DETECTION OF STAPHYLOCOCCAL ENTEROTOXINS AMONG FOODS USING THE REVERSE PASSIVE LATEX AGGLUTINATION AND ENZYME LINKED IMMUNOSORBENT ASSAYS

BY

JOHN MUREITHI MATHENGE (HND)
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DECLARATION

I declare that this thesis is my original work and has not been presented for a degree in any other university or any other award.

JOHN MUREITHI MATHENGE

SIGNATURE..... DATE..26/09/2005

This thesis is submitted for examination with our approval as the university supervisors

DR. ZIPPORAH NG'ANG'A
School of Pure and Applied Sciences
Department of Biological Sciences
Kenyatta University.

SIGNATURE..... DATE..26th September 2005

DR. JACKSON. N. OMBUI
Department of Public Health,
Pharmacology and Toxicology, Faculty of Vet. Medicine
University of Nairobi

SIGNATURE..... DATE 26/9/2005

DEDICATION

Dedicated to my wife **Dorothy**, sons **Ian, Tony** and daughter, **Lynn**

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ABSTRACT

Staphyloccus aureus is a cause of food poisoning in humans. Vomiting, headache, abdominal pain, and diarrhoea that occur 1-6 hours following consumption of contaminated food characterize the poisoning. The illness is caused by five serologically distinct enterotoxins produced by organisms while growing in food. A number of methods have been used to detect Staphylococcal enterotoxins in foods and in *S. aureus* culture filtrates. These include biological assays and immunological methods. Biological methods have varied sensitivities and suffer from cross reactivity problems. This study was carried to determine the rate of contamination of milk and meat products with enterotoxigenic *S. aureus* and compare sensitivity of the two immunological techniques: RPLA and ELISA techniques for detection of enterotoxigenic strains of *Staphylococcus aureus*. A total of 400 milk and meat products were collected around Nairobi. The samples included 200 raw milk, 100 beef carcass swabs, 50 minced meat and 50 chicken carcass. Mannitol salt agar was used as selective and indicator medium for isolation of *S. aureus* from food samples. *S. aureus* were identified using a number of different and diagnostic tests which included oxidative fermentation, mannitol fermentation, coagulase production and deoxyribonuclease (DNase) production assays. Enterotoxin production was dectected using RPLA and ELISA techniques, Staphylococcal enterotoxins reverse passive latex agglutination SET RPLA kit and Staphylococcal enterotoxin enzyme immunoassay SET-EIA Kit. (Labour Dr. W. Bommeli, Bern). *S. aureus* was isolated from a total of 101 (25.4 %) food samples. Of these, 22 were from chicken, 16 minced meat, 55 raw milk and 8 beef carcass. The prevalence of *S. aureus* in the various foods ranged from 8% in beef carcasses to 44% in chicken. A total of 67 *S. aureus* were enterotoxigenic as detected using ELISA, while 62 *S. aureus* were enterotoxigenic using RPLA. There was no significant difference in the number of enterotoxigenic *S. aureus* detected by the two techniques among the food stuffs ($t = 1.324$; $df = 3$, $P = 0.277$). Staphylococcal enterotoxins SEA, SEB, SEC and SED were tested for, and SEC was the most frequent accounting for 59 (88 %) of the 67 enterotoxigenic *S. aureus* identified using ELISA and 61 (98.4 %) of the 62 identified by RPLA. There was however no significant difference in the prevalence of SEC as identified

by the two techniques (t = 0.815, df = 100, P = 0.417). SEA was found in 41 (61.2%) of 67 enterotoxigenic *S. aureus* while SEB was found in 31 (46.3 %), and SED in 26 (38.8 %) isolates as identified using ELISA. SEA was detected in 32 (51.6 %), SEB in 21 (33.9 %) and SED in 13 (21%) of the 62 enterotoxigenic *S. aureus* as identified by RPLA. *S. aureus* strains were found to produce one or more of the enterotoxins types. Twenty four (38.5 %) of the enterotoxigenic *S. aureus* produced a single enterotoxins while 44 (67.7 %) produced one or more with 21 (32.3 %) producing all the four enterotoxins type as identified by ELISA. The results showed that there was a high prevalence of enterotoxigenic *S. aureus* in raw foodstuffs of animal origin with SEC producing strains as the most prevalent, and that RPLA and ELISA were comparable techniques for detection of staphylococcal enterotoxins.

CHAPTER ONE: INTRODUCTION

1.1 Background information

Staphylococci aureus is a facultative anaerobic Gram-positive coccus. It is non-motile, catalase and coagulase positive. The cells appear as spherical single or paired cocci or form grape-like clusters (Cruickshank *et al.,* 1975). *Staphylococcus aureus* forms fairly large yellow colonies on nutrient agar media and is often hemolytic on blood agar. The organisms can grow by aerobic respiration or by fermentation that yields lactic acid. The bacteria can grow at sodium chloride concentration as high as 15%, as well as at high concentration of acid and in a wide range of temperatures (Schmitt *et al.*, 1990). These characteristics enable *S. aureus* to grow in a wide variety of foods. Their ecological niche can easily explain their incidence in foodstuffs that require manipulation during processing including fermented food products (Bergdoll, 1989).

Staphylococcus aureus strains can be classified into biotypes according to their human or animal origin. Devriese (1984) developed a biotype scheme, including six different biotypes i.e human, non-β-hemolytic human, avian, bovine and nonspecific based on biochemical characteristics. *Staphylococcus aureus* is an important pathogen due to a combination of toxin-mediated virulence, invasiveness, and antibiotic resistance (Adcock *et al.*, 1998). The bacteria are a significant cause of nosocomial infections, as well as community-acquired diseases. The spectrum of staphylococcal infections range from pimples, boils and abscesses to oesteomyelitis, pneumonia, meningitis, and toxic shock syndrome, most of which depend on virulence factor.

Some infections such as staphylococcal food poisoning rely on one single type of virulence factor, staphylococcal enterotoxins, which are heat stable (Bergdoll, 1979). The symptoms of staphylococcal food poisoning are abdominal cramps, nausea, vomiting, sometimes followed by diarrhea.

The onsets of symptoms are rapid from 30 minutes to 8 hours and spontaneous remission is observed after 24 hours. *Staphylococcus aureus* has been implicated in many food borne illnesses especially those involving mass catering as practiced is schools, hospitals and parties (Bergdoll, 1979). Bennette *et al.* (1986) identified *Staphylococcus aureus* as the causative agent in many food poisoning outbreaks. Foods implicated in staphylococcal food poisoning include milk and milk products, and meat and meat products (Bergdoll, 1989). Five staphylococcal enterotoxins (SEA, SEB, SEC, SED, and SEE) have been involved in food poisoning. In recent years the existence of new type of staphylococcal enterotoxins (SEG, SEH, SEI, SEK, SEL, SEM, SEN and SEO) has been reported (Jarrand *et al.,* 2001). However, the relationship between these new staphylococcal enterotoxins and human food poisoning is not fully understood. It is known that about 95% of staphylococcal food poisoning outbreaks are caused by type SEA to SEE (Bergdoll, 1983). The remaining 5% of outbreaks may be associated with other newly identified staphylococcal enterotoxins. Individual cultures of *S. aureus* may produce either a single, or a mixture of enterotoxins (Casman *et al.*, 1967). Staphylococcal enterotoxin A (SEA) has been reported to be the most common cause of food poisoning outbreaks in the United States, with staphylococcal enterotoxin D (SED) being next in order of frequency (Gilbert *et al.,* 1981). Staphylococcal enterotoxins C (SEC) has been reported to cause both food poisoning and toxic shock syndrome (Rizkallah *et al.*, 1989).

Specific enterotoxins among *Staphylococcus* strains recovered from various sources vary widely from region to region. Over 50% of the isolates from human specimens in the United States secrete SEA alone or in combination with other enterotoxins (Casman *et al.*, 1967).

In Sri Lanka human isolates producing SEA constitute only 7.8% (Palasuntheram and Beauchamp 1982). Variations are also found among *S. aureus* strains isolated from foods. Harvey *et al.* (1982) found SED to be associated with poultry strains more than human strains. Evans *et al.* (1983) found that 2 out of 3 atypical *S. aureus* isolates that produced a slow, weak positive or negative coagulase reaction and were negative for the anaerobic fermentation of mannitol, produced SED. About 39% of the isolates from Nigeria ready to eat food have been reported to be enterotoxigenic, with 44% of them producing SED (Adesiyun, 1984). Of the 48 isolates from dairy and 134 from meat products studied by Payne *et al.* (1966), 45.8% and 48.5% were found to be enterotoxigenic, respectively.

The enterotoxigenic *Staphylococcus* differ widely depending on the source of the isolate. Casman *et al.* (1967) found only 10% of the 236 raw milk isolates to be enterotoxigenic, while Payne and Wood (1974) found 62.5% of 200 food samples. Simkovicona and Gilbert (1971) found 33% of 36 foods isolate to be enteroxigenic. In Kenya, Kayihura *et al.* (1987) isolated *S. aureus* from both raw and pasteurized milk. All the *S. aureus* isolated from pasteurized milk were positive for enterotoxin A.

A number of methods have been used in analysis of staphylococcal enterotoxins. They include biological assays such as monkey and the kitten feeding and parenteral injection test (Davison *et al.*, 1938). These are inconvenient and expensive.

The microdouble diffusion slide techniques of Crowley (1958) proved to be the most specific as it provides a direct comparison between a reference toxin and the unknown sample. Modification of these techniques includes the optimum sensitivity plate (OSP) method described by Robins *et al.* (1974) and the microslide technique of Casman *et al.* (1967). Using OSP method, upto 1mg of toxin per 1ml can be detected.

Techniques such as Radioimmunoassay (RIA), Agglutination, and Enzyme Linked Immunosorbent Assay (ELISA), which require less concentration of the food extracts and thus save time and are more sensitive can be, used (Bennette, 1986). Akhatar (1998) reported that reversed passive hemaglutination assay (RPHA) is sufficiently sensitive. The RPHA is the simplest to perform but occasionally gives non-specific reactions with certain foods. To overcome this, the reverse passive latex agglutination (RPLA) was developed.

1.2 Statement of the problem

Staphylococcus aureus food poisoning is a common problem in Kenya. *S. aureus* is ubiquitous in the environment especially among animal and food handlers. *Staphylococcus aureus* is also a cause of mastitis in cattle. This makes it easy for contamination of raw foods such as milk and meat. The foods may pose problems of food poisoning if left for long at room temperature. Outbreaks of staphylococcal food poisoning have been reported in Kenya but there is no surveillance of the food borne illness due to lack of a sensitive and reliable test to detect the toxins. Various immunological diagnostic tests including RPLA and ELISA are used for the detection of staphylococcal enterotoxins but their sensitivity has not been compared. There is need therefore to compare RPLA and ELISA in order to identify the most appropriate immunological test for the diagnosis of staphylococcal enterotoxins in food.

1.3 Research questions

1. What is the occurrence of enterotoxigenic *Staphylococcus aureus* in milk and meat products?
2. What are the types of the enterotoxins produced by *Staphylococcus*?

1.4 Null hypothesis

There are no staphylococcal enterotoxion among milk and meat products.

1.5 General objective

To detect staphylococcal enterototions among milk and meat products using Reverse Passive Latex Agglutination (RPLA) and Enzyme Linked Immunusorbent Assay (ELISA) techniques.

1.5.1 Specific objectives

1. To determine occurrence of *Staphylococcus aureus* among milk and meat products.
2. To determine the type of enterotoxin produced by the recovered *Staphylococcus* isolates using Reverse Passive Latex Agglutination and Enzyme Linked immunosorbent assay techniques.
3. To compare the level of detection of enterotoxigenic *S.aureus* using RPLA and ELISA.

1.6 Justification

Staphylococcal food poisoning is now a leading food borne intoxication worldwide. The true incidence of staphylococcal food poisoning is unknown for a number of reasons, including poor responses from victims during interviews by health officers, misdiagnosis of the illness, which may be symptomatically similar to other types of food poisoning such as that caused by *Bacillus cereus* toxins. The incubation period of *Staphylococcus* food poisoning is 1-10 hrs with an average of 2-4 hrs. Recovery generally takes two days. There is need for a reliable and rapid test that will detect the staphylococcal enterotoxins to be able to distinguish staphylococcal food poisoning from other types of food poisonin

1.7 Scope and limitation of the study

Sampling was done at the key slaughterhouses in Dagoretti market. Raw milk was collected in Dairy center directly from the delivery cans where the farmers were given cards with numbers and the one randomly picked was sampled. Due to cost limitation, only 50 chicken carcasses and 50 minced meat were purchased from various butcheries within the study area.

Polymerase Chain reaction (PCR), so a good and reliable method of detecting staphylococcal enterotoxion is expensive and does not prove that a given stain is capable of producing enterotoxion.

CHAPTER TWO: LITERATURE REVIEW

2.1 History of *Staphylococci*

Staphylococci were first described in human pus when Ogoston (1881) showed them to be pathogenic for mice and guinea pigs and reported them to be constantly found in acute and chronic abscesses. Rosenbach (1884) described the two species, *Staphylococcus aureus* (pyogenes) and S*taphylococcus epidermidis* (*albus*), which are now classified in the family micrococcaceae. Interest in the *Staphylococci* as a human pathogen was greatly stimulated in 1928 by a tragic accident in Bundaberg, Austria. Of the 21 children inoculated with the diphtheria toxin-antitoxin from a single rubber capped vial which had been kept at room temperature in subtropical heat for several days, 16 became ill within 5 - 7 hrs with vomiting, high fever, stupor, cyanosis and convulsions. Twelve of the children died within two days and those that survived developed staphylococcal abscesses at the site of injection. The result of the subsequent studies suggested that the early deaths were due to the toxin elaborated by the *Staphylococcus*.

2.2 Description of the *Staphylococcus aureus*

Staphylococcus aureus is a cluster forming gram-positive cocci belonging to the genus *Staphylococcus*. The genus belongs to the family micrococaceae. Five other genera in the same family include *Micrococcus*, *Gaffkya*, *Sarcina*, *Peptococcus* and *Aerococcus*. *Graffkya* and the aerobic species of *Sarcina* are classified in the genus *Micrococcus* (Cruickshank *et al.*, 1975). The distinguishing features are given in table 2.1. The pathogenic species in the family micrococcaceae include *S. aureus* and *S. epidermidis*. The other members of the genera are commonly present either as commensals or contaminants on the surface of the body that lack virulence and primary pathogenicity.

Clinical specimens taken from the body surface such as swabs from the skin, nose, throat, wounds, burns and bedsores are contaminated with these organisms and their presence should not be regarded as being clinically significant. *Gaffkya tetragena* is a rare cause of suppurative lesions in the mouth, neck or respiratory tract (Cruickshamk *et al.*, 1975)

Table 2.1. Characteristics of six genera of the *Micrococcacceae*, the cluster forming Gram positive cocci.

