aureus* and this may be attributed to differences in the management practices at farm level. The isolation of *S. aureus* from hands of milker's, milk buckets and drying towel swab were 25%, 20% and 10%, respectively. These clearly indicated that milk handlers, milk buckets and drying towel could be the potential sources of contamination of milk with *S. aureus*. The isolation rate from milker's hand and milking bucket swab was in agreement with the prevalence rate reported by Ayele *et al.,* (2017) who report prevalence of 32% and 11.1%, respectively at Sebeta. The present study on, isolation of *S. aureus* from milker hand swab was in line with the finding of Lencho (2015) who reported 20% prevalence of *S. aureus* from swabs of milker's hands in Ambo and Guder

town. However, the present finding of *S.aureus* from milking bucket swab is higher when it is compared with a study conducted by Fufa *et al*., (2016) who reported 0% from pooled bucket swab at Asella and Lencho (2015) who reported 9% from milking bucket swab at Ambo and Guder town. The dissimilarity of prevalence of *Staphylococcus aureus* isolates might be related with probable the hygienic status of equipment sampled of the present study was no good than the previous study.

5.3. Risk Factors Associated with Prevalence of *Staphylococcus aureus*

The present study showed significantly high prevalence of *Staphylococcus aureus* in Mukaturi than Sululta and semi-intensive than intensive management system (p=0.035) with prevalence of 21.8% in both Mukaturi and semi-intensive management and 10.5% in both Sululta and intensive management system. Disagree, with Fikru, (2014) from central Ethiopia and with lencho, (2015) from Ambo and Guder town. With regard to farming area this might be associated with cows which were maintained in dirty and muddy common barns with bedding materials and failure to use separate towel for individual cows, there could be high chance of contamination of the udder and milk with pathogenic microorganisms**.** With regard to management system the surrounding air, feed, soil, feces and grass might be possible sources of contamination.

The present study showed significantly high prevalence of *Staphylococcus aureus* in poor than good drainage condition of milking area (p=0.035) with high prevalence of 20.2% in poor and 8.9% in good drainage condition. In line with Abera *et al.,* (2013) at Adama, this is due to association with poor hygiene of milking area; cows which were milking in dirty, muddy and sewage full drainage area increase milk contamination and favor the proliferation and transmission of *S.aureus* to udder of cow**.** It was also found that prevalence of *Staphylococcus aureus* increases as parity number increases with statistically significant variation among the categories (P =0.000) with high prevalence recorded 5.4%, 15% and 45% in few, mid and many parity level respectively. This result is disagreeably with report of Abera *et al.,* (2013) at Adama who reported no statistically significant association. However, it is in agreement with the findings of Befikadu *et al*., (2017) in and around Asella town. This could be due to the reason that as the parity number increases there is high degree of contamination of the udder and milk through milking process. Additionally, this might be due to large amount of milk is

produced and as a result the pressure on the teat canal forces the canals to be opened widely which allows entrance of microbes. The study also revealed that, statistically significant association was observed among age categories (p=0.000) with high prevalence recorded 10.2%, 11.9% and 43.5% in young, adult and old age respectively. This result agrees with the findings of Befikadu *et al.,* (2017) in and around Asella town and Lencho (2015) at Ambo and Guder town. This could be due to the reason that old cows and cow which has many parity levels are more susceptible to udder contamination through milking process, since large amount of milk is produced for long time. As a result the pressure on the teat canal might be forces the canals to be opened widely which allows entrance of microbes.

The present study showed that the prevalence of *S. aureus* showed that lactation stage has no statistically significant variation (p>0.05). This is in line with the report of Abera *et al.,* (2013) at Adama town and Lencho (2015) at Ambo and Guder town. Additionally, Body condition scours of dairy cow has no statistical association (p>0.05). This may be due to the absence of proper udder washing and teat dipping, increased presence of potential pathogens on the skin of the teat which can easily penetrate into the teat canal and multiply and antibiotic resistance ability of most pathogens. In addition, this is due the fact that *S. aureus* is ubiquitous in nature, with humans and animals as the primary reservoirs. They are present in the nasal passages and throat, in the hair, and on the skin of healthy individuals (Mekonnen, 2015).

5.4. *Staphylococcus aureus* Load in Raw Milk from Cow Udder

The present study, indicate that 42.9% raw milk samples had *S.aureus* load above the recommended level which is safe for human consumption. This might be due to *Staphylococcus aureus* has adapted to survive in the udder and establish chronic and subclinical infections. It could be due to the reason that even when drawn under aseptic condition, milk always contains microorganisms which are derived from the milk ducts in the udder and contamination of milk may result from systemic disease in the animal. From there it is shed into the milk, which serves as a source of infection for healthy cows during the milking process (Abera *et al.,* 2013). The present study showed that *S. aureus* count per milliliter in raw milk sample was 1.15×10^5cfu/ml (5.062411logcfu/ml) ,1.06×10^5cfu/ml (5.026793logcfu/ml) and 1.04×10^5cfu/ml (5.015512logcfu/ml) in MSD119, SGtF3 and SWF6 milk sample respectively. This indicates that the number of bacteria, which are present in

