IDENTIFICATION AND ANTIMICROBIAL RESISTANCE PATTERN OF STAPHYLOCOCCI ISOLATED FROM COTTAGE CHEESE (*AYIB*) AND YOGHURT (*ERGO*) IN SELECTED *DISTRICTS* OF JIMMA ZONE, ETHIOPIA

MSc THESIS

SHIMELIS ARGAW FILATTE

MAY, 2015

JIMMA, ETHIOPIA

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MSc THESIS

A thesis submitted to the School of Graduate Studies, Jimma University College of Agriculture and Veterinary Medicine

In partial fulfillment of the requirement for the degree of master in Veterinary Public Health

SHIMELIS ARGAW

May, 2015

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I hereby declare that this thesis is my original work and that all sources of materials used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for MSc Degree in veterinary public health at Jimma University College of Agriculture and Veterinary Medicine, School of Veterinary Medicine and is deposited at the University Library to be made available to borrowers under the 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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BIOGRAPHY

Dr. Shimelis Argaw was born in Dawro zone, SNNPR, Ethiopia on 23rd of Jauary 1987 G.C. He started his primary school in 1994 at Woldehane elementary school in Gena Bossa *district*. He attended his high school and preparatory education at Waka preparatory school, Mareka *district*, from 2001/2 to 2005/6. In 2010, he obtained his degree of Doctor of Veterinary Medicine (DVM) from Haramaya University. From 2010/11 to 2012, he worked in SNNP region, Dawro zone, Gena Bossa *district* Agriculture Office, Livestock Resource Development and Health main work process as a coordinator and Veterinarian. He attended his MSc in Veterinary Public Health at Jimma University College of Agriculture and Veterinary Medicine, School of Veterinary Medicine in department of Veterinay Microbiology and Public Health, from 2013 to 2015.

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LIST OF ABBREVIATIONS

μg	Microgram
a_w	water activity
BAP	Blood Agar Plate
BPW	Buffered Peptone Water
CDC	Center for Disease Control and Prevention
CFU	Colony Forming Units
CNS	Coagulase Negative *Staphylococcus*
CI	Confidence Interval
CLSI	Clinical and Laboratory Standards Institute
CPS	Coagulase Positive *Staphylococcus*
DACA	Drug administration and control authority
DNA	Deoxyribonucleic Acid
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked Immunosorbent Assay
FBD	Food Born Disease
HA-MRSA	Hospital Associated Methicillin resistant *Staphylococcus aureus*
ISO	International Organization for Standardization
KDa	Kilo Dalton
kGy	Kilo Gray
LAB	Lactic Acid Bacteria
MDR	Multi-drug Resistant
MHA	Mueller- Hinton Agar
MIC	Minimium inhibitory concentration
MRSA	Methicillin resistant *Staphylococcus aureus*
MSA	Mannitol Salt Agar
NaCl	Sodium Chloride
NAP	Nutrient Agar Plate
ng	Nanogram
NNIS	National Nosocomial Infection Surveillance

PAB	Purple Agar Base
PCR	Polymerase Chain Reaction
pH	Power of Hydrogen
RIA	Radioimmunoassay
RPLA	Reversed Passive Latex Agglutination
rRNA	ribosomal Ribonucleic Acid
SEA	Staphylococcal enterotoxin -A
SEB	Staphylococcal enterotoxin-B
SEF(TSST)	Toxic shock syndrome toxin
SEl	Staphylococcal enterotoxin like protein
SEs	Staphylococcal Enterotoxins
SFP	Staphylococcal food poisoning
SIG	*Staphylococcus intermedius* group
Spp	Species
SPSS	Statistical Package for Social Sciences
SSSS	Staphylococcal scalded skin syndrome
TSB	Tryptone Soya Broth
TSS	Toxic shock syndrome
U.S.	United State of America
UK	United Kingdom
USD	United States Dollar(s)
WHO	World Health Organization
β	Beta

ABSTRACT

Staphylococci are the main cause of food borne intoxication and food borne outbreaks worldwide due to its ubiquity and ability to persist and grow under various conditions. A cross-sectional study was carried out between the periods of May 2014 to March 2015 with the aims to investigate the occurrence of *Staphylococcus,* determine the prevalence of *Staphylococcus* and evaluate the antimicrobial resistance pattern of the isolates from cottage cheese (*Ayib*) and yoghurt (*Ergo*) collected from selected *districts* of Jimma Zone, Ethiopia. Identification of the staphylococci was undertaken using Gram's staining, catalase, sugar fermentation and coagulase tests on 400 samples (cottage cheese = 200, yoghurt = 200) collected by simple random sampling technique. The overall prevalence of *Staphylococcus* in this study was found to be 14.3% with a specific prevalence of 22% in cottage cheese and 6.5% in yoghurt. The difference on the prevalence of staphylococci between cottage cheese and yoghurt was found to be statistically significant ($P<0.05$). Four species of staphylococci were identified and their prevalence was confirmed to be 5%, 3.5%, 3.3% and 5.8% for *S. aureus, S. intermedius, S. hyicus* and coagulase negative staphylococci respectively. The specific prevalences in cheese and yoghurt for each species respectively were 7% and 3% (*S.aureus*), 5.5% and 1.5% (*S. intermedius*), 4% and 2.5% (*S. hyicus*), and 9.5% and 2% (coagulase negative staphylococci). The identification results showed that the contamination of cottage cheese with *staphylococcus* was more likely to occur than yoghurt (OR = 4.1, 95%CI = 2.1-7.8). The disc diffusion assay of 70 isolates against five biogram revealed the highest resistance to penicillin G (65.7%) followed by tetracycline (41.4%), streptomycin (37.1%), gentamycin (35.7%) and kanamycin (28.6%). A large proportion of the isolates (87.1%) were resistant to one or more antimicrobials. The staphylococci found in cottage cheese and yoghurt was could be due to traditional methods of milking, handling, preparing, storing, selling and hygienic status of the personnel. The high antimicrobial resistance of the staphylococcal isolate observed might be due to indiscriminate use of the drug at the study area. Hence, sanitary measures and awareness creations are needed to improve the hygienic conditions of cheese and yoghurt from farm-to-table continuum as well as proper use of antimicrobials in order to guarantee the health and quality of these highly popular products.

Key words: Antimicrobial Resistance, Cottage Cheese, Jimma Zone, Prevalence, *Staphylococcus,* Yoghurt.

1. INTRODUCTION

Livestock farming in general and milk production in particular play an important socioeconomic role in developing countries (Hamid and Owni, 2007). Dairy products; including milk, cheese, dry milk powder, cream, butter and yoghurt are important and primary sources of nutrition in Ethiopia. The consumption of raw milk and its derivatives or in general dairy products is common in the world as well as in Ethiopia (Yilma, 2003; Abdalla and El-Zubier, 2006; Francesconi *et al.,* 2010). Inspite of this habit, there is no operational hygienic regulation set for smallholder marketed dairy products. This signifies that both the quality of milk and health of the dairy product consuming community will be endangour. This particularly holds true for cottage cheese (*Ayib)* and yoghurt (*Ergo*) or Ethiopian naturally fermented milk products, which are consumed raw with "*Enjera"* and other local foods without further treatment (Ashenafi and Beyene, 1994; Reda, 2001; Yilma, 2012). In-line with consumption of raw milk and milk products is the concern of foodborne diseases due to their neutral pH and rich nutrient composition, suitable for microbs growth (LeJeune and Rajala-Schultz, 2009).

There are about 250 different foodborne diseases described, and out of these two third of outbreaks are caused by bacteria (Loir *et al*., 2003). The World Health Organization (WHO) estimated that in developed countries, up to 30% of the population suffer from food borne diseases each year, whereas in developing countries up to 2 million deaths are estimated per year (WHO, 2007a; WHO, 2007b). According to the estimation by CDC in 1999, around 76 million foodborne outbreaks occur annually, resulting in 325,000 hospitalizations and 5200 deaths and costs annually 5-6 billion USD in the United States every year (Jay, 2000; WHO, 2007a; Buzby and Roberts, 2009). Therefore, it is important that foods and raw ingredients, including dairy products, should be subject to microbiological controls. However, these products have not been subjected to hygiene or sanitary control, because they are made in traditional ways (Ashenafi and Beyene, 1994; Yilma *et al*., 2007).

Staphylococcal food poisoning (SFP) is one of the most common food-borne diseases worldwide (Lina *et al*., 2004; Hennekinne *et al*., 2009). Staphylococcal enterotoxins cause foodborne illness in about 241,000 persons in the U.S. annually (Scallan *et al.,* 2011). Twentyone outbreaks in the

U.S. in 2007 (and 14 in 2008) and 291 outbreaks in Europe in 2008 were attributed to staphylococcal enterotoxin poisoning (EFSA, 2010). The incidence of SFP due to the consumption of dairy products is also common in Ethiopia (Ashenafi and Beyene, 1994; Yilma *et al*., 2007). Prevalence survey of nasal carriage of *S. aureus* in Gondar University revealed that 20.5% of food handlers were positive for *S. aureus* (Dagnew *et al.*, 2012). It is often associated with the ingestion of manually handled foods like raw dairy products which contain one or more highly heat stable staphylococcal enterotoxins (SEs). Many foods support growth of staphylococci and toxin production. Milk, dairy products and meat, especially uncanned foods, are common vehicles that are frequently implicated in staphylococcal food poisoning (SFP) (Jay, 2000; Smith, 2007). As staphylococcal enterotoxins are heat stable, they may be present in food even when *S. aureus* are absent (Balaban and Rasooly, 2000; Soejima *et al*., 2007).

Milk and milk-based food products are good media for growth of potentially pathogenic microbes because of their rich composition, which provides a favorable medium for growth of spoilage agents (De Buyser *et al*., 2001). Unpasteurized milk may become contaminated with enterotoxigenic coagulase-positive *Staphylococcus* species (Carmo *et al*., 2002), either through contact with the cow's udder while milking or by cross contamination during processing (Ramesh *et al.*, 2002; Fujikawa and Morozumi, 2006; Anderson and Pritchard, 2008).

Enumeration and identification of staphylococci in dairy products is a priority in developing public health measures to reduce foodborne disease outbreaks (Palomares *et al*., 2003; Ercolini *et al*., 2004). In addition to that, emergence of antimicrobial resistance is a great concern wordwide now adays, especially among nosocomial pathogens. *Staphylococcus* is one of the most common causes of nosocomial infections. Multidrug-resistant staphylococci pose a serious problem in human health. This is because, there is rise of drug-resistant virulent strains of *Staphylococcus aureus*, and particularly methicillin-resistant *S. aureus* (MRSA) is a serious problem in the treatment and control of staphylococcal infections (Livermore, 2000; Zapun *et al*., 2008).

The uncontrolled application of antimicrobials in the environment is leading to a constant increase in the rate of antimicrobial resistance among community-acquired staphylococci (Harakeh *et al.,* 2005; Harakeh *et al.,* 2006). Emergence of antimicrobial resistance is a result of

the use, overuse and misuse of antimicrobials both in humans and animals. In Ethiopia, there are indications on the misuse of antimicrobials by health care providers, unskilled practitioners, and drug consumers. These coupled with rapid spread of resistant bacteria and inadequate surveillance contributed to the problem. Most pathogenic bacteria that are commonly involved in causing infections to human beings and animals shown considerable degree of resistance to commonly used first line antibacterials in Ethiopia, which is ranged from 0% to 100%. *Staphylococcus* species are one of the most common bacterian that shown high icreanment in resistance level (DACA, 2009). *Staphylococcus* species can rapidly acquire resistance to a broad range of antimicrobials, thereby posing a major concern in the treatment of staphylococcal infections (Bozdogan *et al*., 2004).

Antimicrobial resistance is one of the crucial challenges that public health is facing today. Investigations on antibacterial resistance and on bacterial infections have shown that emerging antibacterial resistance threatens the management of bacterial infections. Some of the major consequences of resistance also include increased mortality, morbidity, costs of treatment, and loss of production in animals (DACA, 2009). Therefore, studying antimicrobial resistance in humans and animals is important for detecting changing patterns of resistance, implementing control measures on the use of antimicrobial agents and preventing the spread of multidrug-resistant strains of bacteria (Van Duijkeren *et al*., 2003). The information available in food poisoning due to *Staphylococcus* and its drug resistance pattern is very limited in selected *districts* of Jimma Zone.

Therefore, this study was carried out with the following general and specific objectives:

General objective:

- To investigate the occurrence of *Staphylococcus* and to evaluate the antimicrobial resistance pattern of the isolates from cottage cheese (*Ayib*) and yoghurt (*Ergo*) collected from selected *districts* of Jimma Zone, Ethiopia.

Specific objectives:

- To detrmine the prevalence of staphylococci in cottage cheese and yoghurt collected from selected *districts* of Jimma Zone.
- To determine antimicrobial resistance pattern of staphylococci isolates.

2. LITERATURE REVIEW

2.1. Historical Background

There is no record of when illnesses similar to staphylococcal food poisoning were first observed, but it is likely that humans have been afflicted with this illness as long as they have been consuming foods in which staphylococci could grow. There are records of illnesses of this type as early as 1830, although the organisms themselves were not recognized until 1878 and 1880 by Koch and Pasteur, respectively. Although Ogston is credited with applying the name '*Staphylococcus*' to these organisms in 1881 because of the grapelike clusters of cocci he observed in cultures, it was Rosenbach who in 1884 obtained pure cultures of the microorganisms on solid media and accepted the name *Staphylococcus* (Bergdoll and Lee Wong, 2006).

Dack (1956), in his book "Food Poisoning*"*, relates several descriptions of foodborne illnesses similar to staphylococcal food poisoning. A number of food items were involved–sausage, rabbit pie, 'pork brawn', milk, ice cream, and of course cheese, where Vaughan and Sternberg first associated micrococci with the illnesses in 1884. Each of these investigators independently examined cheese that had been implicated in food-poisoning outbreaks in Michigan. Sternberg stated: 'It seems not improbable that the poisonous principle is a ptomaine developed in the cheese as a result of the vital activity of the above mentioned *Micrococcus*, or some other micro-organisms which had preceded it, and had perhaps been killed by its own poisonous products.' In 1894, Denys concluded that the illness of a family who had consumed meat from a cow dead of 'vitullary fever' was due to the presence of pyogenic staphylococci, and in 1907 Owen recovered Staphylococci from dried beef implicated in a foodborne illness characteristic of staphylococcal food poisoning.

In 1914, Barber was the first investigator actually to relate staphylococcal food poisoning to a toxic substance produced by the staphylococci. He discovered that milk from a mastitic cow caused illness when left unrefrigerated, and showed that the illness was due to growth in the milk of the staphylococci isolated from the mastitis. The significance of this excellent report was not

recognized and, as a result, this type of food poisoning was ascribed for the most part to other bacterial agents. For example, an outbreak involving 2000 soldiers in the German army during World War I was attributed to *Proteus vulgaris* even though cocci were present in large numbers.

In 1929, Dack rediscovered the role of staphylococci in food poisoning with his classical work on two Christmas cakes that were responsible for the illness of 11 people. These three-layer sponge cakes with thick cream fillings were baked possibly 1 day before delivery and eaten 2 days later. They were presumably refrigerated at the bakery but not after delivery. Dack and his associates showed, with the aid of human volunteers, that the sponge cake substance was responsible for the illness. Staphylococci isolated from this part of the cake produced a substance that caused typical food poisoning symptoms in human volunteers. In essence, this was the beginning of the research on staphylococcal food poisoning (Dack *et al*., 1930).

2.2. Characteristics

2.2.1. Organism

Classification of *Staphylococcus*

The name *Staphylococcus* (staphyle = bunch of grapes in Greece) was introduced in 1883 by Ogston. One year later, Rosenbach used the term in a taxonomic sense and provided the first description of the genus *Staphylococcus* (Todar, 2008).

Taxonomically the staphylococci have been placed in the Family *Micrococcaceae* (Shah, 2003 and Todar, 2008). Baird-Parker (1963) proposed a system of classification of the micrococci and staphylococci based on certain physiological and biochemical tests. He divided the Family *Micrococcaceae* in to Group I (*Staphylococcus* Rosenbach emend. Evans) and Group II (*Micrococcus* Cohn emend. Evans). These groups were then divided in to subgroups on the basis of pigment production, coagulase and phosphatase reactions, acetone production, and formation of acid from glucose (both aerobically and anaerobically) and other sugars. Six subgroups were

recognized within the genus *Staphylococcus* and seven within the genus *Micrococcus*. Hajek and Marsalek (1971) further divided the pathogenic staphylococci found in Baird-Parker's subgroup I in to six biotypes with characteristic biochemical and biological properties.

Staphylococcus can be differentiated from the other three members in the family, *Micrococcus, Stomatococcus*, and *Planococcus*, on the basis of the guanine plus cytosine content of the DNA, cell wall composition, and the ability to grow and ferment glucose anaerobically. Only three species of *Staphylococcus (S. aureus, S. epidermidis* and *S. saprophyticus*) were included in the genus in 1974 (Buchanan and Gibbons, 1974). They were differentiated primarily on the basis of the ability to produce coagulase, ferment mannitol (both aerobically and anaerobically) and produce heat-stable endonuclease, and by the cell wall composition (Baird-Parker, 1974).

Kloos and Schleifer (1975) outlined a simplified scheme for the routine identification of human *Staphylococcus* species. They divided these into 11 species on the basis of coagulase activity, hemolysis, nitrate reduction, and aerobic acid production from several sugars. Since then the number of species and sub-species had increased to 32 as of 1994 (Holt *et al*., 1994). This increase included the elevation of two of the *S. aureus* biotypes to species status, biotype E (from dogs) to *S. intermedius* and biotype F (from swine) to *S. hyicus*. An additional coagulase-positive species, *S. delphini* from dolphins, has been added (Bergdoll and Lee Wong, 2006).

Characteristics of *Staphylococcus aureus*

Staphylococcus aureus is a non-motile, facultative anaerobic, Gram-positive coccus. Cells are spherical single and often form grape-like clusters (Jay, 2000; Shah, 2003). The organism produces catalase and coagulase. Staphylococci survive desiccation and tolerate high levels of salt. The cell wall of staphylococci is resistant to lysozyme and sensitive to lysostaphin, which specifically cleaves the pentaglycin bridges of *Staphylococcus Spp*. 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). 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. The

bacteria can be killed through heat treatment of the food, but the enterotoxins are very heat resistant and survive approved doses of irradiation. Thus, although the bacteria are eliminated, the toxins can cause staphylococcal food poisoning (Genigeorgis, 1989; Loir *et al*., 2003; Aycicek *et al*., 2005).