Genus	Predominant Grouping of cocci	Atmospheric requirement	Catalase production	Breakdown of glucose	Ecological characteristics
Staphylo-Coccus	irregular grapelike Clusters.	aerobic and facultative anaerobic	+	fermentative form acid under aerobic and anaerobic conditions	pathogenic commensals
Micro-Coccus	irregular and rectagular clusters and tetrads	aerobic	+	oxidative	free living saphropytes
Gaffykia	tetrads	aerobic and facultative anaerobe	-	fermentative	pathogenic parasites
Sarcina	cubical packets eight cocci	1.anaerobic 2.aerobic	-	fermentative or inactive oxidative	free living saprophytes
Peptococcus	clusters and tetrads	strictly anaerobic	+	fermentative or inactive	commensal parasites
Aerococcus	small clusters	facultative anaerobe	-, or weak.	fermentative	free living saprophytes

Adapted from Cruichshank *et al.* (1975).

2.2.1 Cellular and colonial morphology of *Staphylococcus aureus*

Rosenbach (1884), described *S. aureus* organisms as Gram positive spherical cells with an average diameter of 0.7- 1.0μm that appear as grape-like clusters with single and paired cocci in films of pus or from solid culture medium. In broth medium, *Staphylococcus aureus* appears as small groups, pairs, singles and short chains (less than five cocci in line). Their characteristic growth in clusters results from irregular divisions in two planes perpendicular to one another in solid media (Davis *et al.,* 1968). When grown on nutrient agar, milk agar or blood agar for 24 hrs at 37°C, individual colonies are circular, 2-3mm in diameter with a smooth shiny surface. Colonies appear opaque and are often pigmented (golden-yellow, fawn or cream), though a few strains are unpigmented (Greenwood *et al.,* 1997).

The main distinctive diagnostic features of *Staphylococcus aureus* include production of an extracellular enzyme coagulase that converts fibrinogen in citrated human or rabbit plasma into fibrin, aided by an activator present in plasma, production of thermastable nuclease that breakdown DNA and production of a surface-associated protein known as clumping factor or bound coagulase that reacts with fibrinogen (Crunshshank, 1975). Various commercial systems are available that rapidly identify coagulase-positive and coagulase-negative species. They are particularly useful for screening large numbers of strains obtained from environmental and food samples (Greenwood *et al.,* 1997). Recovery of the *S. aureus* from stressed cells can be achieved by the use of Baird Parkers agar, which relies on two properties of the organism. First, *S. aureus* utilizes egg yolk lipoprotein which causes clearing around the colonies, often with a white precipitate within this zone due to formation of calcium and magnesium salt of fatty acids.

Secondly, the agar contains tellurite which inhibits many interfering organisms, and which *S. aureus* reduce to give pitch-black colonies. Sodium pyruvate and glycine in the agar acts as a selective growth stimulant and makes the medium non-toxic for stressed populations (Idiziak and Mossel, 1980).

2.2.2 Cultural characteristics of *Staphylococcus aureus*

Staphylococcus is a facultative anaerobe with a growth temperature between 12 - 44°C, with an optimum temperature of 37°C and optimum pH between 7.4 to 7.6 (Alice, 1989). The organism tolerates high concentrations of sodium chloride than many other kinds of bacteria where growth in upto 16-18% sodium chloride has been reported (Genigeorgis and Sadder, 1966). Growth occurs aerobically at levels of water activity down to 0.86 or 0.88. Under anaerobic conditions, Staphylococci grow at a minimum water activity of 0.9 (Qi. and Miller, 2002). *Staphylococcus aureus* ferments glucose and mannitol sugars. Table 2.2 shows the distinguishing characteristics between the species of the genera *Staphylococcus* and *Micrococcus.*

Table 2.2. Characteristics of species of the genera *Staphylococcus* and *micrococcus*.

	Staphylococcus		*Micrococcus*	
Character	*S. aureus*	*S. epidermidis*	*M. luteus*	*M. roseus.*
Acid from glucose				
(1) aerobically	+	+	+	W
(2) anaerobically	+	+	-	-
Coagulase	+	-	-	-
Phosphatase	+	D	-	-
Acetoin (V- P)	+	D	+	-
Acid anaerobically From				
(1) Arabinose	-	-	-	-
(2) Lactose	+	D	-	-
(3) Maltose	+	D	D	W
(4) Mannitol	+	-	-	-

W = weak or negative.

D = different reactions (+ or -) in strains.

Adapted from Baird-Parker (1996).

2.2.3 Genomic variability of *Staphylococcus aureus*

Studies of the genomic deoxyribonucleic acid of S*taphylococcus aureus* subspecies *aureus* strains of various origins, as well as the genomic DNAs of other coagulase-positive *Staphylococcus* species by restriction endonuclease analysis and subjected to pulsed- field gel electrophoresis revealed that the levels of similarity of the small restriction patterns of the *S. aureus* sub species *aureus* strains varied from 30 to 100% (Pantucek *et al.,* 1996). These species were found to belong to the same restriction group. Within this range of similarity values, 13 *S. aureus* intraspecies restriction groups were identified. Each group consisted of strains whose level of similarity ranged from 65 to 100%. *Staphylococcus aureus* sub *S. aureus* CMM88ST (T =type strain) belonged to the major intraspecies restriction group that comprised 39% of the *S. aureus* strains, which were studied. The strains of the other coagulase- positive *Staphylococci*, including S*taphylococcus hyicus, Staphylococcus dolphini* and *Staphylococcus schleigeri* sub species *coagularis,* clustered with their type strains in separate restriction groups.

S. aureus sub species *aureus* exhibited almost no similarity to these species (Pantucek *et al.,* 1996).

2.3 Resistance of the organism to adverse conditions

2.3.1 Effects of chemical and physical agents

Staphylococcus aureus can withstand moist heat at 60°C for 30 minutes but is generally killed in one hour, though some strains may resist 80°C for 30 minutes (Alice, 1989). Because of their resistance to drying, they can be carried on dust particles and live for weeks or months in dried pus or sputum. *Staphylococcus aureus* are resistant to the action of phenols and many other disinfectants, but they are very sensitive to the basic dye (Topley and Miles, 1955).

2.3.2 Resistance of *S. aureus* to high temperature

When compared to Gram negative rods shaped organisms such as *E. coli* and *Salmonella, S. aureus* is resistant to inactivation by temperature below freezing (Georgala and Hurst, 1963). Minor and Marthi (1972) showed that freezing of cells of *S. aureus* strains in trypticase soy broth, storage for 24 hours at -30°C and thawing, had very little effect on viability. Cells treated in this manner were slightly more sensitive to 7.5% concentration of sodium chloride in tryptic soy agar than cells held for similar periods at 37°C. Freezing and thawing may cause injury to cells of *S. aureus* but not death. Injuries that may occur in bacteria stored at subfreezing temperature include membrane damage and changes in metabolic or enzymatic activity (Marth, 1973). Gopalakrishana and Shrivastava (1988) reported absence of coagulase positive *Staphylococci* in frozen lobsters, cattlefish, catfish, starfishand and red snapper.

2.3.3 Resistance of *S. aureus* to pH

Staphylococcus aureus is relatively resistant to the presence of acids in their environment. Minor and Marthi (1972) found appreciable differences on the effects of the survival of 18 hours old cells of *S. aureus* after 24 hours of incubation at 37 °C in tryptic soy broth, acidified with different acids. A 99.9% decrease in number of viable S*taphylococci* occurred with acetic, lactic, phosphoric, citric and hydrochloric acid when the pH of the medium was 4.5, 4.4, 4.2, 3.9 and 3.8 respectively. The undissociated acid molecule was responsible for the enhanced inactivation of the cells and by partially dissociated acids anions of these acids had effect on cell survival. The cells were most sensitive to the effects of hydrogen ions between 12^{th} and 24^{th} hours during 120 hours incubation at 37°C and so at a higher incubation temperature (45°C), and when the initial numbers of bacteria were low.

These workers observed that cells exposed to sub lethal acid treatment appeared to be mildly injured and this injury was repairable when the proper conditions for recovery were provided. Acid inactivation was enhanced in the presence of 7.5% sodium chloride and freezing temperature, but only when the pH of 4.2 and 3.8 were employed. Sensitivity of the survivor to salts increased sharply at the lower pH value.

2.3.4 Resistance to sodium chloride and sugars

The presence of salts and sugars in aqueous environments has direct effects on the water activity of the food. The growth of *S. aureus* occurs aerobically in a media with water activity too low for growth of many species of other microorganisms (Qi. and Miller 2002). *S. aureus* is therefore highly tolerant to the presence of solutes such as salts and sugars in the growth media. According to Genigeorgis and Sandler (1966), growth occurred at 37°C in brain heart infusion broth with 16% sodium chloride at pH 6.9, but no cell survived with pH 5.1 containing 16% salt.

2.3.5 Resistance of *Staphylococci* to antimicrobial agents.

Hospital strains of *S. aureus* are usually resistant to a variety of antibiotics. A few strains are resistant to all clinically useful antibiotics except vancomycin and rarely, vancomycin-resistant strains have been reported. High-level vancomycin resistance was experimentally transferred from *Enterococcus faecalis* to *S. aureus* both *in vitro* and *in vivo* models. Edmond *et al.* (1996) suggested that a patient who is infected or colonized with vancomycin resistant *S. aureus* should be placed in a private room and all persons entering the room should wear clean, nonsterile gloves and disposable gowns. Gloves and gowns should be removed before leaving the room. After the gloves are removed, people are required to wash hands with 4% chlorohexidine or 60% isopropyl alcohol.

A monitor could be placed at the door to prevent unauthorized access and to enforce hand washing and barrier precautions. A plasmid associated with vancomycin resistance has been detected in the enterococci, which can be transferred to *S. aureus* in the laboratory (Edmond *et al.*, 1996). Methicillin resistant *Staphylococcus aureus* (MRSA) are wide spread and most methicillin resistant strains also exhibit multiple resistances to other antibiotics (Al-Massaudie *et al.*, 1988). When Kirby's first description of penicillinase producing strains of *S. aureus* was published in 1944, resistance was infrequently encountered, with only a handful of strains available for study. Staphylococcal resistance was reported shortly after penicillin was introduced and within approximately 60 years, 25% of hospital strains were resistant. One to two decades later, 25% of community isolates were penicillin resistant (Gould and Cruichshank, 1957; Harris and Wise, 1969). Although the rates are only approximate because they are based on reports from numerous locations, a clear correlation exist between the prevalence of penicillin-resistant strain of *S. aureus* reported in hospitals and rates in the community.

2.3.6 Virulence factors of *Staphylococcus aureus*

S. aureus produces coagulase, a soluble enzyme like product, which *in vivo* contributes to the pathogenicity of the organism by inactivating a bacteriocidal substance in normal serum or by protecting the cocci with a fibrin barrier against phagocytosis. The organisms also produce toxins that kill phagocytes and cell surface components that enable the cocci to resist killing by lysosomal enzymes, after ingestion by phagocytic cells. The cell wall components of the coccus include a mucopeptide, a species-specific antigen, protein A that is a precipitant of gamma globulins, alpha and beta ribitol technoic acid antigens, and a type specific antigens (Montie and Kadis, 1970).

The organism produces various types of toxins. Enterotoxins are secreted by growing *S. aureus* cells and are demonstrable of bacteria free filtrates of cultures. Toxins that probably play a role in the pathogenesis of *S. aureus* are alpha toxins and the pento-balentine (PV) leucocidin. Alpha toxins are hemolytic, leucocidal (kill macrophages), cytotoxic dermatonecrotic and show lethal effects. The organism has an antigenic enzyme, which appears to act on the cell wall membranes and probably responsible for some of the symptoms in fatal cases of staphylococcal septicaemia (Montie and Kadis, 1970). Table 2.3 shows some of the virulence factors expressed by *S. aureus*.

Table 2.3. Potential virulence factors expressed by *S. aureus*,

Virulence factor	Activity
Cell wall polymers	
Peptidoglycan	Inhibits inflammatory response: endotoxin-like activity
Teichoic acid	phage adsorption reservoir of bound divalent cations
Cell surface proteins	
Protein A	reacts with Fc region of IgG
Clumping factor	binds to fibrinogen
Fibronectin-binding protein	bind to fibronectin
Collagen-binding protein	binds to collagen
Exoproteins	
α- lysine β-lysine γ-lysine δ-lysine panton- valentine leucocidin	Impairment of membrance permeability: cytotoxic effects on phagocytic and tissue cell
Epidermolytic toxins	cause blistering of skin
Toxic shock syndrome Toxin	induces multisystem effects superantigen effects
Enterotoxins	induce vomiting and Diarrhea: superantigen Effects
Coagulase	Converts fibrinogen to fibrin in plasma
Hyaluronidase	Degrades hyaluronic acid in Connective tissue
Staphylokinase	degrades fibrin
Lipase	Degrades lipid
Phospholipases	Degrade phospholipids
Deoxyribonuclease	Degrades DNA
Proteases	Causes proteolysis

Adapted from Greenwood *et al.* (1997).

2.4 Reservoir host of *Staphylococcus aureus*

Staphylococcus aureus is found in varying numbers in air and dust as well as in water, food, faeces and sewage. Humans are natural reservoirs of *S. aureus,* and asymptomatic colonization is far more common than infection. Colonization of the nasopharynx, perineum, or skin, particularly if the cutaneous barrier has been disrupted or damaged, may occur shortly after birth and may reoccur any time thereafter (Payne *et al.,* 1966). Family members of colonized infants may also become infected. Transmission occurs by direct contact to a colonized carrier. Carriage rates of persons with insulin-dependent diabetes, patients with dermatological conditions, patients 25% to 50% higher than in the general population are observed in injection drug with long-term indwelling intravascular catheters, and health-care workers (Wadlvogel, 2000). Young children have higher colonization rates, probably because of their frequent contact with respiratory secretions (Adcock *et al.,* 1998). Colonization may be transient or persistent and can last for years (Sanford, 1994).

2.5 Food-borne diseases

Food-borne diseases (FBD) are defined by the World Health Organization as "diseases of infectious or toxic nature caused by or thought to be caused by the consumption of food or water". Food – borne diseases are of major concern worldwide. To date around 250 different food-borne diseases have been described, and bacteria are the main causative agents of two thirds of food borne disease outbreaks (Yves *et al.,* 2003).

2.5.1 Staphylococcal food poisoning

Among the predominant bacteria involved in these diseases, *Staphylococcus aureus* is the leading cause of gastro-enteritis resulting from the consumption of food contaminated with *S. aureus* enterotoxions. Enterotoxins are produced by enterotoxigenic strains of *Staphylococcus aureus* while they grow and multiply in food. The term "food poisoning" has been given to a food-borne disease associated with recent consumption of food with a short incubation period ranging from a few minutes to a few days.