freshly drawn raw milk; vary with individual animals, environment of the animal and quarter of the udder. *S. aureus* count range from $5\times10^4$to 1.15×10^5cfu/ml (4.69897 to 5.062411logcfu/ml) in raw milk. The presence of high total *S.aureus* load in raw milk indicates contamination possibly from udder or teat canal of lactating dairy cows. A microbiological study on ready to eat foods in London indicated that, total *S. aureus* count ($\geq10^4$cfu/g/ml) was described as hazardous level of bacterial quality in the foods (Gilbert *et al.,* 2000). According to the present study, the total *S. aureus* count in each *Staphylococcus aureus* positive raw milk sample was $\geq10^4$cfu/ml. Based on the standard level it is hazardous level and milk consumed is a serious risk to the health of the population. The present finding is in line with the findings of (de Oliveira *et al.,* 2011) found counts of *Staphylococcus aureus* varying between 10^2-10^5 cfu/ml in raw milk from *Staphylococcus aureus* positive samples.

5.5. Antimicrobial Susceptibility Test

The antimicrobial susceptibility tests carried out in this study indicated the occurrence of resistance of *S.aureus* to some of the commonly used antimicrobials. The reason for the existence of antimicrobial resistant *S. aureus* isolates could be due to the indiscriminate use of antimicrobials, self-medication, and administration of sub therapeutic dose of antimicrobials to livestock for prophylactic purpose and limited updating of the long used drug groups (Haftay *et al.,* 2018). This study presents the susceptibility of *S. aureus* isolates towards Ampicillin, Amoxacillin and Tetracycline with frequencies of 75.6%, 56.1% and 51.2%, respectively. However, the isolates were found to be highly resistant to Penicillin G (97.6%). The high resistance pattern of the isolates to penicillin G is relatively similar to the findings reported from the country. Thus, Lencho (2015), Ayele *et al.,* (2017), Abebe *et al.*, (2013), and Fikru (2014) reported frequencies of 100%, 98.8%, 96.7%, and 94.6%, respectively. Moreover, the present study showed moderate resistance pattern of *S. aureus* to erythromycin (26.8%) and tetracycline (24.4%). The findings are slightly consistent with the report of Sudhanthiramani *et al.,* (2015) in which (13.95%) tetracycline resistance level seen. However, the current findings are inconsistent with the report of Ayele *et al.,* (2017) in which (69.1%) erythromycin and (64.7%) tetracycline resistance level observed. This is might be these drugs specifically tetracycline is commonly used in the treatment of infections in the previous study area than the present study area. Lacks of stringent regulation and monitoring in the dispensing and use of

antimicrobials in the country might have contributed to the occurrence of high antimicrobial resistance to these drugs (Ayele *et al.,* 2017). Thus, due to the relatively limited access and high price to get the newly developed drugs (like, cephalosporin and quinolone) the reports of prevalence of antimicrobial-resistant to relatively low priced and regularly available antibiotics are alarming for a low-income society living in most developing countries, like Ethiopia (Haftay *et al*., 2018).

In the present study, multi-drug resistance profile of *S. aureus* isolate was reported. Based on analysis of multidrug resistance patterns, 2.4% isolates exhibited resistance to three and four antibiotics with the combination of Ampicillin, Amoxacillin, Penicillin, and Tetracycline. While 21.95% isolates showed resistance to the combination of Amoxacillin and Penicillin. On other hand, 9.76% isolates showed resistance to the combination of Erythromycin, Amoxacillin, and Penicillin. However, 2.4% of isolates were susceptible to all antibiotics used. The probable explanation is, *S. aureus* strains have the capacity to change their resistance behavior to the exposed antimicrobials. The emergence of resistance to many drugs represents public health hazard due to the fact that food borne outbreaks might be difficult to treat and the group of multi-drug resistance *S. aureus* in food supply represents a reservoir for communicable resistant genes (Haftay *et al*., 2018).

6. SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1. Summary

The safety of raw milk and raw milk products with respect to staphylococcal poisoning is of great concern around the world. *S. aureus* is one of the most important causes of milk borne illness associated with the consumption of raw milk. Milk can be contaminated by *S. aureus* when there is infection of the mammary gland and during or after milking by poor hygienic practices, such as improper washing of hands when handling milk storage equipment and coughing or sneezing. Milk and milk product can act as a vehicle for transmitting the *S. aureus* from animal to human to cause the severe food borne intoxication. Antimicrobial resistance is a public health problem due to the persistent circulation of resistant strains of *S. aureus* in the environment and the possible contaminated food.

Milk, equipment and hand of human might contain resistant *S.aureus* posing a potential risk to consumers. A study on the prevalence of *S. aureus* and risk factor contributing for contamination of milk in dairy farm is important in the study area. Thus, there is a need for study on the status of *S. aureus* and milk handling practice in the study area so as to forward the possible management options for *S. aureus.* The aim of the current study was to estimate the prevalence of *S. aureus* in milk of dairy cow and swabs from different contact surfaces and assess milk handling practice among dairy farms, assess associated risk factors for *S. aureus* in dairy cow and *S.aureus* load in raw milk; and determine the antimicrobial susceptibility profile of *S. aureus* in the study area.