Other pathogenic staphylococci

Coagulase-positive staphylococci, other than *S.aureus*, can cause infections in humans and animals. Some veterinary isolates of coagulase-positive staphylococci are classified in the *S. intermedius* group (SIG). *Staphylococcus intermedius* was originally described in 1976 and appeared to be part of the normal micro-flora of the skin and mucosal membranes of dogs and cats. It has also been detected in a variety of other animals, including horses, mink, goats, foxes, raccoons, and pigeons but is not commonly present in humans. Recent molecular analyses demonstrated that isolates of *S. intermedius* detected in a large number of different animals and geographic locations have some significant differences and the species can best be reclassified into three clusters: *S. intermedius*, *S. pseudintermedius*, and *S. delphini* A and B. These three species constitute the *S. intermedius* group (SIG) (Futagawa-Saito *et al*., 2006; Sasaki *et al*., 2007).

Staphylococcus pseudintermedius is the most frequently encountered pathogen in the SIG and was first identified as a novel species in 2005 by examination of rRNA gene sequences in clinical staphylococcal isolates from several animals (Devriese *et al*., 2005). The majority of isolates from dogs are now classified as *S. pseudintermedius* although earlier research papers identified them as *S. intermedius*. *Staphylococcus delphini* was originally isolated from a dolphin but some isolates from horses, pigeons and mink, previously identified as *S. intermedius*, are now classified as *S. delphini* (Sledge *et al*., 2010).

Staphylococcus pseudintermedius has been isolated from pet owners and veterinarians (Morris *et al*., 2010) and occasionally causes infections in humans exposed to dogs carrying these bacteria (Chuang *et al.*, 2010; Stegmann *et al*., 2010). Invasive infections have occurred in persons bitten by dogs (Fitzgerald, 2009) and two recent articles reported *S. intermedius* as the cause of skin

abscesses in an injecting drug user (Kelesidis and Tsiodras, 2010) and meningitis in an infant (Durdik *et al*., 2010).

Staphylococcus intermedius group pathogens produce a number of virulence factors (coagulase, hemolysins, exfoliative toxin and others) similar to those associated with *S. aureus* (Fitzgerald, 2009; Iyori *et al.,* 2010). When animals are injured, sick, or otherwise weakened, these bacteria may cause skin, ear, and wound infections (Weese and Van Duijkeren, 2010). Some SIG isolates also produce enterotoxins and could potentially cause foodborne intoxication (Becker *et al*., 2001). One foodborne outbreak in southwestern U.S. in 1991 affecting over 265 people was traced to *S. intermedius* producing type A enterotoxin in a butter blend (Khambaty *et al*., 1994).

Compared to coagulase-positive staphylococci, coagulase-negative staphylococci are rarely pathogenic and are often considered being opportunistic pathogens, such as *S. epidermidis* is for humans (Cheung and Otto, 2010). However, occasionally coagulase-negative staphylococci produce enterotoxins and have been associated with foodborne outbreaks (Veras *et al*., 2008).

Certain coagulase-negative staphylococci are important components of meat starter cultures (Fadda *et al*., 2010). Recent investigations found that genes coding for staphylococcal virulence factors were rare in coagulase-negative staphylococci isolated from sausage and cheese. Of 129 strains tested, only one contained a gene coding for an enterotoxin and none were capable of producing toxic shock syndrome toxin. Some strains did have genetic information coding for hemolysins and some were capable of producing biogenic amines. Of somewhat greater potential concern was the presence of antimicrobial resistance genes in 71% of isolates, with nearly half the strains resistant to more than one antimicrobial (Even *et al*., 2010).

Hosts and reservoirs

The staphylococci are 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 probably 50 % or more of healthy individuals. The prevalence is usually higher in individuals associated with

hospital environments because many infections and diseases are caused by the staphylococci. These organisms are associated with sore throats and colds, and are found in abundance in postnasal drip following colds. Staphylococci can be isolated from animals, with the bovine being the most important because of the involvement of staphylococci in mastitis. Although animals and humans are the major source, staphylococci also can be found in the air, dust, water, and human and animal wastes (Bergdoll, and Lee Wong, 2006).

2.2.2. Enterotoxins and its characteristics

Staphylococcal enterotoxins are exoproteins produced in food and ingested by humans give rise to symptoms of acute gastroenteritis (responsible for SFP). The toxins have been shown to be proteins of low molecular weight, approximately 27–31 kDa, consisting only of amino acids and are usually produced by CPS species (Ash, 2008; Chiang *et al*., 2008).

The SEs are short proteins belonging to a large family of pyrogenic toxin super antigens encoded by phage, chromosome or plasmid genes with a disulphide bridge secreted in the medium and soluble in water and saline solutions. They are rich in lysine, aspartic acid, glutamic acid, and tyrosine residues. Most of them possess a cystine loop required for proper conformation and which is probably involved in the emetic activity (Loir *et al*., 2003; Salandra *et al*., 2008).

Staphylococcal enterotoxins are highly stable, resist most proteolytic enzymes, such as pepsin, or trypsin, and thus keep their activity in the digestive tract after ingestion. They are highly heat resistant as well, which can resist 100^{o}c for at least 30 minutes and probably longer. Although pasteurization and cooking kills staphylococci cells which are heat labile, thermo-stable SEs generally retain their biological activity. Thus, cases of illness might occur although no viable bacteria can be isolated from the suspected foodstuff and since SEs are more heat stable than the staphylococci bacteria, it is possible to test a food product and obtain negative staphylococci culture results and positive SEs tests (Atanassova *et al*., 2001; Soejima *et al*., 2007).

The effect of gamma irradiation on SEB and SEA has been reported. A dose of 50 kGy (cobalt-60 source) was required to reduce the concentration of SEB in 0.04-M Veronal buffer (pH 7.2) from 31μg/ml to less than 0.7μg/ml. In milk, a dose of 200kGy was needed to reduce the concentration from 30μg/ml to less than 0.5μg/ml. The authors concluded that irradiation processes used for pasteurization or sterilization of foods would not inactivate the enterotoxin (Read and Bradshaw, 1967). A dose of 8kGy was insufficient to inactivate all of 111.1ng/ml SEA (27–34 % remained) in lean minced-beef slurries, although SEA was denatured in gelatin phosphate buffer (Rose *et al*., 1988; Modi *et al*., 1990).

The amount of enterotoxins produced is determined by factors such as the composition of the food, competition from other microorganisms (the presence of other bacteria affects the production of enterotoxin apparently by limiting the multiplication of the staphylococci), temperature and time (Salyers and Whitt, 2002).

A family of 14 different SE types has been identified, which share structure and sequence similarities, of which the antigenic types (named SE-A, B, C, D, and E) are most commonly encountered in SFP (Kerouanton *et al*., 2007). In general, SE-A is recovered from food poisoning outbreaks more often than any of the others, with SE-D being second most frequent and the fewest number of outbreaks are associated with SE-E (Jay, 2000; Shah, 2003; Argudin *et al.,* 2010).

Recently, additional SEs has been identified: SEG, SEH, SEI, SElJ, SElK, SElL, SElM, SElN, SElO, SElP, SElQ, SER, SES, SET, SElU, SElV and SElW. Many of these newly discovered enterotoxins are structurally similar to the classic enterotoxins, which suggest that they also may illicit foodborne illness when consumed in large enough doses. The significance of these SEs in causing foodborne intoxication remains largely unknown and requires both future research and increased surveillance (Rall *et al*., 2008; Ono *et al*., 2008; Argudin *et al.,* 2010).

Generally, different studies indicate that staphylococci produce 22 related but serologically distinct enterotoxins, staphylococcal enterotoxin (SE) A (SEA) to SElW all possesses

superantigenic activity whereas only some (SEA to SEI, SER, SES and SET) have been proven to be emetic (Ono *et al.,* 2008).

There is no enterotoxin F (SEF) because toxic shock syndrome toxin was misidentified as SEF when it was first isolated. Two SEKs were described independently by two different groups at about the same time however they are different proteins based on their deduced amino acid sequences (Jarraud *et al*., 2001; Orwin *et al*., 2001).

The toxins act on the emetic receptors on the abdominal viscera causing stimulation of the emetic center of the brain via vagus and sympathetic nerves. The nerve stimulation ultimately results in causing diarrhoea and vomiting (Atanassova *et al*., 2001; Walderhaug, 2007).

When SEs are expressed systematically, they mediate two illnesses, TSS and SSSS. In both diseases, exotoxins are produced during an infection, diffuse from the site of infection, and are carried by the blood (toxemia) to other sites of the body, causing symptoms to develop at sites distant from the infection. Toxic shock syndrome toxin is produced when SEs are expressed systemically and it is the cause of TSS. It is very weakly related to enterotoxins and does not have emetic activity (Bania, 2006; Smith, 2007). Toxic shock syndrome is an acute life-threatening illness mediated by staphylococcal superantigen exotoxins and can occur as a sequel to any staphylococcal infection if an enterotoxin or TSST is released systemically and the host lacks appropriate neutralizing antibodies (Salyers and Whitt, 2002). Staphylococcal scalded skin syndrome, also known as Ritter's disease characterized by dermatologic abnormalities (Shah, 2003; Todar, 2008).

Staphylococcal toxins could be used as a biological agent (bioterrorist attack) either by contamination of food/water or by aerosolization and inhalation. Breathing in low doses of staphylococcal enterotoxin B may cause fever, cough, difficulty breathing, headache, and some vomiting and nausea. High doses of the toxin have a much more serious effect (CDC, 2010b).

Because of the importance of these toxins in the public health and food sectors, an efficient screening to detect the prevalence of enterotoxic strains in foods is required. Indeed, not all staphylococci produce SEs, and SEs production may be insufficient for food intoxication (Martın *et al*., 2004; Turutoglu *et al*., 2005; Morandi *et al*., 2007*)*.

2.3. Nature of the intoxication in human and animals

Staphylococci are facultative anaerobic, gram-positive, catalase-positive cocci assigned to the family *Micrococcaceae*. They often represent part of the normal bacterial flora of the skin and mucosal surfaces of the respiratory, upper alimentary and urogenital tract of mammals and birds. Thus staphylococci are easily spread between animals and under certain conditions to humans as well by skin to skin contact, but also by contact with excretions which contain staphylococci, such as saliva, or aerosols released during sneezing and coughing. Moreover, staphylococci may be spread by animal products, such as non-pasteurized milk (Kloos, 1980; Bruckler *et al.,* 1994; Yilma and Faye, 2006; Robert *et al.,* 2010).

Staphylococci are among the most significant pathogens causing a wide spectrum of diseases in both humans and animals. There are two kinds of pathogenic staphylococci: those that cause pyogenic infections and those responsible for food poisoning. The same organism may show both these apparently unrelated effects (Johnson *et al*., 2006; Salandra *et al*., 2008).

2.3.1. Disease in food animals

In veterinary medicine, three of the 37 currently known staphylococcal species are of particular importance as a primary cause of specific diseases: *Staphylococcus* (*S*.) *aureus* (mastitis in ruminants, equine botryomycosis), *S. hyicus* (porcine exudative epidermitis), and *S. intermedius* (canine pyoderma) (Euzeby, 1997; Werckenthin *et al.,* 2001; Futagawa-Saito *et al*., 2006).

In food producing animal reservoirs, such as ruminants, *Staphylococcus aureus* presents on the skin and mucosa. In animals, *Staphylococcus aureus* can cause pustular inflammation of the skin

and other organs, mastitis being the most serious. It is frequently associated to subclinical mastitis becoming responsible of contamination of milk and dairy products and is of great economic importance to the dairy industry worldwide (Jones, 1998; Salandra *et al*., 2008). Its large capsule protects the organism from attack by the cow's immunological defenses (Hein *et al*., 2005). The infection occurs through the teat canal with the organisms derived from contaminated environment especially from the skin of the udder and teat (Anderson and Pritchard, 2008).

Staphylococcus aureus causing mastitis is widely distributed in cattle, goats and sheep. The infection is often subclinical in cattle, leading to reduced milk production and milk quality, but acute catarrhal or even gangrenous inflammation may also occur. In goats and sheep however, enzootic acute gangrenous mastitis is commonly seen. *S. hyicus* causes exudative epidermitis (‘greasy pig disease’), an often acute generalised skin infection in piglets. Systemic forms of the disease which result in the death of the animals are also seen. Poor hygienic conditions as well as ectoparasitic infestations favour the onset of the *S. hyicus* infection. Surviving piglets show retarded growth rates. In adult pigs, subacute skin infections, mastitis or metritis, but also septic arthritis may be caused by *S. hyicus* (Bruckler *et al.,* 1994). *S. intermedius* is considered the primary cause of canine and also feline pyoderma. Chronic and recurrent pyoderma often seen in dogs is considered a complex syndrome in which not only the staphylococci, but also cell-mediated hypersensitivity, endocrine disorders, and a genetic predisposition may play an important role in the development of the disease. In dogs and cats, *S. intermedius* has also been reported pyometra, otitis externa and purulent infections of the joints, eyelids and conjunctiva (Werckenthin *et al.,* 2001; Futagawa-Saito *et al*., 2006).

Wash cloths, teat cup liners and flies mechanically transmit the infection from cow to cow. Cattle are often infected by humans and the infection is carried from one cow to another by the milkers' hands. There are estimates that 80-100% of all herds have at least some staphylococcal mastitis, with 5 to 10% of cows infected (Anderson and Pritchard, 2008). Herds with excellent milking hygiene practices and management have lower levels of staphylococcal intramammary infections as compared to those herds with poor hygiene or management (Kaloreu *et al*., 2007). The bacterium produces toxins that destroy cell membranes and can directly damage milk-

producing tissues (Jones, 1998). Staphylococcal infections also develop in to metritis, enteritis, ear infections and conjunctivitis (Anderson and Pritchard, 2008).

2.3.2. Disease in humans

Staphylococcal enterotoxins induce food poisoning after the consumption of food containing enterotoxin produced by the growth of coagulase-positive staphylococci in food especially some strains of *S. aureus*. Although there are some reports about isolation of coagulase-negative enterotoxin producers, very little information is available about food poisoning caused by coagulase-negative staphylococci (Lotter and Genigeorgis, 1977; Bergdoll, 1995; Beneet, 1996; Udo *et al*., 1999; Veras *et al*., 2008).

Staphylococcal infection presents with a wide range of syndromes in human beings affecting many tissues and caused by three mechanisms: local destruction (abscess), blood spread and toxin production (Loir *et al*., 2003; Soejima *et al*., 2007). They cause superficial skin lesions like boils (furuncles), pimples, impetigo, carbuncles and localized abscesses in other sites, deep-seated infections such as osteomyelitis and endocarditis and more serious skin infections such as staphylococcal scalded skin syndrome (SSSS) or furunculosis, hospital acquired (nosocomial) infection of surgical wounds and infections associated with indwelling medical devices. Also result in food poisoning by releasing enterotoxins into food, toxic shock syndrome (TSS) by release of super antigens into the blood stream and urinary tract infections (Loir *et al*., 2003; Shah, 2003; Todar, 2008).

Stsaphylococcal food poisoning occurs with the ingestion of contaminated food in which the enterotoxigenic strains of *S. aureus* can multiply reaching about 10^5 CFU/g of food; this bacterial load allows the production of an amount 20ng to 1µg of SE sufficient to determine symptoms in human beings (Quinn *et al*., 1999; Salandra *et al*., 2008).

The hazard to public health by ingestion of foods contaminated with *S. aureus* is particularly linked to the ability of 50% of these strains to produce thermo-stable SEs associated with food poisoning (Quinn *et al*., 1999; Miwa *et al*., 2001; Aycicek *et al*., 2005; Kerouanton *et al*., 2007).

Staphylococcus aureus is extremely prevalent in atopic dermatitis patients, who are less resistant to it than other people are. It often causes complications. The disease most likely found in fertile active places including, the armpits, hair and scalp. The large pimples that appear in those areas may cause the worst of the infection if popped. This can lead to Scalded skin syndrome (SSSS) (Todar, 2008).

All people are believed to be susceptible to this type of bacterial intoxication. However, the onset and severity of the illness is usually dependent on the individual's susceptibility to the toxin, the amount of contaminated food eaten, the amount of toxin in the food ingested and the general health of the victim (Jay, 2000; Acco *et al*., 2003; Walderhaug, 2007).

2.3.3. Symptoms

All people are believed to be susceptible to this type of bacterial intoxication. However, The onset of symptoms in staphylococcal food poisoning is usually rapid (2–6 hours) and in many cases acute, depending on individual susceptibility to the toxin, the amount of contaminated food eaten, the amount of toxin in the food ingested, and the general health of the victim. The most common symptoms are nausea, vomiting, retching, abdominal cramping, and prostration. Some individuals do not demonstrate all the symptoms associated with the illness. In more severe cases, headache, muscle cramping, and transient changes in blood pressure and pulse rate may occur (Jay, 2000; Acco *et al*., 2003; Walderhaug, 2007). Recovery generally takes 2 days, but it is not unusual for complete recovery to take 3 days or longer in severe cases (Jay, 2000; Aycicek *et al*., 2005). Death from staphylococcal food poisoning is very rare, although such cases have occurred among the elderly, infants, and severely debilitated persons (Bennett and Monday, 2003; Kerouanton *et al*., 2007; Rho and Schaffner, 2007; Walderhaug, 2007).

The variation in severity of staphylococcal food poisoning symptoms experienced by individuals suggests development of resistance to previous exposure to the enterotoxin, but there is no evidence to support this. One unreported attempt to check individuals involved in an outbreak for antibodies was unsuccessful (Bergdoll and Lee Wong, 2006).

2.3.4. Emetic dose

The amount of SE required to produce food poisoning in humans is difficult to determine. Reliable results from the examination of food implicated in food-poisoning outbreaks are difficult to obtain because normally the enterotoxin is not uniformly distributed in the food and it is impossible to know how much food any one individual consumed (Bergdoll and Lee Wong, 2006).

A toxin dose of less than 1.0µg in contaminated food will produce symptoms of staphylococcal intoxication. This toxin level is reached when *S. aureus* populations exceed 10^6cfu. However, in highly sensitive people a dose of 100-200ng is sufficient to cause illness (Bergdoll, 1990; Ananthanarayna and Panikaran, 2001; Stewart *et al.,* 2003).

2.3.5. Diagnosis

Any foodborne illness with the symptoms outlined here, particularly if it involves more than one person, is suspected of being staphylococcal food poisoning. A list of foods consumed at the previous meal or meals is needed to aid in the diagnosis, as there are certain foods that support the growth of staphylococci and are frequently involved in this type of illness. Any suspected food should be examined for the presence of staphylococci; if large numbers are present, it can be concluded with some degree of certainty that the illness is staphylococcal food poisoning. Additional information that can remove any doubt is whether the staphylococci are enterotoxigenic and/or whether enterotoxin can be detected in the suspected food. Although the latter is definite proof of the cause, often an insufficient quantity of food (10g can be used, but larger amounts are better) is available for examination. The presence of enterotoxigenic staphylococci in the food is reasonable assurance that these organisms were the cause of the illness (Johnson *et al*., 2006; Bergdoll and Lee Wong, 2006).