2.5.2 Enterotoxigenic potential of other *Staphylococci*

Several staphylococcal species other than *S. aureus* reportedly produce staphylococcal enterotoxin (Jay, 1992). Among the coagulase negative species are *S. cohnii*, *S. epidermis*, *S. xylosus* and *S. haemolyticus*. These bacteria have been isolated from ewe's milk and were found to produce one or several SEs (Bautista *et al.*, 1988). The coagulase positive *S. intermedius* is the predominant food borne non-*S. aureus* species isolated from food. Some have been shown to produce SEs (Becker *et al.*, 2001). *Staphylococus intermedius* is the only non *S. aureus* species that has been clearly involved in Staphylococcal food poisoning outbreaks (Khambaty *et al.*, 1994).

2.5.3 Staphylococcal enterotoxins

Studies on Staphylococcal enterotoxins (SEs) started from the analysis of *S. aureus* strains involved in staphylococcal food poisoning. In the first SEs identified, the peptide sequence was available before the nucleotide sequence. This was the case for SEA (Huang *et al.*, 1987), SEB (Huang and Bergdoll, 1970) and SEC (Schmidt and Spero, 1983). To date, 14 different SE types have been identified which share structure and sequence similarity. The SEs are short proteins secreted in the medium and soluble in water and saline solutions.

Staphylococcal enterotoxions are rich in lysine, aspartic acid, glutamic acid and tyrosine residue. Most of them possess a cystine loop required for proper conformation and which is probably involved in the emetic activity. They are highly resistant to proteolytic enzymes, such as pepsin or trypsin and thus keep their activity in the digestive tract after ingestion (Bergdoll, 1970). They are also resistant to chymotrypsin, rennin and papain. Staphylococcal enterotoxin B can be destroyed by pepsin digestion at pH 2 but it is pepsin resistant at higher pH, which is a normal condition in the stomach after food ingestion (Bergdoll, 1983). Staphylococcal enterotoxins are highly resistant to heat. They are thought to be resistant in foodstuffs than in a laboratory culture medium (Bergdoll, 1983), but can be inactivated by heat treatment used in the sterilization of canned foods when they are present at low concentrations (Bergdoll, 1983).

Genes encoding SEs have different genetic support most of which are mobile elements, for example, *sea* is carried by a family of temperate phage (Betley and Mekalanos, 1985; Coleman *et al.,* 1989). *Seb* is chromosomally located in some clinical isolate (Shafer and Iandolo, 1978), while it has been found in a 750-Kb plasmid in other *S. aureus* strains (Shalita *et al.,* 1977). Staphylococcal enterotoxin C (SEC) is encoded by a gene located in a pathogenicity island (Fitgerald *et al.*, 2001) and *see* is carried by a defective phage (Couch *et al.*, 1988). The main regulatory system controlling this gene expression of virulence factors in *S. aureus* is the accessory gene regulator *(agr)*. Kornbblum *et al.* (1990) reported agr gene to act in combination with the staphylococcal accessory regulatory gene *sar*; (Cheung *et al.,* 1992;Novick, 2000). Some but not all of the SE genes are controlled by *agr* system. The *seb*, *sec* and *sed* genes have been demonstrated to be agr dependant; where as *sea* and *sef* are *agr* independent (Tremaine *et al.,* 1993; Zhang *et al.,* 1998).

Recent research by Vojtor *et al.* (2002) demonstrated that SEB, like toxic shock syndrome enterotoxin (TSST), is a negative global regulator of exoprotein gene transcription, which acts via the *agr* system. As *agr* expression is tightly linked to quorum sensing the production of *agr*-dependant SEs in foodstuffs is dependent on the ability of *S. aureus* to produce high cell density (estimated at 10cfu/g) in the foodstuffs. Environmental factors play an important role in SEs gene expression (Novick, 2000).

2.5.4 Staphylococcal enterotoxin activities

The SE belongs to the family of the so-called pyrogenic toxins originally from the genera *Staphylococcus* and *Streptococcus*. Pyrogenic toxins include SEs, TSST, exfoliatins A, and B and *Streptococcus* pyrogenic toxins. These toxins share some structural, functional and sequence similarities (Balaban and Rasooly, 2000). Until recently, SEs were discovered in studies of *S aureus* strains implicated in food borne disease outbreaks and they were classified in distinct serological types SEA to E and SEH have been demonstrated as being capable of more or less potent emetic activity. More recently, increasing data resulting from partial or complete genome sequence analyses have allowed the identification of several new SE types.

The new SEs were first identified on the basis of sequence and structural similarities with existing Ses (Balaban and Rasooly, 2000). There is experimental evidence for their superantigenicity *in vitro* and/or *in vivo* activities, but rarely their emetic activities.

Although pyrogenic toxins are involved in distinct pathologies and have common biological activities, they cause immunosuppression and nonspecific T-cell proliferation (Dinges *et al.*, 2000). These activities are referred as superantigen activities. Among the superantigens, only SEs has emetic activity.

Superantigen and emetic activity of the SEs are two separate functions localized on separate domains of the proteins (Horde *et al.*, 1994; Dinges *et al.,* 2000). High correlation exists between superantigen and emetic activities since, in most cases genetic mutation results in loss of superantigen activity (Harris *et al.,* 1993). Superantigen activity results from direct interaction of SEs with T-cell receptors (TCR) and the major histocompatibility complex (MHC) of antigen-presenting cells (APC). Only a few T-cells can recognize a specific antigen presenting in MHC of an APC (Mc McCormick *et al.,* 2001). Superantigen toxins interact with many T-cells by the recognition of specific Variable β chains of the TCR (T cell receptor). This cross-link results in the nonspecific activation and proliferation of T-cell and a massive secretion of interleukins that may be involved in the mechanism of SE toxicity, such as toxic shock syndrome (Mc Cormick *et al.,* 2001). SEB has been well studied due to its potent superantigen activity and are considered as potential microbiological weapons of warfare and terrorism (Greenfield *et al.,* 2002).

The emetic activity of SE's is not as well characterized as superantigen activity. The enterotoxin activity is uniquely characterized by the SE to cause emetic responses when administered orally to monkeys, where as other superantigens are not emetic (Dinges *et al.,* 2000). Little is known about how the SEs causes symptoms of food poisoning. They may have a direct effect on intestinal epithelium and on the vagus nerve, causing stimulation of the emetic center and of gut transit (Bergdoll, 1983; Arbuthnott *et al.*, 1990).

The infective dose required to induce staphylococcal food poisoning in humans is estimated to be approximately 0.1μg, which may vary with patient sensitivity (Evenson *et al.,* 1988). Emetic activity has not been precisely localized.

One common feature of SEs is a cystine loop, thought to be important for emetic activity based on mutant analyses (Horde *et al.*, 1994, Dinges *et al.*, 2000). SEI lacks the cystine loop structure and its emetic activity is nevertheless significantly lower than that of other SEs (Munson *et al.,* 1998). Sequence analysis of two other recently identified SEs SEK and SEL, also indicated absence of the cystine loop (Orwin *et al.,* 2001; Fitzgerald *et al.*, 2001).

2.5.5 Environmental factors that affect production of enterotoxins

Many studies have investigated the conditions in which *S. aureus* is able to produce SEs (Bergdoll, 1989; Genigeorgis, 1989). SEs production has been studied in strains grown in laboratory media and in diverse foodstuffs.

The abundance of accelerating gene regulator *(agr)* is well documented because it regulates most of the virulence factors in *S. aureus* (Novick, 2000). Valine is necessary for growth and arginine and cystine are necessary for both growth and SE production in five strains of *S. aureus* that produce SEA, SEB or SEC. The reqirement for amino acids vary with the strains (Onoue and Mori, 1997).

Glucose has been shown to have an inhibitory effect on SE production, especially for SEB and SEC (Bergdoll, 1989). This effect has been attributed to a drop in pH as a consequence of glucose metabolism. Glucose and low pH have an inhibitory effect on *agr* expression (Regassa *et al.*, 1992; Novick, 2000). Staphylococcal enterotoxin production is optimal in neutral pH and decreases in acidic pH, and usually pH below 5.

Substances used to acidify the medium may have less effect than lactic acid on SE production. High concentration above 12% is independent of the pH (Notermans and Heuvelman, 1983).

Alkaline pH also decreases the production of SEB, SEC, and SED via decreased expression of *agr* (Regassa and Betley, 1992). *Staphylococcus aureus* is sensitive to microbial competition. Genigeorgis (1989) demonstrated that the higher the concentrations of competing microorganisms in milk, the lower the rate of *S. aureus* growth and SE production. Competition with lactic acid bacteria has been reported on cheese (Otero *et al.,* 1988; Vernozy-Rozand *et al.*, 1998) and fermented sauge production (Sameshima *et al.*, 1998).

The effects of lactic acid bacteria are mainly due to lactic production leading to lower pH, and hydrogen peroxide production, competition for nutrients and sometimes the synthesis of antimicrobial substances, such as bacteriocins (Genigeorgis, 1989).

2.6 Foods involved in Staphylococcal poisoning

In all cases of staphylococcal food poisoning, the foodstuff or one of the ingredients was contaminated with an SE-producing *S. aureus* strain and was exposed, at least for a while to temperatures that allows *S.aureus* growth and enterotoxin production (Genigeorgis, 1989).

Most of the time the foodstuff reached this temperature because of failure in the refrigeration process, or because a growth-permissive temperature was required during processing (Bergdoll, 1989).

Many different foods can be used as growth medium for *S. aureus* and have been implicated in staphylococcal food poisoning, including milk and cream, cream-filled pastries, butter ham, cheeses, sausages, canned meat, salads, cooked meal and sandwich filling (Bergdoll, 1989). In one case cheese was involved in an outbreak because it had been made from milk contaminated after pasteurization and before inoculation with lactic starter culture.

The starter culture in this particular case did not grow properly resulting in a fermentation accident that allowed *S.aureus* strains to develop and produce SE (Bergdoll, 1989). In 1985, chocolate milk was the origin of a staphylococcal food poising in Kentucky, USA. This chocolate milk was contaminated and stored at ambient temperature for 4 to 5 hours, before pasteurization. Pasteurization killed the *Staphylococcus aureus* but had no effect on the SEs. In the case of canned food that has been correctly processed, bacteria and SEs are usually destroyed. Some cases of staphylococcal food poisoning involving canned mushrooms that were correctly processed were reported in the USA (Bennet, 1992). Foods that are most often involved in Staphylococcal food poisoning differ widely from one country to another. In the United Kingdom, for example, 53% of the Staphylococcal food poisoning reported between 1969 to 1990 was due to meat products, meat based dishes especially ham.

Twenty two per cent (22%) of the cases were due to poultry-based meals, 8% due to milk products, 7% to fish and shellfish and 3.5% of eggs (Wieneke *et al.*, 1993). In France among the staphylococcal food poisoning reported in a two year period (1999-2000), milk products especially cheese were responsible for 32% of the cases, meat for 22%, sausages and pies for 15%, fish and sea food for 11% and poultry for 9.5% (Haegherbaert *et al.*, 2002).

In the United States, among the staphylococcal food poisoining cases reported between 1975 and 1982, 36% were due to red meat, 12.3% to salads, 11.3% to poultry, 5.1% to pastries and only 1.4% to milk products and seafoods. In 17.1% of the cases, the foods involved were unknown (Genigeorgis, 1989). In France, the consumption of raw milk cheeses is much higher than in Anglo-Saxon countries.

This may explain the importance of milk products involved in staphylococcal food poisoning in France (Wieneke *et al.*, 1993). The main sources of contamination are human (handlers contaminate food via manual contact or via the respiratory tract by coughing and sneezing) and contamination may occur after heat treatment of the food. In foods such as raw meat, sausages, raw milk, and cheese contamination from animal origins are most frequent and due to animal carriage or to infection (Genigeorgis, 1989).

2.7 Frequency of enterotoxigenic strains

Foods and raw ingredients are subjected to regular microbiological control. Among the *S. aureus* strains isolated from food samples, the percentage of enterotoxigenic strains is estimated to be 25% (Bergdoll, 1989). Estimations vary considerably from one food to another. In France, among 61 strains isolated from raw milk cheeses, 15.9% were enterotoxigenic (Rosec *et al.*, 1997).

In Denmark, another study performed on strains isolated from cows with mastitis found that only 1 out of 414 *S. aureus* isolated carried an SE gene (Larsen *et al.*, 2000). A similar study was performed in Minas Gerais, Brazil, where 54 (43%) of 127 *S. aureus* isolates from bovine mastitis were found to be SE producers (Cardoso *et al.*, 1999).

More recently in Germany, similar work on strains isolated from the milk of cows with mastitis showed that upto 72% of the strains were enterotoxigenic when SEA to SEJ were considered (Akineden *et al.,* 2001). There is a great increase in the percentage of enterotoxigenic strains when the newly described staphylococcal enterotoxins are taken into account. A long with the SEA, SEC, and SED often found in the studies, SEG, SEI, and SEJ seem to be the predominant SEs in strains isolated from cattle with bovine mastitis (Akineden *et al.*, 2001).

2.8 Treatment of Staphylococcal food poisoning

Staphylococcal food poisoning is not contagious and cannot be spread from one person to another. There is no medication or antidote that can be given to neutralize the poison or cure the illness caused by *Staphylococcus aureus* but treatment is limited to supporting the body until it can recover on its own. Treatment may include tylenol for fever, cough suppressant for comfort and in some cases intravenous fluid for dehydration. If there are serious breathing problems, management will include oxygen and even mechanical ventilation (http://al hazards. State wy.us.)

Staphylococcus aureus capsular polysaccharides were one of the earliest targets in vaccine studies aimed at preventing staphylococcal infection. Immunization was with polysaccharide antigens extracted from highly encapsulated *S. aureus*. Strains of *Staphylococcus* protected mice against infection with the homologous, but not heterologous capsule type (Ekstedt, 1963). Furthermore protection could be passively transferred to immunologically naïve animals by injecting them with immune serum. Capsule antibodies can protect mice against staphylococcal abscesses in a sublethal injection model (Lee *et al.*, 1988).

In. a study, Jean *et al.* (1997) examined the protective efficacy of antibodies to the *Staphylococcus aureus* type 5 capsular polysaccharide (CP5) in a modified model of catheter - induced endocarditis. The results demonstrated that capsular antibodies elicited by immunization with a polysaccharide protein, conjugate vaccine protected experimental animals against serotype 5 *S. aureus*.

