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**OCCURRENCE, ANTIMICROBIAL SUSCEPTIBILITY AND PUBLIC HEALTH
IMPLICATION OF MRSA IN READY TO EAT DAIRY FOODS IN HARAR TOWN
AND ITS SURROUNDING AREAS**

MSc THESIS



BY

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OCCURRENCE, ANTIMICROBIAL SUSCEPTIBILITY AND PUBLIC HEALTH
IMPLICATION OF MRSA IN READY TO EAT DAIRY FOODS IN HARAR TOWN AND
ITS SURROUNDING AREAS

MSc Thesis



A Thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa
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Veterinary Public Health

By

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Bishoftu, Ethiopia

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IMPLICATION OF MRSA IN READY TO EAT DAIRY FOODS IN HARAR TOWN AND
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First, I declare that this thesis is my *bonafide* work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for MSc degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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LIST OF ABBREVIATIONS

CA-MRSA	Community associated Methicillin Resistant <i>Staphylococcus aureus</i>
CC	Clonal Complex
DNA	Deoxyribonucleic Acid
EFSA	European Food Safety Authority
GHP	Good hygienic practice
GMP	Good manufacturing practice
HACCP	Hazard analysis critical control point
HA- MRSA	Hospital associated Methicillin Resistant <i>Staphylococcus aureus</i>
HCA- MRSA	Health care associated community Methicillin Resistant <i>S. aureus</i>
LA- MRSA	Livestock associated Methicillin Resistant <i>Staphylococcus aureus</i>
MRSA	Methicillin Resistant <i>Staphylococcus aureus</i>
MSSA	Methicillin susceptible <i>Staphylococcus aureus</i>
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
SCCmec	Staphylococcal cassette chromosome <i>mec</i>
ST	Sequence type

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ABSTRACT

Staphylococcus aureus is an important pathogen that recently becomes an emerging zoonotic pathogen that adds another facet to the already existed multi-dimensional challenge of ensuring food safety in developing country. It is highly implicated on its Methicillin-resistant (MRSA) strain and *Staphylococcal* food poisoning. A cross-sectional study was conducted from November 2016 to May 2017 in Harar town and its surrounding areas with the objectives to assess the occurrence of *MRSA* and their antimicrobial susceptibility profiles in ready to eat dairy foods. A semi-structured questionnaire was designed and administered to dairy farm owners and dairy food consumers to assess their consumption behavior and antibiotics usage. Standard bacteriological examination for *Staphylococcal* isolation and identification, *in vitro* antimicrobial susceptibility tests by using disc diffusion method were conducted by following the different standards that were employed and verified in previous studies. A total of 318 ready to eat dairy food products that were obtained from dairy farms, open markets, supermarkets, pastry and cafeteria were analyzed. It was found that 50.3 of the dairy foods were contaminated by *Staphylococci* species. Among the ready to eat dairy foods: cakes, cream cakes, raw milk and retailed milks were frequently contaminated by *S.aureus* as 23.1%, 29.2%, 25% and 27.5% respectively. Among 47 out of 58 isolates of *S. aureus* tested for susceptibility, 76.6 % of the isolates were found to be Methicillin resistant. Moreover, 34.0% of *S. aureus* isolates were resistant to two or more drugs and 10 (21.3%) were multi-drug resistant. The dairy food consumers interviewed in this study indicated that 51.2% of them consume raw/undercooked milk and milk products in different forms such as cheese, yoghurt, raw milk, boiled milk in cafeteria. Moreover, this survey documented different predisposing factors that can significantly contribute for further spread of multi drug resistance *S.aureus* in the study area. Some of these factors include; antibiotics usage without prescription; self-administration of drugs without proper clinical examination or medical consultation; cessation of drug usage before complete dose and common use of antibiotics for minor and unidentified cases such as coughing, nasal discharge, diarrhea, pneumonia and so on. Considering the consumption habit of consumers in the area and the irrational drug usage, the study clearly demonstrated that ready to eat dairy foods can be considered as an important vehicles for the transmission of antibiotic resistant

Staphylococci particularly *S.aureus* to the consumers which weigh the public health significance of MRSA. The finding from this study warrants further investigation on the sources of contamination in the ready to eat dairy foods and to elucidate the public health importance of *S.aureus* and MRSA.

Key words: Ready to eat dairy foods; *S. aureus*; Multi drug resistance; Harar, MRSA

1. INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a versatile pathogen in humans and animals which is responsible for a diverse spectrum of diseases ranging from minor skin infections to life threatening diseases, such as pneumonia and meningitis (Ferber, 2010). *S. aureus* can also produce toxin-mediated diseases, such as scalded skin syndrome (SSS) and toxic shock syndrome. In these cases, the organisms may remain relatively localized, but production of potent toxins causes systemic or widespread effects (Forbes *et al.*, 2007). Antimicrobial resistance is a major public health problem in many countries because of the persistent circulation of resistant strains of bacteria in the environment and the possible contamination of water and food. *S. aureus* has been reported to frequently show multiple antimicrobial resistance patterns (Enright, 2003).

The primary habitats of *S. aureus* are the nasal passages, the skin and hair of warm blooded animals and hence the sources from which this organism enters foods are mostly human or animal origin (Forbes *et al.*, 2007, Quinin *et al.*, 2002). About 40 to 44% of healthy humans carry staphylococci in the nose (Ferber, 2010). Strains present in the nose often contaminate the back of hands, fingers and face and so, nasal carriers can easily become skin carriers. Sometimes, food processors experience problems with elevated numbers of this microorganism, which can lead to problems with market acceptance. Food handlers can be a source of the spread of food-borne disease caused by poor personal hygiene or cross-contamination (Lues and van Tonder, 2007).

Milk and milk products are highly susceptible to microbial attack because of their rich nutrient composition, which provides a favorable medium for growth of many microorganisms, especially bacterial pathogens, hence they have a potential to transmit pathogenic organisms to humans (Chye *et al.*, 2004). *S. aureus* and other coagulase-positive *Staphylococcus* spp commonly contaminate the milk and its products and affect their quality (Chye *et al.*, 2004). They can contaminate the products either through contact with the cow's udder during milking or by cross contamination during handling and processing (Zouhairi *et al.*, 2010)

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a Gram-positive bacterium that is resistant to methicillin (a member of the penicillin family) and many other β -lactam antimicrobials; β -lactam antimicrobials include penicillins and cephalosporins (Chambers and Deleo, 2009; Chambers, 2001). Currently, the term MRSA is often used to describe multi-drug resistant *S. aureus*. Regrettably, β -lactam antimicrobial therapy is the most commonly prescribed treatment for general infections; thus, resistance to this broad class of antimicrobials poses a challenge for human and animal practitioners and represents substantial health risks as a result of treatment failure and lead to higher mortality and morbidity due to invasive infections (Chambers, 2001).

Different dairy food products that could lead to Staphylococcal food intoxication or serve as vehicle for MRSA transmission to humans, are reported with various research that isolate MRSA strains in different areas (Normanno *et al.*, 2007; Guven *et al.*, 2010; Zouhairi *et al.*, 2010; Sasidharan *et al.*, 2011). As these strains were found to harbour genes for expression of common Staphylococcal enterotoxins, they also had the potential to cause food poisoning (Normanno *et al.*, 2007). As indicated in Figure 1, it is obvious to find MRSA strains all over the continents, but there are no sufficient data or report available to find the distribution pattern of MRSA in the majority of African and Asian countries including Ethiopia (Grundmann *et al.*, 2006).

Even though the molecular analysis and detailed studies of MRSA are not well described in Ethiopia, different published as well as unpublished studies conducted in different parts of Ethiopia has shown that *S. aureus* was one of the predominant bacterial isolate that cause clinical and subclinical mastitis as well as isolated from dairy products (Lemma *et al.*, 2001; Workeneh *et al.*, 2002; Kero and Tareke, 2003; Hundera *et al.*, 2005; Mekonnen *et al.*, 2005; Almaw *et al.*, 2008 and 2009; Lakew *et al.*, 2009; Abera *et al.*, 2010; Bitew *et al.*, 2010; Tesfaye *et al.*, 2010; Addis *et al.*, 2011a and 2011b; Sori *et al.*, 2011, Daka *et al.*., 2012). Even though, yet there are limited data available on the status of MRSA in Ethiopia but there are some indication on the existence of MRSA both in human and dairy animals (Daka *et al.*, 2012; Lemma *et al.*, 2015; Eshetie *et al.*, 2016; Beyene *et al.*, 2017)

The practical application of these studies in mastitic cow and in dairy products warrants a great attention as far as food safety issue is concerned. This is because the organism can gain access to raw milk and milk products either by the direct excretion from udder having clinical and sub-clinical staphylococcal mastitis or by contamination from food handlers (Yilma *et al.*, 2007). The other fact that aggravates the situation is, most dairy foods in Ethiopia are produced by ‘cottage industry’ using traditional techniques with little emphasis on sanitary practices (Ashenafi, 2002).

Dairy sectors all over the world are challenged by expanding populations and economic growth. Growing demand for dairy products has led to a significant increase in public and private involvement in dairy cattle production. Hence, dairy cattle owners, consumers, institutions promoting dairy industry, public health professionals and policy makers require baseline information about the health of dairy cattle and the safety of dairy products. Antibiotics on dairy operations are used to treat highly prevalent infections, such as subclinical mastitis, and as a preventive measure during dry cow therapy. Monitoring the emergence of resistant pathogens in animal reservoirs is important particularly for those with zoonotic potential. Although different antibiotic classes of drugs are used in animal health management and in human medicine, the selection of resistance to one drug class may lead to cross-resistance to another (Haran, *et al.*, 2012). Antibiotics on dairy operations are used to treat highly prevalent infections, such as subclinical mastitis, and as a preventive measure during dry cow therapy. Monitoring the emergence of resistant pathogens in animal reservoirs is important particularly for those with zoonotic potential. According to different evidences, the occurrence of MRSA has been increasing at an alarming pace throughout the world with showing considerable variation in prevalence according to geographical area or region (Bertrand, 2010). Understanding the overall epidemiology of MRSA at country level is so substantial to support effective prevention and control strategies. Despite the inadequate study conducted in Ethiopia in general and Harar town in particular, it is clear that MRSA is a potentially important in veterinary and public health concern that requires further study to enhance understanding and effective response.

Therefore, the objectives of this research project are:-

General Objectives

- To assess the occurrence and antimicrobial susceptibility profiles of *S. aureus* in ready to eat dairy products in Harar town and its surrounding areas.
- To determine the occurrence of Methicillin resistance *S. aureus* (MRSA).

Specific objectives

- To assess the occurrence of *Staphylococcus* species in ready to eat dairy foods.
- To determine the antimicrobial susceptibility profiles of *S. aureus* including rate of occurrence of MRSA.
- To assess the public health significance of *S. aureus*/MRSA isolates in the study area.

2. LITERATURE REVIEW

Staphylococci are Gram positive cocci that commonly cause human and animal infections. *Staphylococcus* species colonize the skin and respiratory tract as a normal flora. In humans, especially in hospital, *Staphylococcus aureus* (*S. aureus*) and *Staphylococcus epidermidis* are the main *Staphylococci* causing health problems. *S. aureus* is a versatile and dangerous pathogen in humans. It causes skin infections, fatal septicemia, pneumonia and food poisoning as well as life threatening postsurgical infections. In animals, *S. aureus* is the most notorious pathogen that causes mastitis in dairy cows. It also causes morbidity and asymptomatic colonization in pigs (Butaye *et al.*, 2007; Greenwood *et al.*, 2002).