2.4. Food products commonly implicated with staphylococcal food poisonings

Any food that provides a good medium for the growth of staphylococci may be involved in this type of foodborne illness. The foods involved in different countries vary with the diet as well as the local conditions (Bergdoll and Lee Wong, 2006). Foods that are frequently incriminated in staphylococcal food poisoning include meat and meat products, poultry and egg products, salads such as egg, tuna, chicken, potato, and macaroni, bakery products such as cream-filled pastries, cream pies, and chocolate éclairs, sandwich fillings, and milk and dairy products. Foods that require considerable handling during preparation and that are kept at slightly elevated temperatures after preparation are frequently involved in staphylococcal food poisoning (Tatini and Bennett, 1999; Bennett and Monday, 2003). The possibility of occurrence of SFP due to consumption of dairy products is common in Ethiopia (Ashenafi and Beyene, 1994; Yilma *et al*., 2007).

2.4.1. Yoghurt *(Ergo)*

Fermented foods and beverages constitute a major portion of peoples' diets all over the world and provide 20 – 40% of the total food supply (Campbell-Platt, 1994). Apart from lactose providing variety to foods, fermented foods have the advantage of prolonged shelf-life due to organic acids such as lactic acid, acetic acid and other acids produced during fermentation which lowers the pH thus inhibiting the growth of spoilage microorganisms (Fields, 1981; Sanni, 1993; Byaruhanga, 1999). Generally, fermented food products are usually considered safe because of the low pH and production of antimicrobial substances by the fermenting organisms. However, some entero-pathogens have been reported to survive and grow in fermented milks (Caro and Garcia-Armesto, 2007). Although low pH is expected to inactivate bacterial pathogens, acid adaptation was reported to promote the survival of different foodborne pathogens in different types of cheeses (Ramsaran *et al.,* 1998; Vernozy-Rozand *et al.,* 2005).

Milk is processed primarily to convert it into a more stable product, e.g. fermented milk can be stored for about 20 days compared with less than one day for fresh milk. Milk products are more stable than fresh milk because they are more acidic and/or contain less moisture. Fermented

milks have been prepared for more than 2000 years. Allowing milk to ferment naturally gives an acidic product that does not putrefy. Fermented milks are wholesome and readily digestible; examples of such products are yoghurt, *kefir, koumiss* and acidophilus milk (O'Connor, 1995).

In Ethiopia, a considerable proportion of milk is consumed in the fermented form and the smallholder farmers produce fermented milk by traditional methods. The major fermented milk products produced by smallholder farmers by traditional methods include "*Ergo*" (fermented sour milk), "*Kibe*" (traditional butter), "*Nitir kibe*" (ghee or clarified butter), "*Ayib*" (cottage cheese), "*Arerra*" (sour defatted milk), and "*Aguat*" (whey) (Ashenafi, 2006).

The fermented milk product (Yoghurt) has different vernacular names in Ethiopia such as "*ergo"*, "*ititu"*, "*geinto"* or "*meomata"* among the Amhara, Oromo, Sidama, or Wolayta people, respectively (Ashenafi, 2006). *"Ergo*" is mainly produced on farm by women who may further process in to more stable products which may be sold in the market, and thus generates income by which other household items purchased (Lemma, 2004). As the major fermented dairy product, Yoghurt is one of the most widely consumed milk product (Grieger *et al*., 1990). Yoghurt *(Ergo)* is popular and is consumed in all part of the Ethiopia and by every member of the family. Yoghurt *(Ergo)* is considered as a special food which serves as a basis for further processing and it is particularly used as a nutritional support to sick people, children and to pregnant and lactating mothers of the family (O'Connor, 1994).

In Ethiopia, "*Ergo"* (sour milk): is made from raw milk, which is milked in washed and well smoked container (clay pot, calabash) using filtering materials like muslin cloth. Raw milk will be left at ambient temperatures (2-3days) or kept in warmer places to ferment naturally and milk from a previous fermentation serves as inoculum. It is a traditional naturaly fermented milk product, which has some resemblance to yoghurt (Ashenafi, 2002). Yoghurt *(Ergo)* is made by natural fermentation of milk under ambient temperature, without the addition of starter cultures (Assefa *et al*., 2008; Alemayehu, 2008). The use of a portion of yoghurt (*Ergo*) from a previous batch as a starter in highland areas where ambient temperature is relatively low is reported (Kassa, 2008). This practice is technically adapted to overcome the effect of low ambient temperature, which slows down the growth of lactic acid bacteria (LAB) in the absence of starter

culture thus prolongs the fermentation time (Assefa *et al.*, 2008). Fermented milk is reported to have a storage stability of 15 to 20 days at a temperature of about 16 to 18^{o}C (O'Connor, 1994; Yilma, 2012).

Yoghurt is one of the oldest fermented milk products that involve the action of microorganisms, principally the lactic acid bacteria, e.g. Lactobacillus, Streptococcus (Saint-Eve *et al*., 2006). Microbial contamination of yoghurt might occur during the manufacturing process or due to the lack of personal hygienic practice. The microbial spoilage of milk and milk products are due to unclean processing lines and unhygienic handling (Soomro *et al.,* 2002; Aly and Galal, 2002; Lues *et al.,* 2003; Chye *et al*., 2004; Rashed *et al.,* 2014).

The consumption of raw milk and its derivatives is common in Ethiopia (Yilma, 2003), which is not safe from consumer health point of view as it may lead to the transmission of various diseases (Spreer, 1998; Yilma, 2012).

2.4.2. Cottage cheese *(Ayib)*

"Ayib" is a traditional Ethiopian cottage cheese made from sour milk after the fat is removed by churning then by slowly heating until a distinct curd mass forms and floats over the whey. It is an acidic product. "*Ayib"* is an important source of nutrients and serves as a staple diet. It may be consumed fresh as side dish, or it may be spiced with hot spices, salt and other herbs (Ashenafi, 1992; Almaz *et al*., 2001; Ashenafi, 2006).

Cheese provides a useful service in extending the shelf life of valuable human food stuff milk (Hamid and Owni, 2007). In Ethiopia, smallholder milk processing is based on sour milk resulting from high ambient temperatures, while meeting consumers' preferences and improving keeping quality. Cottage cheese is a popular milk product consumed by the various ethnic groups of the country (Ashenafi, 1990; Yilma *et al*., 2007).

Milk for churning is accumulated in a clay pot over several days and kept in a warm place about 30^0C for 24 to 48 hours to sour spontaneously. Butter is made by churning naturally fermented whole milk (*Ergo*) using traditional churns such as clay pot and bottle gourd after filling the ergo to about half of the capacity of a churn. The churn is then agitated after covering and securely tying it with materials such as a false banana leaf, a piece of skin or hide. After butter granules have coalesced into large grains, the churn is rotated on its base to help collect butter grains and form lumps of butter in the center. The butter is then skimmed off, kneaded in cold water and washed to remove visible residual buttermilk. Cottage cheese known as "*ayib"* is made by heating buttermilk (a by product of butter-making) in a clay pot or other material on a low fire at a temperature of 40 to 70^0C. Heat treatment does not appear to affect yield but gives the product a cooked flavor. When the curd and the whey separate, the heating is stopped and the contents of the pot are allowed to cool. After draining off the whey using materials such as muslin cloth, the cheese curd *(Ayib)* is kept in a clean bowl or pot until it is served with different dishes (Ashenafi, 1990; Almaz *et al*., 2001; Ashenafi, 2006).

During preparation of "*ayib",* the high initial count of microorganism in milk, which increases during the fermentation process, is shown to decrease by the combined action of cooking and low pH (Ashenafi, 2006). The presence of high microbial load of ready-to-consume "*ayib"* is believed to be introduced from plant parts used for packaging and imparting flavor; and from handlers, too (Ashenafi, 1990). The safety of cheeses with respect to food-borne diseases is of great concern around the world and especially true in developing countries, where production of milk and various dairy products often take place under unsanitary conditions (Ashenafi, 1992; Yilma, 2012).

The production of cheese takes place under unsanitary (unsatisfactory hygiene) conditions rendering it a potential carrier of pathogenic microorganisms, such as enterotoxin-producing *Staphylococcus* species (Ashenafi, 1990; Yilma *et al*., 2007). Staphylococcal enterotoxins have been detected in cow's milk cheeses with a relatively high frequency (2.8 to 5.4%) of contaminated samples (Bautista *et al*., 1988; Jorgensen *et al*., 2005).

2.5. Vehicles of transmission

Staphylococci exist in air, dust, sewage, water, milk, food, or on food equipment, environmental surfaces, humans, and animals (Maria *et al*., 2010; Bennett and Monday, 2003). Staphylococci are present in the nasal passages and throats and on the hair and skin of 50% or more of healthy individuals. This incidence is even higher for those who associate with or who come in contact with sick individuals and hospital environments. Although food handlers are usually the main source of food contamination in food-poisoning outbreaks, equipment and environmental surfaces can also be sources of contamination with *S. aureus*. Human intoxication is caused by ingesting enterotoxins produced in food by some strains of *S. aureus*, usually because the food has not been kept hot enough (60°C, 140°F, or above) or cold enough (7.2°C, 45°F, or below) (Acco *et al*., 2003; Bennett and Monday, 2003).

2.6. Public health and economic importance

Staphylococcal Food Poisoning (SFP) is one of the most common Food borne diseases (FBD) and is of major concern in public health programs worldwide (Balaban and Rasooly, 2000; Loir *et al*., 2003; Hennekinne *et al.,* 2012). Staphylococcal infections are frequent but are usually contained by immune mechanisms to the site of entry. The highest incidence of disease usually occurs in people with poor personal hygiene, overcrowding and in children (Hobbs and Gilbert, 1981; Rho and Schaffner, 2007). In developing countries, the surveillance system of FBD hardly exists and it is therefore, difficult to estimate the real magnitude of the problem (Rowland *et al*., 1994; Hocking and Doyle, 1997; Boschi-Pinto *et al*., 2008). Even in countries where surveillance services are very efficient, the precise incidence of food poisoning is not known, as outbreaks are often not reported to public health authorities. Hence, the incidence of FBD caused by staphylococci is thought to be much higher than reported since many cases remain undeclared (Jay, 2000; Walderhaug, 2007).

Food borne diseases are a serious and growing problem in the world (Loir *et al*., 2003; Baron, 2007). World Health Organization and the US Centers for Disease Control and Prevention (CDC) report every year a large number of people affected by foodborne illnesses (Busani *et al.*,

2006). Globally, an estimated 2 million people died from diarrheal diseases in 2005; approximately 70% of diarrheal diseases are foodborne. It is estimated that up to 30% of the population suffer from foodborne illnesses each year in some industrialized countries (WHO, 2011). According to the estimation by CDC in 1999, around 76 million foodborne illnesses occur annually, resulting in 325,000 hospitalizations and 5200 deaths and costs annually 5-6 billion USD in the United States each year (Jay, 2000; WHO, 2007a; Buzby & Roberts, 2009). However, a decrease in the incidence rates of notified foodborne illness was noticed from 1996 to 2005, but these rates have remained static since 2005 (Anderson *et al*., 2011). Identified pathogens account for an estimated 14 million illnesses, 60,000 hospitalizations, and 1800 deaths. *Salmonella, Listeria,* and SFP organisms are responsible for 1500 deaths. Unidentified pathogens account for the remaining 62 million illnesses, 265,000 hospitalizations, and 3200 deaths. Overall, FBD appear to cause more illnesses but fewer deaths than previously estimated (Loir *et al*., 2003; Baron, 2007).

Among FBD, SFP is of major concern in global public health programmes. Staphylococcal organisms alone have found to cause hospitalization rates as high as 14%. Although not considered especially lethal, death can ensue if large amounts of SE are ingested: fatality rates range from 0.03% in the general population to as high as 4.4% for highly sensitive persons such as immunocompromised persons, elderly persons and children (Atanassova *et al*., 2001; Aycicek *et al*., 2005; Kerouanton *et al*., 2007).

In the United States, latest available data on foodborne disease outbreaks reported by the Centre for Disease Control and Prevention showed that *S. aureus*, together with Shiga toxin-producing *Escherichia coli*, ranked as the third commonest bacterial causative agents (9.8%), following *Salmonella* (39.7%) and *Clostridium perfringens* (11.5%)(CDC, 2009). The disease burden attributed by *S. aureus* seemed to become smaller when compared with the mean annual total for the previous 5 years (15.0%), though it was similar to that for 1998 to 2002 (8.5%)(CDC, 2006).

The latest report produced by European Food Safety Authority, which received data from 27 European Union Member States, showed that *S. aureus* was the fourth most common causative agent for the reported foodborne outbreaks in 2008, following *Salmonella*, foodborne viruses and

Campylobacter. *S. aureus* caused 291 foodborne outbreaks which constituted 5.5% of total number of reported outbreaks in the European Union (EFSA, 2010).

2.7. Isolation and identification of staphylococci

Symptoms of FBD associated with staphylococci are not suggestive and have little importance to warrant diagnosis (Loir *et al*., 2003; Johnson *et al*., 2006; Baron, 2007). In the diagnosis of SFP, proper interviews with the victims and gathering and analyzing epidemiologic data are essential (Hobbs and Gilbert, 1981). Incriminated foods should be collected and examined for staphylococci or the SEs produced. The latter is especially important when foods that have been heated before consumption are implicated in the outbreak. Food handlers are also tested to ensure whether they are carriers of the strain responsible (Bautista *et al*., 1988; Rho and Schaffner, 2007).

Incorrect identification of an isolate can impact on the implementation of effective treatment and/or control measures (Rowland *et al*., 1994; Salyers and Whitt, 2002; Smith, 2007). In the diagnosis of SFP, detailed history, including the duration of the disease, characteristics and frequency of bowel movements, and associated abdominal and systemic symptoms, may provide a clue to the underlying cause. The presence of a common source, types of specific food, travel history, and use of antimicrobials always should be investigated. Diagnosis is confirmed by isolation of the organism or SE from relevant specimens (Jay, 2000; Baron, 2007; Walderhaug, 2007).

The presence of relatively large numbers of enterotoxigenic staphylococci is a good circumstantial evidence that the food contains SEs. The most conclusive test is the linking of an illness with a specific food or in cases where multiple vehicles exist, the detection of the toxin in the food samples (Martın *et al*., 2004; Hein *et al*., 2005; Chiang *et al*., 2008). In cases where the food may have been treated to kill the staphylococci, as in pasteurization or heating, direct microscopic observation of the food may be an aid in the diagnosis (Hagstad and Hubbert, 1986; Bania *et al*., 2006; Walderhaug, 2007).

The isolation and identification of *Staphylococcus* species is conducted on the basis of colony morphology, haemolytic properties, Gram-stain, catalase production, coagulase production and biochemical profile or sugar fermentation (Quinn *et al*., 2002; Aycicek *et al*., 2005). Samples were inoculated aseptically on the surface of the BAP medium by spreading with a sterile loop in such a way that bacteria are ultimately deposited singly because when the bacteria are at a sufficient distance from each other, the whole progeny of each accumulates locally during growth to form a discrete mass or colony which is readily visible to the naked eye. Each colony was presumed to be a pure culture, consisting exclusively of the descendants of a single cell (Loir *et al*., 2003; Shah, 2003; Todar, 2008).

On agar plates, staphylococcal colonies appear opaque to golden yellow in colour, glistening, smooth and in circular form. Blood agar is the medium of choice for isolation of the organism from specimens, and on 24 hours incubation staphylococci give good growth of creamy, often deeply pigmented colonies that is surrounded by the narrow zones of clear haemolysis, a broader zone of incomplete haemolysis or none depending on the species (Freeman, 1985; Quinn *et al*., 2002; Bendahou *et al*., 2008). Some species of *Staphylococcus* synthesize the enzyme haemolysin. Haemolysin is an exoenzyme that lyses red blood cells. If a colony of bacterial cells is producing haemolysin and secreting it into the medium, there will be a round, clear zone surrounding the colony because the red blood cells in that area have been lysed. The presence or absence of haemolytic properties, therefore, cannot be used as a definitive identification of *Staphylococcus* species as some species and strains of *Staphylococcus* species may not cause haemolysis (Quinn *et al*., 2002; Salandra *et al*., 2008).

Preparation and examination of Gram stained smears from typical colonies shows Gram positive spherical bacterium (coccus), which on microscopic examination appears in pairs, short chains, or bunched, grape like clusters (Aycicek *et al*., 2005; Todar, 2008).

Catalase test is important to distinguish streptococci (catalase-negative) from staphylococci, which are catalase-positive. The catalase test determines if the organism produces the enzyme catalase that breaks down hydrogen peroxide (H_2O_2) to water and oxygen (Rowland *et al*., 1994; Shah, 2003; Sandel and McKillip, 2004). When mixed with 3% H_2O_2, catalase-positive

organisms will generate bubbles of oxygen, which are visible to the naked eye while catalase negative organisms do not. This enzyme allows organisms to breakdown harmful metabolites of aerobic respiration and may be seen in aerobic and facultatively anaerobic organisms. It is preferable to test colonies for catalase production from media without blood since erythrocytes possess catalase activities (Quinn *et al*., 2002; Todar, 2008).

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + O_2 \text{ (gas bubbles)}$$

Pathogenic organisms require mechanisms to help them overcome host defense mechanisms. One mechanism involves coating the bacterial cells in a body substance, such as fibrin, to fool the immune system. The coating of a natural body substance will not trigger an immune response and this is accomplished through the production of coagulase. Coagulase is an exoenzyme that causes fibrin of blood plasma to be deposited on bacterial cells resulting in clot formation. Pathogenic staphylococci produce coagulase, while non-pathogenic strains are coagulase negative (Shah, 2003; Turutoglu *et al*., 2005; Morrison, 2008).

The ability of *Staphylococcus* to produce coagulase, an enzyme capable of clotting plasma, was first reported by Loeb in 1903 (Clark and Nelson, 2002; Morrison, 2008). The most widely used and generally accepted criterion for identification of pathogenic staphylococci is usually by their ability to produce coagulase (the coagulase test correlates well with pathogenicity). The presence of this highly specific enzyme was a fairly certain indication that a *Staphylococcus* was pathogenic. A pathogenic *Staphylococcus* has become an organism which produces coagulase and *vice versa.* Fresh or reconstituted commercial freeze dried rabbit plasma is the reagent used. Rabbit plasma contains fibrinogen that is converted to fibrin by the staphylococcal coagulase enzyme (Quinn *et al*., 2002; Lamprell *et al*., 2004).

A range of selective and diagnostic media have been developed to assist in the detection and enumeration of staphylococci in routine food surveillance programmes and food poisoning investigations (Baird and Lee Wong, 1995). Selective bacteriological media containing one or more agents that are inhibitory to microorganisms other than the target pathogen (staphylococci) can be applied. The microorganism of interest is not inhibited by the presence of these

components in the medium, and will therefore, form visible colonies during incubation (Quinn *et al*., 2002). The two selective agents most commonly used for these pathogens are sodium chloride (NaCl) and potassium tellurite (Baird and Lee Wong, 1995; Pal, 2007).