2.9 Detection of *Staphylococcus* enterotoxins

2.9.1 History of enterotoxin detection

Various methods have been used to detect enterotoxins in food and in culture supernatant or filtrates of *S. aureus* strains. The first methods applied for detection of *S. aureus* enterotoxins were biological assays such as monkey and the kitchen feeding and parenteral injection test (Davidson *et al.,* 1938). However, these were inconvenient and expensive. Therefore immunological techniques were elaborated, based on immuno-diffusion techniques following Ouchterlony (1958). Precipitation in gels of the enterotoxin-antibody complex has been the most frequently employed serological method. The microdouble diffusion slide technique of Crowley (1958) proved to be the most specific as it provides a direct comparison between a reference toxin and the unknown sample. Modification of these techniques include the optimum sensitivity plate (OSP) method described by Robbins *et al.* (1974) and the microslide technique of Casman *et al.* (1967).

Using OSP method, up to 1µg of toxins/ml can be detected. The OSP technique proved to be a simple method for testing strains of *S. aureus* for enterotoxin production.

However, for satisfactory results, culture methods have to be used that favour synthesis of enterotoxins. For this, the dialysis-sac culture (Donnelly *et al.,* 1967) and the cellophane-over agar culture (Hallander, 1965) have been used.

2.9.2 Electrophoretic and immunoblot analysis of staphylococcal enterotoxins in food

Sodium dodecyl sulphate-polyacryamide gel electrophoresis (SDS-PAGE) is a common protein separation method (Orden *et al.,* 1992). An electrical field is applied so that charged molecules migrate through a polyacrylamide matrix to the electrode bearing the opposite charge.

The negatively charged detergent, SDS, denatures and strongly binds proteins. SDS-bound proteins migrate to the positive pole at rates inversely proportional to their molecular weights. In general, two part discontinous gel are used (Laemmli, 1970). The sample is loaded onto the upper portion (stacking gel), which has a low acrylamide concentration, low pH, and low resolving ability. When the sample runs through the stackling gel, all protein are concentrated into a narrow band. The narrow band enters the lower portion (resolving gel) that separates protein by size.

The acrylamide concentration chosen for the resolving gel depends on the size of protein to be separated. SEs is 25-30 Kda and 12.5% acrylamide is useful for separating protein in that range. Immunoblots have two important advantages for food testing. First even though heat and other treatments during food processing can cause proteins to aggregate, the aggregates are solubilized and unfolded in SDS gels. Other antibody-based methods of food analysis, such as ELISA, do not have a SDS solubilization step. Instead, the sample is applied directly to the antibody, because SDS in the sample would denature the detecting antibody (Orden *et al.,* 1992).

Second, cross-reacting antigens usually can be distinguished from the desired antigen on the basis of molecular weight in a western blot (Rasooly, 1998). In ELISA, and other assays in which samples are evaluated without separation or purification, cross-reacting antigens increase the background.

Western blotting has some limitations, which are important to recognize when applying the method to food analysis. First, inactive and active SE is nearly indistinguishable by Western blotting (or any other antibody-based method). Secondly only small sample volume (30-50ml) can be loaded onto a gel, which may limit the sensitivity of the method.

When compared directly with ELISA using contaminated mushroom samples, Western blotting was as sensitive as ELISA with native samples and much more sensitive with heat-denatured samples (Rasooly, 1998). A third limitation of western blots is that cross-reactive bands potentially could co-migrate with the antigen. Cross-reactivity is an inherent problem with all immunological methods, because antibodies recognize small regions of proteins and similar epitopes may occur in other proteins (Rasooly, 1998).

Increasing the specificity of the reaction by using monoclonal antibodies minimizes this problem. Several independently isolated antibodies and control samples of uncontaminated similar food can be used to determinate whether the bands represents toxin or unrelated antigens (Rasooly, 1998).

2.9.3 Rapid latex agglutination test for detection of staphylococcal enterotoxins

A polystyrene latex particle sensitized with purified rabbit antiserum raised against purified staphylococcal enterotoxin is used latex particles agglutinate on the presence of the corresponding enterotoxin. The control reagent used consists of latex particles sensitized with non-immune rabbit globulins (Nancy *et al.*, 1986).

The test is performed in v-well microlitre plates. The presence of a staphylococcal enterotoxin is indicated by agglutination that occurs in the form of a diffuse lattice structure, which upon settling forms a diffuse layer on the base of the well. (Nancy *et al.*, 1986). Cenci *et al.* (2003), isolated *S. aureus* strains to determine whether one or more enterotoxins in milk and daily products was linked to staphylococcal food poisoning.

The research isolated 160 *S. aureus* out of 2,343 routine submissions of a composite quarter over a period of 9- month from 18 daily farms in California.

The production of staphylococcal enterotoxins A through D (SEA, SEB, SEC and SED) was evaluated using reverse passive latex agglutination enterotoxins kit. Twenty-two of 160 *S. aureus* isolates produced enterotoxin. Seven produced SEC, 12 produced SED, and 3 produced both SEC and SED. None of the isolates produced SEA or SEB.

Four lots of the Set RPLA kit (Denka Seiken Co. Ltd. Tokyo) a commercial reverse passive latex agglutination test kit for detection of *staphylococcal* enterotoxin A, B, C and D in food have been evaluated for their efficacy (Park and Szabo, 1986). The kits showed high specificity and sensitivity with a detection limit of 0.75ng enterotoxin/g of food. The test is simple and can be completed within 24h, and does not require complicated extraction or concentration procedures or expensive equipment.

These workers found no significant difference in the enterotonigenicity of *S. aureus* from different food source using ELISA.

Fujikawa and Igarashi (1988) used rapid reverse passive latex agglutination method that used high-density latex particles for incubation. It took 3 hours much less than the 16h needed with a customary latex agglutination test.

The rapid test was also efficient in SE detection in foods and culture supernatants of staphylococcal strains, similar to the SET-RPLA test

2.9.4 Enzyme linked immunosorbent assay for detection of staphylococcal enterotoxins in foods

Enzyme linked immunosorbent assay (ELISA) for detection of *S. aureus* enterotoxin (SE) has an enzyme label conjugated either to the enterotoxin as in competitive ELISA (Shifter- Rosenberg and Fey, 1978) or conjugated to the antibody as in sandwish ELISA (Notermans *et al.,* 1978; Koper *et al.*, 1980). Both competitive and sandwish ELISA are performed using, as a solid phase, polystyrene plates coated with anti- SE.

After incubation with the sample, the amount of absorbed toxin is measured using either anti-SE conjugated to an enzyme i.e. sandwich ELISA (Berdall *et al.,* 1981) or SE conjugated to an enzyme (competitive ELISA). The sandwich method was found very sensitive and convenient for use in detecting staphylococcal entertoxin A, B and C (Olsvik *et al.,* 1982). Both polyclonal and monoclonal antibodies have been used in ELISA. The rabbit polyclonal antibody systems have been widely used. The monoclonal antibodies are capable of detecting 1.0ng of enterotoxin per milliliter of food sample.

Both systems provide an unlimited supply of highly uniform reagents and cross-reacting monoclonal antibodies can be adapted to these assay systems (Nancy *et al.,* 1986).

Lenz *et al.* (1983) isolated 340 *S. aureus* strains either from routine food test samples or from food left over from healthy or sick persons in cases of suspected food poisoning in various places of the Federal Republic of Germany. The staphylococcal enterotoxin types A, B and C (SEA, SEB and SEC) was determined both by the ELISA and the microslide test (MS-test) and the enterotoxin type D and E by the MS- tests only. Comparison of the two test methods clearly showed that the ELISA technique was superior to the MS method.

The sensitivity of the ELISA was at 2.5nanogram, where as that of the MS-test was limited to approximately one microgram. ELISA revealed 6.2% more of the strains as enterotoxin producers. Further more the ELISA is far more efficient, consuming less test reagents and with a capacity for testing more strains in a shorter time.

Fey (1987) described a semi-quantitative Staphylococcal Enterotoxins Enzymes immuno Assay (SET-EIA). The SET-EIA is a semi-quantitative set, which detects SE in food and in culture supernatants. A test kit based of this method contains coloured coded polystyrene balls coated with antibodies specific for SET-A, SET-B, SET -C and SET -D. Balls used as negative controls were coated with immunoglobnlins from normal rabbit serum. The cultures suspected to contain staphylococcal enterotoxin were incubated with the coated balls for 4 hours.

The balls were washed with buffer solution to remove unbound non-specific proteins. The balls were washed individually into tubes and incubated with phosphatase labeled second antibody (enzyme conjugate). After washing, p-nitrophenylphosphate substrate (pNPP) was added. Phosphatase labeled antibodies bound to the ball cleaved pNPP to the yellow compound p-nitrophenolate.

The intensity of the yellow colour was proportional to the antigen concentration the detection limit of the test lied between 0.1 to 1 ng staphylococcal enterotoxin 1 ml of the extract depending on the type of food specimens.

2.9.5 Polymerase chain reaction (PCR) for detection of staphylococcal enterotoxins

Polymerase chain reaction (PCR) is one of the most widely used target nucleic acid amplification methods. The method combines the principle of complementary nucleic acid replication that is applied repeatedly through numerous cycles.

By this method, a single copy of a nucleic acid target often undetectable by standard hybridization method is multiplied to 10^7 or more copies within a relatively short period.

This provides ample target that can be readily detected by numerous methods. The PCR involves 30 to 50 repetitive cycles with each cycle comprising three sequential reactions. Denaturation of target nucleic acid, primer annealing to single-stranded target nucleic acid, and extension of primer target duplex (Persing, 1996).

To investigate the distribution of staphylococcal enterotoxin (SE) A to I (SEA to SEI) genes (*sea* to *sei*) in *Staphylococcus aureus*, 146 isolates obtained in Japan from humans involved in samples from food poisoning outbreaks, healthy human cows with mastitis, and bovine raw milk were analyzed by multiplex PCR (Omoe *et al.*, 2002). One hundred thirteen (77.4%) *S. aureus* isolates were found to be positive for one or more *se* genes.

The *se* genotype was classified into 14 genotype *seg* and *sei* coexisted in the same

S. aureus stains SEs have been divided into five serological types (SEA through SEE) on the basis of their antigenicities (Bergdoll, 1983).

In recent years the existence of new types of SEs (SEG, SHE, SEI, SEJ, SEK, SEM, SEN and SEO) has been reported (Jarraud *et al.,* 2001).

Staphylococcus aureus isolates harbouring *seg* and about 60% of the isolates harbouring *sei* did not produce a detectable levels of SEG or SEI, while reverse transcription PCR analysis proved that the mRNAs of SEG and SEI were transcribed in *S. aureus* strains habouring *seg* and *sei* genes.

These results suggest the importance of quantitative assessment of SEG and SEI production in food in order to clarify the relationship between the new SEs and food poisoning (Omoe *et al*, 2002). However the relationship between these new SE,s and human poisoning is not fully understood at present.

It is known that about 95% of staphylococcal food poisoning outbreaks are caused by SE types SEA to SEE (Bergdoll, 1983). The remaining 5% of the outbreaks may therefore be associated with other newly identified SEs.

To clarify the role-played by these newly identified SEs in food poisoning the development of reliable method of detection of SE proteins is essential. Several reports have described the development of a multiplex PCR for the detection of *se* genes (Mclauchlin *et al.,* 2000). However, it is noteworthy that the PCR is only able to demonstrate the capability of *se* genes in *Staphylococcus aureus* isolates and does not prove that the production of SE proteins occurs. To demonstrate the capability of a strain to produce an amount of SE protein that is sufficient to induce disease, bioassays or immunological methods for detecting the SE protein are still important.

Omoe *et al.* (2002) using PCR and Ming-Haung *et al.* (2003) showed that there are three major antigenically distinct SEC subtypes; SECI, SEC2, and SEC 3. The nucleotide sequence homology between SEC 1, SEC 2 and SEC 3 genes is higher than 97%.

Based on the nucleotide sequences of the subtypes of SEC, Ming-Haung (2003) designed SEC subtype specific primers for the detection of individual genes. These were used to investigate the distribution of SEC 1, SEC 2 and SEC 3 *Staphylococcus aureus* strains in staphylococcal food poisoning outbreaks that occurred in central Taiwan between 1995 and 1997.

In an attempt to show that more than one PCR primer set could be designed and used for the differentiation of these SEC genes that are highly homologous to each other, Ming-Haung *et al.* (2003) designed a second set of PCR primers for SEC 1, SEC 2 and SEC 3 enterotoxins.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study area

The study was carried in and around Nairobi City. The area included was Dagoretti Westlands and the Nairobi Central district (Appendix 1).

3.2 Samples for isolation of *Staphylococcus*

3.2.1 Chicken

Fifty (50) chicken carcasses were purchased from various butcheries within the study area. They were wrapped in polythene bags and packed into a cool box containing ice packs, before being transported to laboratory for analysis.

3.2.2 Swabs from beef carcasses

Swabs (100) were taken aseptically from the surfaces of 100 beef carcasses from five slaughterhouses within the study area, just after dressing operations. Wet and dry swabs were used to swab the general body surface of the carcasses but with emphasis on sites that are usually highly contaminated like the brisket, flank, neck and the forequarter. The swabs were then aseptically transferred into universal bottles containing 10 milliliters of 1.0- percent peptone water and 0.5 percent sodium chloride as a transport medium. The universal bottles were then tightly capped.

3.2.3 Raw milk

A total of 200 raw milk samples were collected into sterile universal bottles at the dairy centers within the study area. These were transported to the laboratory and cultured within one hour of collection.

3.2.4 Minced beef

Fifty (50) minced beef samples were bought from butcheries in the study area. The samples were placed in plastic bags and packed in a cool box containing ice packs.

3.3 Isolation of *Staphylococcus aureus*

3.3.1 Cleaning and sterilization of glassware.

Petri dishes, universal bottles, Bijou bottles, pipettes, conical flasks, beakers, and screw-capped test tubes were thoroughly washed and dried in an oven at 80°C. These were then dried and sterilized at 160°C for 2 hours.

3.3.2 Preparation of culture media

Unless otherwise, all media were sterilized by autoclaving at 121°C for 15 minutes. Incubation was done at 37°C for 18-24 hrs (Appendix 2.1 – 2.9).

3.3.3 Isolation of *S. aureus* from raw milk.

Manittol Salt Agar (MSA) was used as the selective and indicator media for isolation of *S. aureus*. The contents of the samples bottle were thoroughly mixed by shaking the bottle. A loopful of milk was streaked directly onto MSA plates. Four suspect *S. aureus* colonies were then picked and sub cultured onto sterile BA plate.

3.3.4 Isolation of *S. aureus* from beef carcasses.

MSA was used as the selective and indicator media for isolation of *S. aureus* from beef carcasses. The contents of the samples bottle were thoroughly mixed by shaking the bottle. A loopful of fluid from carcass swabs was streaked directly onto MSA plates. Four suspect *S. aureus* colonies were then picked and sub cultured onto sterile BA plate.