2.1 Historical background of Methicillin Resistant *S. aureus* (MRSA)

Staphylococcus aureus was discovered in the late 1880s and caused about 80% mortality before the discovery of penicillin around 1940. Even though the introduction of penicillin for treatment against gram-positive bacteria gave a great hope, in the 1950s there were persistent reports on the rise of penicillin resistant *Staphylococcus aureus*. In 1962, MRSA was reported after different clinical trials. That was two years after methicillin was on market for the first time in 1960. However, microbiologists remained confident over the use of methicillin as anti-staphylococcal agent, because methicillin resistance remained rare and was expressed by resistant organisms under different conditions that seemed to be very different from those prevailing at the site of infections (such as low temperature and high salt concentration). The MRSA developed combined resistance to other broad spectrum antibiotics such as streptomycin, tetracycline, erythromycin and gentamycin (Grundmann *et al.*, 2006).

The description “methicillin-resistant” was first used in 1961, based on the discovery of a human *S. aureus* infection in the United Kingdom that was resistant to methicillin. Since that time, MRSA has emerged as a significant problem worldwide, and the term has evolved to include resistance to additional β -lactam antimicrobials. Currently, the term MRSA is often used to describe multi-drug resistant *S. aureus*. Regrettably, β -lactam antimicrobial therapy is the most commonly prescribed treatment for general infections; thus, resistance to this broad class of antimicrobials poses a challenge for human and animal practitioners and represents substantial health risks as a result of treatment failure to those infected with this resistant organism (Chambers, 2001).

2.2. Mechanism of development of resistance

Penicillin and β -lactam antibiotic resistance can be achieved in two main ways; first bacteria can acquire a gene (through horizontal gene transfer) encoding beta-lactamase enzyme which cleaves the critical ring structure of the antibiotics preventing them from binding the penicillin-binding proteins and disrupting cell wall and peptidoglycan formation. Second, some bacteria can produce a modified penicillin-binding protein that no longer binds to the antibiotic and prevents the desired effects of the drug (Slonczewski and Foster, 2009).

Resistance in MRSA is related to Staphylococcal Chromosomal Cassete (SCC) encoded with *mecA* and *mecC* genes that specifies the production of an abnormal penicillin binding protein called PBP2a or PBP2'. Penicillin-binding proteins are membrane-bound enzymes, which targets for β -lactam antibiotics that exert antibacterial activity by binding and inhibiting enzymes necessary for bacterial cell wall synthesis. PBP2a has a decreased affinity for binding β -lactam antibiotics resulting in resistance not only to methicillin but also to most of β -lactams including penicillins and cephalosporins (Weems, 2001). The *mecA* gene which confers resistance to methicillin encodes a variant penicillin-binding protein, PBP2a. Native PBP2 catalyses a key step in the synthesis of the bacterial peptidoglycan cell wall, and is bound and inactivated by

penicillin-type antibiotics including methicillin. PBP2a is not inhibited by penicillin and can function instead of PBP2 (Pinho *et al.*, 2001). The *mecA* gene is carried within a larger family of DNA sequences called *SCCmec* (mobile staphylococcal chromosomal cassette), which can also encode other antibiotic resistance genes, and inserts into a specific site on the *S. aureus* chromosome called *orfX*. There are eight major *mecA* variants so far identified (*SCCmec* types I-VIII) (Katayama *et al.*, 2003; Grundmann *et al.*, 2006).

2.3. Epidemiology of MRSA

2. 3.1. Classification of MRSA

Different types of MRSA may be distinguished based on epidemiological groups. It should be noted that virulence may differ between strains within groups and that toxin producing strains may be found in any of the groups.

1. *Health Facility Associated MRSA*: includes the nosocomial infections likely to be acquired in health care settings when they emerge at least 48 hours after admission in patients having particular risk factors such as prolonged hospital stay, care in intensive care units (ICUs), prolonged antibiotic treatment, surgical interventions, and/or close contact with MRSA-positive individuals (Salgado *et al.*, 2003). It also describes infection associated with outpatients with MRSA infection and previous hospitalization, such as residence in a nursing home, receiving of home nursing, attending centers for dialysis and/or centers for diabetes where MRSA of hospital origin has been introduced. *SCCmec* types I-III are the most common in *Health facility Associated MRSA* (de Lencastre *et al.*, 2007).

2. *Community Associated MRSA (CA-MRSA)*: emerge in the community, infections occur in otherwise healthy people without a recent history of hospitalization or medical procedures, and are usually associated with skin and soft tissue infection. CA-MRSA typically harbor the smaller

and possibly more mobile *SCCmec* type IV but also type V, while multi resistance is less common (de Lencastre *et al.*, 2007; David *et al.*, 2008).

3. *Livestock Associated MRSA (LA-MRSA)*: refers mainly to the clonal spread of a certain MRSA strain (ST398) that colonise different food animal species (including horses) and may cause infections in humans. LA-MRSA carry *SCCmec* types III, IV or V. This MRSA type is the main concern of this review paper because LA-MRSA is not host specific and can transfer between animals and humans (EFSA, 2009).

2.3.2. Distribution of MRSA around the world

MRSA strains are distributed all over the continents (Figure 1). Moreover, over the past years according to the surveillance initiatives such as the National Nosocomial Infection Surveillance System and European Antimicrobial Resistance Surveillance System, MRSA rates have been promptly increasing worldwide. As indicated in Figure 1, it is obvious to find MRSA strains all over continents, but there are no sufficient data or report available to find the distribution pattern of MRSA in the majority of African and Asian countries including Ethiopia (Grundmann *et al.*, 2006).

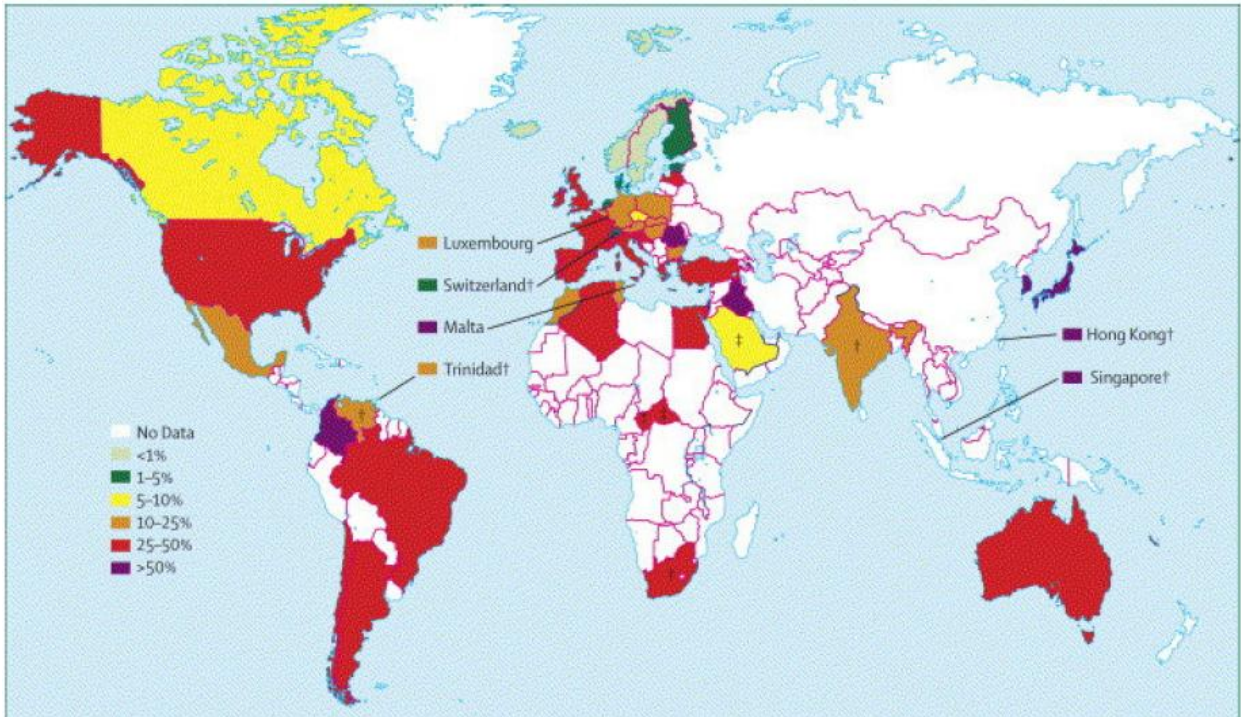


Figure 1: Worldwide distribution of MRSA (Source: Grundmann *et al.*, 2006)

2.3.3. Risk factors for colonization and infections

i. Risk factors for animals

Antimicrobial use

A causal relationship between the use of antimicrobial drugs and MRSA has been demonstrated in human medicine for different antimicrobial compounds. It is probable that similar conditions apply also to animals, in particular since LA-MRSA are often co-resistant to several other antimicrobial agents (Tacconelli *et al.*, 2008).

Other risk factors

A lower prevalence of MRSA was found among sows compared with piglets and finishers. In addition, a marked difference in the number of MRSA positive animals between open and closed farms. This is in line with a Dutch survey which indicated transmission of LA-MRSA within the production chain, e.g. from multiplier to finisher farms (Denis *et al.*, 2008; Van Duijkeren *et al.*, 2008). MRSA infections in small animals have also been associated with exposure to medical hospitals, extensive wounds, prolonged hospitalization and immunosuppression (Duquette and Nuttall, 2004).

ii. Risk factors for humans

Farmers are now considered a defined risk group which is screened upon admission to hospitals (Wulf *et al.*, 2008a). The occupational hazard for LA-MRSA colonization through pig contact has been confirmed in Europe (Denis *et al.*, 2008; Meemken *et al.*, 2008; Wulf *et al.*, 2008b). Occupational or recreational exposure to horses has been incriminated as a risk factor for human MRSA colonization (Weese *et al.*, 2006). It is likely that MRSA will behave similarly to MSSA if these become established in slaughter and processing plants. The origin of *S. aureus* found on carcasses at slaughter may be the animals entering the abattoir or the human handlers. The organism may become endemic in the environment of the slaughterhouse (EFSA, 2009). It has been shown that even apparently healthy animals may be MRSA reservoirs, and therefore may pose a risk to their handlers (Yasuda *et al.*, 2000). Several reports suggest that human *S. aureus* may become established as part of the endemic flora of food handlers, with subsequent contamination of carcasses and meat. Other livestock derived food product could lead to MRSA food intoxication or serve as vehicle for MRSA (Normanno, 2007).