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

2.7.1. Principles of detection of *Staphylococcus aureus*

No specific test may be useful in every case to isolate the staphylococci from the wide variety of foods in which they are found. As result, attempts have been made to find a combination of selective and enrichment media that will support the growth of the staphylococci and at the same time suppress the growth of other microflora present that tends to overgrow the staphylococci. A three-tube isolation procedure using trypticase soy broth with 10% sodium chloride and 1% sodium pyruvate was accepted as the official method for recovery of the largest numbers of coagulase positive staphylococci from the widest variety of foods (Lancette and Lanier, 1987); however, thermally stressed cells of *S. aureus* are unable to grow in the medium. As a result, food samples likely to contain a small population of injured cells were incubated in double-strength trypticase soy broth before the addition of 13% NaCl and spread-plating on Baird-

Parker agar (Lancette and Tatini, 1992). For detecting small numbers of *S. aureus* in raw food ingredients and non-processed foods expected to contain large numbers of competing organisms, incubation is in trypticase soy broth containing 10% NaCl and 1% sodium pyruvate before transferring to Baird-Parker agar plates. For detecting relatively large numbers of staphylococci, the food extract is plated directly on Baird-Parker agar (Bergdoll and Lee Wong, 2006).

Typical colonies of *S. aureus* on Baird-Parker agar are circular, smooth, convex, moist, ~1.5 mm in diameter on uncrowded plates, gray-black to jet-black, smooth with entire margins and off-white edges, and may show an opaque zone with a clear halo extending beyond it. Normally those colonies that appear to be *S. aureus* will be counted, and one or more of each type tested for coagulase and TNase production. However, the upgrading of biotypes E and F to *S. intermedius* and *S. hyicus* complicates the species classification because all three species can be coagulase- and TNase positive. If at least one test is positive and the food being examined is from a food-poisoning outbreak, the staphylococci are probably *S. aureus* from human contamination. An additional positive anaerobic mannitol fermentation test will confirm *S. aureus*. The number of colonies on the triplicate plates represented by the *S. aureus* positive colonies is multiplied by the dilution factor, and the result reported as the number of *S. aureus* per gram of food (Bergdoll and Lee Wong, 2006).

Agglutination kits employing the clumping factor, protein A, and specific antigens of *S. aureus* are available for identification of *S. aureus* strains. However, these kits are designed primarily for use in the clinical field where large numbers of staphylococci are being examined and where large numbers of coagulase-negative species are also encountered. The clumping factor test is not satisfactory because *S. intermedius* and some coagulase-negative species can be positive for this factor (Wilkerson *et al*., 1997).

An alternative method has been proposed by Roberson *et al*. (1992) in which P agar supplemented with acriflavins and the β-galactosidase tests are used. Of the coagulase positive species, only *S. aureus* will grow on the supplemented P agar and is negative with the β-galactosidase test. *S. intermedius* does not grow on the modified P agar and is 100% positive with the β-galactosidase test, whereas *S. hyicus* is negative by both tests. This method is useful if

the staphylococci being tested are from sources other than clinical (Bergdoll and Lee Wong, 2006).

Another method that has been proposed to identify *S. aureus* from non-clinical sources employs an immunoenzymatic assay using a monoclonal antibody prepared against endo-β-acetyl-glucosaminidase-an enzyme produced by all isolates of this species. Comparison of this method with six kits available for identification of *S. aureus* has shown it to be specific for *S. aureus,* whereas the kits were positive for *S. intermedius, S. schleiferi,* and *S. lugdunensis* (Guardati *et al*., 1993; Bergdoll and Lee Wong, 2006).

2.8. Principles of detection of the enterotoxins

It was not possible to develop specific methods for the detection of the enterotoxins before Bergdoll *et al*. (1959a) identified and purified the first enterotoxin. Until that time, the only means of detecting the presence of the enterotoxins was by the use of animals that gave emetic reactions to the toxin, either intragastrically or intravenously. Fortunately, at the time Surgalla and Bergdoll began their research to identify the enterotoxin; immunological methods were being developed for the specific detection of individual proteins. These investigators were able to show that specific antibodies could be produced to the enterotoxin when the emetic reaction in monkeys was neutralized by antisera produced against the crude toxin (Surgalla *et al*., 1954; Bergdoll *et al*., 1959b). Subsequently, all laboratory methods for the enterotoxins have been based on the use of specific antibodies to each of the enterotoxins for their detection, because it is almost impossible to detect individual proteins by chemical methods (Bergdoll and Lee Wong, 2006).

The most conclusive test is the linking of an illness with a specific food or in cases where multiple vehicles exist, the detection of the toxin in the food samples (Martin *et al*., 2004; Chiang *et al*., 2008).

2.8.1. Biological methods

Before the first enterotoxin was purified, many types of animals (such as pigs, dogs, cats and skitters, and monkeys) were tested in the search for an inexpensive specific test method. All of these animals, with the exception of monkeys, were relatively insensitive to the enterotoxins, unless the toxin was injected intraperitoneally or intravenously. Emesis is the most readily observable reaction to enterotoxin; hence animals without a vomiting mechanism, such as rodents, were of little value as test subjects (Hammon, 1941; Schantz *et al.*, 1965; Bergdoll and Lee Wong, 2006).

Because antibodies are specific for each enterotoxin, it is necessary to continue the use of animal testing until each new enterotoxin has been purified and antibodies produced against it. Animal testing is also necessary for assessing the effect of various treatments, such as heat, on the enterotoxins (Bergdoll and Lee Wong, 2006).

2.8.2. Immunological methods

The most specific and sensitive tests for the enterotoxins are based on their 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 radioimmunoassay (RIA) was applied, and later the enzyme-linked immunosorbent assay (ELISA) and the reversed passive latex agglutination (RPLA) method were developed. The gel-diffusion methods have been used primarily for the detection of enterotoxin production by staphylococcal strains, although the RPLA method is used for testing strains for low production of enterotoxin. The RIA method was used for testing for enterotoxin in foods until the ELISA and RPLA were available (Bergdoll and Lee Wong, 2006).

2.8.3. Detection in foods

The detection of enterotoxin in foods requires methods that are sensitive to less than 1ng/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 ELISA method, the RPLA method and screening methods (Hein *et al*., 2005; Bergdoll and Lee Wong, 2006).

2.9. Antimicrobial resistance pattern

Antimicrobial resistance is a form of drug resistance whereby some (or, less commonly, all) sub-populations of a microorganism, usually a bacterial species, are able to survive after exposure to one or more antimicrobials; pathogens resistant to multiple antimicrobials are considered multidrug resistant (MDR) (CDC, 2013b). Antimicrobial resistance is a major clinical obstacle in medicating disease especially in the developing countries (Tenover, 2006; Jilani *et al*., 2008).

In the 1940s, penicillin was introduced for the treatment of infection; as early as 1942, strains of *S. aureus* resistant to penicillin had been detected in hospitals. Within 2 decades, ~80% of both hospital and community acquired *S. aureus* isolates were penicillin resistant. The introduction of methicillin in 1961 was rapidly followed by reports of methicillin resistance in *S. aureus.* Today, MRSA strains are found world wide, and most are multidrug resistant (Appelbaum, 2006).

Antimicrobial resistance is a serious and growing phenomenon in contemporary medicine and has emerged as one of the pre-eminent public health concerns of the 21st century, in particular as it pertains to pathogenic organisms (the term is especially relevant to organisms that cause disease in humans). In the simplest cases, drug-resistant organisms may have acquired resistance to first-line antimicrobials, thereby necessitating the use of second-line agents (Arias *et al.,* 2009).

The resistance to antimicrobial agents is an increasingly global problem worldwide, especially among nosocomial pathogens. Staphylococci have become one of the most common causes of nosocomial infections. Multidrug-resistant staphylococci pose a growing problem for human health. The rise of drug-resistant virulent strains of *Staphylococcus aureus*, particularly methicillin-resistant *S. aureus* (MRSA) is a serious problem in the treatment and control of staphylococcal infections (Livermore, 2000; Zapun *et al*., 2008).

The liberal use of antimicrobial agents at hospitals and treatment centers, as well as their sub-therapeutic use in livestock for growth promotion and prophylaxis, greatly contributes to the emergence and persistence of resistant strains (Helmuth, 2000; Ray, 2004). The bacterial resistance against antimicrobial agents may be driven by the interplay of several genetic factors (Bennett, 2008; Canton, 2008; Hung and Kaufman, 2010). Moreover, the practice of addition of antimicrobials to livestock feed may heighten the drug-resistance and hence limits the usefulness of antimicrobials to eliminate bacterial infections (Gales *et al.*, 2001; Mathew *et al.*, 2007; Allerberger and Mittermayer, 2008). The main concern is that the pool of resistance genes is increased and can be spread through bacterial plasmids and other mobile genetic elements. The use of antimicrobials in animal husbandry often leads to a higher level of resistance against that drug. In pathogens with zoonotic potential, like *Salmonella* and *S. aureus*, antimicrobial resistance reduces our ability to treat both humans and animals, increasing their public health significance (Helmuth, 2000; Thaker, 2013).

Staphylococci are naturally susceptible to most antimicrobials but are also very prone to the development of resistance genes through mutation and DNA transfer (Livermore, 2001). Resistance in *S. aureus* developed very quickly after the introduction of penicillin in the 1940s. Today there are strains of *S. aureus* that are resistant to the most commonly used antimicrobials (Todar, 2011). MRSA is a strain that is resistant to beta-lactams, including methicillin and often oxacillin, penicillin, and amoxicillin (CDC, 2010a). MRSA was originally associated with nosocomial infections and still remains a problem in hospitals throughout the world today (Voss and Doebbeling, 1995). Within the healthcare setting, risk factors include patients with a weakened immune system, recent surgery, and insertion of a catheter among other things. Hospital Associated (HA)-MRSA is commonly associated with surgical wound infections,

urinary tract infections, bloodstream infections, and pneumonia (CDC, 2010a). Antimicrobial resistance in general continues to grow in hospitals. The National Nosocomial Infection Surveillance (NNIS) system has shown an increase in the proportion of *S. aureus* strains isolated from patients in U.S. hospitals that are resistant to methicillin, oxacillin, or naficillin (60%) (CDC, 2003).

Not only is there still a concern with HA-MRSA, but the prevalence of community associated (CA) MRSA is growing. Everyone is at risk for CA-MRSA and can be infected through skin-to-skin contact, cuts and abrasions, contaminated items or surfaces, living in a crowded space, and poor hygiene (CDC, 2010a). MRSA in livestock animals is another contributing factor in human infections. A study in the Netherlands found a new MRSA strain from an animal reservoir that entered the human population and now is responsible for more than 20% of all MRSA strains in the Netherlands. The study also showed that the strain was more likely to be isolated from pig and cattle farmers (Loo *et al.,* 2007).

As previously mentioned, resistance among *S. aureus* is a public health issue worldwide. A study conducted in the 1980s in Addis Ababa, Ethiopia, sampling hospital and non-hospital populations, isolated *S. aureus* from 32.4% and 21.6% respectively. Over 96% of the hospital strains and 88% of the non-hospital strains showed resistance to at least one antimicrobial and 45% of the hospital strains were multiply resistant (Tewodros and Gedebou, 1984). A more recent study in south western Ethiopia determined 8.3% of *S. aureus* isolates from clinical specimens at the Jimma hospital were MRSA and over 90% were resistant to penicillin and ampicillin (Gebre-Sealassie, 2007).

Resistant *S. aureus* strains have also been isolated from cattle throughout Ethiopia. Studies isolating *S. aureus* from bovine mastitic milk show high levels of resistance to ampicillin, penicillin, polymixin B, and streptomycin (Abera *et al.*, 2010; Sophia, 2011). Considering the large portion of the Ethiopian population that lives in close proximity to their livestock, there is potential for transmission of resistant *S. aureus* from livestock to humans through the consumption of milk and milk products (Sophia, 2011).

Antimicrobial resistance is a worldwide problem. New forms of antimicrobial resistance can cross international boundaries and spread between continents easily. Many forms of resistance spread with remarkable speed (CDC, 2013a).

2.9.1. Different test methods in detecting antimicrobial resistance

There are several antimicrobial susceptibility testing methods available today, and each one has their respective advantages and disadvantages. They all have one and the same goal, which is to provide a reliable prediction of whether an infection caused by a bacterial isolate will respond therapeutically to a particular antimicrobialt reatment. This data may be utilized as guidelines for chemotherapy, or at the population level as indicators of emergence and spread of resistance based on passive or active surveillance. Some examples of antimicrobial sensitivity tesing techniques are: Dilution method (broth and agar dilution method), Disk-diffusion method, E-test, Automated methods, Mechanism-specific tests such as beta-lactamase detection test, and chromogenic cephalosporin test and Genotypic methods such as PCR and DNA hybridization methods (CLSI, 2007).

Selection of the appropriate method will depend on the intended degree of accuracy, convenience, urgency, availability of resources, availability of technical expertise and cost. Interpretation should be based on veterinary standards whenever possible, rather than on human medical standards, which may not always be applicable. Among these available tests, the two most commonly used methods in veterinary laboratories are the agar disc-diffusion method and the broth microdilution method (CLSI, 2007).

Disk diffusion method

Because of convenience, efficiency and cost, the disc diffusion method is probably the most widely used method for determining antimicrobial resistance in private veterinary clinics (CLSI, 2007; Sahu, 2013).

A growth medium, usually Mueller-Hinton agar, is first evenly seeded throughout the plate with the isolate of interest that has been diluted at a standard concentration (approximately 1 to 2 x 10^8 colony forming units per ml). Commercially prepared discs, each of which are pre-impregnated with a standard concentration of a particular antimicrobial, are then evenly dispensed and lightly pressed on to the agar surface. The test antimicrobial immediately begins to diffuse outward from the discs, creating a gradient of antimicrobial concentration in the agar such that the highest concentration is found close to the disc with decreasing concentrations further away from the disc. After an overnight incubation, the bacterial growth around each disc is observed. If the test isolate is susceptible to a particular antimicrobial, a clear area of "no growth" will be observed around that particular disk (CLSI, 2007; Mohanty *et al.*, 2010; Sahu, 2013).

The zone around an antimicrobial disc that has no growth is referred to as the zone of inhibition since this approximates the minimum antimicrobial concentration sufficient to prevent growth of the test isolate. This zone is then measured in mm and compared to a standard interpretation chart used to categorize the isolate as susceptible, intermediately susceptible or resistant. MIC measurement cannot be determined from this qualitative test, which simply classifies the isolate as susceptible, intermediate or resistant (Bonev *et al*., 2008; Mohanty *et al.*, 2010).

2.10. Management strategies

There is no significant growth of staphylococci at temperature below 4.4^0C and the organisms are destroyed when kept at 77^0C for 20 minutes (Ash, 2008). Storing foods at temperature less than 4.4^0C or greater than 60^0C effectively prevents replication of staphylococcal organisms and significant toxin production (Hocking and Doyle, 1997; Salyers and Whitt, 2002; Ash, 2008).

2.10.1. Treatment

The objective of treatment in human patients is to replace fluids, salt, and minerals that are lost by vomiting or diarrhoea (Foster, 1991; Sandel and McKillip, 2004). Some strains of

Staphylococcus have acquired genes making them resistant to multiple antimicrobial agents. These organisms are uniformly resistant to penicillins and cephalosporins. Penicillinase resistant penicillins such as oxacillin and flucloxacillin are used for serious infections. First or second generation cephalosporins such as cephalothin, cephalexin and cefuroxime are usually safe in patients who are hypersensitive to penicillins. Vancomycin is usually effective for methicillin-resistant staphylococci. Erythromycin and its newer relatives are used in milder infections. The infections can also be treated with combination therapy using sulfa drugs and minocycline or rifampin (Kloos and Bannerman, 1994; Rho and Schaffner, 2007).

2.10.2. Prevention and Control measures

Staphylococci are ubiquitous and are impossible to eliminate from the environment. However, SFD is preventable (Byrd-Bredbenner *et al*., 2013). The total destruction or significant reduction in the bacterial load in foods during growth, harvesting, processing, packaging, and storage prior to consumption has always been a general goal. However, the wide array of parameters for proliferation of foodborne pathogens is staggering. Some of the same methods for the control of organisms in the food supply are used separately or in combination in the preservation of foods. Staphylococci may be totally destroyed or injured when subjected to lethal or sublethal doses, respectively, of heat, cold, drying, irradiation, or chemicals. While total destruction of these organisms might be ideal, sublethal injury may occur, thus providing the organism an opportunity to recover and proliferate, if conditions are conductive (Martin and Myers, 1994; Bennett and Monday, 2003).

There is no effective long term decolonization therapy for *S. aureus* carrier. Even with the use of antimicrobials, *S. aureus* can only be removed from the nose over a few weeks, but relapses are common within several months (Coates *et al*., 2009). Although post treatment eradication may be initially high, sustained decolonization drops to half of cases 6 to 8 months after treatment (Preston, 2010). Only pre-employment or routine medical and laboratory examinations of food handling personnel are of no value in the prevention of foodborne diseases but in addition, providing education and training in good hygienic practices to all food handling personnel have high importance (WHO, 1989 and CDC, 2011).

Prevention of staphylococcal infections/intoxication requires strategies to interrupt various modes of transmission. Essentially these control programs include improvements in personal hygiene practices among healthcare workers and food handlers, decontamination of equipment, surfaces, and clothing, judicious use of antimicrobials, proper cooking and storage of foods, and screening programs (Doyle *et al.*, 2011).

To prevent food-poisoning outbreaks, it is necessary to keep foods either refrigerated (-10°C) or hot (45°C) to prevent proliferation of the organism to such numbers (10^5 cells/g) necessary for detectable toxin formation (Bennett and Monday, 2003). The permissive temperature for the growth and toxin production by *S. aureus* is between 6°C and 46°C. Thus, the ideal cooking and refrigerating temperature should be above 60°C and below 5°C, respectively (Kadariya *et al.*, 2014). Additionally, foods should be refrigerated in shallow layers or small portions to facilitate quick cooling (Bennett and Monday, 2003).