3.3.5 Isolation of *S. aureus* from minced beef.

Approximately 10 grams of minced beef were transferred into 5 mls of 1.0% bacteriological peptone water, containing 10% sodium chloride, mixed well, and incubated at 37°C for 18-24 hours. A loopful of the enrichment broth was then streaked onto MSA plates and incubated. Four suspect *S. aureus* colonies were then picked, sub cultured onto bovine blood agar plates.

3.3.6 Isolation of *S. aureus* from chicken carcasses.

Peptone water sodium chloride (PWNaCl) was used as an enrichment and selective medium. The carcasses were aseptically skinned, and the skin was placed in 500 mls glass capped bottle containing 100 mls of sterile 1.0% PWNaCl and thoroughly shaken. These were then incubated at 37°C for 18-24 hours. A loopful of the broth was then streaked on MSA plates. Four suspect *S. aureus* colonies were then subcultured on sterile BA.

3.4. Differential and Diagnostic Tests.

3.4.1 Oxidative fermentation test (O/F).

The test was used to differentiate *Staphylococcus* from the genus *Micrococcus*. The test was performed according to Baird-Parker (1966). Suspected *S. aureus* colonies were picked from BA plates and inoculated, in duplicate, into cooled molten glucose tryptone agar in test tubes and the tubes gently swirled to mix the contents. One tube was then covered with a layer of sterile paraffin to a depth of about one centimeter to provide anaerobic conditions, while the other one was left uncovered to allow aerobic growth. Tubes were examined daily for 5 days. *Staphylococci* break down glucose both oxidatively and fermentatively with production of acid that turns the medium yellow in both tubes, while *Micrococci* only do so oxidatively turning the upper part of one of the tubes yellow.

3.4.2 Mannitol fermentation test

The test was used to differentiate coagulase negative *S. aureus* from the species *S. epidermidis.* OFMM medium was used and the test performed according to Baird Parker (1966). *Staphylococcus aureus* breaks down Mannitol both oxidatively and fermentatively with production of acid that turns the medium yellow in both tubes, while *S. epidermidis* breaks down mannitol only oxidatively with production of lactic acid.

3.4.3 Tube coagulase test

A one in ten (1:10) dilution of rabbit plasma in sterile saline (0.85% NaCl) was prepared and distributed in one-milliliter amounts into small tubes. The strains to be tested were inoculated into tubes by adding 0.1 ml each of an 18-24 hours broth culture. Control tests of known coagulase positive and negative cultures and one tube of un-inoculated plasma were incubated together at 37°C in a waterbath and examined for coagulation at 1, 3, and 6 hours. Negative tubes were left in the water bath overnight and re-examined for coagulation. The conversion of the plasma into a stiff gel, best seen on tilting the tube to the horizontal or vertical position, was recorded as positive for coagulase.

3.4.4 Deoxyribonuclease (DNase) production

Suspect *S. aureus* colonies were inoculated on tryptose agar medium containing 2 g/litre of deoxyribonucleic acid by spotting them onto the surface of the agar so that a thick growth was evident after 18-24 hours of incubation at 37°C. The plates were then flooded with 1 N hydrochloric acid, which precipitates DNA and turned the plate cloudy. The appearance of a zone of clearance (absence of turbidity) around the colony denoted a Dnase production and was recorded as a positive test. The coagulase and Dnase tests were used to indicate the potential for the *S. aureus* isolates to produce enterotoxins.

3.4.5 Storage of the isolates

The isolated colonies were inoculated in cooked meat medium and stored at room temperature awaiting analysis for enterotoxins.

3.5 Assay for enterotoxin production

3.5.1 Preparation of entrotoxin for testing by latex agglutination.

Staphylococcus aureus isolates were inoculated into Bacto-tryptic soy broth and incubated at 37°C for 24 hours. The broth culture was filtered through a membrane filter of pore size 0.22µm. The filtrate was then retained for toxin assay.

3.5.1.1 Reverse passive latex agglutination assay

The reverse passive agglutination (SET –RPLA; Oxoid) test kit was used to assay for the presence of staphylococcal enterotoxins in broth culture. The kit consists of polystyrene latex particles sensitized with purified rabbit antiserum raised against purified staphylococcal enterotoxin A, B, C and D. The V-well shaped microlitre plates were arranged such that each row consisted of 8 wells. Five of such rows were used for each sample and 25µl of the diluents (TD910; appendix 3.1) was dispensed into each well of the five rows; and 25µl of the test sample was added to the first well of each of the five rows. Doubling dilution was performed along each of the five rows using a pipette diluter. The dilution was stopped at the 7^{th} well to leave the last well containing the diluents only. Twenty five microlitres of latex sensitized with anti-enterotoxin A was added to each well in the first row, and 25µl of latex sensitized with anti-enterotoxin B to each well of the second row, 25µ l of latex sensitized with anti-enterotoxin C to each of the wells in the third row, 25 µl of latex sensitized with anti-enterotoxin D into each of the wells in the fourth row, and finally 25µl of control latex into each of the wells in the fifth row. Rotating the plates in the micromixer mixed the contents of each well.

Plates were then covered with a lid to avoid evaporation, and left undisturbed in a vibration free surface at room temperature for 20-24 hours. The wells were examined for agglutination using a reflecting mirror. The agglutination patterns were judged and classified as positive if there was an observable diffuse layer of agglutination particles at the base of the well. The test was termed as negative if a tight button formed at the base of the well. Results in the row of the control latex were interpreted as negative in all cases, together with all the wells in the 8th column containing the diluent but not test sample.

3.5.2 Enterotoxin assay by SET-EIA.

Staphylococcus aureus strains were tested for enterotoxin production using SET-EIA test kit according to the manufactures (Labor Dr. W. Bommeli Langgass-strasse 7 Ch- 3012 Bern). One coated plastic all of each antibody SET-A, SET-B, SET-C and SET-D were put into an empty vial. For each enterotoxin type, a control normal rabbit serum plastic ball was also placed in a 50 ml vial. Each of the vials containing the respective balls was washed with sodium chloride-Tween. 4 mls of diluted culture supernant was added to the 8 balls. The vial was then laid on a shaker and agitated at 100rpm at room temperature for 4 hours. After incubation, culture supernatant was poured off and the balls were first washed two times in the vial with wash solution and then transferred into the respective colour coded tubes. The balls were once more washed together with the polystyrene tubes with wash solution. 0.5mls of conjugate was added into the respective tubes and each of the four NRS-control balls equally processed with one of the four conjugates. The tubes with each content were incubated for 2 hours at room temperature without shaking. The plastic balls were then washed with wash solution three times by filling the tube completely and removing the wash solution by use of a cannula attached to an aspirator.

One ml of the substrate solution was added to each tube containing a plastic ball and incubated at room temperature for 60 minutes. For a simple differentiation between positive and negative value, the tube was visualized for a yellow colour.

3.6 Statistical analysis

The data was analyzed using student's t-test to determine the occurrence of *S. aureus* in various foods, the occurrence of SE and also the type of enterotoxin recovered from the isolates using RPLA and ELISA at 0.05 levels of significance.

CHAPTER FOUR: RESULTS

4.1 Prevalence of *Staphylococcus aureus* in foodstuffs

A total of 400 samples of different foodstuffs were tested for the presence of *S. aureus.* They included 50 (12.5%) minced meat (Appendix 4. 1), 100 (25%) beef carcass (Appendix 4.2), 50 (12.5%) chicken (Appendix 4.3) and 200 (50%) raw milk (Appendix 4.4). One hundred and one foodstuffs accounting for 25.4% were contaminated with *S. aureus*. The *S. aureus* were isolated from 22 chicken samples, 16 minced meat, 55 raw milk and 8 beef carcasses. Figure 4.1 shows the prevalence of *S. aureus* in the various foods ranging from 8% in beef carcasses to 44% in chicken. There was significant difference in the prevalence of *S. aureus* in the four different food sources ($t = 4.295$; $df = 3$; $P = 0.023$).

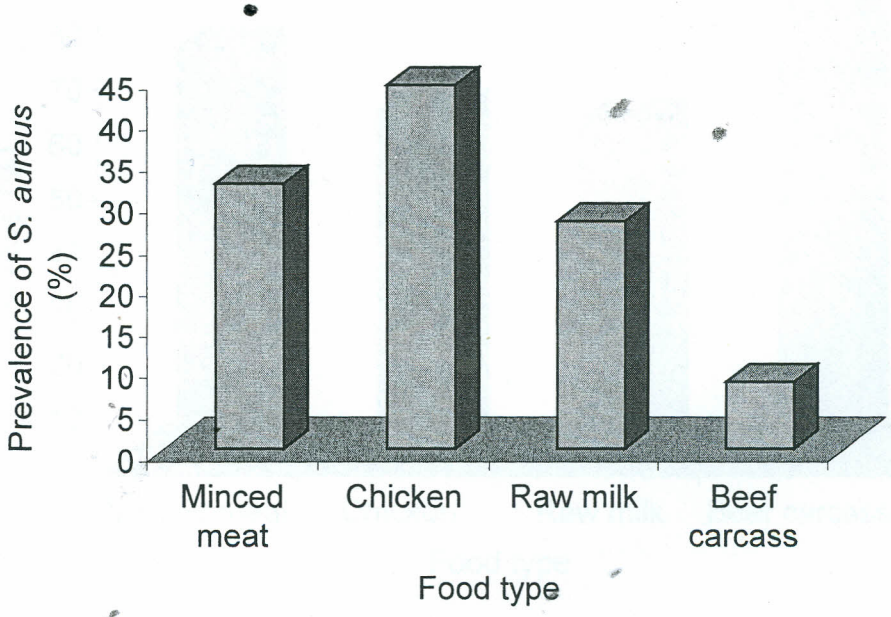

Figure 4.1: Prevalence of *S. aureus* isolated from the four foodstuffs

4.2 Detection of enterotoxigenicity of *S. aureus* using ELISA

All the 101 *S. aureus* isolates were tested for the presence of *S. aureus* enterotoxins (SE). Out of these 67 (66.3%) were positive for the presence of enterotoxins using Enzyme Linked Immunosobent Assay (ELISA). Thirty-four (61.8%) *S. aureus* with enterotoxins were isolated from raw milk while 14 (63.6%), 12 (75%) and 7 (87.5%) were isolated from chicken, minced meat and beef carcass, respectively. The prevalence of enterotoxigenic *S. aureus* among the foods is given in Fig. 4.2. There was a significant difference in the prevalence of enterotoxigenic *S. aureus* from different food sources ($t = 12.111$; $df = 3$; $P = 0.001$).

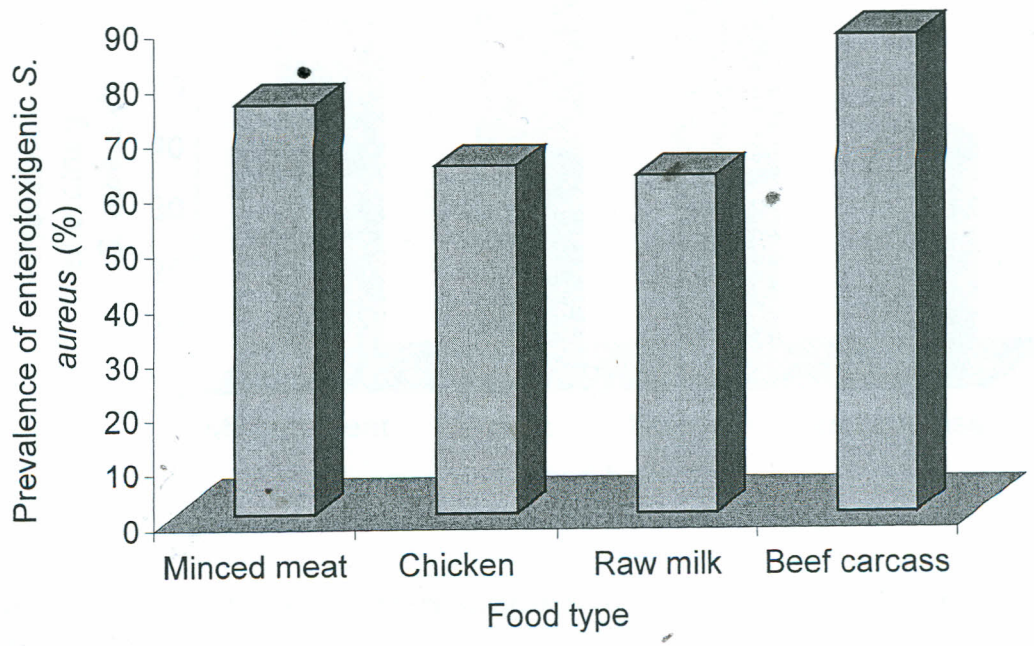

Figure 4.2: Prevalence of enterotoxigenic *S. aureus* from different foods as identified using ELISA

4.3 Detection of enterotoxigenicity of *S. aureus* using RPLA

Out of the 101 *S. aureus* isolated from the four foodstuffs, 62 (61.9%) had the enterotoxins as identified using Rapid Passive Latex Agglutination (RPLA) test. Thirty-six (65.5%) of *S. aureus* with enterotoxin were isolated from raw milk while 14 (63.6%), 9 (56.3%) and 3 (37.58%) were isolated from chicken, minced meat and beef carcass, respectively. The prevalence of enterotoxigenic *S. aureus* from different foods is shown in Fig. 4.3. There was a significant difference in the enterotoxigenicity of *S. aureus* from different food sources ($t = 8.720$; $df = 3$; $P = 0.003$).