A cross- sectional study was carried out from November 2017 to June 2018 in selected dairy farms of Mukaturi and Sululta town, Oromia Regional State, Ethiopia. The possible risk factors for *S. aureus* contaminations in milk were evaluated face to face through a structured questionnaire. A total of 247 samples(183 raw milk from lactating cow by simple random sampling technique collected from purposively selected dairy farms and depending on number of worker, frequencies of farm visit and material used 64 swab samples) were examined using standard microbiological techniques. The antimicrobial susceptibility profiles of the isolates were also investigated using disc diffusion method. From the total of 77 respondents in the study area with regard to milk handling, 72.1% of respondents (milk consumers) used plastic

containers for milk handling meanwhile only 37.2% kept milk in refrigeration before consumption. Overall, 16.6% (n= 41) of the samples were positive for *S. aureus*. The prevalence of *S. aureus* was 15.3% from udder milk and 25%, 20% and 10% from milkers' hand, milking bucket and drying towel swab respectively. According to this study, 12(42.9%) raw milk samples had $\geq 10^4$cfu/ml *S.aureus* count, which is above the recommended level for human consumption. The isolates were found to be resistant to penicillin G (97.6%), and Amoxicillin (43.9%). Generally, the study revealed a prevalence of antimicrobial resistant *S. aureus* from raw cow milk and swabs; poor milk handling practices and raw milk consumption behavior in study area.

6.2. Conclusion and Recommendations

Milk wished-for human consumption has to be free from potentially harmful bacteria. Based on observations made throughout the study period, improper hygiene and poor farm management practices contributed to the presence of *S. aureus* in dairy cow milk samples. During the sampling some hygienic practices have been evaluated in dairy farms of study area. They apply hand milking in a cow barn without use a separate milking room. In the study area, there is no standard hygienic conditions followed by milk consumer and milking personnel on milk handling practice. The hygienic conditions are different according to the dairy farmers and consumers, adapted practices, level of awareness, and availability of resources.

In most of the cases, the common hygienic actions taken during milking and milk handling practice are limited to udder washing and dried after milking, use drying towel separately for udder, and antiseptic use during milking. Additionally, in Mukaturi dairy farm the common hygienic actions also taken during milking and milk handling practice are somewhat limited to store milk under appropriate temperature before sell or transport to collection center, barn cleaning attitude, and hand washing before milking and between milking while compared with Sululta dairy farm. With regard to consumers there are the habit /adaptation of frequently used plastic container, raw milk consumption and handle the milk at home without means of cooling aid or refrigerator for some temporal time before consumption.

The study also has shown raw milk sample has *S.aureus* count, which is above the recommended level for human consumption from contaminated raw milk samples by *S. aureus*. Considering the standard level, the *S. aureus* counted in raw milk sample indicates the product is improper for human consumption or the raw milk is hazardous/toxic level for consumption. In general, the study has revealed the way of milk contaminated by *S.aureus* and possibility of the public health concern posed by raw milk consumption in study area. Based on the present study the following recommendations are forwarded.

- Proper handling of milk at farm level and maintain the hygiene of milking area, equipment, milking personnel hand and using drying towel separately to be exercised to decrease milk contamination by *S.aureus* and make it safe for human consumption.
- Further study has to be conducted and taking in to account the control options that would make possible to reduce milk contamination by antimicrobial resistant *S. aureus* and so, the associated public health risks.
- Milk should be stored in refrigerator at required temperature until consumption.
- Creation of public awareness about good milk handling practices, milk borne diseases and their prevention is important.
- Besides, monitoring the use of drugs based on reason and alternative measurement of the antimicrobial sensitivity of drugs before use are recommended.

7. REFERENCES

Abebe, M., Daniel, A., Yimtubezinash, W. and Genene, T. 2013. Identification and antimicrobial susceptibility of *Staphylococcus aureus* isolated from milk samples of dairy cows and nasal swabs of farm workers in selected dairy farms around Addis Ababa, Ethiopia. *African Journal of Microbial, Research*, 7(27):3501-3510.

Abera, M., Demie, B., Aragaw, K., Regassa, F. and Regassa, A. 2013. Isolation and identification of *Staphylococcus aureus* from bovine mastitic milk and their drug resistance patterns in Adama town, Ethiopia. *African Journal of Dairy Farming and Milk Production,* 1 (2):19-23

Aberra, A. 2010. Microbiological Safety of Pasteurized and Raw Milk from Milk Processing Plants In and Around Addis Ababa. MSc Thesis, Addis Ababa University, Addis Ababa, Ethiopia.

Abunna, F., Fufa, G., Megersa, B. and Regassa, A. 2013. Bovine mastitis; prevalence, risk factors and bacterial isolation in small-holder dairy farms in Addis Ababa city. Ethiopia, *Global Veterinaria,* 10 (6):647–52.

Amanu, M., Gebremeskel, M. and Tadesse, B. 2016. Prevalence and Antibiotic Resistance of *Staphylococcus aureus* Mastitis in Holeta Area, Western Ethiopia. *Global Veterinaria,* 16 (4): 365-370.

Andremont, A. 2015. What to do about resistant bacteria in the food-chain In: Bull World Health Organization, pp. 217-218.

Anagaw, B., Shiferaw, Y., Biadglegne, F., Moges, F. and Kassu, A. 2013. Frequency of Methicillin-resistant *Staphylococcus aureus* isolates from clinical Specimens in Gondar University Hospital, Northwest Ethiopia. *Asian Journal of Medical Science*, 5(3):59–64.