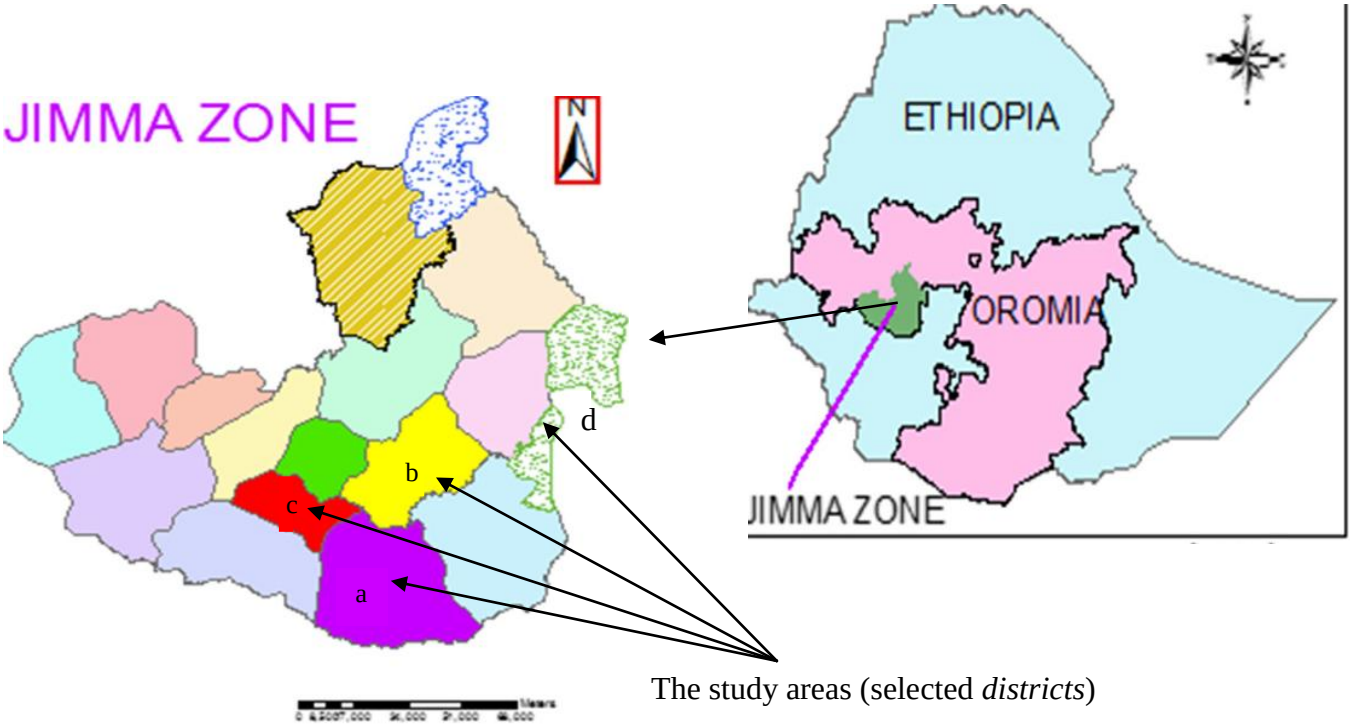
3. MATERIALS AND METHODS

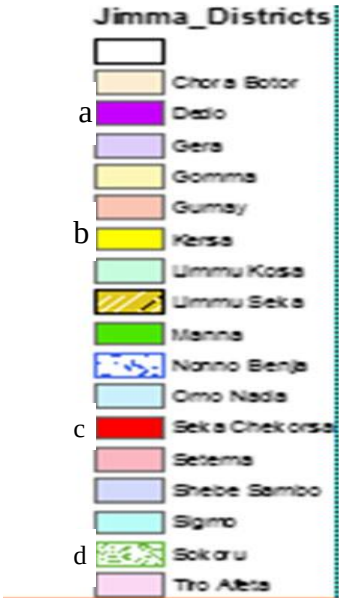
3.1. Study Period and Area

The study was carried out between May 2014 and March 2015 in selected *districts* of Jimma zone, Oromia Regional State, Ethiopia. Jimma zone is one of the thirteen zones of Oromia regional state which geographically lies at southwestern part of Ethiopia. Jimma town is the capital of the zone that is 345 km South-west of Addis Ababa, capital city of Ethiopia. It covers a total surface area of 19,305.5 km^2. According to the 2007 Population and Housing Census of Ethiopia, the total population of Jimma zone was 2,486,155. From the total population in the zone, 2,204,225 (88.66%) is the rural population, which directly depends on agricultural activities for domestic use and exchange of commodities with urban residents. The zone bordered in Northwest by Illubabor, in East by Wellega and in West by Shewa zones as well as in south by Southern Nations and Nationalities People's Regional state. In general, the zone lies at 7ºC 41'N latitude and of 36°50'E longitude and topographical features elevation varies from 1000 to 3360m above sea level with the maximum and minimum temperatures in range of 25–30°C and 7–12°C, respectively. Annual rainfall of the zone is one of the highest in the country reaching up to 1200–2800mm per year. The predominant economic activities involve mixed farming, which broadly includes cultivation of cereal crops, cash crops including primarily coffee and production of livestock.

According to the Central Agricultural Census Commission 2003, Jimma zone has an estimated of 1,718,284 heads of cattle, 466,154 sheep, 194,677 goats, 74,774 horses, 40,555 donkeys and 30,541 mules populations.

The present study was conducted in 4 selected *districts* of Jimma zone*:* namely, Seka chekorsa 20 km in Southeast, Dedo 20 km in South, Sekoru 75 km in Northeast and Kersa 18 km in Northwest, of Jimma town. According to recent zoning system, the zone is divided into 17 *districts* (Figure 1)





Source: Yigezu *et al*. (2014)

Figure 1: Map of the study area

3.2. Study Design

3.2.1. Study type, type of samples and source of samples

A cross-sectional study design was used. Cottage cheese and yoghurt made from cow's milk were the sample types used in this study. Sampling was carried out repeatedly in four selected *districts* of the zone until the allocated sample size was fulfilled. The *districts* selected for the study were Kersa, Dedo, Sekoru and Seka chekorsa. These *districts* were selected purposely due to their high production potential of cheese and yoghurt. Besides the *districts* are the major suppliers of cheese and yoghurt to Jimma town. Samples were collected from retailers, restaurants and households for cow milk based cottage cheese samples. Yoghurt samples from cow milk were taken from different cafeterias' and households' storage containers in selected *districts* of Jimma zone which were supposed to be the major risk areas for the consumers as many people may share the pooled product. Restaurant in this study refers to any small houses (bistros) which sale ready to eat foods, including cheese for consumers; retailers refers to any person who sale cheese products to public use or consumption rather than for resale; households means any farmer's house who produce cheese and yoghurt and sale for consumers and retailers where as cafeteria means any small scaled houses which sale coffee, tea, yogurt, for breakfast or any time for consumers in study areas.

3.2.2. Sampling method and sampling size determination

Simple random sampling technique was used to take cottage cheese and yoghurt samples from households, restaurants, retailers and cafeterias. The sample size was determined by considering the following parameters: 95% level of confidence (CL), 5% desired level of precision and no same previous study in the areas so by taking 50% expected prevalence of *Staphylococcus* in cottage cheese and yoghurt. The sample size was determined using the formula given in Thrusfield (2007).

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

Where: n = required sample size,

P_{exp} = expected prevalence,

d = desired absolute precision.

Using the above formula, the sample size was calculated to be 384 (cottage cheese = 192, yoghurt = 192). However, to increase the precision 200 cottage cheese and 200 yoghurt samples were collected and examined. The distribution of ottage cheese and yoghurt samples among each *districts* were made to be 50 samples (Table 1).

Table 1: Number and sources of samples

No.	***District***	**No. of samples**		**Sources of samples**				
		Cottage cheese	Yoghurt	Cottage cheese			Yoghurt	
				Household	Retailers	Restaurant	Household	Cafeteria
1	Dedo	50	50	17	17	16	25	25
2	Seka chokorsa	50	50	17	17	16	25	25
3	Sekoru	50	50	17	17	16	25	25
4	Kersa	50	50	17	17	16	25	25
Total		200	200	68	68	64	100	100
					200		200	
		400			**400**			

3.3. Sample collection and transportation

Cottage cheese samples were collected in selected Jimma zone *districts*' retailers, households and restaurants randomly. After removing the external surface of approximately 2 cm depth, about 100 gram of cheese was sampled from each retailer, restaurant and household selected for the study. Yoghurt samples were collected from containers of households and cafeteria, which are critical control point. Pooled yoghurt (yoghurt from different cow's milk colleceted in one container) samples were collected from different households and cafteria aseptically in the study period after thoroughly mixing the yoghurt.

All samples were correctly labeled using the date of collection, sources (retailers, cafeteria, houses and restaurant), *districts*, sample type and container type (Annex-2). All samples were aseptically collected and put into a sterile screw capped bottles and was kept in an ice-box containing ice pack and taken immediately to the laboratory of Microbiology and public health in School of Veterinary Medicine, Jimma University, Jimma, Ethiopia. Upon arrival, the samples were stored overnight in a refrigerator at $+4^0$C until processing (14 to 24 hours).

3.4. Study methodology

3.4.1. Cultural procedure

Isolation and identification of *Staphylococcus* species from cottage cheese and yoghurt samples were conducted in the Microbiology and Public Health Laboratory of the school of Veterinary Medicine of Jimma University. Isolation and identification were performed following the standard microbiological technique recommended by Quinn *et al.* (1999) and the techniques recommended by the International Organization for Standardization, ISO 6888-3: 2003 (Annex-1). Samples which were kept for overnight in a refrigerator at 4^0C were thawed for 3-5 hours at room temperature. The bacteriological media used for the study were prepared following the instructions of the manufacturers (Annex10). In order to get discrete separate colonies, the surface of the agar media was made dry by keeping the medium in the incubator for overnight.

Twenty-five grams of each cottage cheese and twenty-five ml of each yoghurt sample was stirred separately into 225 ml of sterile buffered peptone water (BPW) in a sterile stomacher bag. The pre-enriched samples were homogenized in a stomacher for 2 minutes and incubated aerobically at 37^0C for 24 hours. Following this, 0.1 ml or a loopful of the pre-enriched broth of the various dilutions were streaked (seeded) aseptically onto sterile blood agar plates (BAP) enriched with 7% heparinized sheep blood and incubated at 37^0C for 24-48 hours under aerobic culture conditions. The plates were examined for the presence of *Staphylococcus* colonies. Isolates were supposed to belong to *Staphylococcus* species on the basis of their morphological aspects (creamy, greyish, white or yellow colonies) and haemolytic pattern on the surface of BAP was collected. Presumed Staphylococcal colonies were then sub-cultured on nutrient agar plates

(NAP) and incubated at 37^0C for 24-48 hours to get a pure culture (clone of cells derived from a single cell). The pure isolates from NAP were preserved and maintained for biochemical differentiation tests and characterizing the isolates. The biochemical differentiation of mixed contamination (contamination of single sample by different species of *Staphylococcus*) was carried out by taking pure colonies from different directions of the plate. Pure cultures of a single colony type from the NAP was inoculated in to nutrient agar slants and incubated at 37^0C for 24-48 hours under aerobic culture conditions. The pure isolates in the nutrient slant were preserved and maintained for 15 to 30 days at 4^0C until antimicrobial resistance test had carried out.

3.5. Isolation and identification of *Staphylococcus* species

Prsumptive identification of staphylococci organism and species confirmation were done based on Gram's staining, catalase test, sugar fermentation and coagulase tests (Annex-5 and Annex-6).

3.5.1. Gram's staining

All suspected cultures of *Staphylococcus* species were subjected to Gram's stain and observed under a light microscope for Gram's reaction, size, and shape and cell arrangements. The Gram stained smears from typical colonies that were shown Gram-positive cocci occurring in bunch, grape like irregular clusters were taken as presumptive *Staphylococcus* species.

3.5.2. Biochemical tests

Catalase test

Pure culture of the isolates were picked using a sterile loop from the agar slant and mixed with a drop of 3% H_2O_2 on a clean glass slide. If the organism was positive, bubbles of oxygen were liberated within a few seconds and the catalase negative isolates did not produce bubbles. The catalase positive cocci were considered as staphylococci (Quinn *et al.,* 1999) (Annex-3).

Mannitol salt agar (MSA)

The colonies that had the characteristics of *Staphylococcus* in Gram-staining reaction and catalase test as a positive were streaked on MSA plates, incubated at 37^0C and examined after 24-48 hours for growth and change in the colour of the medium. The presence of growth and change of pH in the media (red to yellow colour) were regarded as confirmative identification of staphylococci. Phenol red pH indicator detects the acidic metabolic product of mannitol. Fermentation of mannitol by *S. aureus* causes yellow discoloration of the medium. Colonies that develop weak or delayed yellow colour after 24 hours of incubation were taken as *S. intermedius* and colonies that failed to produce any change on the medium were considered as *S. hyicus* and Coagulase-negative *Staphylococcus (*CNS) (Quinn *et al.,* 1999).

Coagulase test

Coagulase test was determined by the method described by Quinn *et al.* (1999). Tube coagulase test was performed in sterile tubes by adding 0.5ml of selected isolates of *Staphylococcus* from sub cultured NAP and grown on tryptone soya broth (TSB) at 37^0C for 24 hours and mixed with 0.5ml of citrated rabbit plasma (Annex-4). After mixing by gentle rotation, the tubes were incubated at 37^0C along with a negative control tube containing a mixture of 0.5ml of sterile TSB and 0.5ml of citrated rabbit plasma. Clotting was evaluated at 30 minutes intervals for the first 4 hours of the test and then after 24 hours incubation. The reaction was considered positive, if any degree of clotting from a loose clot to a solid clot that is immovable when the tube is inverted (tilted) was visible within the tube and no degree of clotting was taken as negative.

Purple agar base

According to the method of Quinn *et al.* (1999), Purple agar base (PAB) with the addition of 1 percent maltose was used to differentiate the pathogenic staphylococci, particularly the coagulase-positive isolates. The suspected culture was inoculated on PAB media plate with 1% of maltose and incubated at 37^0C for 24-48 hours. The identification was based on the fact that *S. aureus* rapidly ferment maltose and the acid metabolic products cause the pH indicator

(bromocresol purple) to change the medium and colonies to yellow. *S. intermedius* gave a weak or delayed reaction while *S. hyicus* did not ferment maltose but attacked the peptone in the medium producing an alkaline reaction (a deeper purple) around the colonies.

3.6. Antimicrobial resistance pattern test

Antimicrobial resistance pattern of the isolates were tested by the disk diffusion method (Annex 9) and performed according to Clinical and Laboratory Standards Institute; Performance Standard for Antimicrobial Susceptibility Testing (CLSI, 2007): seventeenth informational supplement guidelines in the Mueller-Hinton agar. The antimicrobials tested for resistance pattern in this study were those which were proved to be often available and routinely used in the study areas for the treatment of animals. Four to five positively identified pure *Staphylococcus* species' colonies of the same morphological type were selected from nutrient agar plate (NAP) and emulsified in 5 ml sterile tryptone soya broth in a sterile test tube. The turbidity of the suspension was then adjusted by comparision with 0.5 McFarland turbidity standards which was in same amount in a similar test tube, in order to standardize the size of inoculums. A sterile cotton swab on an applicator stick was dipped into the standardized suspension of the bacterial culture, squeezed firmly against the insides of the test tube above the fluid level to remove the excess fluid and streaked and continuously brushed over the Mueller-Hinton agar plate and allowed to stand for 5 minutes to dry the flood. Thereafter, five different antimicrobial discs: Penicillin-G (P^{10}), Gentamycin (Gen^{10}), Tetracycline (TE^{30}), Kanamycin (K^{30}) and Streptomycin (S^{10}) were placed on the agar by distance of 24mm (center to center) using sterile forceps and gently pressed down with the point of a sterile forceps to ensure complete contact with the agar surface. The plates were then allowed to stand for 30 minutes for diffusion of active substance of the agents. Plates were inverted and incubated at 35^0C-37^0C for 16-24 hrs. An inhibition zone diameter of each antimicrobial was then measured by using caliper and interpreted as ‘Resistant’, ‘Intermediate’ and ‘Sensitive’ by comparing with recorded diameters of a control organism, ATCC25923 (Annex 10).

3.7. Data management and statistical analysis

Microsoft excel spread sheet program was used to store all the data and Statistical Package for Social Sciences (SPSS, 2013) version 22.00 software was used to analyze the data. Prevalence of *Staphylococcus* and the respective *Staphylococcus* species in cottage cheese from houses, retailers and restaurants as well as yoghurt from households and cafeteria, were computed as the number of each food items positive for *Staphylococcus,* and its species divided by total number of the samples examined. Analysis of resistance test was by category agreement, where the zone diameters were divided into different categories (susceptible, intermediate and resistant). The percentages of antimicrobial resistance patterns were calculated as number of staphylococci and it's species resistant to the tested antibiogram devided by total isolated number of staphylococci or its species. The 95% confidence interval (CI) of a proportion was used to calculate the lower and upper limits of the proportion of *Staphylococcus* and *Staphylococcus* species in the samples examined. Chi-square (χ^2) was used to test the presence of association between variables and generally, descriptive statistics was used to summarize the data in tables. When P value was less than 0.05, the presence of significance difference was considered. Odds ratio was calculated to determine the degree of association between the types of sample and staphylococcal coccurrence.

4. RESULT

4.1. Prevalence and distribution of *Staphylococcus* in cottage cheese and yoghurt

The examination of 400 samples of cottage cheese and yoghurt originating from retailers, housholds, cafeteria and restaurant of selected *districts* in Jimma zone revealed 57 positive and 343 negative results with the overall prevalence of 14.3% (95%CI = 11-18%) of *Staphylococcus.* The identification results showed that the contamination of cottage cheese with *staphylococcus* was more likely to occur than yoghurt (OR = 4.1, 95%CI = 2.1-7.8). There was statistically significant difference (P<0.05) observed among the prevalence of isolates in cottage cheese and yoghurt (Table 2).

Table 2: Distribution of staphylococci in cottage cheese and yoghurt

Sample type	Examined samples	Positive samples	Prevalence (%)	χ^2(Df,P-value)	OR(95%CI)
Cottage cheese	200	44(22%)	11	19.66(1, 0.000)	4.1(2.1-7.8)
Yoghurt*	200	13(6.5%)	3.3		1
Total	**400**	**57(14.3%)**	**14.3**		

* = *Reference category*

4.2. *Staphylococcus* species isolates from cottage cheese and yoghurt samples

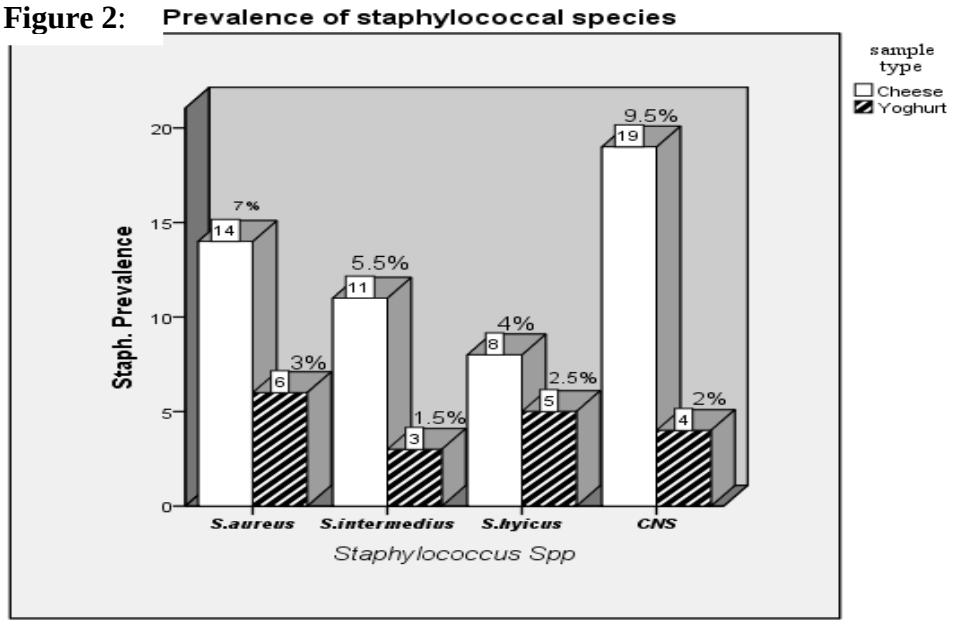
The study on fermented dairy products (cottage cheese and yoghurt) confirmed four species categories of staphylococci which were found to be 5% of *S. aureus*, 3.5% of *S. intermedius*, 3.3% of *S. hyicus* and 5.8% of CNS in both types of samples. In the confirmation, mixed contamination of the sample (presence of different species of *staphylococcus* in single sample) was also observed. Hence, the isolates number was found to be increased from 57 to 70. The identification results showed a predominance of CNS with a total of 23 isolates, comprising 32.9% (23/70) of the total isolates of *Staphylococcus* species followed by *S. aureus* (28.6%) (20/70), *S. intermedius* (20.0 %) (14/70) and *S. hyicus* (18.6%) (13/70) (Table 3).