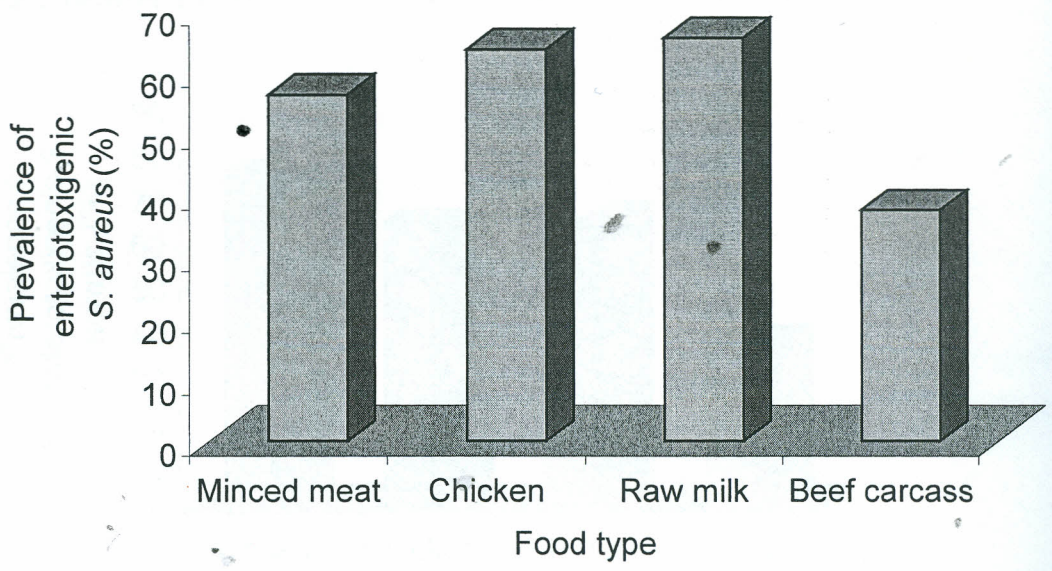

Figure 4.3: Prevalence of enterotoxigenic *S. aureus* from different foods as identified using RPLA

4.4 Comparison of level of detection of enterotoxigenicity of *S. aureus* between ELISA and RPLA techniques

A comparison of the two enterotoxin identification techniques showed that out of the16 *S. aureus* isolated from minced meat, 12 (75%) were found to be enterotoxigenic by ELISA while 9 (56.3%) were by RPLA. Similarly, out of 22 *S. aureus* isolated from chicken ELISA and RPLA identified each 14 (63.6%) as being enterotoxigenic, while of the 55 *S. aureus* isolated from raw milk, 34 (61.8%) and 36 (65.5%) had enterotoxins as identified by ELISA and RPLA, respectively. Similarly, the 8 *S. aureus* isolated from beef carcass, 7 (87.5%) and 3 (37.5%) were enterotoxigenic as identified by ELISA and RPLA, respectively (Fig. 4.4). There was however no significant deference in enterotoxin detection between ELISA and RPLA ($t = 1.324$; $df = 3$; $P = 0.277$).

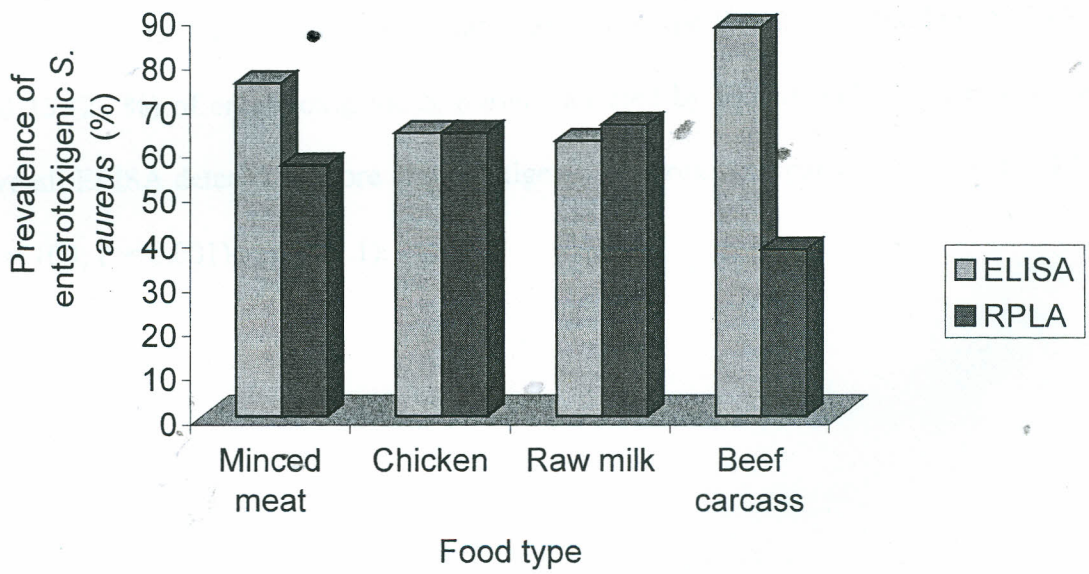

Figure 4.4: Prevalence of enterotoxigenicity of *S. aureus* isolated from the different foods as identified by ELISA and RPLA

4.5 Diversity of enterotoxins among the *S. aureus* isolates

Four different enterotoxins types were tested for among *S. aureus* isolates. These were enterotoxin types A, B, C and D. The most frequent enterotoxin type was C, which was detected in 59 (88.1 %) of the enterotoxigenic *S. aureus* by ELISA and 61 (98.4 %) by RPLA. There was no significant difference in the prevalence of enterotoxin type C as identified by the two tests ($t = 0.815$; $df = 100$; $P = 0.417$). Enterotoxin type A was found in 41 (61.2 %) of enterotoxigenic *S. aureus* detected by ELISA and 32 (51.6 %) detected by RPLA. The prevalence of enterotoxin type A between the two tests was significantly different ($t = 2.601$; $df = 100$; $P = 0.011$). Enterotoxin type B was identified in 31 (46.3 %) and 21 (33.9 %) of enterotoxigenic *S. aureus* detected by ELISA and RPLA respectively. There was a significant difference between the two tests ($t = 3.315$; $df = 100$; $P = 0.001$). The least common enterotoxin was type D that was found in 26 (38.8 %) and 13 (21 %) of enterotoxigenic *S. aureus* detected by ELISA and RPLA respectively. Overall ELISA detected 5 more enterotoxigenic *S. aureus* compared to RPLA ($t = 4.176$; $df = 100$; $P = 0.001$) (Table 4.1).

Table 4.1: Comparison of isolates testing for various enterotoxins types using ELISA and RPLA

Enterotoxin type	No. (%) positive by ELISA. (N = 67)	No. (%) positive by RPLA (N = 62)
SEA	41(61 %)	32 (51.6 %)
SEB	31(46.3 %)	21 (33.9 %)
SEC	59 (88.1%)	61(98.4 %)
SED	26 (38.8 %)	13 (21 %)

4.6 Distribution of enterotoxin types in *S. aureus* isolated from the four food sources

4.6.1 Enterotoxin type A

Out of 101 *S. aureus* isolated from the four different food sources, 32 (47.9%) had enterotoxin type A. Seventeen (50%) of these enterotoxigenic *S. aureus* with enterotoxin type A were isolated from raw milk while 12 (100%), 9 (64.3%) and 3 (42.9%) were from minced meat, chicken and beef carcass, respectively (Table 4.2). The occurrence of enterotoxin type A was more common in *S. aureus* isolated from raw milk as identified using ELISA ($t = 14.070$; $df = 3$; $P = 0.001$). Out of the 101 *S. aureus* 32 (51.6 %) had the enterotoxin type A as identified by RPLA. Fifteen (41.7%) of enterotoxin type A producers were *S. aureus* isolated from raw milk, while 7 (50%), 7 (77.8%) and 3 (100%) were isolated from chicken, minced meat and beef carcass respectively (Table 4.3). There was a significant difference in the occurrence of enterotoxin type A among the food sources as identified by RPLA ($t = 5.001$; $df = 3$; $P = 0.015$).

4.6.2 Enterotoxin type B

Thirty-one (46.3%) of enterotoxigenic *S. aureus* had enterotoxin type B as identified by ELISA. Fifteen (44.1%) of the enterotoxin type B were *S. aureus* isolated from raw milk while 7 (50.7%), 5 (41.7%) and 4 (57.1%) were isolated from chicken, minced meat and beef carcass respectively (Table 4.2). The occurrence of enterotoxin type B was significantly different among the foodstuffs as detected by ELISA ($t = 8.099$; $df = 3$; $P = 0.004$). Twenty-six (41.9%) of enterotoxigenic *S. aureus* had enterotoxin type B as identified by RPLA. Eight (22.2%) of these were *S. aureus* isolated from raw milk while 5 (35.7%), 5 (55.6%) and 3 (100%) were isolated from chicken, minced meat and beef carcass respectively (Table 4.3). The occurrence of enterotoxin type B was significantly different among the foods as detected RPLA ($t = 3.142$; $df = 3$; $P = 0.05$).

4.6.3 Enterotoxin type C

Fifty-nine (88%) of enterotoxigenic *S. aureus* produced enterotoxin type C as identified using ELISA. Thirty-three (97%) enterotoxin type C *S.aureus* producers were isolated from raw milk, while 13 (93%), 9 (75%) and 4 (57%) were isolated from chicken, minced meat and beef carcass respectively (Table 4.2). The occurrence of enterotoxin type C was significantly different among the isolates from the four sources ($t = 8.770$; $df = 3$; $P = 0.003$). Sixty-one (98.4%) of enterotoxigenic *S. aureus* produced enterotoxin type C as identified using RPLA. Thirty-five (97.2%) of enterotoxin *C S. aureus* producers were isolated from raw milk, while 14 (100%), 9 (100%) and 3 (100%) were isolated from chicken, minced meat and beef carcasses, respectively (Table 4.3). The occurrence of enterotoxin type C was significantly different as detected by RPLA ($t = 132.33$; $df = 3$; $P = 0.001$) among the foods.

4.6.4 Enterotoxin type D

Twenty-six (38.8 %) of enterotoxigenic *S. aureus* isolates had enterotoxin type D as identified using ELISA. Twelve (35%) of the isolates with this enterotoxin were isolated from raw milk, while 5 (35.7%), 5 (41.7%) and 4 (57%) were isolated from chicken, minced meat and beef carcass respectively (Table 4.2). There was a significant difference in the distribution of this enterotoxin among the isolates from different foods ($t = 8.288$; $df = 3$; $P = 0.004$). Thirteen (21%) of enterotoxigenic *S. aureus* had enterotoxin type D as identified using RPLA. Eight (22.2%) of these were isolated from raw milk, while 3 (21.4%) and 2 (22.2%) were isolated from chicken and minced meat, respectively, as shown in Table 4.3. The occurrence of enterotoxin type D as detected by RPLA was not significantly different with the food source ($t = 2.998$; $df = 2$; $P = 0.058$). No enterotoxin type D was detected by RPLA from beef carcass.

Table 4.2: The distribution of *S. aureus* enterotoxins types among isolates from various foods as identified by ELISA

FOOD TYPE	SEA	SEB	SEC	SED	Total *S.aureus* isolated
Raw milk	17 (50 %)	15 (44.1%)	33 (97 %)	12 (35 %)	34 (100 %)
Chicken	9 (64.3%)	7 (50%)	13 (93 %)	5 (35.7 %)	14 (100 %)
Minced meat	12 (100%)	5 (41.7 %)	9 (75 %)	5 (41.7 %)	12 (100 %)
Beef carcass	3 (42.9 %)	4 (57.1%)	4 (57 %)	4 (57 %)	7 (100 %)
Total enterotoxion	41(61.2%)	31 (46.3 %)	59 (88 %)	26 (38.8 %)	67 (100 %)

Table 4.3: The distribution of *S. aureus* enterotoxins types among isolates from various foods as identified by RPLA

FOOD TYPE	SEA	SEB	SEC	SED	Total *S.aureus* isolated
Raw milk	15 (41.7 %)	8 (22.2 %)	35 (97.2 %)	8 (22.2 %)	36 (100 %)
Chicken	7 (50 %)	5 (35.7 %)	14 (100 %)	3 (21.4 %)	14 (100 %)
Minced meat	7 (77.8 %)	5 (55.6 %)	9 (100 %)	2 (22.2 %)	9 (100 %)
Beef	3 (100 %)	3 (100 %)	3 (100 %)	0	3 (100 %)
Total enterotoxion	32 (51.6 %)	26 (41.9 %)	61 (98.4 %)	13 (21 %)	62 (100 %)

4.7 Frequency distribution of the enterotoxin combination types as identified by ELISA and RPLA

A total of four enterotoxin combination types were present in the *S. aureus* isolates. These included one four-enterotoxin combination (quadruplet), 2 triple, 3 double and 4 single enterotoxin types.

In general, ELISA identified 25 (38.5%) of enterotoxigenic *S. aureus* as single enterotoxin producers, 13 (20%) of the isolates as two enterotoxin type producers, 6 (9.2 %) three enterotoxin types and 21 (32.3%) all the four enterotoxin types (Table 4.4). A total of seven enterotoxin combination types were identified using RPLA. Twenty-nine of enterotoxigenic *S. aureus* produced a single enterotoxin type, 10 (16.1%), 14 (22.6%) and 9 (14.5%) produced two, three and all the four enterotoxin types, respectively (Table 4.5). RPLA did not detect any *S. aureus* isolate to produce SEA and SEB alone.

Table 4.4: The number of *S. aureus* producing SE combination types as identified by ELISA

FOOD TYPE	Raw milk	Chicken	Minced meat	Beef carcass	Total isolates
Single combination					
SEA	1 (3 %)	1 (7.1 %)	3 (27.3 %)	0	5
SEB	0	0	0	1 (14.3 %)	1
SEC	13 (39.4 %)	3 (21.4 %)	0	1 (14.3 %)	17
SED	0	0	0	2 (28.6 %)	2
Subtotal	14	4	3	4	25 (38.5 %)
Double combination					
A + C	3 (9.1 %)	2 (14.3 %)	3 (27.3 %)	0	8
B + C	2 (6.1 %)	2 (14.3 %)	0	0	4
C + D	1 (3 %)	0	0	0	1
Subtotal	6	4	3	0	13 (20 %)
Triple combination					
A + B + C	2 (6.1 %)	1 (7.1 %)	0	1 (14.3 %)	4
A + C + D	0	1 (7.1 %)	1 (9.1 %)	0	2
Subtotal	2	2	1	1	6 (9.23 %)
Quadruple combination					
A + B + C + D	11 (33.3 %)	4 (28.4 %)	4 (36.4 %)	2 (28.6 %)	21
Subtotal	11	4	4	2	21 (32.3 %)
TOTAL	33 (100 %)	14 (100 %)	11 (100 %)	7 (100 %)	65

Table 4.5: The number of *S. aureus* producing SE combination types as identified by RPLA

FOOD TYPE	Raw milk	Chicken	Minced meat	Beef carcass	Total isolates
Single combination					
SEA	1 (2.8 %)	0	0	0	1
SEC	20 (55.6 %)	6 (42.9 %)	2 (22.2 %)	0	28
Subtotal	21	6	2	0	29 (46.8 %)
Double combination					
A + C	3 (8.3 %)	3 (21.4 %)	0	0	6
B + C	1 (2.8 %)	1 (7.1 %)	2 (22.2 %)	0	4
Subtotal	4	4	2	0	10 (16.1 %)
Triple combination					
A + B + C	3 (8.3 %)	1 (7.1 %)	3 (33.3 %)	3 (100 %)	10
A + C + D	4 (11.1 %)	0	0	0	4
Subtotal	7	1	3	3	14 (22.6 %)
Quadruple combination					
A + B + C + D	4 (11.1 %)	3 (21.4 %)	2 (22.2 %)	0	9
Subtotal	4	3	2	0	9 (14.5 %)
TOTAL	36 (100 %)	14 (100 %)	9 (100 %)	3 (100 %)	62

4.8 Diversity of enterotoxin isolated among the foods.

4.8.1 Single enterotoxin combination

Four single enterotoxin types were produced by *S. aureus* isolates as identified by ELISA. Enterotoxin type C was the most common produced by 17 isolates, followed by SEA produced by 5, SED, produced by 2 and SEB produced by one of the isolates. Of the 17 SEC producers, 13 were isolates from raw milk, 3 from chicken and one from beef carcass. Of the 5 SEA, 3 were from minced meat, and one each from raw milk and chicken. All the two SED were isolated from beef carcasses as well as the one SEB producer.