Ash, M. 2008. *Staphylococcus aureus* and Staphylococcal Enterotoxins. In: Food borne microorganisms of public health importance, 5th Edition, AIFST (NSW Branch), Sydney, Australia, Pp 313-332.

Ayele, Y., Fanta, D., Bedaso, M., Robel, G., Takele, B., Tariku, J., Fanos, T., Mesula, G. and Ashenafi, F. 2017. Assessment of *Staphylococcus aureus* along milk value chain and its public health importance in Sebeta, central Oromia, Ethiopia. *BMC Microbiology,* Pp 17:141.

BAMO (Bacteriological Analytical Manual Online). 2001. *Staphylococcus aureus*. Chapter 12, Pp 1-5.

Bedada, B.A. and Hiko, A. 2011. Mastitis and Antimicrobial Susceptibility Test at Asella, Oromia Regional State, Ethiopia. *Journal of Microbiology Antimicrobial,* 3: 28-232.

Befikadu, S., Hailemariam, K. and Yimer, M. 2017. Prevalence, Associated Risk Factors and Antimicrobial Susceptibility of *Staphylococcus aureus* Isolated from Bovine Mastitis Milk in and Around Asella town, Ethiopia. *Advances in Biological Research,* 11 (5): 295-301.

Baron, S. 1996. editor. Medical Microbiology 4th edition; Galveston (TX): University of Texas Medical Branch at Galveston; Chapter 12. Available from: *https://www.ncbi.nlm.nih.gov/books/NBK7627/* [accessed at 11:24pm 5/5/2017]

Baron, F. 2007. Review on *Staphylococcus aureus* and food poisoning. *Genetics and Molecular Research, 2*: 163-176.

Benić, M., Habrun, B. and Kompes, G. 2011. Clinical and epidemiological aspects of cow mastitis caused by *Staphylococcus aureus* and its methicillin-resistant strains. *Medical Science,* 37: 113-122.

Bergdoll, M. and Lee Wong, A. 2006. Staphylococcal intoxications. In: Foodborne Infections and Intoxications; Reimann, P. and Cliver. D. 3rd Ed.; Academic Press, Elsevier: New York, NY, USA, Pp. 523–525.

Brown, D.F.K., Edwards, D. I., Hawkey, P.M., Morrison, D., Ridgway, G.L., Towner, K.J. and Wren, M.W.D. 2005. Guidelines for The Laboratory Diagnosis and Susceptibility Testing of Methicillin-resistant *S. aureus* (MRSA). *Journal of Antimicrobial Chemotherapy, 56: 1000–1018.*

CHP (Centre for Health Protection). 2011. Scientific Committee on Enteric Infections and Food borne Diseases. Review of Staphylococcal food poisoning in Hong Kong. pp 1-18.

CLSI(Clinical laboratory Standard Institute). 2017. Performance Standards for Antimicrobial Susceptibility Testing; Twenty seven edition; CLSI Supplement M100.Waye.PA; clinical laboratory standard institute, 2017.

CSA (Central Statistical Authority). 2017. Annual report data.

De Oliveira, L.P., Soares, E., Barros, L.S., Silva, V.C. and Cirqueira, M.G. 2011. Study of *Staphylococcus aureus* in raw and pasteurized milk consumed in the Reconcavo area of the State of Bahia, *Brazil. Journal of Food Protect. Technology, 2: 128.*

Deurenberg, R.H and Stobberingh, E.E. 2008. The evolution of *Staphylococcus aureus. Infectious Genet Evolution,* 8: 747-763.

Fanta, D. 2010. Quantitative Risk Assessment of Consuming Milk Contaminated with *Staphylococcus aureus* in Debre-Zeit. MSc Thesis, Addis Ababa University, Addis Ababa, Ethiopia.

Fikru, G. 2014. Staphylococcus: Epidemiology and Its Drug Resistence in Cattle, Food Chains and Human in Central Ethiopia, MSc Thesis, Addis Ababa University, Addis Ababa, Ethiopia.

FSA (Food Standards Agency). 2017. Risk Assessment on Methicillin-Resistant *Staphylococcus aureus* (MRSA), with a focus on Livestock-associated MRSA, in the UK Food Chain. Pp 1-61.

Fufa, A, Tekeste, A., Fikru, G., Takele, B., Ashenafi, F., Dinka, A., Bedaso, M. and Reta D. 2016. *Staphylococcus*: Isolation, Identification and Antimicrobial Resistance in Dairy Cattle Farms, Municipal Abattoir and Personnel in and Around Asella, Ethiopia. *Journal of Veterinary Science and Technology*, 7: 383.

Gilbert, R.J, J. de. Louvois, T. Donovan, C. Little, K. Nye, C.D. Ribeiro, J. Richards, D. Roberts and F.J. Bolton. 2000. Guidelines for the microbiological quality of some ready-to-eat foods sampled at the point of sale, PHLS Advisory Committee for Food and Dairy Products, London. *Journal of Communicable Disease Public Health,* (3): 163-7

Girma, K., Tilahun, Z., and Haimanot, D. 2014. Review on Milk Safety with Emphasis on Its Public Health. *World Journal of Dairy and Food Science,* 9 (2): 166-183.