2.3.4. Transmission in animals and humans

MRSA is transmitted from human to human, animal to human and vice versa and through viable or inanimate vectors (Kawada *et al.*, 2003). Reports have indicated that owners and veterinary personnel who come into contact with MRSA-colonized or MRSA-infected animals may become colonized by MRSA. There is then a risk that subsequent contact with susceptible animals or human beings will transfer MRSA infection (Weese, 2005; Lloyd, 2006). Skin to skin contact is probably the main route of transmission between humans, humans to animals, animals to human and between animals, however contaminated materials, surfaces, food or dust can play also a role in transmitting the agent (Lee, 2003). Airborne bacteria in livestock buildings are attached to dust particles which originate from the animals (e.g. epithelial cells, hair, and feathers), the feed, the litter and the faeces. They are emitted from the animal house by the ventilation system together with the exhaust and can contaminate soil and plants close to the building or can be transmitted by the airborne route to residential dwellings or other farms in the vicinity (Gibbs *et al.*, 2006). Dust is identified as a vehicle for the airborne transmission of *S. aureus* and may play a role in the spread of infections. Once exposed to MRSA, animals can become colonized, and may serve as reservoirs to transmit the infection to other animals and also to their human handlers (Weese, 2005). It has been shown that even apparently healthy animals may be MRSA reservoirs, and therefore may pose a risk to their handlers (Yasuda *et al.*, 2000).

2.4. Laboratory Diagnosis of MRSA

Reliable microbiological diagnostics of MRSA are essential for treatment, surveillance and control. Clinical microbiology laboratories play a central role in the detection, identification, antibiotic susceptibility testing, and confirmation of MRSA. These diagnostic techniques are discussed below.

2.4.1. Phenotypic detection methods

Phenotyping is based on the expression of genes, and it may be influenced by environmental factors. Several conditions used in phenotypic *in-vitro* laboratory tests, including medium, inoculum, incubation time and temperature, and test agent, are known to affect the expression of resistance (Brown and Walpole, 2001). It comprises the following common techniques.

i. Primary identification of *S. aureus*

S. aureus forms golden or white colonies on blood agar. Mannitol salt agar (MSA) or variations of this medium have been used as a primary isolation medium for MRSA (Smyth and Kahlmeter, 2005). All the strains produce catalase, an enzyme which converts hydrogen peroxide (H₂O₂) to water and oxygen. The catalase test is useful to distinguish *Staphylococci* from *Enterococci* and *Streptococci*. Most of these strains ferment mannitol and trehalose anaerobically which, among other tests to identify *S. aureus*. *S. aureus* is able to clot extracellular plasma, and can thereby be differentiated from most other staphylococci (Bannerman, 2003) by the coagulase tests. For routine testing, more rapid coagulase tests are used, especially commercial latex agglutination tests (Van Griethuysen *et al.*, 2001).

ii. Antimicrobial susceptibility test

The disc diffusion method and minimal inhibitory concentration (MIC) are used to determine the resistance towards antimicrobial agents. The strains are then categorized as sensitive, intermediate or resistant. The Clinical and Laboratory Standard Institute (CLSI, formerly NCCLS; USA) is a leading committee which recommends standards for antimicrobial susceptibility testing for global application (CLSI, 2015).

iii. Phage Typing

Phage typing is based on the capabilities of different bacteriophages to lyse different MRSA isolates. In phage typing, 23 standard phages are applied to an agar plate covered with the isolates. Areas of lysis (plaques) caused by each phage are noted as either a weak or a strong reaction. A strong reaction is defined as more than 50 plaques, is used to distinguish between isolates. A weak reaction is defined as a lower number of plaques is recorded but should not influence the final phage type. If the isolate shows no lysis at the routine test dilution (RTD), then a concentration of 100 times greater (RTD X 100) is carried out (Kerttula and Anne-Marie, 2007).

2. 4.2. Genotypic methods

Genotyping is based on analysis of chromosomes or extra chromosomal DNA. It allows direct comparisons of genotypes between isolates. It is also useful in establishing clonal relationship between isolates. The following are some of the methods used to genotype *Staphylococcus* species and MRSA.

i. Plasmid analysis

Plasmid analysis was one of the techniques used for the epidemiological studies of MRSA. This technique differentiates isolates according to the number and size of the plasmids. Plasmid analysis has been used successfully, but it has some disadvantages as plasmids can be lost and acquired easily and thus the analysis is often unreliable (Kerttula and Anne-Marie, 2007).

ii. Pulsed-Field Gel Electrophoresis (PFGE)

PFGE is the most widely used method in genotyping of MRSA which involves a few steps, namely embedding the microorganism in the agarose, lysing the organism in-situ, and digestion of chromosomal DNA with appropriate restriction enzymes. The restricted DNA fragments are then separated by PFGE. PFGE enables the separation of large fragments of DNA up to 10 Mb in size, where the direction of electric field periodically changes for short 'pulse' time. The change in the direction of electric causes the DNA to switch its course and to re-orient itself in a new course. Larger fragments take longer to re-orient itself, and thus separation of large and small fragments occur. The most commonly used PFGE format is the contour clamped homogenous electrophoresis (CHEF). CHEF uses an electrophoresis chamber that consists of six electrodes arranged in a hexagonal pattern. The current is then applied in each of three directions, at 120° apart, and in turn, for short periods of time. This short period of time is also known as pulse time. The DNA patterns produced are then compared with each other, usually using software, to establish the clonal or epidemiological relationship (Kerttula and Anne-Marie, 2007).

iii. Polymerase chain reaction typing (PCR)

Based on the polymerase chain reaction principle there are different methods that are used to detect and characterize the MRSA clones for epidemiological studies. The following are some of the most widely used techniques to detect MRSA in different research such as: Arbitrarily primed PCR, Coagulase gene typing, Protein A typing and Staphylococcal Cassette Chromosome *mec* (SCC*mec*) typing (Enright *et al.*, 2000; Oliveira and Lencastre, 2002; Kerttula and Anne-Marie, 2007).

2.4.3. Recent advances in bacterial strain typing

Methods discussed above are non-sequence based typing. Sequence based nucleotide typing is the latest advancement in bacterial strain typing in the last decade. Sequence based nucleotide typing is rapid and reliable, thus making it a feasible typing method. Sequence based typing include multi-locus sequence typing (MLST), multiple-locus variable number of tandem repeats analysis (MLVA), DNA microarray, whole genome sequencing; genomic analysis such as genome comparison of resistant and sensitive strains, and identification of resistant mechanism and mass spectrophotometry (Enright *et al.*, 2000; Kerttula and Anne-Marie, 2007).

2.5. Food Safety Concern of MRSA

Besides its importance as hospital and community pathogen, *S. aureus* is also a well-known cause of food intoxication. *S. aureus* food poisoning is the result of the production of staphylococcal enterotoxins, of which many types have been found in strains of *S. aureus* (Le Loir *et al.*, 2003). In contrast, MRSA food poisoning is very rare. The only report on MRSA food poisoning comes from the United States, where three adults became mildly ill after they had eaten coleslaw contaminated with MRSA producing enterotoxin C. This strain probably came from a food handler in the market place where the coleslaw was bought and was possibly of hospital origin (Jones *et al.*, 2002; kerouanton *et al.*, 2007).

MRSA of human origin found on meat of animal origin in different part of the world for example MRSA strain isolated from raw pork in the Netherlands, on raw pork and on a sample of raw beef in United States, in chicken meat in South Korea and Jordan. All these studies did not report whether the detected strains were capable of producing enterotoxins. In Japan however, a MRSA strain of human origin isolated from raw chicken samples appeared capable of producing enterotoxin C (Lee, 2003; Kitai *et al.*, 2005; Kwon *et al.*, 2006; EFSA, 2009; Pu *et al.*, 2009).

The emergence of LA-MRSA in food animal raises the question whether these strains are also present on derived meat and via this way could find an entry key for a larger spread in the human

population. A Dutch study proved that LA-MRSA could be present on pork. In other study MRSA strains were isolated from 11.9% raw meat products. An overwhelming 85% of the strains were LA-MRSA. The highest isolation percentages were found in turkey, chicken and veal meat. (de Boer *et al.*, 2009). Two reports describe MRSA isolation from healthy and sick chickens (Lee 2003, 2006), but there are limited prevalence and incidence data. Two recent studies reported isolation of ST398 from healthy chickens (Nemati *et al.*, 2008; Persoons *et al.*, 2009), and a third study characterized isolates from infected poultry, reported the predominance of a common human epidemic clone (Clonal Ccomplex 5) (Hasman *et al.*, 2010).

In addition to meat, different dairy products that could lead to MRSA food intoxication or serve as vehicle for MRSA transmission are reported with various research that isolate MRSA strains (Normanno *et al.*, 2007; Guven *et al.*, 2010; Zouhairi *et al.*, 2010; Sasidharan *et al.*, 2011). As these strains were found to harbor genes for expression of common *Staphylococcal* enterotoxins, they had the potential to cause food poisoning (Normanno *et al.*, 2007).

It is reasonable to suspect that MRSA food poisoning could become more common with an increase in food contamination and colonization of food handlers. As with methicillin-susceptible *Staphylococci*, classical *Staphylococcal* “food poisoning” caused by ingestion of preformed enterotoxins can occur. While MRSA isolates can possess various enterotoxin genes there is only one report of *Staphylococcal* food poisoning caused by MRSA (Jones *et al.*, 2002; Kerouanton *et al.*, 2007). As already outlined above, both farm and companion animals and foods of animal origin are the potential reservoirs of MRSA which aggravate the public health significance of MRSA (Weese *et al.*, 2005; Lee, 2006; de Neeling *et al.*, 2007). It has been demonstrated that LA-MRSA is able to colonize the nasal cavity of farmers, pig attendants and their family members, veterinarians, veterinary students, laboratory personnel and meat inspectors who have been exposed to colonized or infected animals as well as to the dust from animal houses (EFSA, 2009). Infections due to MRSA are associated with worse outcome in addition to prolonged

hospital stay, higher cost of treatment and increased mortality (Zahar *et al.*, 2005; Shorr *et al.*, 2006).

Another important aspect of LA-MRSA in humans is that, although infrequently reported and not (yet) substantiated by epidemiological data, LA-MRSA appears to be capable to transfer between humans. In the Netherlands, a six-months-old daughter of pig farmers, who presumably had not had direct contact with pigs, appeared colonized. In that same study, the son of a veterinarian and the nurse treating the son in the hospital to which he was admitted also appeared to be carrying LA-MRSA (Voss *et al.*, 2005).

It has become clear that MRSA exposure is an occupational risk in veterinary medicine. Most, but not all, studies of colonization of veterinary personnel have indicated relatively high colonization rates. The strains present in veterinary personnel further support the hypothesis of occupational exposure, at least for equine and food animal veterinarians (Weese, 2005).

2.6. The Status of MRSA in Ethiopia

Different published as well as unpublished studies conducted in different parts of Ethiopia showed that *S. aureus* was one of the predominant bacterial isolate that cause clinical and subclinical mastitis (Lemma *et al.*, 2001; Workeneh *et al.*, 2002; Kero and Tareke, 2003; Hundera *et al.*, 2005; Mekonnen *et al.*, 2005; Almaw *et al.*, 2008 and 2009; Lakew *et al.*, 2009; Abera *et al.*, 2010; Bitew *et al.*, 2010; Tesfaye *et al.*, 2010; Sori *et al.*, 2011; Abera *et al.*, 2012). The result of these reports indicated the significance of this bacterium which deserves great attention from food safety and public health point of view as a main cause of toxin-mediated virulence, invasiveness, and antibiotic resistance (EFSA, 2009). Either of these can occur since the organism can gain access to raw milk and milk products either by the direct excretion from udder having clinical and sub-clinical staphylococcal mastitis or by contamination from food handlers (Yilma *et al.*, 2007). Study conducted in Debre Zeit and surrounding areas, for isolation

and identification of *Staphylococcus* species in raw bovine milk and cottage cheese indicated the prevalence of *S. aureus* to be 10% and 5% respectively (Abera *et al.*, 2010; Addis *et al.*, 2011a and 2011b). Indirectly some other reports indicated the resistance pattern of *S. aureus* to other commonly available antimicrobials like ampicillin, penicillin and tetracycline (Mekonnen *et al.*, 2005; Abera *et al.*, 2010; Sori *et al.*, 2011, Daka *et al.*, 2012). Even though, yet there are limited data available on the status of MRSA in Ethiopia but there are some indication on the existence of MRSA both in human and dairy animals (Daka *et al.*, 2012; Lemma *et al.*, 2015; Eshetie *et al.*, 2016; Beyene *et al.*, 2017).