Two single enterotoxin types were identified by RPLA among the different food sources. Enterotoxin C was produced by 28 isolates, of which 20 were from raw milk 6 from chicken and 2 from minced meat, while SEA was produced by only one isolate from raw milk (Table 4.5).

4.8.2. Double enterotoxin combination.

There were 13 *S. aureus* isolates with double enterotoxin combinations as identified by ELISA. The most common combination was A and C produced by 8 of the isolates, 3 of which were from raw milk, 3 from minced meat and 2 from chicken. B and C enterotoxin combination were 2 produced by 4 *S. aureus* isolates from raw milk and chicken. C and D combination was produced by only one isolate from raw milk. Two different double enterotoxin combinations were identified by RPLA. A and C combination type was produced by 6 isolates, 3 of which were from raw milk and 3 from chicken. The other combination type was B and C, which were produced by 2 isolates, isolated from chicken (1) and beef carcasses (1), respectively, (Table 4.5).

4.8.3. Triple enterotoxin combination type.

Two triple combination types were identified using ELISA. The ACD combination type was produced by 4 isolates 2 of which were from raw milk, one from chicken, and one from beef carcasses. ABC combination was also produced by 2 isolates, one of which was isolated from chicken and one from minced meat (Table 4.4).

Ten isolates produced ABC enterotoxin combination as identified by RPLA. Three isolates each with this combination were from raw milk, beef carcasses an minced meat, while one was from chicken. Combination ACD was produced by 4 isolates, all from raw milk (Table 4.5).

4.8.4 Quadruple enterotoxin combination

There were 19 *S. aureus* isolates, with all the ABCD enterotoxins as identified by ELISA. Of these, 9 were isolated from raw milk, while 4, 4, and 2 were from minced meat, chicken, and beef carcass respectively. (Table 4.4). Using the RPLA, 9 *S. aureus* had the quadruple enterotoxin combination, which were produced by 4, 3 and 2 S. *aureus* isolated from raw milk, chicken and minced meat, respectively (Table 4.5).

CHAPTER FIVE: DISCUSSION

5.1. Isolation of *Staphylococcus aureus* from milk and meat products.

The *S. aureus* isolated from chicken accounted for 22 (44%). This was relatively high in comparison with the other foodstuffs. This may have been attributed to the high moisture content of the chicken carcasses, which favours the growth of *Staphylococcus aureus*. When the chicken carcasses are being processed, there is increased human handling especially when plucking off the feathers and transporting. Unhygienic and high temperature conditions favours multiplication of *S. aureus* (Bergdoll, 1889). Using water that is highly contaminated with *S. aureus* during processing may also be a contributing factor. Kitai *et al.* (2005) collected a total of 444 samples of raw chicken meat (thighs, wings, livers, gizzards, heart and ovaries) that retailed at 145 super markets in Japan. The researchers isolated 292 (65.8%) *S. aureus* from the samples. The study showed no significant difference in the rate of detection of *S. aureus.*

In this study, 8 out of 100 (8%) beef carcasses were positive for *S. aureus* in comparison with minced meat, which accounted for 16 out of 50 (32%). Minced meat is a product of beef carcasses and during processing there is increased human handling. Since human beings are natural reservoirs for *S. aureus*, sneezing, coughing, laughing and touching the meat, may transmit the organism. As the grinder cuts the meat, bacterial lumps are broken and microorganisms become redistributed. Muscle cells are broken down releasing nutrients, which become readily available to bacteria and the increased surface area created by mincing gives a bigger room for increased multiplication of *S. aureus*.

There was low rate of isolation of *S. aureus* from freshly slaughtered beef carcasses in comparison with the other foodstuffs. This indicates that the meat is not highly contaminated immediately after slaughter. Factors, which may contribute to low isolation, may include the short period of time between slaughter and sampling, which may not allow high contamination. The washing of the carcasses with chlorinated water would have reduced the number of the strains on the surface. Hygienic conditions maintained by the veterinary officer in the slaughterhouse and little human handling before the sample was taken would have reduced surface contamination.

The study revealed that 55 (27.5%) of *S. aureus* were isolated from raw milk. Milk is a suitable medium for growing of many groups of microorganism such as coagulase-negative *Staphylococci* and *S. aureus*. Dudrikova and Pilicincova (2003) found a total of 41.8% of raw sheep milk samples to be positive for S. *aureus*, which is higher in comparison with what is reported in this study.This shows that the organism is common in raw milk. Travnicek *et al.* (2003) isolated 7.1% *S. aureus* from clinically healthy ewes from Slovak breeds. Abo Elnaga *et al.* (1985) confirmed the presence of *S. aureus* in 16% of milk samples from sheep. One of the frequent explanation of higher *S. aureus* incidence in milk can be as a result from subclinical mastitis, as reported by Al Maljali and Jawabreh (2003). Higher incidence may also be due to contamination of milk from improper hand processing. Kayihura *et al.* (1987) found that properly pasteurized milk could free *S. aureus* contamination except a few cases that were attributed to underpasteurization or post pasteurization contamination. Tondo *et al.* (2000) showed that 19 out 51(35.2%) of food handlers were asymptomatic carriers of *S. aureus* and that 19 out of 21(90.4%) of raw milk sampled was contaminated. *Staphylococcus aureus* was isolated from only 10 samples from more than 3200 investigated dairy products.

5.2. Occurrence of enterotoxigenic *S. aureus* in milk and meat products.

The isolated *S. aureus* were tested for staphylococcal enterotoxin (SE). In total, sixty seven (67) out of 101 (66.3%) *S. aureus* isolates were found to produce enterotoxins using ELISA. Beef carcass had the highest numbers of *S. aureus* producing enterotoxin, which accounted for 87.5% followed by minced meat (75%), chicken (63.6%) and raw milk (61.8%).

There was a significant difference in the enterotoxigenicity of *S.aureus* from different food sources using ELISA. Using reverse passive latex agglutination, out of 101 *S. aureus* isolated from the four foodstuffs, 62 (61.9%) produced enterotoxins. Thirty-six (65.5%) of *S. aureus* with enterotoxin were isolated from raw milk while 14 (63.6%), 9 (56.3%) and 3 (37.5%) were isolated from chicken, minced meat and beef carcasses respectively.

Using ELISA, Rosec *et al.* (1997) found that out of 121 *S. aureus* isolated from foodstuff only 15.9% were enterotoxigenic from raw milk and cheeses while 43% were from other foodstuff. Cenci-Goga *et al.* (2003) found that twenty-two of 160 (13.75%) milk and dairy products were linked to staphylococcal food poisoning. Studies by Isigid *et al.* (1986) found that 100% of the 59 *S. aureus* strains from minced meat were enterotoxigenic. Casman *et al.* (1970) found 23 out of 157 (15%) of *S. aureus* strains isolated from cases of bovine mastitis to produce enterotoxin. Umoh *et al.* (1988) isolated enterotoxigenic *S. aureus* strains from Nigerian fermented milk products.

They reported 6.5% (37 out of 568) *S. aureus* strains from three fermented milk products, i.e. fura (fermented millet, or sorghum milk products), nono (fermented milk) and Manshanu (local butter products from fermented milk) to be enterotoxigenic.

5.3 Detection of types of staphylococcal enterotoxin produced by *Staphylococcus aureus* using RPLA & ELISA.

Out of 101 *S. aureus* isolated from the four different food sources, 32 (47.9%) had enterotoxin type A (SEA). The occurrence of SEA was more common in *S. aureus* isolated form minced meat as identified by ELISA, which accounted for 100%. Using RPLA, minced meat had still the highest percentage of SEA. There was however a significant difference in the occurrence of enterotoxin type A among the food sources as identified by RPLA. Kayihura *et al.* (1987) isolated 250 *S. aureus* strains from milk, 65.6% (164 of the 250) of which produced SEA. The workers found that 3 out of the 99 pasteurized milk samples yielded SEA producing *S. aureus* strains.

In this study, beef carcass had the highest percentage of staphylococcal enterotoxin type B (57.1%). Among the *S. aureus* isolated from beef carcasses, all the 3 (100%) were identified by RPLA as enterotoxin B producers. The occurrence of enterotoxin type B was significantly different among the food sources ($t=3.142$; $df = 3$; $P=0.05$). Nader Fildo *et al.* (1988) found enterotoxin B producing *S. aureus* from milk drawn from cows with subclinical mastitis. The potential for the production of enterotoxins was obvious in those cases in which raw milk containing enterotoxigenic type was poorly handled, or received sub-lethal heat treatment.

Holeekova *et al.* (2002) investigated the production of staphylococcal enterotoxin A and B (SEAand SEB) and the presence of respective staphylococcal enterotoxin genes from *S. aureus* isolated from foods and food industry manufacturers in East Slovakia. The workers used Radioimmunoassay (RIA), polymerase chain reaction (PCR), and dot-blot hybridization.

The ability to synthesis enterotoxins was found in 20(39.2%) of the total number of 51 isolates. Production of SEA was recorded in 3(5.9%), production of SEB in 12(23.5%), and production SEA together with SEB in 5(9.8%) staphylococcal isolates. These researchers recorded no differences in results between RIA and PCR as well as PCR and dot-blot hybridization. Fifty-nine (88%) *S. aureus* had enterotoxin type C as identified by ELISA while sixty-one (98.4%) *S. aureus* had enterotoxin type C as identified by RPLA.

Raw milk had the highest percentage of enterotoxin type C (SEC) as detected by ELISA while 100% of enterotoxigenic *S. aureus* isolates from chicken, minced meat, and beef carcasses produced SEC as detected by RPLA. Among the enterotoxigenic strains from the various food types, ELISA frequently encounter the most SE producing *S. aureus* strains with 97-100% by RPLA and 57-97% . In this study, SED was the least detected by ELISA, which accounted for 38.8% and 21% by ELISA and RPLA, respectively. This is contrary to reports from other parts of the world where SEA and SED have been found to be the most commonly encountered enterotoxin strains (Casman *et al.,* 1967; Evan *et al.*, 1983; and Adesiyun, 1984). In this study it was found that a single isolate of *Staphylococcas aureus* could produce more than one enterotoxin type. Thirty two percent of the isolates produced all the enterotoxin as studied by ELISA while 14.5% produced all the four enterotoxin by RPLA.

Studies by Cenci-Goga *et al.* (2003) on the ability of *S. aureus* strains to produce one or more enterotoxin in milk and dairy products showed that twenty-two of 160 *S. aureus* isolates produced enterotoxin. Seven produced both SEC and SED and none of the isolates produced SEA or SEB.

Production of one, or more than one enterotoxin combinations relies on regulation of the gene expressing the enterotoxin by accessory gene regulator *(agr)* locus. It is interesting that the production of SEB (agr+. *agr*-dependent) is dependent on the ability of *S. aureus* to grow to a high cell density upto (10^6 CFU/ ml) as reported by Leloir *et al.* (2003). On the contrary, SEA (agr-*agr*-independent) is present on the medium after 180 minutes of cultivations, meaning that SEA production is a function of the growth stage (Rasooly, 1998).

According to Grieger *et al* (1990), a low percentage (10-20%) of enterotoxigenic strains have been found in bovine and other animal *S. aureus* biotypes, in comparison with high percentage (70%) in human biotypes. On the contrary, Bautista *et al.* (1988) detected synthesis of 4 enterotoxin type (SEA, SEB, SEC and SED) in 62.9% of Staphylococcal strains isolated from sheep milk, with the highest production of SEA and SED. Novick (2002) suggested that environmental factors play an important role in SEs gene expression.

5.4 A comparison of RPLA and ELISA for the detection of staphylococcal enterotoxins.

The two enterotoxin identification techniques showed that out of the 16 *S. aureus* isolated from minced meat,12(75%) were found to be enterotoxigenic by ELISA while 9(56.3%) were positive by RPLA.

Out of 22 *S. aureus* isolated from chicken, ELISA and RPLA identified each 14 (63.3%) as being enterotoxigenic. There was no significant difference in enterotoxin detection by the two techniques (t= 1.324; df=3; P=0.277).

Adesiyun *et al.* (1992) isolated a total of 1015 strains from human beings, animals and food and tested for staphylococcal enterotoxin SEA, SEB and SEC. Of these, 495 (48.8%), 467 (46.0%) and 204 (20.1%) were classified as enterotoxigenic by the ELISA, RPLA, and modified Onchterlony precipitation test (MOPT), respectively.

The difference in the number of strains classified as enterotoxigenic by ELISA and RPLA tests were not significant (P>or = 0.05, chi 2), but both tests detected significantly (P<0.001; χ^2) more enterotoxigenic strains than the MOPT. All factors considered, the RPLA test appears most suitable for quantitatively screening a large number of strains for staphylococcal enterotoxin because it is less cumbersome and few reagents are used.

Staphylococcal food poisoning can be caused by consumption of food sources of animal origin. The problem can be complicated due to the organisms producing other staphylococcal enterotoxin due to the organism processing genes that coexist in the same *S. aureus* strains. The onset of symptoms in staphylococcal food poisoning is usually rapid and in many cases acute, depending on individual susceptibility to the toxin, the amount of toxin in the food ingested and the general health of the victim.

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATION

6.1. Conclusions

The following conclusions can be made from the results of the study.

1. Chicken carcass accounted for the highest percentage of *Staphylococcus aureus* isolated in the four foodstuffs and therefore it poses a high risk of staphylococcal food poisoning.
2. Beef carcass had the highest number of *S. aureus* producing enterotoxin, therefore there is high risk of staphylococcal intoxication due to consumption of beef meat, which may result from improper handling of meat and meat products during processing hence increasing levels of contamination and thus high chances of food poisoning.
3. Beef carcass, chicken, minced meat and milk mainly produced SEC and SEA while even some produces SEB and SED. Some strain of *Staphylococcus aureus* produced one or more enterotoxins.
4. There was no difference in detection of staphylococcal enterotoxin using RPLA and ELISA.