Haftay, A., Geberemedhin, H., Belay, A., Goytom, E. and Kidane, W. 2018. Antimicrobial resistance profile of *Staphylococcus aureus* isolated from raw cow milk and fresh fruit juice in Mekelle, Tigray, Ethiopia. *Journal of Veterinary Medicine and Animal Health,* 10 (4):106-113.

Hudzicki, J. 2009. Kirby-Bauer Disk Diffusion Susceptibility Test Protocol.ASM Microbe Library.AmericanSocietyforMicrobiology.*http://www.microbelibrary.org/component/resource/laboratory-test/3189-kirby-bauer-disk-diffusionsusceptibility-test-protocol. [Accessed at 2:45pm, 14/7/2017]*.

Kadariya, J., Smith, T.C. and Thapaliya, D. 2014. Review Article *Staphylococcus aureus* and Staphylococcal Food-Borne Disease: An Ongoing Challenge in Public Health. *Biomedical Research International,* 2014:1-9.

Khan, M.T.G., Zinnah, M.A., Siddique, M.P., Rashid, M.H.A., Islam M.A., and Choudhury, K.A, K.A, K.A., 2008. Physical and microbial qualities of raw milk collected from Bangladesh agricultural university dairy farm and the surrounding villages. *Bangladesh Journal of Veterinary Medicine,* 6 (2): 217-221.

Kitara, L.D., Anywar, A.D., Acullu, D., Odongo, A. E., Aloyo, J. and Fendu, M. 2011. Antibiotic susceptibility of *Staphylococcus aureus* in suppurative lesions in Lacor Hospital, Uganda. *African Health Science,* 11 (3): 34-39.

Kümmel, J., Stessl, B., Gonano, M., Walcher, G., Bereuter, O., Fricker, M., Grunert, T., Wagner, M, and Ehling-Schulz, M. 2016. *Staphylococcus aureus* Entrance into the Dairy Chain: Tracking *S. aureus* from Dairy Cow to Cheese. Front. *Microbial*, **7**:1603.

Le Loir, Y., Baron, F and Gautier, M. 2003. *Staphylococcus aureus* and food Poisoning, *Genetics and Molecular Research,* 2: 63-76.

Lencho, M. 2015. 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, Addis Ababa University, Addis Ababa, Ethiopia.

MOARD (Ministry of Agriculture and Rural Development). 2005. Standard Veterinary Laboratory diagnostic manual; animal health department Addis Ababa, *Bacteriology,* 2:1-186.

Mekibib, B., Furgasa, M., Abunna, F., Megersa, B. and Regassa, A. 2010. Bovine Mastitis: Prevalence, Risk Factors and Major Pathogens in Dairy Farms of Holeta Town, Central Ethiopia. *Veterinarian World,* 3 (9):397-403.

Mekonnen, A., Malondra, P and N-kyule, M. 2011. Isolation and Identification of *Staphylococcus* species from Raw Bovine Milk in DebreZeit, Ethiopia. *Veterinary Research,* 4 (2):45-49.

Mekonnen, A. 2015. A Review on Staphylococcal Food Poisoning. Food Science and Quality Management, 40:1-14.

Melese, A. and Addisu, H. 2015. Microbiological Quality Assessment of Raw and Pasteurized Milk. *Review International. Journal of Food Science and Microbiology,* 2 (6):087-091.

Melese, A. and Tesfaye, W. 2015. Bacteriological Quality and Safety of Raw Cow's Milk in and around Jigjiga City of Somali Region, Eastern Ethiopia. *International Journal of Research Studies in Biosciences,* 3 (5):48-55.

MWLPO (Mukaturi Woreda Livestock Production Office). 2017. Author record information data.

Mullarky, I. K., Wolfe, P.C. S. and Jones, G. M. 2010. *Staphylococcus aureus* Mastitis: Cause, Detection, and Control; Paper Review Virginia Cooperative Extension: Virginia Polytechnic Institute State University. Pp. 404-229.

PHE (Public Health England). 2016. Enumeration of Coagulase-Positive Staphylococci (*Staphylococcus aureus* and other species), National Infection Service, Food, Water & Environmental Microbiology Standard Method FNES8 (F12), *version* 4.

Quinn, P.J., Markey, B., Donnelly, W.J., Leonard, F.C. and Maghire, D. 2005.Veterinary Microbiology and Microbial Disease. Blackwell Science, Ltd., London, pp 468-470.

Quinn, P.J., Carter, M.E., Markey, B. and Carter, G.R. 2002. Veterinary Microbiology Microbial Diseases- Bacterial causes of Bovine Mastitis, 8th Edition. Mosby international Limited, London, pp 465-475.

RAMS (Risk Assessment Microbiology Section). 2009. Microbiological Risk Assessment of Raw Cow Milk. pp 1-119.

SDAO (Sululta district agricultural office). 2012. Annual report record.

Sharad, M., Kiran, K. and Ashutosh, D. 2016. Body Condition Scoring of Dairy Cattle: *A Review. Research & Reviews: Journal of Veterinary Sciences, 2 (1*):1-8.

Shimelis, T. 2014. Study on Isolation and Antimicrobial Susceptibility of *Staphylococcus aureus* and Occurrence of Methicillin resistant *Staphylococcus aureus* (mrsa) in

Mastitis Dairy Cows in the Selale/Fitche Area. MSc Thesis, Addis Ababa University, Addis Ababa, Ethiopia.