2.7. Control and Prevention of MRSA

2.7.1. Reduction of selective pressure of the use of veterinary antimicrobial agents

A reduction of the selective pressure by avoiding routine mass medication could be a major potential control measure. An additional benefit of this measure would be to preserve the efficacy of the current antimicrobials for veterinary and human use. To confirm a reduction of antimicrobial consumption, detailed information on the applied therapies is necessary, with respect to animal species and the route of administration. Preferably the indication, the production system (e.g. broiler versus layer), and the regimens applied needs to be documented in detail (dose, duration, formulation, treatment interval) (EFSA, 2009).

2.7.2. Husbandry interventions and management organization of animal and food production

General control options on farms, in slaughterhouses and in food production areas are likely to be the same for MSSA as well as MRSA, and include good husbandry practices, Hazard Analysis and Critical control Point (HACCP), Good Hygienic Practice (GHP), and Good Manufacturing Practice (GMP).

LA-MRSA may also be introduced by contaminated or colonised humans. Reduction of the number of visitors and implementation of control measures such as shower-in, dedicated clothing and other measures for employees and visitors who are allowed entry may help to prevent introduction of MRSA into a herd/flock, or food production area (Ribbens, *et al.*, 2008).

2.7.3. Options for control of transfer of MRSA from animals to humans

Vulnerable patients (including the immunocompromised, recently hospitalized, elderly, postsurgical patients, and known MRSA carriers) that have contact with small animals, especially those who may be infected or colonised with MRSA or have received antibiotics recently, should be educated about potential zoonotic transfer and hygiene. Basic hygiene measures are key, especially hand washing before and after pet contact, and if possible, avoiding direct contact with nasal secretions, saliva and wounds (Anderson *et al.*, 2008).

2.7.4. Monitoring and surveillance of MRSA

Monitoring and surveillance are not control options as such, however these processes are essential for determining control strategies and for the evaluation of their effectiveness. The complex epidemiology of MRSA makes monitoring and surveillance necessary at local, regional and national levels (EFSA, 2009).

3. MATERIALS AND METHODS

3.1 Description of the study area

The study was conducted in Harar, a fortified historic town in Harari National regional state, Eastern Ethiopia and surrounding areas such as Babile and Haramaya towns which are the source of dairy products for the dwellers in Harar town. The region lies between $9^{\circ} 11' 49''$ and $9^{\circ} 24' 42''$ N latitude and between $42^{\circ} 03' 30''$ and $42^{\circ} 16' 24''$ E longitude. The elevation in the Harar city varies from 1,600 to over 1,920 masl (Meter above sea level). Harar, the capital of the Region is located at between $09^{\circ} 18' 43''$ N latitude and $42^{\circ} 07' 23''$ E longitude at 515 km east of Addis Ababa, and lies in the south-western part of the region just off the southern edge of the southeastern plateau dividing the Great Rift Valley from the plains of the Ogaden lowlands. Administratively it is divided in to 19 town kebeles and 17 rural administrative units called peasant associations (PAs). Based on the available records, the mean annual daily temperature at the Harar weather Station is 19.2°C , while the annual mean minimum and mean maximum daily temperatures are 13.0°C and 25.2°C , respectively. The mean annual rainfall can be between 667 mm to 714.6 mm. Topographically, the Harari Region may be divided in to highlands (> 1400 masl) and lowlands/valley bottoms (<1400 masl) with elevation of 1400 masl as limit, only 3670 ha (10.7%) fall in the lowlands and 30,650 ha (89.3%) under the highlands (Ishot, 2009).

Growing demand for dairy products has led to a significant increase in private involvement in dairy cattle production in small holder farming system (Harari Livestock and Fisheries office). Similar to other parts of the country people in the town consume raw dairy products and also the preparation of some traditional milk products occur in home and in some 'cottage industry' by using traditional techniques with little sanitary practices. The people in the town can consume or get the ready to eat dairy foods from different sources such as from open/retail markets, supermarkets, pastry, cafeteria and dairy farms that processed or sell dairy products such as raw,

boiled, pasteurized, cheese, yoghurt, cakes and ice creams for the consumers in the town (Personal observation and communication).

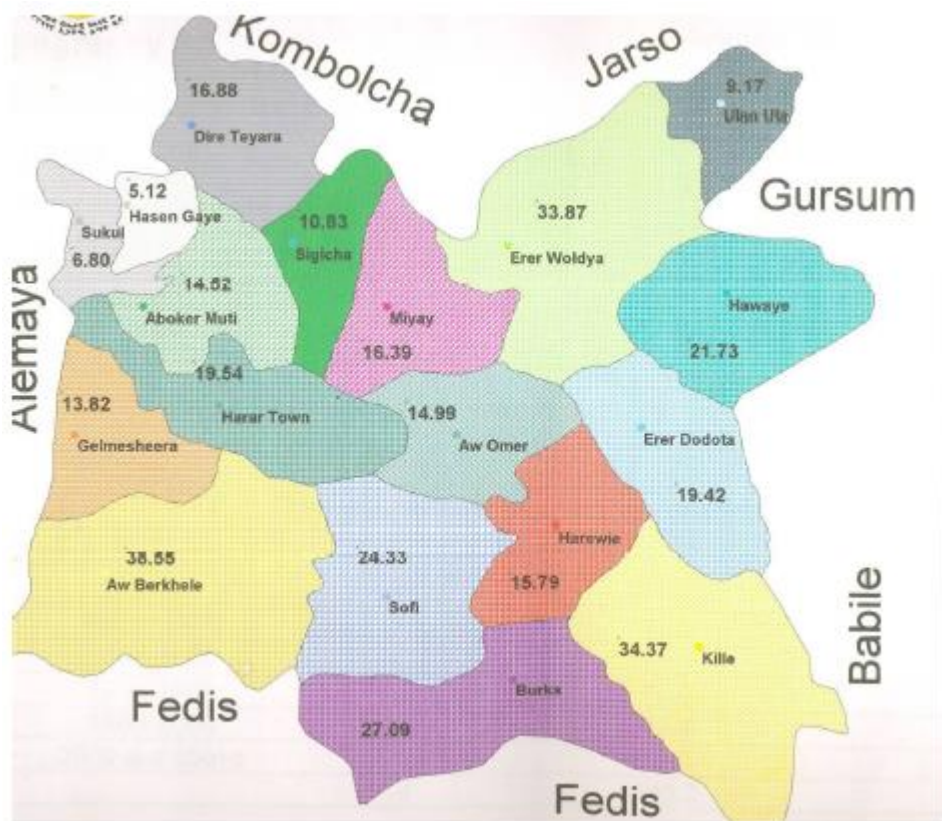


Figure 2: Map of the Harari National Regional State and surrounding districts of the East Hararghe zone of Oromia (Ishot, 2009).

3.2. Description of Study population

The study was conducted on those dairy food products that were commonly consumed by the people in Harar town and surrounding areas which were raw, boiled and pasteurized milk, cheese, yoghurt, cakes and creams (Both ice-cream and cream cakes). Retailed milk samples were

collected from retail outlets by purchasing from retailers from open market. Retailed milk is raw milk that was originated from various vicinities of the towns and pooled together and sold at open market. The vendors displayed the retailed milk in plastic bottles. Boiled milk which are moderately boiled by tea machine, cakes and ice cream samples were purchased from cafeterias and pastries that sold these ready to consume dairy food products for the consumers in the towns. However the pasteurized milk samples were obtained from supermarkets that purchased directly from factories around Addis Ababa and keep them cooled at 4⁰ C temperatures and delivered to consumers.

3.3. Study design

A cross-sectional survey was conducted from November 2016 to May 2017.

3.4. Determination of Sample size and Sampling Methods

The sample size was determined by considering the Central Limit Theorem which recommended for normal distribution of population provided the sample size to be sufficiently large (usually $n \geq 30$). By considering this and to increase the precision a total of 318 ready to consume dairy food samples were collected and analyzed microbiologically as indicated in Table 1. Simple random sampling technique was used to take the dairy food samples from the target locations such as open/retail markets, supermarkets, pastry, cafeteria and dairy farms.

Table 1: Dairy food type and their number used for this study

Sample type	No analyzed
Boiled milk	62
Cake	39
Cheese	9
Cream cake	48
Ice cream	19
Raw milk	32
Pasteurized milk	33
Retailed milk	40
Yoghurt	36
Total	318

3.5. Sample collection and transportation

The dairy food samples were purchased randomly from the open/retail markets, supermarkets, pastry, cafeteria and dairy farms as sold for consumers. They were properly identified by date of collection, sources and sample type. After purchase, the samples were put into sterile bottles and kept in an icebox containing ice packs and transported to the Microbiology laboratory of the College of Veterinary Medicine, Haramaya University. Upon arrival at the laboratory, the samples were stored in a refrigerator at 4^oC until processed on the day of collection.

3.6. Isolation and Identification of *Staphylococcus aureus*

3.6.1. Bacteriological Culturing

The techniques and interpretation of findings recommended by the International Organization for Standardization, ISO 6888-3: 2003 and Quinn *et al.* (2002) were employed for the isolation and identification of *Staphylococcus* species (Annex- 1). A loop full of samples enriched by buffer peptone water (BPW) (Oxoid, Hampshire, UK) was streaked on sterile 7% sheep blood agar plate (BAP) ((Oxoid, Hampshire, UK) and the plates were incubated aerobically at 37°C and examined after 24 to 48 hrs of incubation for growth. Then changes observed on blood agar were; growth, no hemolysis, and hemolysis (Annex 10A). If there is no hemolysis, negative, if there is growth and hemolysis it is positive and sub cultured to nutrient agar (HiMedia, Mumbai, India) for further examination. Finally the isolation and identification of *Staphylococcus aureus* was done as indicated below.

3.6.1.1. Gram's staining

All suspected cultures of *Staphylococcus* species were subjected to gram's stain and observed under light microscope for gram's reaction, size, and shape and cell arrangements. The gram positive cocci that were arranged in bunch of grape like clusters were considered as presumptive *Staphylococcus* species.

3.6.1.2. Catalase test

Catalase test were done by picking colony using a sterile loop from nutrient agar (HiMedia, Mumbai, India) and mixed thoroughly with a drop of 3% H₂O₂ on a glass slide. If the organism was positive, bubbles of oxygen liberated within a few seconds but the catalase negative isolates did not produce bubbles. The catalase positive cocci were considered as *Staphylococci* (Quinn *et al.*, 2002) (Annex 10 C).