Table 3: Distribution of each category of species of staphylococci in examined samples

Species identified	Examined samples	Positive samples	Prevalence (%)	95% CI
S. aureus	400	20	5.0	2.8-7.2
S. intermedius	400	14	3.5	2.0-5.5
S. hyicus	400	13	3.3	1.8-5.2
CNS	400	23	5.8	3.8-8.3
Total	**400**	**70**	**17.5**	**14.1-20.9**

The identification of *Staphylococcus* species by their biochemical characterization was resulted four species groups and which comprises: *S. aureus* (7% in cheese and 3% in yoghurt), *S. intermedius* (5.5% in cheese and 1.5% in yoghurt), *S. hyicus* (4% in cheese and 2.5% in yoghurt) and coagulase negative *Staphylococcus* species (CNS) (9.5% in cheese and 2% in yoghurt samples) as described in (Figure 2).

Figure 2:



4.3. Prevalence of *Staphylococcus* based on sources, *districts* and types of container

The overall prevalence of staphylococci in cottage cheese and yoghurt based on sources, *districts* and container types was 14.3%. The identification results showed that cottage cheese's (22%) of sample type, retailer's (26.5%) of sample sources, Sekoru *district*'s (22%) and False banana leaf's (*Inset* leaf) (30.7%) from types of container were the most contaminated with *Staphylococcus.* The results of prevalence showed statistically significant difference (P<0.05) among sources and types of container of samples. However, the prevalence difference between *districts* was not statistically significant (P>0.05) (Table 4).

Table 4: Prevalence of *Staphylococcus* based on sources, *districts* and types of containers

Variables		**Examined samples**	**Positive samples**	**Prevalence (%)**	**χ^2(Df,P-value)**
Source	Restaurant	64	12	18.8	18.01(3, 0.000)
	Household	168	23	13.7	
	Retailer	68	18	26.5	
	Cafeteria	100	4	4.0	
	Total	**400**	**57**	**14.3**	
District	Dedo	100	12	12.0	6.94(3, 0.074)
	Seka	100	10	10.0	
	Sekoru	100	22	22.0	
	Kersa	100	13	13.0	
	Total	**400**	**57**	**14.3**	
Types of Container	Plastic	168	17	10.1	31.6(5, 0.000)
	Inset leaf	88	27	30.7	
	Clay pot	48	9	18.8	
	Metal pan	16	2	12.5	
	Gourd (*kil*)	32	1	3.1	
	Aluminum	48	1	2.1	
	Total	**400**	**57**	**14.3**	

4.3.1. Distribution of coagulase positive staphylococci isolates

Out of the total examined samples of dairy fermented products (cottage cheese and yoghurt) three species of the staphylococci were categorized under coagulase positive staphylococci (CPS) based on coagulase test which comprises *S. aureus, S. intermedius* and *S. hyicus.* The proportional distribution of these categories of bacteria (CPS) in cottage cheese and yoghurt were found to be 16.5% and 7.0% respectively. The odds of recovery of CPS in cheese were more than yoghurt (OR= 2.6, 95%CI= 1.7-7.8). The odds ratio for sources and types of container in respect to organism recovery chance were not calculated due to the difference in sources and containers depending on sample type and size. The identification results also indicated that samples colleceted from restaurant (21.9%), Sekoru *district* (17%) and Clay pot (22.9%) from container type were the most contaminated with CPS. The difference in CPS prevalence among types, sources and types of container of samples indicated significance (P<0.05). However, the difference of prevalence among *districts* was not statistically significant (P>0.05) (Table 5).

Table 5: Distribution of CPS based on types of sample, source, *district* and types of container

Variables		Examined samples	Positive samples	Prevalence (%)	χ^2(Df,P-value)	OR(95%CI)
Types of sample	Cheese	200	33	16.5		2.6(1.7-7.8)
	Yoghurt*	200	14	7.0	8.7(1, 0.003)	1
	Total	**400**	**47**	**11.75**		
Source	Restaurant	64	14	21.9		
	Household	168	20	11.9		
	Retailer	68	10	14.7	15.1(3, 0.000)	
	Cafeteria	100	3	3.0		
	Total	**400**	**47**	**11.75**		
District	Dedo	100	12	12.0		
	Seka	100	8	8.0		
	Sekoru	100	17	17.0	3.7(3, 0.054)	
	Kersa	100	10	10.0		
	Total	**400**	**47**	**11.75**		
Types of Container	Plastic	168	15	8.9		
	I*nset* leaf	88	18	20.5		
	Clay pot	48	11	22.9		
	Metal pan	16	1	6.3	21(5, 0.001)	
	Gourd (*kil*)	32	2	6.3		
	Aluminum	48	0	0.0		
	Total	**400**	**47**	**11.75**		

Distribution of *S. aureus*

As the result shown in table 6, the total prevalence of staphylococci, *S. aureus*, in cottage cheese and yoghurt based on types, sources of sample, *district* and types of container was 5%. The prevalence of this species of staphylococci in cottage cheese and yoghurt were found to be 7% and 3% respectively. The identification results showed that cottage cheese's (7%) of sample type, retailer's (7.4%) of sample sources, Sekoru of *district*'s (8%) and false banana leaf's (*Inset* leaf) (10.2%) from types of containers were the most contaminated with *S. aureus*. Even though, there is variation between prevalence's of *S. aureus* among types, sources, *districts* and types of containers of samples, these differences were not statistically significant (P>0.05).

Table 6: Distribution of *S. aureus* based on sample types, sources, *district* and container

Variables		**Examined samples**	**Positive samples**	**Prevalence (%)**	**χ^2(Df,P-value)**
Types of sample	Cheese	200	14	7.0	3.37(1, 0.066)
	Yoghurt	200	6	3.0	
	Total	**400**	**20**	**5.0**	
	Restaurant	64	5	8.0	3.77(3, 0.28)
	Household	168	8	4.8	
	Retailer	68	5	7.4	
	Cafeteria	100	2	2.0	
	Total	**400**	**20**	**5.0**	
District	Dedo	100	5	5.0	2.95(3, 0.400)
	Seka	100	4	4.0	
	Sekoru	100	8	8.0	
	Kersa	100	3	3.0	
	Total	**400**	**20**	**5.0**	
Types of Container	Plastic	168	6	3.6	8.8(5, 0.12)
	Inset leaf	88	9	10.2	
	Clay pot	48	3	6.2	
	Metal pan	16	1	6.3	
	Gourd (*kil*)	32	1	3.1	
	Aluminum	48	0	0.0	
	Total	**400**	**20**	**5.0**	

Distribution of *S. intermedius*

The total prevalence of *S. intermedius* in cottage cheese and yoghurt was 3.5%. The proportional distribution of this species of staphylococci in cottage cheese and yoghurt were calculated to be 5.5% and 1.5% respectively. Based on prevalence of *S.intermedius*, cottage cheese (5.5%) of sample type, restaurants' (9.4%) of sample sources, Sekoru's of *districts* (6%) and false banana leaf's (*Inset* leaf) (8.0%) from types of container were the most contaminated with *S. intermedius*. The difference in prevalence of *S. intermedius* among types and sources of samples indicated significant difference (P<0.05). However, the difference of prevalence among *districts* and types of container were not statistically significant (P>0.05) (Table 7).

Table 7: Distribution of *S. intermedius* by sample types, sources, *district* and types of containers

Variables		**Examined samples**	**Positive samples**	**Prevalence (%)**	**χ^2(Df,P-value)**
Types of sample	Cheese	200	11	5.5	4.7 (1, 0.030)
	Yoghurt	200	3	1.5	
	Total	**400**	**14**	**3.5**	
Source	Restaurant	64	6	9.4	8.6(3, 0.035)
	Household	168	5	3.0	
	Retailer	68	2	2.9	
	Cafeteria	100	1	1.0	
	Total	**400**	**14**	**3.5**	
District	Dedo	100	3	3.0	3.9(3, 0.28)
	Seka	100	1	1.0	
	Sekoru	100	6	6.0	
	Kersa	100	4	4.0	
	Total	**400**	**14**	**3.5**	
Types of Container	Plastic	168	4	2.4	10.4(5, 0.07)
	Inset leaf	88	7	8.0	
	Clay pot	48	3	6.2	
	Metal pan	16	0	0.0	
	Gourd (*kil*)	32	0	0.0	
	Aluminum	48	0	0.0	
	Total	**400**	**14**	**3.5**	

Distribution of *S. hyicus*

The total prevalence of *S. hyicus* in cottage cheese and yoghurt was 3.3%. The identification results showed that cottage cheese's (4%) of sample type, restaurant's (4.7%) of sample sources, Dedo of *district*'s (4%) and Clay pot's (10.4%) from types of container were the most contaminated with *S. hyicus.* Even though, there is variation between prevalence's of *S. hyicus* among types, sources, *districts* and container types of samples, these differences were not statistically significant (P>0.05)(Table 8).

Table 8: Distribution of *S. hyicus* by types of sample, source, *district* and types of container

Variables		**Examined samples**	**Positive samples**	**Prevalence (%)**	**χ^2(Df,P-value)**
Types of sample	Cheese	200	8	4.0	0.72(1, 0.39)
	Yoghurt	200	5	2.5	
	Total	**400**	**13**	**3.3**	
Source	Restaurant	64	3	4.7	4.5(3, 0.21)
	Household	168	7	4.2	
	Retailer	68	3	4.4	
	Cafeteria	100	0	0.0	
	Total	**400**	**13**	**3.3**	
District	Dedo	100	4	4.0	0.24(3, 0.625)
	Seka	100	3	3.0	
	Sekoru	100	3	3.0	
	Kersa	100	3	3.0	
	Total	**400**	**13**	**3.3**	
Types of Container	Plastic	168	5	3.0	10.3(5, 0.067)
	Inset leaf	88	2	2.3	
	Clay pot	48	5	10.4	
	Metal pan	16	0	0.0	
	Gourd (*kil*)	32	1	3.1	
	Aluminum	48	0	0.0	
	Total	**400**	**13**	**3.3**	

4.3.2. Distribution of coagulase negative staphylococci

The total prevalence of coagulase negative staphylococci (CNS) in cottage cheese and yoghurt was 5.8%. The proportional distribution of this category of staphylococci in cottage cheese and yoghurt were found to be 9.5% and 2% respectively and based on source, *districts* and types of container were listed below in table 9. In this study, higher chance of CNS to be recovered from cottage cheese than yoghurt (OR= 5.1, 95%CI = 1.7-15.4). The odds ratio for sources and types of container in respect to organism recovery chance were not calculated due to the difference in sources and containers depending on sample type and size. The identification result also showed that retailer's (14.7%) of sample sources, Sekoru's (8%) of *district* and false banana leaf's (*Inset* leaf) (12.5%) from types of containers were found to be the most contaminated with CNS. Prevalence of CNS on types and sources of samples indicated significant difference (P<0.05) but among *districts* and types of container were not (Table 9).

Table 9: Distribution of CNS based on types of sample, source, *district* and types of container

Variables		Examined samples	Positive samples	Prevalence (%)	χ^2(Df,P-value)	OR(95%CI)
Types of sample	Cheese	200	19	9.5	10.4(1, 0.001)	5.1(1.7-15.4)
	Yoghurt	200	4	2.0		1
	Total	**400**	**23**	**5.8**		
Source	Restaurant	64	2	3.1	13.5(3, 0.004)	
	Household	168	9	5.4		
	Retailer	68	10	14.7		
	Cafeteria	100	2	2.0		
	Total	**400**	**23**	**5.8**		
District	Dedo	100	5	5.0	1.3(3, 0.74)	
	Seka	100	5	5.0		
	Sekoru	100	8	8.0		
	Kersa	100	5	5.0		
	Total	**400**	**23**	**5.8**		
Types of Container	Plastic	168	8	4.8	11.1(5, 0.05)	
	Inset leaf	88	11	12.5		
	Clay pot	48	2	4.2		
	Metal pan	16	1	6.2		
	Gourd(*kil*)	32	0	0.0		
	Aluminum	48	1	2.1		
	Total	**400**	**23**	**5.8**		

4.4. Distribution of mixed contamination

The contamination of single sample in two and above Staphylococcal species in cottage cheese and yoghurt was 3.3%. The proportional distribution of this mixed species of staphylococci in cottage cheese and yoghurt wre found to be 4% and 2.5% respectively. The identification results showed that cottage cheese's (4%) of sample type, restaurant's (6.2%) of sample sources, Dedo of *district*'s (5%) and clay pot's (8.3%) from containers were the most contaminated samples with mixed staphylococcal species. Even though, there is variation between prevalence's of mixed contamination among types, sources, *districts* and container types of samples', these differences were not statistically significant (P>0.05)(Table 10).

Table 2: Prevalence of mixed contamination by sample types, source, *districts* and container

Variables		Examined samples	Positive samples	Prevalence (%)	χ^2(Df,P-value)
Types of sample	Cheese	200	8	4.0	0.7(1, 0.558)
	Yoghurt	200	5	2.5	
	Total	**400**	**13**	**3.3**	
Source	Restaurant	64	4	6.2	3.7(3, 0.296)
	House	168	6	3.6	
	Retailer	68	2	2.9	
	Cafeteria	100	1	1.0	
	Total	**400**	**13**	**3.3**	
District	Dedo	100	5	5.0	0.69(3, 0.876)
	Seka	100	3	3.0	
	Sekoru	100	3	3.0	
	Kersa	100	2	2.0	
	Total	**400**	**13**	**3.3**	
Types of Container	Plastic	168	6	3.6	4.07(5, 0.54)
	Inset leaf	88	2	2.3	
	Clay pot	48	4	8.3	
	Metal pan	16	0	0.0	
	Gourd(*kil*)	32	1	3.1	
	Aluminum	48	0	0.0	
	Total	**400**	**13**	**3.3**	

4.5. Antimicrobial resistance pattern of *Staphylococcus*

4.5.1. Antimicrobial resistance pattern of staphylococci based on species category

The 70 staphylococci isolates obtained in study were analyzed for antimicrobial resistance. Of the 70 staphylococci isolates, 61(87.1%) showed resistance to one or more antimicrobials while 100% of *S. aureus*, 78.6% of *S. intermedius*, 84.6% *S. hyicus*, 82.6% CNS and 89.4% of CPS isolates were found to be resistant to one or more antimicrobials. The isolates showed highest resistance to Penicillin G (P^{10}) (46(65.7%)) followed by Tetracycline (TE^{30}) (29(41.4%)), Streptomycin (S^{10}) (26(37.1%)), Gentamycin (Gen^{10}) (24(35.7%)) and Kanamycin (K^{30}) (20(28.6%)). In species group antimicrobial test, out of 20 isolates of *S. aureus*, 19 was resistant to Penicillin G (P^{10}) and Tetracycline (TE^{30}) (90%) followed by Streptomycin (S^{10}) (65%), Gentamycin (Gen^{10}) (55%) and Kanamycin (K^{30}) (40%) than other species groups. The test analysis also showed *S. intermedius* (of 14 isolates) was highly resistant to Penicillin G (P^{10}) (64%) followed by Gentamycin (Gen^{10}) (36%), Kanamycin (K^{30}) (29%), Tetracycline (TE^{30}) and Streptomycin (S^{10}) (14%). Out of 13 isolates of *S. hyicus* resistance were found to be 54%, 54%, 46%, 31% and 23% to Penicillin G, Streptomycin, Gentamycin, Tetracycline and Kanamycin respectively. Similarly CNS (of 23 isolates) resistance to Penicillin G (52%) followed by Tetracycline and Kanamycin (22%), Gentamycin (17%) and Streptomycin (13%) was depicted. In group, the resistant of CPS species out of 47 isolates were 72% to Penicillin G, 51% to Tetracycline, 47% to Gentamycin, 47% to Streptomycin and 32% to Kanamycin **(**Table 11).