Singh, P. and Prakash, A. 2010. Prevalence of Coagulase positive pathogenic *S. aureus* in milk and milk products collected from unorganized sector of Agra Acta Agriculture*, Slo,* 96: 37–41.

Smith, K. 2007. Hand book of Food borne pathogenic microorganisms and natural toxins. Food and Drug Administration, *Center for Food Safety and Applied Nutrition,* 10: 119 150.

Solomon, M., Mulisa, M., Yibeltal, M., Desalegn, G. and Simenew, K. 2013. Bacteriological quality of bovine raw milk at selected dairy farms in Debre Zeit town, Ethiopia, *Comprehensive Journal of Food Sciences and Technology Research,* 1(1), 1-8

Sudhanthiramani, S., Swetha, C.S. and Bharathy, S. 2015. Prevalence of antibiotic-resistant *Staphylococcus aureus* from raw milk samples collected from the local vendors in the region of Tirupathi, *India, Veterinary World,* 8(4): 478- 481.

Tessema, D. and Tsegaye, S. 2017. Study on the Prevalence and Distribution of *Staphylococcus aureus* in Raw Cow Milk Originated from Alage ATVET College Dairy Farm, Ethiopia. *Journal of Nutrition and Food Science,* 7: 586.

Teshome, G., Fekadu, B. and Mitiku, E. 2014. Handling Practices and Microbial Quality of Raw Cow's Milk Produced and Marketed in Shashemene town, Southern Ethiopia. *International Invention Journal of Agricultural and Soil Science,* 2 (9):153-162.

Teshome G. 2016. Microbial Quality and Associated Public Health Hazards of Raw Cow's Milk Produced and Marketed in Ethiopia: A review. *Biomedicine and Nursing,* 2(1) *http://www.nbmedicine.org* [Accessed at 7:14am 4/2/2018].

Thaker, H.C., Brahmbhatt, M.N., and Nayak, J.B. 2013. Isolation and identification of *Staphylococcus aureus* from milk and milk products and their drug resistance patterns in Anand, Gujarat *Veterinary World,* 6(1):10-13.

Thrusfield, M. 2007. Veterinary Epidemiology 3rd ed. London: Blackwell Science Ltd. Pp: 227-24.

Todar, K. 2008. Todar's online Textbook of Bacteriology. Available online at: *http://www.textbookofbacteriology.net*. [Accessed at 11:27pm 9/5/2017].

Tsegalem, A., Yoseph L., Behar, M. and Befekadu, U. 2016. Bacteriological quality of raw camel milk along the market value chain in Fafen zone, Ethiopian Somali regional state. *BMC Research Notes*, 9:285.

WHO (World Health Organization). 2011. Initiative to estimate the Global Burden of Forborne Diseases: Information and publications; Available online at:*http://www.who.int/foodsafety/foodborne_disease/ferg/en/index7.html/.* [Accessed at 4:15pm 31/5/2017].

Wubete, A. 2004. Bacteriological Quality of Bovine Milk in Small Holder Dairy Farms in DebreZeit, Ethiopia. MSc Thesis, Addis Ababa University, Addis Ababa, Ethiopia.

Yilma, Z., Loiseau, G. and Faye, B. 2007. Manufacturing Efficiencies and Microbial Properties of Butter and Ayib, a Traditional Ethiopian Cottage Cheese. *Livestock Research for Rural Development,* Pp 19: 67.

Zdolec, N.V., Dobranić, I., Butković, A., Koturić, I.I., and Filipović, V.M. 2016. Antimicrobial susceptibility of milk bacteria from healthy and drug-treated cow udder, *Veterinarski Arhiv,* 86(2), 163-172.

8. ANNEX

Annex 8.1.**Questionnaire I**: Information related with farm management, individual animal history and milk hygiene practices

1. ***General information***

Dairy Farm name: _____Owners Name: _____

Address_____ Date of sample Collection_____

1. What is the name of farms?
2. What type of management system used? 1. Intensive 2. Semi intensive 3. Extensive

2. ***Animal information***

1. What breed of the dairy cow/cattle in your farm? 1. Local 2. Cross 3. Exotic
2. What age of the sampled cattle? Answer(s)…….
3. What parity level of the sampled cattle? Answer(s)…….
4. What lactation stage of the sampled cattle? Answer(s)…….
5. What animal Body condition score? Answer(s)…….
6. What is the drainage condition of milking area? A, good b. poor

Questionnaire I*: Information of milking personnel about *milking and milk handling practices

Date: _____ Address (farm name): _____ Sex _____

Education……

1. Is hand washing practiced before milking? 1. Yes 2. No
2. Is hand washing practiced between milking? 1. Yes 2. No
3. Is that udder washed and dried before milking? 1. Yes 2. No
4. Is the udder washed and dried after milking? 1. Yes 2. No
5. Is that you used drying towel separately for drying udder? 1. Yes 2. No

6. Is that antiseptic used for cleaning during milking? 1. Yes 2. No
7. What kind of milking utensils do you use? a. plastic b pot c. Stainless steel
8. What kind of storage containers do you use? 1. Plastic 2. Pot 3. Stainless steel
9. Do you use detergents for washing of milk containers? a. yes b. no
10. How long is milk stored after milking at home under room temperature before sold?