3.6.1.3. Mannitol salt agar (MSA)

The colonies that were identified by gram staining and catalase test were streaked on mannitol salt agar (MSA) (Oxoid, Hampshire, UK) plate and incubated at 37°C and examined after 24-48 hr for growth and change in the colour of the medium. The presence of growth and change in the pH of media (red to golden yellow color) were considered as confirmative identification of *Staphylococci*. The golden yellow discoloration due to manitol fermentation shows the *Staphylococcus aureus*. The delayed yellow discoloration was caused by *Staphylococcus intermedius* and colonies that fail to produce any change on the medium were considered as *Staphylococcus hyicus* or Coagulase Negative Staphylooccus (CNS) (Smyth and Kahlmeter, 2005). (Annex 10 B).

3.6.1.4. Coagulase test

The tube coagulase test was performed in sterile tubes by adding 0.5 ml of selected isolates of *Staphylococcus* Spp. grown on Tryptone Soya Broth (TSB) (Oxoid, Hampshire, UK) at 37°C for 24 hrs to 0.5 ml rabbit plasma (National Veterinary Institute (NVI), Bishoftu, Ethiopia). After mixing by gentle rotation, the tube was incubated at 37°C along with a negative control tube containing a mixture of 0.5 ml of sterile TSB and 0.5 ml of rabbit plasma. Clotting was evaluated at 30 minutes intervals for the first 4 hrs of the test and then after 24 hrs of incubation. If there was an extent of clotting observed from loose clot to a solid clot that immovable when the tube was tilted (inverted), considered as positive. However if no degree of clotting observed it was negative (Lamprell et al., 2004) (Annex 10 D).

3.6.1.5. Purple agar plate

Finally the suspected culture was inoculated on Purple Agar Base (PAB) ((Difco™, Becton, Dikson, USA) media plate supplemented with 1% of maltose and incubated at 37°C for 24-48hr

to differentiate coagulase- positive *Staphylococci* isolates. The identification was based on the fact that *S. aureus* rapidly fermented maltose and gave yellow discoloration of the media. *S. intermedius* gives weak or delayed reaction that produced weak yellow discoloration and *S. hyicus* did not ferment maltose but formed deeper purple around the colony (Quinn et al., 2002) (Annex 10 E).

3.6.2. Antibiotics susceptibility Testing

Antibiotic susceptibility testing was performed on *S. aureus* isolates. They were tested for their susceptibility profiles on Mueller Hinton Agar (Oxoid, Hampshire, UK) to 5 antibiotics by using Kirby–Bauer disk diffusion method as indicated by the Clinical and Laboratory Standards Institute (CLSI, 2015). Antibiotic discs were gently pressed onto the inoculated Mueller Hinton agar to ensure intimate contact with the surface and the plates were incubated aerobically at 37°C for 18 to 24 hrs. The antimicrobial agents tested include tetracycline (30µg), cephalothin (30µg), gentamicin (10µg); Penicillin-G (10 IU) and cefoxitin (30µg). Inhibition zone diameters were measured and values interpreted (Annex 8). Reading from CLSI (CLSI, 2015) was used to interpret the results obtained. *S. aureus* isolates were then classified as resistant, intermediate or susceptible to a particular antibiotic. Isolates displaying resistance to three or more antimicrobial classes were considered to be multidrug-resistant (MDR) (CLSI, 2015) (Annex 10 F). The use of the cefoxitin disc indicated for the screening of mec-A mediated Methicillin/Oxacillin resistance profiles of *S.aureus*.

3.7. Questionnaire survey

Two semi-structured questionnaire were designed. The first was administered to dairy cow owners and milking personnel of selected farms. The second questionnaire was used to interview

randomly selected consumers on their dairy foods consumption habits and the use of antibiotics. Brief checklist was also prepared and administered to the veterinary clinics in Babile, Harar and Haramaya towns in order to collect information on the antibiotics consumption for the last 1 year (Annex 11). Majority of the questions were closed ended but some open ended questions were added to assess the practice and experience of the respondents. The questions were forwarded for the participants after translation to one of their local languages including Amharic, Harari, Orommiffa and Somali.

3.8. Ethical Considerations

This study involved a questionnaire based survey of dairy farmers as well as dairy food consumers. Seventy dairy farmers and 41 dairy food consumers were randomly selected. Volunteer participants, from whom their verbal consent was obtained, were interviewed. Following detailed discussion about the objectives of the study with each participant, the face-to-face interview was conducted.

3.9. Data management and analysis

Laboratory and questionnaire data collected were coded, entered into Microsoft Excel spreadsheet and analyzed by using SPSS version 20.0 software. Descriptive statistics was executed to summarize the information collected in the different stages of this study. The occurrence or the proportion of *S. aureus* was calculated as the number of positive isolates divided by the total number of samples examined. Finally the proportion of MRSA calculated as the proportion of the total MRSA isolates with the total number of *S. aureus* isolates.

4. RESULTS

4.1. The occurrence of *Staphylococcus* species in ready to eat dairy foods

This study revealed the occurrence of *Staphylococci* species in 50.3% of the microbiologically analyzed ready to eat dairy foods obtained from Harar town and surrounding areas. The three most isolated species were *S.aureus*, CNS and *S.hyicus* as 18.2 % (58/318); 14.8 % (47/318) and 11.3 % (36/318), respectively. Among the ready to eat dairy foods: cakes, cream cakes, raw milk and retailed milks were commonly contaminated by *S.aureus* as 23.1%, 29.2%, 25% and 27.5% respectively (Table 2). Surprisingly there were no dairy foods not harboring *S.aureus*.

Table 2: Proportion of *Staphylococci* species in ready to eat dairy foods (n=381)

Dairy food type	No of samples examined	<u>Staphylococci species</u>			
		CNS (%)	<i>S.hyicus</i> (%)	<i>S.aureus</i> (%)	<i>S.intermidus</i> (%)
Boiled milk	62	4.8	1.6	3.2	3.2
Cake	39	15.4	30.8	23.1	5.1
Cheese	9	66.7	11.1	11.1	0
Cream cake	48	18.8	18.8	29.2	10.4
Ice cream	19	15.8	5.3	15.8	0
Raw milk	32	21.9	6.3	25	15.6
Pasteurized milk	33	3	0	12.1	3
Retailed milk	40	15	12.5	27.5	5
Yoghurt	36	16.7	13.9	16.7	5.6
Total	318	14.8	11.3	18.2	6

4.2 Antibiotics Susceptibility profiles of *S. aureus* isolates

Among 58 isolates of *S. aureus* identified in this study, antimicrobial susceptibility test was conducted on 47 of them. Twenty six of the isolates were tested with 5 antibiotic discs including Cefoxitin. However the remaining 21 were tested only by Cefoxitin that is a screening test for mec-A mediated Methicillin (Oxacillin) resistance. Among 47 isolates tested for susceptibility 36 (76.6 %) of the isolates were found to be Methicillin (Oxacillin) resistant. However, 16 (34.0%) of *S. aureus* isolates were resistant to two or more drugs and 9 (19.1%) were multi-drug resistance which were resistance to three or more antibiotics (Table 3 & 4).

Table 3: Antimicrobial susceptibility profiles of *S. aureus* isolates from ready to eat dairy foods in Harar town and surrounding areas (n=47)

Antimicrobials	<u>Susceptible (S)</u> <u>N₀ (%)</u>	<u>Intermediate</u> <u>(I) N₀ (%)</u>	<u>Resistant (R)</u> <u>N₀ (%)</u>
Gentamicin (G) (10 µg)	19 (40.4)	0 (0)	7 (14.9)
Penicillin G (10 units)	1(2.1)	0 (0)	25 (53.2)
Tetracycline (30 µg)	10 (21.3)	0 (0)	16 (34.0)
Cephalothin (CEP) (30µg)	12(25.5)	5(10.6)	9 (19.1)
Cefoxitin (30µg)	11 (23.4)	0 (0)	36 (76.6)

Table 4: Resistance Pattern of *S. aureus* isolates from ready to eat dairy foods in Harar town and surrounding areas (n=47)

Antimicrobials	Resistant (R) No (%)
Penicillin G & Tetracycline	7 (14.9)
Gentamicin, Penicillin G & Tetracycline	3 (6.4)
Penicillin G, Tetracycline & Cephalothin	2 (4.3)
Gentamicin, Penicillin G, Tetracycline & Cephalothin	4 (8.5)
Cefoxitin	36 (76.6)

4.3. Questionnaire Survey

4.3.1 Results on antibiotics usage of dairy farms in the study area

This study identified that a total of 2962 vials of antibiotics were used in the three towns for treatment and for prophylaxis purposes. As indicated by the dairy animal owners these antibiotics were purchased from government clinics or private vet clinics that were available in the areas (Table 5).

Table 5: Commonly available antibiotics and number of vials used in the last 1 year in the veterinary clinics (Government or private) in the study area

Study area	Antibiotics used (Dosage in vials)				Total
	Oxytetracycline (20%)	Oxytetracyc- line (10%)	Penicillin- Streptomycin	Sulfa drugs	
Haramaya	266	220	240	120	846
Harar	564	175	265	117	1121
Babile	384	225	260	126	995
Total	1214	620	765	363	2962

The survey conducted on the dairy animal owner showed that the entire study participant uses antibiotics for their animal for the last one year by purchasing either from government veterinary clinic or private drug stores found in the area. Moreover 14.3% uses drug without prescription; 10% of the respondent administered the drugs by themselves without any proper clinical examination and authorization/training to do so. However 32.9% of them stopped the administration of drugs before attaining the complete dose recommended by the clinician. Surprisingly 72.9% of the respondent prefers to use antibiotics for minor and unidentified cases like cough, diarrhea, nasal discharge, skin infections and so on (Table 6).

4.3.2. Results of antibiotics usage and practices by dairy food consumers in the study area

The dairy food consumers interviewed in this study indicated that 51.2% of them consume raw/undercooked milk and milk products in different forms such as cheese, yoghurt, raw milk, boiled milk in cafeteria. Among them, 53.7% explained their knowledge on the side-effects of raw/undercooked milk consumption. However, 46.3% of the respondents encountered abnormal/sick consequences after consumption of raw/undercooked milk products (Table 7).

Likewise the results of the questionnaire on the dairy food consumers has shown that, 58.5% of the respondents have taken antibiotics for the last 1 year but 29.3% of them used it without prescription and proper medical consultation. Moreover 63.4% of the consumers reported that they stop taking of the drug before reaching the full dose recommended or supposed to be taken. About 58.5% of the study participants preferred to take antibiotics for minor and unidentified cases like cough, diarrhea, pneumonia, nasal discharge, joint pain and soon (Table 7).