Table 3: Antimicrobial resistance pattern of staphylococci and its species

Species	Penicillin-G (P^{10})		Tetracycline (TE^{30})			Kanamycin (K^{30})			Gentamycin (Gen^{10})			Streptomycin (S^{10})			Resistance ≥ 1 drugs
	R	S	R	I	S	R	I	S	R	I	S	R	I	S	
S. aureus **(n=20)**	18 (90%)	2 (10%)	18 (90%)	2 (10%)	0 (0%)	8 (40%)	5 (25%)	7 (35%)	11 (55%)	1 (5%)	8 (40%)	13 (65%)	2 (10%)	5 (20%)	20 (100%)
S. intermed-ius (n=14)	9 (64%)	5 (36%)	2 (14%)	7 (50%)	5 (36%)	4 (29%)	3 (21%)	7 (50%)	5 (36%)	3 (21%)	6 (43%)	2 (14%)	7 (50%)	5 (36%)	11 (78.6%)
S. hyicus **(**n=13)	7 (54%)	6 (46%)	4 (31%)	3 (23%)	6 (46%)	3 (23%)	2 (15%)	8 (62%)	6 (46%)	2 (15%)	5 (39%)	7 (54%)	4 (31%)	2 (15%)	11 (84.6%)
CNS (n=23)	12 (52%)	11 (48%)	5 (22%)	7 (30%)	11 (48%)	5 (22%)	3 (13%)	15 (65%)	4 (17%)	6 (26%)	13 (57%)	3 (13%)	8 (35%)	12 (52%)	19 (82.6%)
CPS (n=47)	34 (72%)	13 (28%)	24 (51%)	12 (26%)	11 (23%)	15 (32%)	10 (21%)	22 (47%)	22 (47%)	6 (13%)	19 (40%)	22 (47%)	13 (28%)	12 (26%)	42 (89.4%)
Total (n=70)	46(65. 7%)	24(34. 3%)	29(41 .4%)	19(27. 1%)	22(31. 4%)	20(28. 6%)	13(18.6 %)	37(52.9 %)	26(37. 1%)	12(17. 1%)	32(45. 7%)	25(35. 7%)	21(30 %)	24(34. 3%)	61 (87.1%)
Mean	***0.657***	***0.343***	***0.414***	***0.271***	***0.314***	***0.286***	***0.186***	***0.529***	***0.371***	***0.171***	***0.457***	***0.357***	***0.30***	***0.343***	***0.871***
SD	***0.478***	***0.473***	***0.496***	***0.448***	***0.468***	***0.455***	***0.392***	***0.503***	***0.483***	***0.403***	***0.502***	***0.483***	***0.462***	***0.478***	***0.321***

Key: R = Resistant I = Intermediate S = Susceptible

4.5.2. Antimicrobial resistance pattern of staphylococci based on *districts*

Of the 17 staphylococci isolates from Dedo *district*, 16(94%) showed resistance to one or more antimicrobials while 70.6%, 52.9% and 29.4% showed resistance to Penicillin-G, Tetracycline and Kanamycin, and Gentamycin and streptomycin respectively. Out of the 13 staphylococci isolates from Seka *district*, 9(69%) showed resistance to one or more antimicrobials while 53.8%, 46.2%, 38.5% and 30.8% showed resistance to Penicillin-G, Tetracycline and streptomycin, Gentamycin and, Kanamycin respectively. The study detected 25 staphylococci isolates from Sekoru *district*, out of these staphylococcal isolates 23 (92%) showed resistance to one or more antimicrobials and 60%, 44%, 32% and 28%, showed resistance to Penicillin-G, Tetracycline, streptomycin, and Kanamycin and Gentamycin respectively. Similarly in Kersa *district*, 15 staphylococci was identified and of which 13(86%) showed resistance to one or more antimicrobials while 80% to Penicillin-G, 33% to Gentamycin, and streptomycin and 20% to Tetracycline and Kanamycin resistant (Table 12).

Table 4: Antimicrobial resistance pattern of staphylococci based on different *districts*

Districts	**Antimicrobials**										**Resistance**	
	Penicillin-G		**Tetracycline**		**Kanamycin**		**Gentamycin**		**streptomycin**		**≥ 1 drugs**	
	n	%	n	%	n	%	n	%	n	%	n	%
Dedo (n=17)	12	70.6	9	52.9	5	29.4	9	52.9	5	29.4	16	94
Mean(SD)	*0.17(0.381)*		*0.13(0.337)*		*0.07(0.259)*		*0.13(0.337)*		*0.07(0.259)*		*0.23(0.423)*	
Seka (n=13)	7	53.8	6	46.2	4	30.8	5	38.5	7	53.9	9	69
Mean(SD)	*0.1(0.302)*		*0.9(0.282)*		*0.06(0.234)*		*0.07(0.259)*		*0.10(0.302)*		*0.13(0.337)*	
Sekoru (n=25)	15	60	11	44	8	32	7	28	8	32	23	92
Mean(SD)	*0.21(0.413)*		*0.16(0.367)*		*0.11(0.321)*		*0.10(0.302)*		*0.11(0.321)*		*0.33(0.473)*	
Kersa (n=15)	12	80	3	20	3	20	5	33	5	33	13	86
Mean(SD)	*0.17(0.381)*		*0.04(0.204)*		*0.04(0.204)*		*0.07(0.234)*		*0.07(0.259)*		*0.19(0.392)*	
Total (n=70)	**46**	**65.7**	**29**	**41.4**	**20**	**28.6**	**26**	**37.1**	**25**	**35.7**	**61**	**87.1**

4.5.3. Antimicrobial resistance pattern of staphylococci based on species category and *districts* level

In this study all 20 (100%) *S. aureus* isolates were showed resistance to one or more antimicrobials in all four *districts*. Of *S. intermedius* isolates from Seka and Kersa, 100% and 66.7% from Dedo and Sekoru *districts* were resistant to one or more antimicrobials. In contrast, of *S. hyicus* isolates, 100% from Dedo, and Sekoru and 66.7% from Seka and Kersa were resistant to one or more antimicrobials. The attempt to test the antimicrobial resistance pattern between CNS isolates was showed that 100% of Dedo and Sekoru *districts* and also 80% and 40% of Kersa and Seka *districts* respectively were resulted resistance to one or more tested antimicrobials. Cumulatively, from CPS isolates, 91.7%, 87.5%, 88.2%, and 90% of Dedo, Seka Sekoru and Kersa revealed resistance to one or more tested antibiograms respectively. The Prevalence of antimicrobial resistance pattern of staphylococcal species based on different *districts* is briefly described in Table13.

Table 5: Antimicrobial resistance pattern of staphylococcal species based on different *districts*

Staphylococci	***District* (*districts*)**	**Antimicrobials**										**Resistance ≥ 1 drugs**	
		Penicillin-G		**Tetracycline**		**Kanamycin**		**Gentamycin**		**Streptomycin**			
	(n)	n	%	n	%	n	%	n	%	n	%	n	%
	Dedo (5)	4	80	5	100	2	40	3	60	1	20	5	100
S.aureus	Seka (4)	4	100	4	100	1	25	2	50	3	75	4	100
(n=20)	Sekoru (8)	7	87.5	7	87.5	3	37.5	4	50	6	75	8	100
	Kersa (3)	3	100	2	66.7	2	66.7	2	66.7	3	100	3	100
	Total (20)	**18**	**90**	**18**	**90**	**8**	**40**	**11**	**55**	**13**	**65**	**20**	**100**
	Mean(SD)	*0.26(0.44)*		*0.26(0.44)*		*0.11(0.32)*		*0.16(0.37)*		*0.19(0.39)*		*0.29(0.46)*	
	Dedo (3)	2	66.7	1	33	1	33	2	66.7	1	33	2	66.7
S.intermedius	Seka (1)	1	100	1	100	1	100	1	100	1	100	1	100
	Sekoru (6)	3	50	0	0	1	16.7	1	16.7	0	0	4	66.7
(n=14)	Kersa (4)	3	75	0	0	1	25	1	25	0	0	4	100
	Total (14)	**9**	**64.3**	**2**	**14.3**	**4**	**28.6**	**5**	**35.7**	**2**	**14.3**	**11**	**78.6**
	Mean(SD)	*0.13(0.34)*		*0.03(0.17)*		*0.06(0.23)*		*0.07(0.26)*		*0.03(0.17)*		*0.16(0.37)*	
	Dedo(4)	3	75	2	50	1	25	2	50	2	50	4	100
	Seka(3)	1	33	1	33	1	33	2	66.7	1	33	2	66.7
S. hyicus	Sekoru (3)	1	33	1	33	1	33	1	33	2	66.7	3	100
(n=13)	Kersa (3)	2	66.7	0	0	0	0	1	33	2	66.7	2	66.7
	Total (13)	**7**	**53.8**	**4**	**30.8**	**3**	**23**	**6**	**46.2**	**7**	**53.8**	**11**	**84.6**
	Mean(SD)	*0.10(0.30)*		*0.06(0.23)*		*0.04(0.20)*		*0.09(0.28)*		*0.10(0.30)*		*0.16(0.37)*	
	Dedo(5)	3	60	1	20	1	20	2	40	2	40	5	100
	Seka(5)	1	20	0	0	1	20	0	0	1	20	2	40
CNS	Sekoru (8)	4	50	3	37.5	3	37.5	1	12.5	0	0	8	100
(n= 23)	Kersa (5)	4	80	1	20	0	0	1	20	0	0	4	80
	Total (23)	**12**	**52.2**	**5**	**21.7**	**5**	**21.7**	**4**	**17.4**	**3**	**13**	**19**	**82.6**
	Mean(SD)	*0.17(0.38)*		*0.07(0.26)*		*0.07(0.26)*		*0.06(0.23)*		*0.04(0.20)*		*0.27(0.45)*	
	Dedo(12)	9	75	8	66.7	4	33	7	58	4	25	11	91.7
CPS	Seka(8)	6	75	6	75	3	37.5	5	62.5	5	62.5	7	87.5
(n=47)	Sekoru(17)	11	64.7	8	47	5	29.4	6	35.3	8	47	15	88.2
	Kersa (10)	8	80	2	20	3	30	4	40	5	50	9	90
	Total (47)	**34**	**72**	**24**	**51**	**15**	**31.9**	**22**	**46.8**	**22**	**46.8**	**42**	**89.4**
	Mean(SD)	*0.49(0.50)*		*0.34(0.48)*		*0.21(0.41)*		*0.31(0.47)*		*0.31(0.47)*		*0.60(0.49)*	

4.5.4. Multi-drug resistance pattern

Out of 70 *Staphylococcus* isolates, 39 (55.7%) found to be multi-drug resistant or showed resistance to two or above antimicrobials which is alarming level of resistance of *Staphylococcus* to commonly used antimicrobial agents in the study area. Higher proportion of the isolates (61/87.1%) was exhibited resistance to one or more than one antimicrobials (Table 14).

Table 14: Percentage and frequency of Multi-drug resistance pattern of staphylococci for tested antimicrobials

No. of antimicrobial agents	**No. of staphylococci (n= 70)**	**Precent (%)**
No resistance (0)	9	12.9
One	22	31.4
One and above	61	87.1
Two	15	21.4
Two and above	39	55.7
Three and above	24	34.3
All	4	5.7

5. DISCUSSION

The safety of dairy products with respect to food-borne diseases is a great concern around the world. This is especially true in developing countries where production of milk and various dairy products take place under unsanitary conditions and poor production practices (Ashenafi, 1990; Yilma and Faye, 2006). This fact is also true in Ethiopia where the smallholder farmers produce milk and fermented milk products by traditional methods (Ashenafi, 2006). *Staphylococcus* species are prevalent food-borne bacterial pathogens that cause food poisoning in humans when ingested in contaminated foods, including dairy products like cheese and yoghurt. They cause SFP by toxin production (Salandra *et al*., 2008). *Staphylococcus* species can indeed be easily eliminated from foods by heat treatment (in pasteurized foods) or by competition with other flora (in fermented foods), whereas SEs resist most of the treatments used during food processing. Hence, the surveillance of food for microbial contamination is vital for the protection of public health and consumer interests. Production of safe food also has important economic implications in an increasingly competitive global market (Mekonnen *et al.*, 2011b).

The overall prevalence of *Staphylococcus* in this study was found to be 14.3% (95% CI=11-18%) in cottage cheese and yoghurt with a specific prevalence of 22% in cottage cheese and 6.5% in yoghurt samples collected and examined from selected *districts* of Jimma zone. The high prevalence of staphylococci in cottage cheese and yoghurt in this study could be explained by the fact that preparation procedures for most products are still traditional arts, the fermentation is uncontrolled and starter cultures are not normally used. Traditional handmade dairy products may be produced from raw milk heated for only a few minutes to temperatures that are not enough to kill many pathogenic bacteria (Zouhairi *et al*., 2010). In Ethiopia the smallholder farmers produce fermented milk by traditional methods (Ashenafi, 2006). Also, the method of their sale is entirely based on tradition which may expose the products to the organism repeatedly and the organism get chance to survive the unfavorable conditions. Staphylococci are halophilic bacteria and are able to survive in conditions of very low moisture content (Zouhairi *et al*., 2010). Zouhairi *et al*. (2010) again indicated in their studies on fermented dairy products that locally made fermented dairy-based foods act as vehicles for the transmission of antimicrobial-resistant *Staphylococcus* species.

The overall prevalence of *Staphylococcus* species in cottage cheese and yoghurt in this study (14.3%) was found to be in-line with the previous studies who reported prevalence of 15% in dairy products (Martin *et al.*, 2004), 10% (Sasidharan *et al*., 2011) in yoghurt and cheese, 6.7% of Thaker *et al*. (2013) in yoghurt and cheese, 22.6% of Zouhairi *et al*. (2010) in dairy products, 17.4% by Tambekar and Bhutda (2010) in dairy products, 7.3% by Fagundes *et al.* (2010) in dairy products, 6.6% by Kumar and Prasad (2010) in dairy products, 13% *S. aureus* in yoghurt and cheese of EI-Jakee *et al*. (2013) and was found to be lower than the 50% prevalence of staphylococci species in yoghurt and cheese by EI-Jakee *et al*. (2013), 62.9% by Bautista *et al.* (1988) in dairy products, 28.6% by Morandi *et al*. (2007) in dairy products and 40% by Zakary *et al.* (2011) in dairy products from different areas. The difference in result might be due to the differences in geographical location of the study area, sample size, education status of the local people and availability of the technology to collection, preparation, storage etc and preparation methods of the dairy products.

Coagulase production was described as one of the most reliable criteria for the identification of pathogenic *Staphylococcus* species. Staphylococci producing coagulase are usually pathogenic (Quinn *et al*., 2002; Lamprell *et al*., 2004; Morrison, 2008). The results in this study showed that coagulase-positive staphylococci (CPS) species were more frequently occurred in cottage cheese and yoghurt (11.8%) than coagulase negative staphylococcal species (5.8%). This study showed that CPS were a common cause of contamination of cottage cheese and yogurt in study area. This contamination of cottage cheese and yoghurt by CPS may be attributed to the poor conditions of hygiene during milking and lack of hygienic measures in the preparation, handling, selling, and storage of cottage cheese and yoghurt. The prevalence of CPS in this study (11.8%) was known to be in agreement with the previous studies who reported prevalence of CPS to be 15.9% in Lebanon by Zouhairi *et al*. (2010) in dairy products, 13.3% of *S. intermedius* in France by Lamprell *et al.* (2004) and 14.5% in Debrezeit, Ethiopia by Mekonnen *et al.* (2011a). The prevalence of CPS in this study was comfirmed to be lower than that reported as 47.75% of CPS by Lamprell *et al.* (2004) in France and 59.3% of CPS by Bendahou *et al.* (2008) in northern Morocco. The lower prevalence of CPS in our study could be explained by the different techniques used in those studies, acidic nature of the sample types, differences in the origin of the samples or by geographical area differences.

The prevalence of CNS in cottage cheese and yoghurt (5.8%) was in line with the prevalence of CNS reported by Lamprell *et al.* (2004), Zouhairi *et al*. (2010) and Mekonnen *et al.* (2011a) who reported the prevalence of CNS to be 4.5% in France, 6.7% in Lebanon and 9.5% in Debre ziet, Ethiopia respectively in cottage cheese and yoghurt samples. The prevalence of CNS in our study was found to be lower than 54% prevalence of CNS in milk products confirmed by Bendahou *et al.* (2008) in north Morocco. The lower prevalence of CNS of our study might be explained by the different techniques used in those studies, acidic nature of the sample types, differences in the origin of the samples or by geographical area differences.

The prevalence of *Staphylococcus* species in cottage cheese in this study was confirmed to be 22%, of which 7%, 5.5%, 4% and 9.5% were *S. aureus, S. intermedius, S. hyicus* and CNS respectively. Odds of recovery of *Staphylococcus* was found to be more in cheese than yoghurt (OR = 4.1, 95%CI = 2.1-7.8). This could be explained as the high level of acid (low pH) in yoghurt than cheese and difference in storage containers. The prevalence of staphylococci in cottage cheese in our study was found to be in line with the finding of Mekonnen *et al.* (2011a) who reported the prevalence of *Staphylococcus* species to be 24% in cottage cheese samples in Debre Zeit, Ethiopia. Similarly, the prevalence of *S. aureus* in the current study (7%) was found to be in agreement with the findings of Mekonnen *et al.* (2011a), EI-Jakee *et al*. (2013), Thaker *et al.* (2013) and Zinke *et al.* (2012) who found the prevalence of *S. aureus* to be, 5% in Debre zeit, Ethiopia, 4% in Egypt, 10% in Gujarat, India and 5.5% in Germen respectvelly. The prevalence of *S. intermedius* in cottage cheese in this study (5.5%) was found to be in-line with the findings of Mekonnen *et al.* (2011a), Lamprell *et al.* (2004) and Bendahou *et al.* (2008) who reported the prevalence of *S. intermedius* to be 5.5% in Ethiopia, 13.3% in France and 3.7% in north Morocco in cheese samples.

Contamination of cheese by *S. hyicus* in this study (4%) was found to be similar with the report of 4% in Debrezeit, Ethiopia by Mekonnen *et al.* (2011a) and 3.7% in Morocco by Bendahou *et al.* (2008) from cottage cheese samples. Similarly the prevalence of CNS in our study (9.5%) was in-line with those findings reported as 9.5%, by Mekonnen *et al.* (2011a) in Debrezeit, Ethiopia, 6.7% by Zouhairi *et al*. (2010) in Lebanon and 4.5% by Lamprell *et al.* (2004) in France. The prevalence of *S. aureus* in cheese (7%) in this study was found to be lower than

28.6% by Morandi *et al.* (2007), 41.7% by Kav *et al.* (2011), 51.9% by Bendahou *et al.* (2008), 82.2% by Lamprell *et al.* (2004), 92.3% by Singh and Prakash (2008), 42.2% by Zouhairi *et al*. (2010) prevalence of *S. aureus* reports in different study areas. The prevalence of CPS in cheese (14.5%) in this study was lower than 38% CPS in cheese collected from West Darfur State by Hamid and Owni (2007). Additionally, 22% overall prevalence of *Staphylococcus* species in cheese found to be lower than 48.9% of all *Staphylococcus* species in cheese reported by Zouhairi *et al*. (2010) in Libanon. These lower contamination prevalence findings of cheese by different staphylococcal species in this study could be due to the difference in sample size, and study areas, hygienic measures of preparation, handling, nature, sources and storage of cheese in different area and local people.

The findings of current study in yogurt sample revealed a lower prevalence of staphylococci (6.5%) than cheese sample (22%). The reason for this variation could be the higher acidic property of yoghurt which is supported by the concept of Ashenafi (2006), "the effect of lower pH of yoghurt (*Ergo)* in controlling the proliferation of undesirable microorganisms is more effective after 24 hours of incubation". The 6.5% prevalence of yoghurt (*Ergo)* contamination with *Staphylococcus* species in this study was in agreement with the 3.3% prevalence of *Staphylococcus* by Thaker *et al.* (2013) in Gujarat, India, however, it was proved to be lower than 22% of *S. aureus* and 50% of all species of *Staphylococcus* prevalences reported by EI-Jakee *et al*. (2013) in Egypt and 76.9% by Rashed *et al.* (2014) in Bangladish. The differences in finding could be explained by the different levels of acidity of the yoghurt, different sources and size of the sample, types of sample and types of container, difference in hygienic measures of preparation, handling, storage and different starter cultures used to initiate the fermentation processes.