    a. <2 hr b. 2-6 hr c. 6-12hr

11. Where do you store unsold milk? 1. with no cooling 2. in the refrigerator
12. Is there any means other than cooling for preservation of milk? a. yes b. no
13. Do you pool/bulk milk of different sources/cows? a. yes b. no

***Questionnaire* II*:* Information of consumers about milk handling and related illness**

Date: ____Name:_____ Address: _____Sex _____

Questions;

1. Where do you buy the milk? 1. Direct from farm 2. From collection shop 3.other
2. What kind of containers do you use? a. plastic b pot c. stainless steel
3. When do you buy? 1. Morning 2. Afternoon 3. Evening 4. Morning and evening

4. How long the milk stay at home under room temperature prior consumption?

    a. <2 hr b. 2-6 hr c. 6-12hr

4. Where do you put? 1. with no cooling 2. in the refrigerator
5. Habit of milk consumption: a. raw b. boiled c. other
6. What is the rationale? Answer(s)……..
7. Do you mix fresh and left over milk for consumption? a. yes . no
8. Do you know any health risk associated with raw milk consumption? a. yes b. no

9. Do you know any GIT disturbance associated with drinking of raw milk? a. yes b. no

10. Which age of people show more GIT disturbance associated with drinking of raw milk? 1. Young children 2. Adult 3. Infant

11. What is the rationale? Answer(s)……..

12. What are the symptoms? Answer(s)……..

13. Did you suffered from milk borne infections? a. yes b. no

Disease name.....?

14. What drug has been given? Answer (s)……..

15. Was its curative? 1. Yes 2. No

Annex 8.1.1. Sample collection sheet for bacteriological analysis

serial number	Date of collection	Type of sample	Source	Sample code	Area

Annex 8.1.2. 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	25	19	-	19
Ampicillin	10	28	-	29
Erythromycin	15	13	14-22	23
Penicillin	10	28	-	29
Tetracycline	30	14	15-18	19

Source: (CLSI, 2017)

Annex 8.2. Milk Sample Collection Procedure

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. The teat end was scrubbed gently with cotton swabs moistened with 70% ethyl alcohol. The first streams of milk were discarded
4. Remove the cap from the tube or vial but do not set the cap down or touch the inner surface of the cap.
5. Maintain the tube or vial at approximately a 45 degree angle while taking the sample.
6. 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. Approximately 6 to 6.5 ml of milk collected from each quarter of the udder.
7. Store samples immediately in ice box
8. Lastly, the teat and udder was washed after sample taken.
9. Then sample was taken to laboratory for culture.

Source: (Quinn *et al.,* 2002)

Annex 8.3. The *Staphylococcus aureus* isolation (protocol) flow chart was;

25ml Milk samples and swab was collected

▼

0.01ml of milk (loop full of milk) and swab was streaked on blood agar plate (BAP) with 7% sheep blood)

▼

Incubation at 37 ^{O}C for 24-48 hours

▼

(Haemolysis and colony characterization observation)

▼

Sub- culturing on Tryptone Soya Agar (TSA)

▼

Incubation at 37 ^{O}C for 24 hours

▼

- Gram staining
- Catalase test
- Growth on Mannitol Salt Agar (MSA)
- Coagulase test
- Growth on Purple Base Agar (PBA)

Annex 8.4. Biochemical tests and procedures used were:

A, 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. Counterstain 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 colour indicate Gram positive and pinkish colour indicate Gram negative bacteria

B, 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% H2O2 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 1)

Figure 1: Slide catalase test

C, Growth on mannitol salt agar

The colonies that were confirmed by gram's staining reaction, haemolysis on the blood agar and catalase positive was selected and streaked on Mannitol salt agar plate, incubated at 37^0 C and examined after 24-48 hr for growth. The presence of growth and fermentation of mannitol by *S. aureus* causes yellow discolouration of the medium (figure 2).

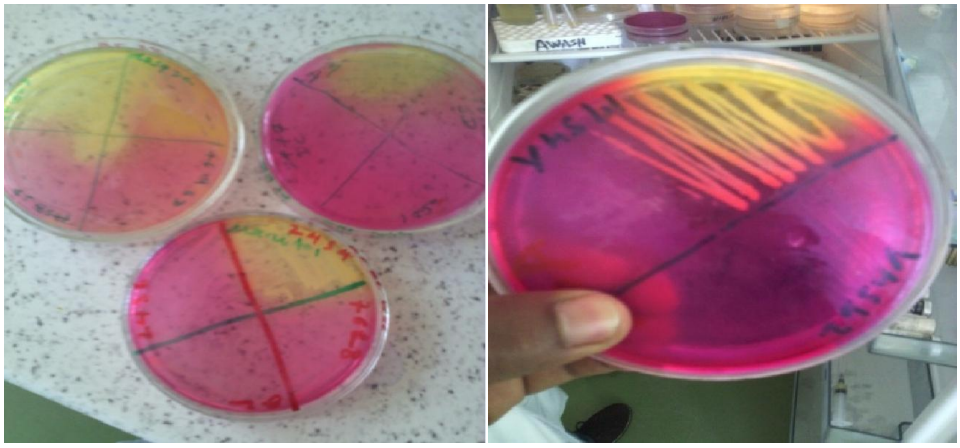


Figure 2: Growth on mannitol salt agar

D, Coagulase test procedure

1. Two test tubes are taken and labeled as "test", and "negative control"

2. Each tube is filled with 0.5 ml of rabbit plasma.

3. To the tube labeled test, 0.5ml of overnight broth culture of test bacteria is added.

4. To the tube labeled negative control, only 0.5 ml of sterile tryptone soya broth is added.

5. 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.