Table 6: Dairy farm owner’s knowledge and practice on the use of antibiotics in the study area

Parameters	Number of respondents	
	Total	Yes (%)
Antibiotic usage for the last 1 year	70	70(100)
Practice on antibiotic purchase	70	
With prescription		60(85.7)
Without Prescription		10(14.3)
Self-administration of antibacterial to your animals (without prescription)	70	7(10)
Antibiotic therapy without proper clinical examination of animal	70	11(15.7)
Administration of proper dose as recommended by clinician and manufacturers	70	46(65.7)
Action taken on milk if your cow is being treated with antibiotics	70	
Feeding to calves		45(64.3)
Consumed by the family members		15(21.4)
Continue selling to the consumers		10(14.3)
Knowledge on risk of using antibiotics treated milk for consumers	70	37(52.9)
Knowledge on withdrawal period	70	0(0)
Knowledge on frequent use of the same antibiotics and its negative effect on efficacy	70	20(28.6)
Cessation of prescribed antibiotics usage in the middle or before complete dosage.	70	23(32.9)
Action taken when treatment doesn’t work/ ineffective	70	
Change drug		9(12.9)
Increased dose of the drug		3(4.3)
Take to the clinic		9(12.9)
Contact Veterinarian		45(64.3)
Use traditional medicine		4(5.7)
Knowledge about antibiotic resistance	70	23(32.9)
Perception on misuse of antibiotics (overdose, under dose) as cause of reduction in efficacy	70	39(55.7)
Use of antibiotic for minor/unidentified cases (cough, diarrhea, wound, Pneumonia, poor appetite)	70	51(72.9)
Occurrence of antibiotic treatment failure?	70	23(32.9)
Any information or advice received on unnecessary use of antibiotics for your animals	70	43(61.4)

Table 7: Consumption habit and antibiotics usage in dairy food consumers in Harar and surrounding areas

Parameters	Number of respondents	
	Total	Yes (%)
Consumption of Raw/undercooked milk	41	21(51.2)
Knowledge on any side effects of raw milk consumption	41	22(53.7)
Disease or abnormal condition occurred after consuming raw or undercooked milk	41	19(46.3)
Use of antibiotics for the last 1 year	41	24(58.5)
Practice on antibiotic purchase	41	
With prescription		29(70.7)
Without Prescription		12(29.3)
Knowledge on frequent use of the same antibiotics and its negative effect on efficacy	41	14(34.1)
Cessation of prescribed antibiotics usage in the middle or before complete dosag	41	26(63.4)
Action taken when treatment doesn't work/ ineffective	41	
Change the drug using		13(31.7)
Increased the recommended dose of the drug		10(24.4)
Go back to the clinic/Consult physician		18(43.9)
Knowledge about antibiotic resistance	41	11(26.8)
Perception on misuse of antibiotics (overdose, under dose) as cause of reduction in efficacy	41	18(43.9)
Use of antibiotic for minor/unidentified cases (cough, diarrhea, wound, Pneumonia, poor appetite)	41	24(58.5)
Occurrence of antibiotic treatment failure?	41	15(36.6)
Any information or advice received on unnecessary use of antibiotics for your animals	41	7(17.1)

5. DISCUSSIONS

Livestock can carry *S. aureus* in nasal cavity and on skin. Foods derived from these animals can be contaminated during and after slaughtering, during milking and processing. Studies on the prevalence and characteristics of *S. aureus*, especially MRSA from dairy foods derived from Ethiopia, are inadequate. Hence, surveillance of dairy foods for microbial contamination is crucial for the protection of public health and consumer interest. The production of safe and wholesome food has important economic implications in an increasingly competitive global market.

This study describes the isolation and antibiotic susceptibility characterization of *S. aureus* from ready to consume dairy products. The result indicated that 50.3 % of ready to eat dairy foods were harboring *Staphylococci* species, 18.2 % (58/318) of the dairy foods were contaminated by *S. aureus*. It indicated that *S. aureus* is the most frequently occurred isolates among *Staphylococci* species in milk and milk products. Many studies conducted in bovine mastitis in Ethiopia reported this fact (Lemma *et al.*, 2001; Workeneh *et al.*, 2002; Kero and Tareke, 2003; Hundera *et al.*, 2005; Mekonnen *et al.*, 2005; Almaw *et al.*, 2008 and 2009; Lakew *et al.*, 2009; Abera *et al.*, 2010; Bitew *et al.*, 2010; Tesfaye *et al.*, 2010; Addis *et al.*, 2011a and 2011b; Sori *et al.*, 2011; Abera, *et al.*, 2012; Daka *et al.*, 2012). The finding from this study found to be higher than the reports of Beyene *et al.*, 2017 who reported 16.1% *S.aureus* and 12.4% CNS in Addis Ababa. The occurrence of *S.aureus* in the present study was higher than the report from Debre Zeit which indicated 5% in cottage cheese and 10 % in raw milk (Addis *et al.*, 2011a and 2011b). This result was found to be lower than a study conducted in Hawassa which reported 40.6 % of *S. aureus* in milk (Daka *et al.*, 2012). Similarly in a recent study from Hawassa *S. aureus* was isolated from 51.2% of the milk samples taken from mastitic herds (Abebe *et al.*, 2017). A report from Adama, central Ethiopia showed 42.1 % occurrence of *S. aureus* in mastitic dairy cows (Abera *et .*, 2010). A recent report showed that, 16.1 % occurrence of *S.aureus* in dairy farms in Addis Ababa (Beyene *et al.*, 2017). Apart from Ethiopia a study in Morocco isolated 40% of *S.*

aureus in whey and traditional cheese (Bendahou *et al.*, 2008). A study conducted in Poland reported 35.7% *S. aureus* from ready to eat foods (Podkowik *et al.*, 2008). Among the ready to consume dairy foods cakes, cream cakes, raw milk and retailed milks were found to be commonly contaminated by *S.aureus*. Because of their rich nutritive value and complex chemical composition make these products more suitable for growth and multiplication of microorganisms (Chye *et al.*, 2004). Our result demonstrated that ready to eat dairy foods can be considered as an important source of antibiotic resistant *Staphylococci* particularly *S.aureus*. Hence they are considered as vehicles for its transmission to consumers.

Even though, the animal health and public health significance of *S. aureus* in mastitis and dairy food varies from country to country and between studies, a high proportion of these bacteria in milk and milk products can be significantly associated with poor hygienic practices like unclean hands of milkers and unhygienic milking utensils and milking environment (Mehmeti *et al.*, 2016). Based on observations made during the collection of samples, we therefore observed improper hygienic practices like selling milk in an open market with frequent contact to dust and flies, presenting milk in plastic jars (Plastic bottles). Besides this, the study conducted in and around Haramaya university reported an evidence of poor farm management practices like poor animal house hygiene, poor udder hygiene and poor husbandry and milking practices which were favoring high prevalence of coagulase positive *Staphylococci* species in the milk (Tafa *et al.*, 2015). Because *S.aureus* is a part of the normal teat skin flora and mucosa of humans and animals, and also found free-living in the environment (Normanno *et al.*, 2007), definitely it can be a common cause of contamination of milk and milk products. In addition, raw milk may contain it if the cow suffers from mastitis as it is indicated by many studies in Ethiopia. Bacterial contamination of milk and its products usually occur during the milking and handling process and this depends on the sanitary condition of the environment, utensils used for milking and the hygiene of milking personnel. It could also result from pathogens that enter the udder through the teat opening (Kalsoom *et al.*, 2004).

This study disclosed a high occurrence of cefoxitin resistance *S.aureus* isolates (76.6%) from ready to eat dairy food products in the study area, which is an indication of mec-A gene mediated methicillin or Oxacillin resistance. Besides this, the highest resistance was identified for penicillin G (53.2%) and followed by Tetracycline (34%). Due to a difference in study design from other studies, almost all of them were conducted in mastitis; it seems difficult for us to compare the result precisely. However we tried to compare the status of the *S.aureus* isolates with other findings from other sample sources. The study conducted by Abera *et al.*, 2010 in Adama reported that *S.aureus* isolates from mastitic cow samples were 94.4% resistant to Pencillin. *S.aureus* isolates that were obtained from quarter milk samples in Hawassa revealed 67.9% and 60.3% resistance to Pencillin G and Oxacillin respectively (Daka et al., 2012). A latest report from Addis Ababa has discovered that 95.3% and 55.8% of resistant profiles of *S.aureus* for Pencillin G and Cefoxitin respectively (Beyene *et al.*, 2017). A study from pediatrics patients indicated 51.5% of *S.aureus* and 16.8% of MRSA occurrence in Amhara regional state (Lemma et al., 2015). A metanalysis report from Ethiopia in human shown that methicillin resistant *S. aureus* was 32.5% (Eshetie *et al.*, 2016), which is lower than the current finding in dairy foods. *S.aureus* isolates from Morocco that were obtained from whey and cheese product shown 50%, 25% and 15% resistance level for Penicillin G, Tetracycline and Oxacillin respectively (Bendahou *et al.*, 2008). This high occurrence may be as a result of an easy access to these commonly used antibiotics for the treatment of infections both in human and animal. As it was investigated in the questionnaire survey dairy animal owners as well as consumers involved in the study clearly indicated their use of available antibiotics without prescription and appropriate clinical examination/medical consultation. The other justification for the high occurrence of resistant *S.aureus* can be linked with the high occurrence of CNS in this study (14.8 %). CNS can also be implicated as sources for multi-drug or methicillin resistance profile of *S.aureus*. A study conducted in Dublin, Ireland explained this hypothesis as “Could CNS be an evolutionary source of resistance genes for *S. aureus*?” And this study tried to test and confirmed CNS as a possible source of resistance gene through horizontal transfer of gene (Jayadev-Menon *et al.*, 2015).

The presence of resistant *S.aureus* in ready consumed milk products might pose a serious health risk to humans especially those consumers that indicate the habit of consuming raw/undercooked dairy products can be in higher risks. From the consumer consumption habit we learned that a high number of people can consume raw/undercooked milk products. This aggravates the situation when geared with the irrational use of drugs as investigated in this study. Although different antibiotic classes of drugs are used in animal health management and in human medicine, the selection of resistance to one drug class may lead to cross-resistance to another (Haran *et al.*, 2012). Besides improvement of health and agricultural systems, understanding of how people prepare, process and preserve foods, as well as how people use various antimicrobials throughout the food value chain is crucial to preventing the emergence of food-borne antimicrobial resistance as well as designing effective public health interventions (Lauren *et al.*, 2017).

This survey documented different predisposing factors that can significantly contribute for further spread of multi drug resistance *S.aureus* in the study area. Some of the irrational practices in antibiotics usage in the study areas such as antibiotics usage without prescription; self-administration of drugs without proper clinical examination; cessation of drug usage before complete dose and common use of antibiotics for minor and unidentified cases such as coughing, nasal discharge, diarrhea and so on. Antibiotic use is the key factor in the selection of resistant bacteria, with community and hospital settings forming the principal ecological niches of emergence in human health (WHO, 2015). However, it is the use of antibiotics in animals that has contributed to the magnitude of the global challenge of antibiotic resistance (FAO, 2015). In addition to these factors which increase the occurrence of resistant, the consumption habit of the people in the study area can facilitate the circulation of the resistant strains in the human ecology. As described by Chang *et al.*, 2015; resistant bacteria may reach humans indirectly along the food chain through consumption of contaminated food or food derived products. The usage of antibiotics correlates with the emergence and maintenance of antibiotic resistant traits within pathogenic strains (WHO, 2015; FAO, 2015). Antibiotic-resistant *S. aureus* isolates pose a severe

challenge to both veterinary and health professions and dairy cattle producers because they have a negative impact on therapy (Brouillette and Malouin, 2005).