The highest prevalence of staphylococci was observed in the samples taken from retailers (26.5%) and the lowest was from samples taken from cafeteria (4%). Similarly the highest prevalence of staphylococci was recoreded on those samples which was handled by *inset* leaf (30.7%) whereas the lowest was recored in those samples which were kept by aluminium containers (2.1%). Statistically significant difference ($P<0.05$) in the prevalence of *Staphylococcus* between source of samples and container types used to handle cheese and

yoghurt were observed which might be attributed to the different acidic condition and nature of cheese and yoghurt, survival differences of staphylococci and different hygienic measures in different containers as well as sources (Gadaga *et al.,* 2004; Ashenafi, 2002; Ashenafi, 2006). The differences in the prevalence of staphylococci could be due to difference in containers used for fermentation, quality of the water used for washing, ingredients added with the intention of improving the flavor of the product, cooking temperatures that may not be enough to kill spoilage as well as pathogenic microorganisms, materials used for "*Ayib"* making, other utensils such as that used for draining the whey or ladling out the "*Ayib"*, and filtration, keeping temperature and personal hygiene of people handling the product (Soomro *et al.,* 2002; Yilma, 2012). However, there was no statistically significant difference (P>0.05) in prevalence was observed between *districts* of the sample origin. This could be justified by the fact that ecological similarity in *districts* has same effect on bacterial survivality (Kurmann, 1994; Tamime and Robinson, 1988).

The antimicrobial resistance patterns determination of *Staphylococcus* isolated from cottage cheese and yoghurt in our study showed that 87.1% of the organisms were resistant to one or more tested antimicrobials which were similar with that reported as 89% in Dessie, Ethiopia by Agumas *et al.* (2014), 88% in Addis Ababa by Tewodros and Gedebou (1984) and 97.8% by Zouhairi *et al*. (2010) in Libanon. The antimicrobial resistance in this study was higher than 50% resistance report by Duran *et al.* (2012) in India. The resistance level of all *Staphylococcus* species identified in this study to Penicillin-G, Tetracycline, Kanamycin, Gentamycin and Streptomycin were known to be 65.7%, 41%, 28.6%, 35.7%, 34.3% respectively. The resistance to Gentamycin (35.7%), Tetracycline (41.4%) in this study was in-line with the finding of Duran *et al.* (2012) who justified resistance as staphylococci to be 31.5% for Gentamycin and 35.6% for Tetracycline in India, 51.5% for Tetracycline in Jimma by Mama *et al.* (2014), and this result was tends to be lower than that of 97% resistance reported by Dorgham *et al.* (2013) from Egypt and 86.8% of penicillin G in Jimma by Mama *et al.* (2014). The staphylococcal resistance pattern to Gentamycine (35.7%) in this study was higher than 8.8% report of Mama *et al*. (2014) in Jimma. The difference in the resistance pattern of staphylococci could be explained by the fact in the difference of study area and empirical use of broad-spectrum antibacterials, size of inoculums (turbidity), the test mediums, incubation conditions and the bacterium dgree of resistance.

In this study, all (100%) *S. aureus* isolates were resistant to one or more tested antimicrobials and of which Penicillin G and Tetracycline resistance (90%) was registered as highest resistance than other drugs. This result (90% of Penicillin G) was in agreement with previous studies by Tariku *et al.* (2011), Kejela and Bacha (2013), Zerfie *et al*. (2014), Agumas *et al.* (2014), Mama *et al.* (2014), Abebe *et al.* (2013), Shittu *et al.* (2011), Duran *et al.* (2010), Thaker *et al*. (2013), Gebre-Sealassie (2007) and Rashed *et al.* (2014) who reported the resitance of staphylococci to Penicillin G to be 87.2% in Jimma town, 100% in Jimma town, 90% in Dessie, 100% in Dessie, 91.5% in Jimma, 92.2% around Addis Ababa, 88% in Nigeria, 92.8% in India, 100% in India, 90% in Jimma, Ethiopia and 87% in Bangladish respectively. However, 90% resistance to Pencillin G was proved to be greater than 21.9% in Denmark by Aaestrup *et al.* (1998), 43.9% in Brazil by Lange *et al.* (1999), 14.3% in Schweizland by Stephan *et al.* (1999) reports. Similarly the resistance to Tetracycline (90%) was higher than 5.3% finding in Jimma town by Kejela and Bacha (2013), 37.1% in Dessie by Zerfie *et al.* (2014), 51% in Jimma by Mama *et al.* (2014), 73.5% in Dessie by Agumas *et al.* (2014), 66.7% around Addis Ababa by Abebe *et al.* (2013), 55.9% in Nigeria by Shittu *et al.* (2011), 30.8% in India by Duran *et al.* (2012), 30% in India by Thaker *et al*. (2013) and 32.3% in Central Ethiopia by Sophia (2011), however in agreement with 100% resistance to tetracycline in Jimma by Getnet and Wondwosen (2011). The resistance of *S. aureus* to kanamycin in this study (40%) was found to be similar to that reported to be 37.9% by Zerfie *et al.* (2014) in Dessie and 45% by Rashed *et al.* (2014) in Bangladish whereas it was higher than 12.1% resistance report by Lange *et al.* (1999) in Brazil and 4.7% by Kejela and Bacha (2013) in Jimma town. Gentamycin resistance of *S. aureus* identified as 55% in this study was in-line with the 58.8% in Dessie by Agumas *et al.* (2014) but was higher than 27.8% finding by Kejela and Bacha (2013) in Jimma town, 7.1% by Zerfie *et al.* (2014) in Dessie, 4% by Mama *et al.* (2014) in Jimma, 10% by Thaker *et al*. (2013) in India, 17.7% by Shittu *et al.* (2011) in Nigeria and 12% by Rashed *et al.* (2014) in Bangladish. The *S. aureus* resistance to streptomycin (65%) was higher than 12.1% by Lange *et al.* (1999) and 4.1% by Stephan *et al.* (1999) findings in Brazil and Schweizland respectively. The difference in these finging reports might be due to different uncontrolled application culture of antimicrobial agents of various classes for therapeutic intervention of diseases in animals, and for prophylactic measures, size of inoculums of the tests, antimicrobial concentration in the disc, study area period differences.

The antimicrobial resistance test revealed a high resistance of *S. intermedius* (76.6%) to one or more antimicrobials and of which resistance to Penicillin G (64.3%) was the highest record than other drugs (Gentamycin (35.7%), Kanamycin (28.6%), Tetracycline (14.3%) and Streptomycin (14.3%)). The 64.3% resistance of *S. intermedius* to Penicillin G in this study was in-line with the 60% resistance to Penicillin G reported by Pedersen and Wegener (1995), and lower than 82.7% in USA reported by Medleau *et al*. (1986) and 80.2% in UK by Noble and Kent (1992). Pedersen and Wegener (1995) were reported 20% Tetracycline resistance in Denmark which coincided with 14.3% of this study but our result was lower than 52.8% in USA by Medleau *et al*. (1986) and 52.1% in UK by Noble and Kent (1992) and 30% in German by Greene and Schwarz (1992) reports. Resistance to Gentamycin (35.7%) in this study was higher than that of 0.9% in German by Greene and Schwarz (1992) and 0% in USA by Medleau *et al*. (1986). These reisistance result variation could be explained due to the difference in study period, geographical location of the area and culture of antimicrobials use. Streptomycin resistance of *S. intermedius* (14.3%) in this investigation was incommon with the study results of 6.3% in UK reported by Noble and Kent (1992) and 23.3% in German by Greene and Schwarz (1992).

84.6% of *S. hyicus* was resistant to one or more antimicrobials and of which resistance to Penicillin-G, Tetracycline, Kanamycin, Gentamycin and Streptomycin found to be 53.8%, 30.8%, 23%, 46%, and 46.2% respectively. These results were found to be in-line with those studies reported as 44%, 15%, and 56% resistance to Penicillin-G, Kanamycin and Streptomycin by Wegener and Schwarz (1993) in German respectively. The *S. hyicus* resistance to Tetracycline (40.5%), to Streptomycin (51.4%) by Noble and Allaker (1992) in UK and 42.8% to Streptomycin by Schwarz and Blobel (1989) in Denmark were known to be similar with our finding. In contrast, 32.4% to Penicillin-G by Noble and Allaker, 47% to Tetracycline by Wegener and Schwarz (1993) in German, 0% to Gentamycin by Schwarz and Blobel (1989) in Denmark, Noble and Allaker (1992) in UK and Wegener and Schwarz (1993) in German were the contradicting records. These result variations could be explained due to the difference in study period, geographical location of the area, due to indiscriminated use of antimicrobials as empirical treatment, test bacterium strain difference and different methods used to test antimicrobials.

Coagulase-negative staphylococci isolate in this study were more resistant to penicillin G (52.2%) than other tested antimicrobials (Tetracycline (21.7%), Kanamycin (21.7%), Gentamycin (17.4%) and Streptomycin (13%)). The penicillin G (52.2%) resistance finding was in-line with 57.6% by Lilenbaum *et al.* (1999) in USA, hawever, was different from 76% by Mama *et al.* (2014) in Jimma and 37.8% over all resistance to penicillin G in Morocco by Bendahou *et al.* (2008) report. Resistance of CNS to Tetracycline (21.7%) was tends to be lower than 52% by Mama *et al.* (2014) in Jimma. The overall resistance of CNS in this study was 82.6%. This result was in support with the idea of Werckenthin *et al.* (2001) which was CNS are an increasing problem in nosocomial infections and this may be due to the increase of multiresistant CNS isolates. The finding was also in-line with 10% resistant to gentamycin by Bendahou *et al.* (2008) in Morocco, Duran *et al.* (2012) findings of 25.8% to Gentamycin in India, 30.8% to Tetracycline and 57.6% to penicillin G reported by Lilenbaum *et al.* (1998) in USA.

6. CONCLUSION AND RECOMMENDATIONS

In this study the occurrence of staphylococci in cottage cheese (*Ayib*) and yoghurt (*Ergo*) was found to be very high in selected *district*s of Jimma zone, Ethiopia. The antimicrobial susebtbilty test of the isolates in this study revealed that there was high level antimicrobial resistance pattern. The occurrence of antimicrobial resistant staphylococci in cottage cheese and yoghurt in the study warrant public health concern since consumption of these products could lead to a severe SFP as low contamination levels with *Staphylococcus* organisms that favour growth and multiplication could induce SFP. The occurrence of multidrug resistant pathogenic staphylococci in cottege cheese and yoghurt among selected *district*s of Jimma zone indicates the need to avoid the indiscriminate usage of antimicrobials for the treatment of animals and implement strict hygienic measures in the manufacturing, handling, storage and selling of cottage cheese and yoghurt to improve the quality of these highly popular products and minimize or eliminate the risk of staphylococcal food poisoning and health hazard in the study area. Based on the above conclusion the following recommendations are forwarded:

- Sanitary measures and awareness creations must be given to the community to improve the hygienic conditions during milking, preparation, selling and storing of cheese and yoghurt.
- The hygienic handling and processing of cottage cheese and yoghurt among food handlers, judicious use of antimicrobials and screening programs are needed to ensure the microbiological quality as well as the consumer safety.
- Considerable research effort is still required for better understanding of the interactions between *Staphylococcus* and dairy products, and of the drug resistance pattern of the bacteria in the studied area.
- Rapid and reliable methods of genotyping the *Staphylococcus* for antimicrobial reistance and its toxin amount determination are needed to find out the appropriate resistance pattern and therapy decisions.

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8. ANNEXES

Annex 1: Procedure of the ISO 6888-3 protocol

1. Isolation and identification of *Staphylococcus.*
2. Add twenty-five grams (25ml) samples in a stomacher bag containing 225ml of buffered peptone water.
3. Homogenize the mixture using a laboratory blender and incubate aerobically at 37^0C for 24 hours.
4. Strike aseptically a loopful of the cultures of the various dilutions onto sterile BAP and incubate at 37^0C for 24-48 hours under aerobic culture conditions.
5. Colonies will be 0.5 to 1.5 μm in diameter, grey or grey-white to golden-yellow.
6. Gram-positive spherical bacteria that occur in microscopic clusters resembling grapes.
7. Biochemical tests for confirmation fo *Staphylococcus*:
   - ❖ Catalase test 3% H_2O_2 (positive)
   - ❖ Inoculate colonies on MSA and incubate at 37^0C 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 (*S. intermedius*) and non fermentative (*S. hyicus* and CNS).
   - ❖ Coagulase test to identify the most pathogenic CPS (*S. aureus, S. intermedius* and *S. hyicus*) from CNS.
   - ❖ Inoculate CPS isolates on PAB media plate with 1% of maltose and incubate at 37^0C for 24-48 hours to differentiate the pathogenic staphylococci, particularly the coagulase-positive isolate. The identification is 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. hyicus* do not ferment maltose.

Annex 2: Sample collection sheet for bacteriological analysis

Serial number	Date of collection	*District*	Type of sample	Source of sample	Container type	Sample code	Remark

Annex 3: Procedure for catalase test

1. Place a drop of 3% H_2O_2 on a glass slide.
2. Touch a sterile loop to a culture of the organism to be tested and pick up a visible mass of cells (colony).
3. Mix the organism in the drop of hydrogen peroxide.
4. Observe for immediate and vigorous bubbling.

Interpretation: Bubbling indicates a positive test and no bubbling indicates a negative test.

Annex 4: Procedure for coagulase test

1. Using a sterile pipette, add 0.5ml of the rehydrated plasma to a 12x75mm test tube.
2. Using a sterile serological pipette, add 0.5ml of the overnight broth culture of the test organism to the tube of plasma or, using a sterile bacteriological loop, thoroughly emulsify 2-4 colonies (one loopful) from a noninhibitory agar plate in the tube of plasma.
3. Mix gently and Incubate at 37^0C
4. Examine periodically for coagulation by gently tipping the tube after the first hour and once every hour thereafter until four hours have elapsed. If no clot is observed at the end of this period, examine at 24 hours. 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: - any degree of clotting - from a loose clot suspended in plasma to a solid clot that is immovable when the tube is inverted. Negative - no degree of clotting

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

Serial number	Type of sample	Sample code	Colony charac-teristics on BAP	Haemolysis	Gram stain	Catalase test	Coagulase reaction	Growth on MSA	Mannitol fermentation(MSA	Maltose fermentation (PAB)	*Staphylococcus*

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

Serial number	***Staphylococcus* species**	**Haemolysis**	**Pigment production**	**Coagulase test**	**Fermentation of sugar**	
					MSA	PAB
1	*S. aureus*	+	+	+	+	+
2	*S. intermedius*	+	_	+	±	±
3	*S. hyicus*	_	_	+	_	_
4	CNS	_	_	_	_	_

Source: Quinn *et al*. (1999)

Key: + = 90% or more strains are positive, ± = 90% or more strains are weakly positive, - = 90% or more strains are negative.

Annex 7: Record sheet for identification of coagulase positive and negative staphylococci

Serial No.	Date of collection	Type of sample	Source	Sample code	CPS	CNS

Annex 8: Record sheet for drug resistance pattern of staphylococci

Sample code	Species of the bacteria	Types of antimicrobials				Remark

Annex 9: Procedure for antimicrobial sensitivity test (disc diffusion testing method) (CLIS, 2007).

1. Select colonies
2. Prepare inoculum suspension

3. Standardize inoculum suspension
4. Inoculate plate
5. Add antimicrobial discs
6. Incubate plate
7. Measure inhibition zones
8. Interpret results

Annex 10: Zone diameter interpretive standards for *Staphylococcus* species

Antimicrobial agents	**Disc contents**	**Zone Diameter, nearest whole mm**		
		Resistant	Intermediate	Susceptible
Penicillin	10 units	≤ 28	-	≥ 29
Gentamicin	10 µg	≤ 12	13-14	≥ 15
Kanamycin	30 µg	≤ 13	14-17	≥ 18
Tetracycline	30 µg	≤ 14	15-18	≥ 19
Streptomycin	10 µg	≤ 23	24-27	≥28

Source: CLIS, 2007 and Rosco, 1994

Annex 11: Bacteriological media preparation used for the study

- **Buffered peptone water (HiMedia, India)**

Composition of medium (g/l):

Peptic digest of animal tissue...…….…….10.0

Sodium chloride…….…..5.0

Final pH 7.2 ± 0.2 at 25^0C

Instructions for use:

Dissolve 15.0g in 1 liter of distilled water. Stirr and dissolve completely. Sterilize by autoclaving at 121^0C for 15 minutes. Cool to room temperature before use.

- **Blood agar (Sisco, India)**

Composition of medium (g/l):

Pancreatic digest of casein …….…….…..….13.0

Yeast ……...5.0

Sodium chloride…….....5.0

Agar……...15.0

Heart muscle solid structure infusion …….…....2.0

Final pH 7.3 $\pm$ 0.2 at 25^0C

Instructions for use:

Suspend 40g in 1 litre of demineralized (distilled) water. Bring volume to 950ml and mix thoroughly. Gently heat and bring to boiling to dissolve completely. Sterilize by autoclaving at 121^0C for 15 minutes. Cool to 45-50^0C and add 50ml of sterile defibrinated sheep blood and mix thoroughly.

- **Nutrient agar (Sisco, India)**

Composition of medium (g/l):

Beef extract……...1.0

Yeast extract ……..2.0

Peptone……...5.0

Sodium chloride ……....5.0

Agar ……15.0

Final pH 7.4 $\pm$ 0.2 at 25^0C

Instructions for use:

Suspend 28g in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121^0C for 15 minutes.

- **Mannitol salt agar (HiMedia, India)**

Composition of medium (g/l):

Pancreatic digest of casein ……....5.0

Peptic digest of animal tissue ……5.0

Beef extract ……..…..1.0

D-Mannitol……...10.0

Sodium chloride …….…..…....75.0

Phenol Red …….……0.025

Agar…….…….15.0

Final pH 7.4 $\pm$ 0.2 at 25^0C

Instructions for use:

Suspend111g in 1 litre distilled water and bring to the boil to dissolve completely. Sterilize by autoclaving at 121^0C for15 minutes. Mix well before pouring in to sterile Petri dishes.

- **Purple agar base (Difco, France)**

Composition of medium (g/l):

Proteose peptone …….……..10.0

Beef extract……... ……1.0

Sodium chloride……..5.0

Brom-cresol Purple……0.02

Agar……..15.0

Final pH 6.8 $\pm$ 0.2 at 25^0C

Instructions for use:

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

- **Tryptone soya broth (HiMedia, India)**

Composition of medium (g/l):

Pancreatic digest of casein……17.0

Papaic digest of soybean meal……3.0

Sodium chloride……..5.0

Di- potassium hydrogen phosphate…….2.5

Dextrose (Glucose)……..……2.5

Final pH 7.3 $\pm$ 0.2 at 25^0C

Instructions for use:

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

- **Mueller- Hinton Agar (Sisco, India)**

Composition of medium (g/l):

Acid hydrolysate of casein ……...……17.5

Beef extract ……...2.0

Starch……..……1.5

Agar …… 17.0

Final pH: 7.3 $\pm$ 0.2 at 25^0C

Instructions for use:

Suspend 38g in 1 litre of distilled/deionized water. Mix thoroughly. Gently heat and bring to boiling to dissolve completely. Sterilize by autoclaving at 121^0C for 15 minutes.