6. 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 3)

Figure 3: Tube coagulase test

E, Procedure for the disk diffusion methods

1. A well isolated colonies of the same morphological type are selected and just the top of the colonies are touched.

2. Then a suspension is made in 5 ml 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 will be placed no closer together than 24 mm (centre to centre).

11. After incubation, the diameter of the zones of inhibition is measured to the nearest mm using a ruler or caliper.

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

13. The diameter of the zones will be read across the centre of the discs.

14. An interpretation of the size of the zones of inhibition (figure 4) is made with reference

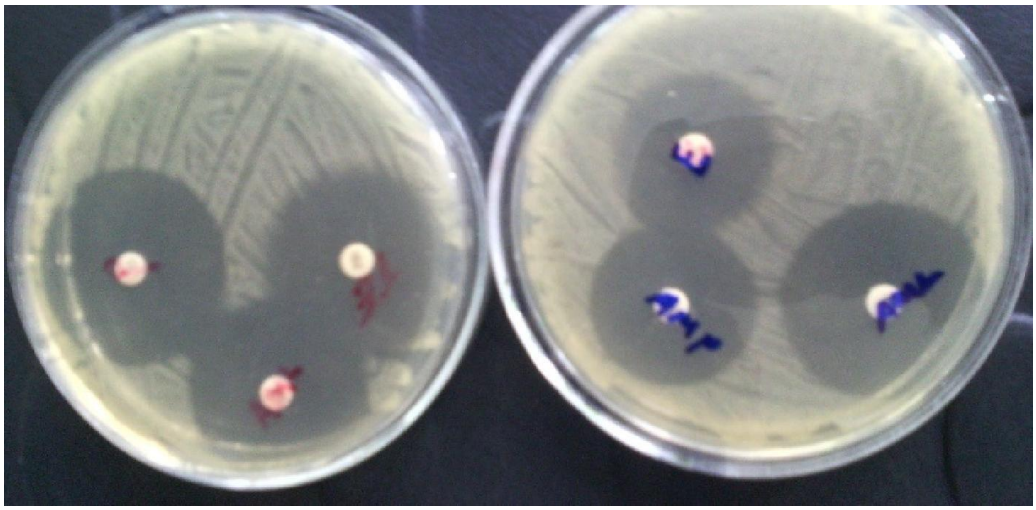


Figure 4: Antibiotic sensitivity test

F, Procedure for *Staphylococcus aureus* enumeration

1. 60 g of the Baird parker agar (BPA) was added into one liter of purified water.

2. Then heated with frequent agitation and boiled for one minute to completely dissolve the medium.

3. It was autoclaved at 121°C for 15 minutes.

4. After cooling to 45 - 50°C, 50 ml of Egg Yolk Tellurite Supplement was added. The ratio of Egg yolk emulsion to Tellurite supplement (1%) was 5ml: 1ml.

5. It was mixed thoroughly prior to dispensing and 18-20ml of the prepared BPA (7-10^{o}C) was added into series of plates

6. Test samples were mixed using vortex& prepared with a series of dilution by using normal saline water as diluents as follows (figure 5)

7. From each critical dilution best for countable range one ml of sample suspension was aseptically transferred to a series of plates that contain prepared Baird Parker agar plate as follows and the suspension were distributed over the surface using a sterile, bent glass rod.

8. Inoculums were allowed to be absorbed by the medium before inverting the plates. This activity was made within 5 minutes.

9. Then, the plates were incubated with 35-37°C for 48 hours.

10. Colonies with black, shiny, convex appearance in the medium were taken as *Staphylococcus aureus*. Plates with 20-200 colonies were selected for counting *Staphylococcus aureus* and total *S. aureus* count was calculated using the formula employed by PHE (2016)

Figure 5: Serial dilution and inoculated Baird parker agar plates for ***S****. aureus* enumeration

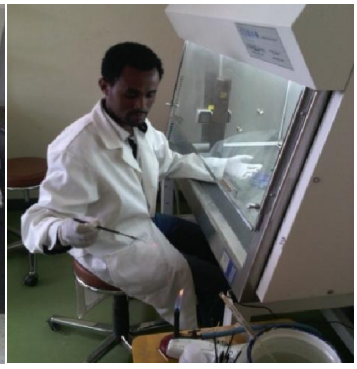
Annex 8.5. Site journey for data and sample collection (Figure A, B and C); and laboratory activity (Figure D, E, F, G, H and I)

A

B

C

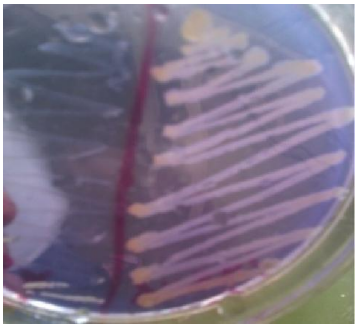
D



E

F

G



H

I