6. CONCLUSION AND RECOMMENDATIONS

Antibiotics resistance is a global public health challenge with severe health and socio-economic consequences that is significantly influenced by antibiotic use in food animals. This study revealed a high level of *S. aureus* (18.2%) and MRSA (76.3%) contamination in the ready to eat dairy food products in Harar and surrounding area. It also reveals evidences of the irrational use of antibiotics in the dairy production and consumer's habit. The presence of multi drug resistant *S. aureus* in dairy products poses a health hazard and rise concerns about the safety of these food products.

Therefore, based on the findings of the current study and the above conclusion the following recommendations are forwarded:

- ❖ Holistic research and surveillance should be strengthened to know details about *S.aureus* and its food safety implication.
- ❖ Strategy should be designed to improve the awareness of the consumers and farm owners on proper/rational use of antibiotic, antibiotic resistance and and on the behavior of raw milk consumption.

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8. ANNEXES

Annex 1: Flow chart of the ISO 6888-3 protocol

Isolation and identification of *Staphylococcus*



Twenty five grams (25 ml) samples added in a stomacher bag containing 225 ml of buffered peptone water (BPW).



Homogenized the mixture by using laboratory blender and incubated at 37°C for 24 hours.



A loopful of the cultures streaked aseptically onto sterile BAP (Blood Agar Plate) and incubated at 37°C for 24- 48 hours under aerobic condition.



Colonies are 0.5 to 1.5 µm in diameter, grey or grey-white to golden- yellow.



Gram positive spherical bacteria that occur in microscopic clusters resembling grapes



Biochemical tests for confirmation



Catalase test- 3% H₂O₂ (Positive)



Inoculate colonies on MSA and incubate at 37°C for 24- 48 hours, growth and change in the pH of the medium is confirmative for *Staphylococcus* classified as highly fermentative (*S. aureus*), weakly fermentative for (*S. intermedius*) and non-fermentative (*S. hicus* and CNS).



Coagulase test to identify the pathogenic CPS (*S. aureus*, *S. intermedius* and *S. hicus*) from CNS.

Inoculate CPS isolates on PAB media plate with 1% of maltose and incubate at 37°C for 24- 48 hours to differentiate the coagulase positive isolates. The identification will be based on the fact that *S. aureus* rapidly ferment maltose to change the medium and colonies to yellow. *S. intermedius* gives a weak or delayed reaction and *S. hicus* did not ferment maltose.

Annex 2: Sample collection sheet for microbiological analysis

Serial number	Date of collection	Sample Type	Sample Source	Sample code

Annex 3: Procedure for catalase test

1. Place a drop of 3% H₂O₂ on glass slide
2. By using a sterile loop pick up a visible mass of cells (colony) from the culture of organism.
3. Mix the organism thoroughly with the drop of 3% H₂O₂.
4. Observe for immediate and vigorous bubbling.

Interpretation: The presence of bubbling indicates a positive test

Annex 4: Procedure for coagulase test

1. Using a sterile pipette, add 0.5ml of rehydrated rabbit plasma to test tube.
2. Add 0.5ml of the overnight broth culture of the test organism to the tube of rabbit plasma, or using a sterile bacteriological loop; thoroughly emulsify 2-4 colonies from a non-inhibitory agar plate in the tube of plasma.
3. Mix gently and incubate at 37⁰C.
4. Examine periodically for coagulation by gently tipping the tube at 30 minutes intervals for the first 4 hours of the test and then after 24 hours of incubation. Avoid shaking or agitating the tube during reading. Doubtful or false negative results may occur due to breakdown of the clot.
5. Record results: **Positive**: - if there is any degree of clotting that form a loose clot suspended in plasma to a solid clot that is immovable when the tube is inverted.

Annex 5: Record sheet for laboratory isolation and identification of *Staphylococcus*

Serial number	Type of sample	Sample code	Colony morphology on on BAP	Hemolysis	Gram's stain	Catalase test	Coagulase test	Growth on MSA	Mannitol fermentation (MSA)	Maltose fermentation (PAB)	<i>Staphylococcus Spp.</i>

Annex 6: Differential tests used for identification and confirmation of *Staphylococcus* species

Species	Haemolysin Result	Catalase test	Mannitol Agar	Salt	Coagulase test	Purple Agar Base
<i>S. aureus</i>	α or β	positive	Golden yellow		positive	Fast fermented
<i>S. intermedius</i>	β or only growth	positive	Delayed yellow		positive	Late fermented
<i>S. hyicus</i>	β or only growth	positive	growth		positive	Growth but no fermentation
CNS	β or only growth	positive	negative		negative	No growth

Annex 7: Record sheet for identification of CPS *Staphylococcus* on purple agar base

Serial number	Type of sample	Source	Sample code	<i>S. aureus</i>	<i>S. intermedius</i>	<i>S. hyicus</i>

Annex 8. Procedure for performing the disc diffusion test

Inoculum preparation

- ☞ At least three to five well-isolated colonies of the same morphological type are selected from an agar plate culture.
- ☞ The top of each colony is touched with a loop, and the growth is transferred into a tube containing 4 to 5 ml of a suitable broth medium, such as tryptic soy broth.
- ☞ The broth culture is incubated at 35°C until it achieves or exceeds the turbidity of the 0.5 McFarland standards (usually 2 to 6 hours)
- ☞ The turbidity of the actively growing broth culture is adjusted with sterile saline or broth to obtain turbidity optically comparable to that of the 0.5 McFarland standards. This results in a suspension containing approximately $1 \text{ to } 2 \times 10^8$ CFU/ml for *E.coli* ATCC 25922.

Inoculation of Test Plates

- ☞ Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab is dipped into the adjusted suspension.
- ☞ The swab should be rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This will remove excess inoculum from the swab.
- ☞ The dried surface of a Müeller-Hinton agar plate is inoculated by streaking the swab over the entire sterile agar surface.
- ☞ This procedure is repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of the agar is swabbed.
- ☞ The lid may be left ajar for 3 to 5 minutes, but no more than 15 minutes, to allow for any excess surface moisture to be absorbed before applying the drug impregnated discs.

Application of Discs to Inoculated Agar Plates

The predetermined battery of antimicrobial discs is dispensed onto the surface of the inoculated agar plate.

- ☞ Each disc must be pressed down to ensure complete contact with the agar surface.
- ☞ Whether the discs are placed individually or with dispensing apparatus, they must be distributed evenly so that they are no closer than 24 mm from the center to the center.
- ☞ Ordinarily, no more than 12 discs should be placed on one 150 mm plate or more than 5 discs on a 100 mm plate.
- ☞ The plate are inverted and placed in an incubator set to 35⁰ c within 15 minutes after the disc are applied.

Reading Plates and Interpreting Results

- ☞ After 16 to 18 hours of incubation, each plate is examined.
- ☞ If the plate was satisfactorily streaked, and the inoculum was correct, the resulting zones of inhibition will be uniformly circular and there will be a confluent lawn of growth.
- ☞ The diameters of the zones of complete inhibition (as judged by the unaided eye) were measured, including the diameter of the disc.
- ☞ Zones are measured to the nearest whole millimeter, using sliding calipers or a ruler, which is held on the back of the inverted Petri plate.
- ☞ The Petri plate is held a few inches above a black, nonreflecting background and illuminated with reflected light.
- ☞ If the test organism is a *Staphylococcus* or *Enterococcus* spp., 24 hours of incubation are required for vancomycin and oxacillin, but other agents can be read at 16 to 18 hours.
- ☞ The zone margin should be taken as the area showing no obvious, visible growth that can be detected with the unaided eye
- ☞ The sizes of the zones of inhibition are interpreted according to CLSI, 2015 (Table 8).

Table 8: Details of the antibiotics that were used in the study to test the antibiotic resistance of *S. aureus* isolates

Antibiotics	Abbreviation	Disc content (µg)	Inhibition Zone (mm)		
			R	I	S
Penicillin-G	Pen-G	10 unit	≤28	-	≥29
Tetracycline	TTC	30	≤14	15-18	≥19
Gentamicin	G	10	≤12	13-14	≥15
Cephalothin	CEP	30	≤14	15-17	≥18
Cefoxitin	FOX	30	≤21	-	≥22

Annex 9: Composition and preparation of media that was used by this study

❖ **Buffered peptone water (Oxoid, Hampshire, UK)**

Typical formula (g/l)

Peptone ----- 10.0

Sodium chloride ----- 5.0

Final pH 7.3 ± 0.2 at 25°C

Instruction for use:

Dissolve 15.0g in 1 liter of distilled water. Stir and dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes. Cool to room temperature before use.

❖ **Blood agar (Oxoid, Hampshire, UK)**

Typical formula (g/l)

“Lab- Lemco” powder ----- 10.0

Peptone ----- 10.0

Sodium chloride ----- 5.0
Agar ----- 15.0

Final pH 7.3 ± 0.2 at 25°C

Instruction for use:

Suspend 40g in 1 liter of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes. Cool to $45 - 50^{\circ}\text{C}$ and add 7% sterile defibrinated blood.

❖ **Nutrient agar (HiMedia, Mumbai, India)**

Typical formula (g/l)

“Lab- Lemco” powder ----- 1.0
Yeast extract ----- 2.0
Peptone ----- 5.0
Sodium chloride ----- 5.0
Agar ----- 15.0

Final pH 7.4 ± 0.2 at 25°C

Instruction for use:

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

❖ **Mannitol salt agar (Oxoid, Hampshire, UK)**

Typical formula (g/l)

“Lab- Lemco” powder ----- 1.0
Peptone ----- 5.0
Mannitol ----- 10.0
Sodium chloride ----- 75.0
Phenol red ----- 0.025
Agar ----- 15.0

Final pH 7.5 ± 0.2 at 25°C

Instruction for use:

Suspend 11g in 1 liter distilled water and bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes. Mix well before pouring into sterile petri dishes.

❖ **Purple agar base (Difco™, Becton, Dikson, USA)**

Typical formula (g/l)

Proteose peptone ----- 10.0

Beef extract ----- 1.0

Sodium chloride ----- 5.0

Bromocresol purple ----- 0.02

Agar ----- 15.0

Final pH 6.8 ± 0.2 at 25°C

Instruction for use:

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

❖ **Tryptone soya broth (Oxoid, Hampshire, UK)**

Typical formula (g/l)

Pancreatic digest of casein----- 17.0

Peptic (enzymatic) digest of soybean meal ----- 3.0

Sodium chloride ----- 5.0

Di-basic potassium phosphate ----- 3.5

Glucose ----- 2.5

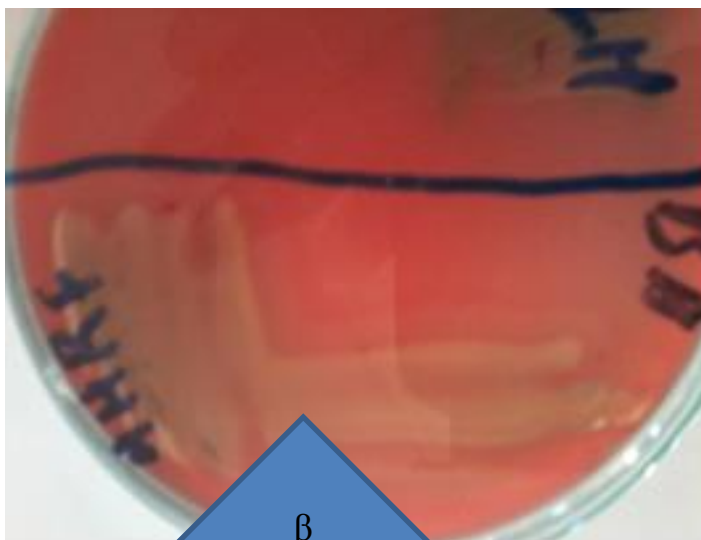
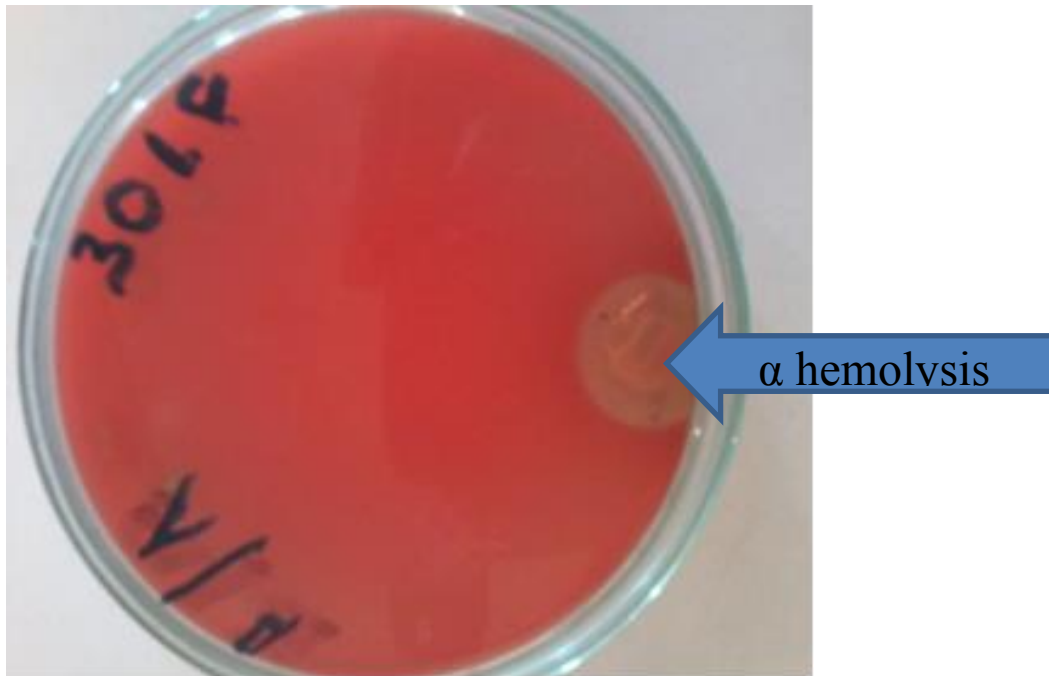
Final pH 7.3 ± 0.2 at 25°C

Instruction for use:

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

Annex 10: Pictures of Laboratory Findings of this study

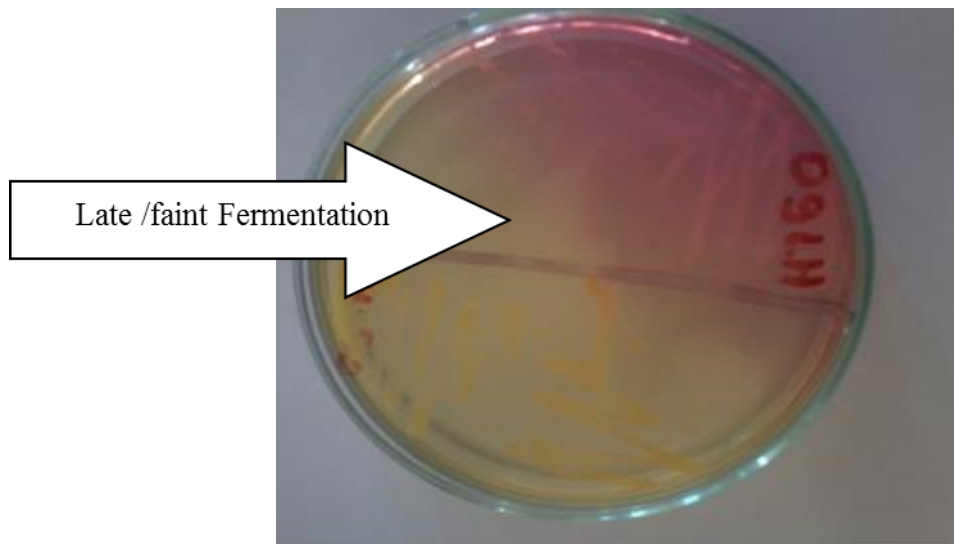
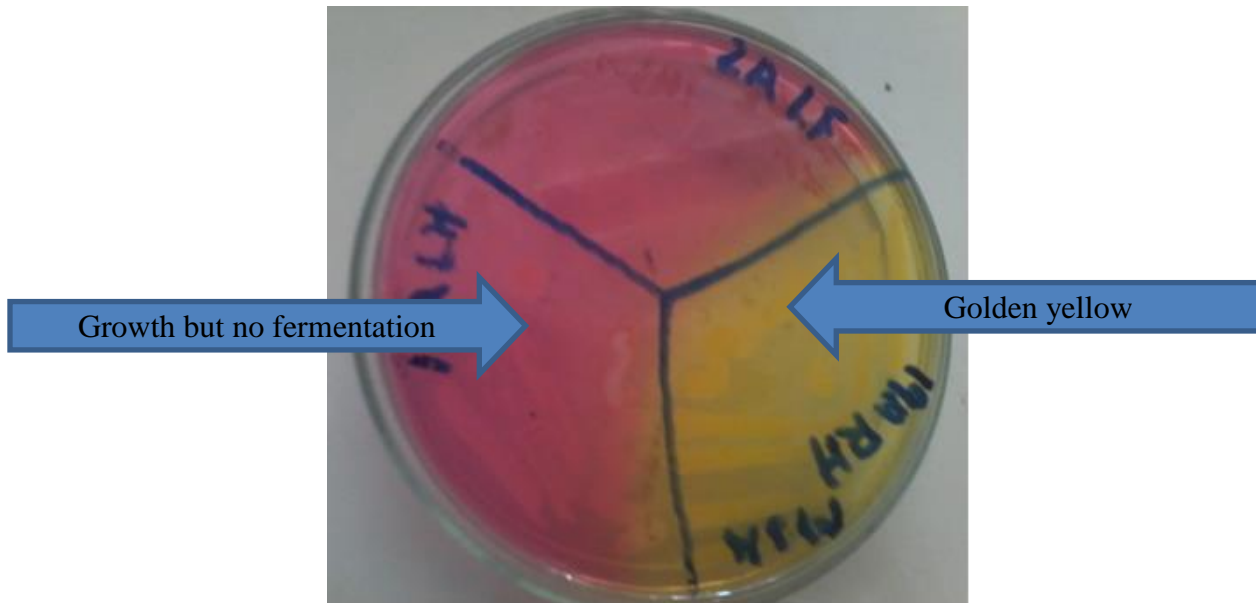
A. Blood agar hemolysin



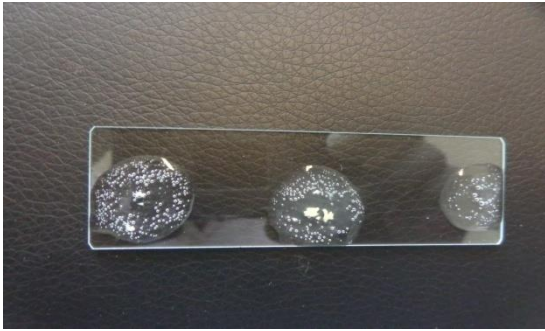
β
hemolysis

B. Manitol fermentation on Mannitol Salt Agar

B. Growth and manitol fermentation on Mannitol Salt Agar



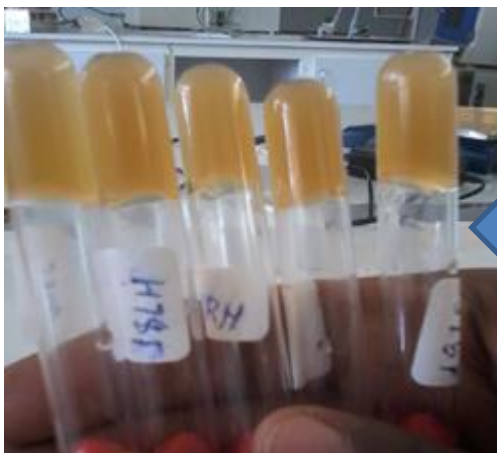
C. Catalase positive reactions (production of bubbles)



D. Coagulase test

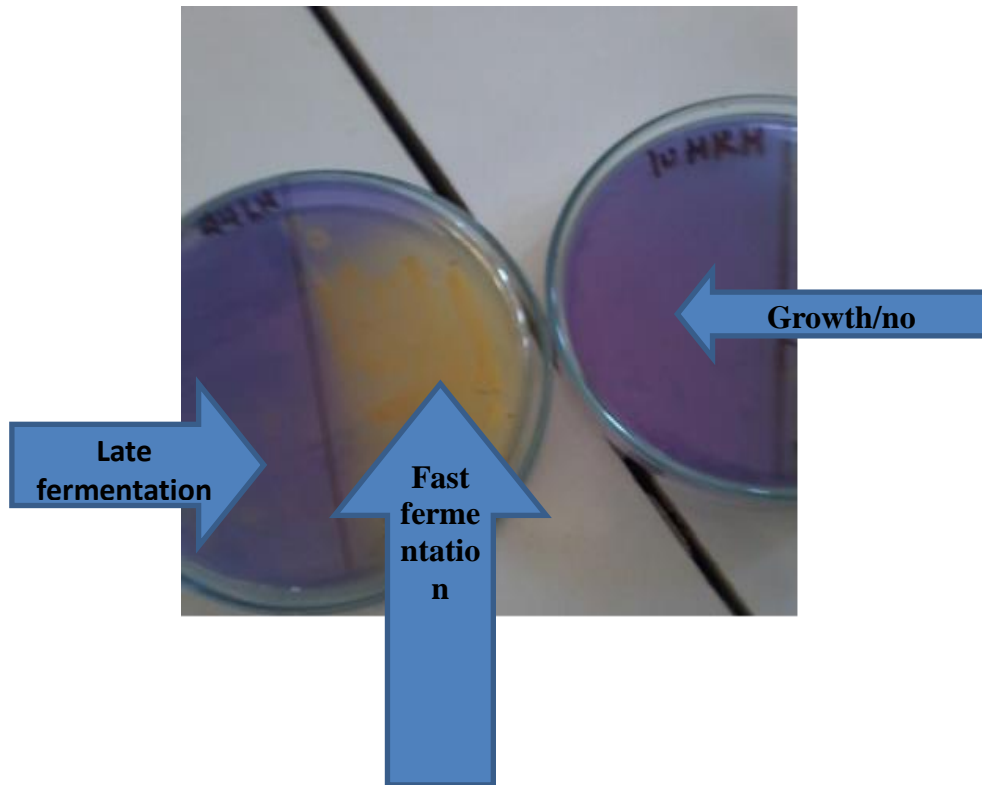


Coagulase negative



Coagulase positive

E. Purple Base Agar fermentation



F. Antibiotics Susceptibility test