6.2. Recommendation

1. There is need for educating consumer, food handlers, and processors so as to avoid high levels of *Staphylococcus* contamination and consequently food poisoning.
2. In the diagnosis of Staphylococcal foodborne illness, proper interviews with the victims and gathering and analyzing epidemiological data are essential so as to know the cause of the disease.
3. There is need for considerable research efforts for better understanding of the interaction between *S. aureus*, the food matrix and the mechanism of staphylococcal enterotoxins
4. In the investigation for staphylococcal enterotoxins either ELISA or RPLA can be used for the detection of the toxins.
5. There is need for more research for the identification of new staphylococcal enterotoxins and the role they play in food poisoning.

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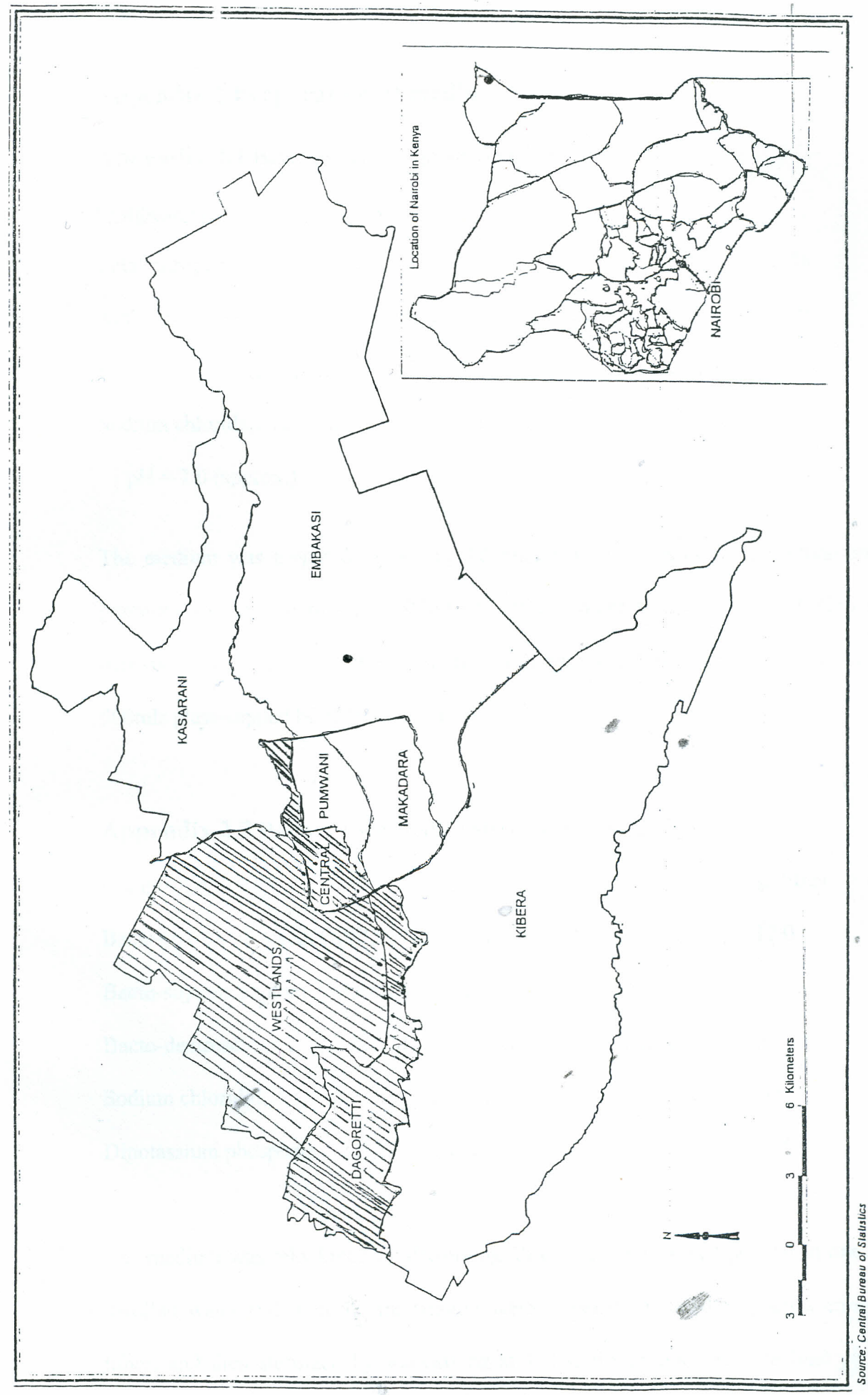
APPENDIX

Appendix 1. Map of Nairobi showing the study area.



Source: Central Bureau of Statistics

Appendix 2 Preparation of media

Appendix 2.1 Bacteriological peptone (Lab M)

Composition

Total nitrogen.....12%

Amino nitrogen.....5.0%

Ash.....9.0%

Sodium chloride.....4.0%

pH = 7.0 (approx.)

The medium was prepared by adding 10 grammes of bacteriological peptone and 100 grammes sodium chloride to 1000ml of distilled water to give a concentration of 1% peptone water and 10% NaCL. Hundred milliliters of the medium were dispensed into 500mls glass-capped bottle and sterilized.

Appendix 2.2 Bacto-Tryptic broth (TSB) (Difco Laboratories)

Composition	g/ litres
Bacto-trytone of casein (USP)	17.0
Bacto-soytone peptone (Soybean)	3.0
Bacto-dextrose	2.5
Sodium chloride	5.0
Dipotassium phosphate	2.5

The medium was rehydrated by dissolving 30 g of the dehydrated powder in one liter of distilled water and then 5.0 ml amount were dispensed into 10 mls screw capped test tubes, and then sterilized by autoclaving at 121°C for 15 minutes. The final pH of the medium was 7.3 at room temperature.

Appendix 2.3 Blood Agar base (lab M)

Composition	g/l
Lab M Beef Extract	10.0
Lab M Balanced peptone No.1	10.0
Sodium chloride	5.0
Lab M. agar No.2	12.0

pH = 7.3 approximately.
This was prepared by dissolving 37 grams of blood agar base (BAB) in one litre of distilled water, and sterilized. The molten BAB was cooled to about 50°C. The 50 ml of sterile defibrinated bovine blood, warmed at 37°C, was added to give 5% blood (v/v) final concentration.

Appendix 2.4 DNase Agar

Composition	g/litre
Tryptone	20.0
Deoxyribonucleic acid	2.0
Sodium chloride	5.0
Agar	12.0

pH 7.3(approx.).

The medium was prepared by dissolving the DNase test agar in distilled water at a rate of 42 g/litre, sterilized and then dispensed into petri dishes. The final pH of the medium was 7.3.

Appendix 2.5 Mannitol salt agar (Lab M)

Composition

Lab M Beef extract.....1.0g

Lab M balanced peptone No. 1.....10.0g

Sodium chloride.....75.0g

D-Mannitol.....10.0g

Lab M agar No. 2.....12.0g

Phenol red.....0.025g

pH = 7.4

Hundred and eight grams (108g) of MSA were added to 1000ml of distilled water and the mixture brought to boil with frequent mixing to dissolve the solids. The medium was sterilized, cooled in the waterbath, and distributed into Petridishes.

Appendix 2.6 Nutrient broth (Oxoid)

Composition

Peptone.....10.0

Lab lemco powder.....10.0

Sodium Chloride.....5.0

Dextrose.....2.0

28 grams of powder was suspended in 1 liter of distilled water. It was brought to boil to dissolve completely and sterilized by autoclaving at 121°C for 15 minutes.

Appendix 2.7 Oxidative fermentation glucose medium (OFGM)

Composition	% w/v
Tryptone (Lab M)	1.0
Yeast (Gibco)	0.1
Glucose (BDH chemicals)	1.0
Bacteriology agar No.1	0.5
Bromocresol purple (BDH)	0.004
Distilled water	100mls.

This was made by dissolving 1.0% tryptone, 0.1% yeast, 1.0% glucose 0.5% bacteriological agar No. 1 and 0.004% bromocresol purple in 100 ml of distilled water and the medium brought to boil. The pH of the solution was then adjusted to 7.2 at 50°C, and 5-ml amounts dispensed into 10-ml screw capped tubes, which were then sterilized.

Appendix 2.8 Oxidative fermentation mannitol medium (OFMM)

Composition	% w/v
Tryone (Lab M)	1.0
Yeast (Gibco)	0.1
D-mannitol (Lab M)	1.0
Bacteriological agar N.o.1	0.5
Bromocresol purple (BDH)	0.004
Distilled water	100ml

This was made by dissolving 1.0% tryptone, 0.1% yeast 1.0% Mannitol 0.5% bacteriological agar No. 1 and 0.004% bromocresol purple in 100 ml of distilled water and the medium brought to boil. The pH of the solution was then adjusted to 7.2 at 50°C, and 5-ml amounts dispensed into 10-ml screw capped test tubes and sterilized.

Appendix 2.9 Trytone (Lab M)

A pancreatic digest of lactose-free casein

Composition

Total Nitrogen 12.7%

Amino nitrogen.....5.1%

Ash.....6.1%

Sodium chloride.....0.5%

pH 7.2(2% soln)

30 grams of the powder was dissolved in 1 liter of distilled water and mixed well to dissolve. It was heated to dissolve completely and distributed into final containers and then sterilized by autoclaving at 121°C for 15 minutes.

Appendix 2.10: Preparation of rabbit plasma

New Zealand white rabbits were bled into sterile universal bottles containing 0.3ml of membrane sterilized 20% (w/v) sodium citrate as anti-coagulant. The blood was then centrifuged at 1062g for 15 minutes. The plasma was aseptically transferred to clean sterile screw capped tubes and stored at 4°C, if it was not to be used immediately.

Appendix 2.11: Physiological Saline.

Sodium chloride was dissolved in distilled water at a rate of 0.85% (w/v) and sterilized.

Appendix 2.12: Cooked meat medium

The medium was prepared by adding 0.3g of meat granules to 4.0 ml of nutrient broth per bijou bottle.

Appendix 3: Components of test kits

Appendix 3.1 Component of the SET-RPLA kit

TD 901

-A latex sensitized with anti-enterotoxin A
-A latex suspension sensitized with specific antibodies
-Rabbit IgG against SEA

TD 902

-Latex sensitized with anti-enterotoxin B
-Latex suspension sensitized with specific antibodies (rabbit IgG) against SEB

TD 903

-Latex sensitized with anti-enterotoxin C
-Latex suspension sensitized with specific antibodies (rabbit IgG) against SEC

TD 904

-Latex sensitized with anti-enterotoxin D
-Latex suspension sensitized with specific antibodies (rabbit IgG) against SED.

TD905

-Control latex
-Latex suspension coated with non-immune rabbit globulin

TD906 SEA

TD907 SEB

TD908 SEC

TD909 SED

TD910 -Diluent

-PBS containing bovine serum albumin and sodium hexaphospate

Appendix 3.2 Component of the Staphylococcal Enterotoxin SET-EIA

Package of SET-EIA (Cat.No.40010).

1 tube with 10 anti SET-A plastic balls

1 tube with 10 anti SET-B plastic balls

1 tube with 10 anti SET-C plastic balls

1 tube with 10 anti SET-D plastic balls

1 tube with 20 control plastic balls, coated with rabbit immunoglobulins free of SET-antibodies

2 x 4ml anti SET-A enzyme-conjugate

2 x 4ml anti SET-B enzyme-conjugate

2 x 4ml anti SET-C enzyme-conjugate

2 x 4ml anti SET-D enzyme-conjugate

2 x 4ml normal rabbit serum (NRS), for the absorption of staphylococcal protein A.

100 ml stock solution of the wash solution (10-times concentration) NaCL-Tween

(Diluted): 0.14 M NaCL, 0.1% Tween "20"

80ml sublets of 20mg p- nitrophenyl phosphate each (pNPP)

60 plastic tubes with different color codes

SUPPLEMENTARY REAGENTS

Cat. No 4-0060 SET (A, B, C and D resp.) culture supernatant, 1 µg/ml.

Appendix 4: *Staphylococcus* isolate

Appendix: 4.1. *S. aureus* isolated from minced meat.

S. aureus isolate No.	SET EIA				SET RPLA			
	A	B	C	D	A	B	C	D
MN 5	+	-	-	-	-	-	-	-
MN 9	-	-	-	-	-	-	-	-
MN 14	+	-	-	-	-	-	-	-
MN 18	-	-	-	-	-	-	-	-
MN 22	+	-	-	-	-	-	-	-
MN 26	+	+	+	+	+	+	+	-
MN 27	+	+	+	+	+	+	+	+
MN 28	+	-	+	+	+	-	+	-
MN 30	+	-	+	-	+	-	+	-
MN 31	+	+	+	+	+	+	+	-
MN 32	+	+	+	-	+	+	+	-
MN 33	+	+	+	+	+	+	+	+
MN 35	+	-	+	-	-	-	+	-
MN 36	-	-	-	-	-	-	-	-
MN 38	+	-	+	-	-	-	+	-
MN 45	-	-	-	-	-	-	-	-

Appendix 4.2. *S. aureus* isolated from beef carcass

S. aureus isolate No.	SET EIA				SET RPLA			
	A	B	C	D	A	B	C	D
C 2	-	-	-	-	-	-	-	-
C 15	+	+	+	+	+	+	+	-
C 19	+	+	+	+	+	+	+	-
C 27	-	-	-	+	-	-	-	-
C 31	-	-	-	+	-	-	-	-
C 55	-	-	+	-	-	-	-	-
C 67	+	+	-	-	-	-	-	-
C 95	+	+	+	-	+	+	+	-

Appendix: 4.3. *S. aureus* isolated from chicken

S. aureus isolate No.	SET EIA				SET RPLA			
	A	B	C	D	A	B	C	D
CH 1	-	-	-	-	-	-	-	-
CH 6	+	-	-	-	-	-	-	-
CH 17	+	+	+	+	+	+	+	+
CH 18	-	-	+	-	-	-	-	-
CH 20	+	+	+	+	+	+	+	+
CH 21	-	+	+	-	-	+	+	-
CH 22	+	+	+	-	+	-	+	-
CH 25	-	-	+	-	-	-	+	-
CH 27	-	-	-	-	-	-	-	-
CH 28	+	+	+	+	+	+	+	+
CH 30	-	-	-	-	-	-	-	-
CH 31	-	-	-	-	-	-	+	-
CH 32	-	-	-	-	-	-	-	-
CH 33	-	-	-	-	-	-	-	-
CH 34	+	-	+	+	+	-	+	-
CH 35	-	+	+	-	-	-	+	-
CH 36	+	-	+	-	+	-	+	-
CH 40	+	+	+	+	+	+	+	-
CH 41	-	-	+	-	-	-	+	-
CH 42	-	-	-	-	-	-	-	-
CH 44	-	-	-	-	-	-	+	-
CH 50	+	-	+	-	-	-	+	-

Appendix: 4.4. *S. aureus* isolated from raw milk

S. aureus isolate No.	SET EIA				SET RPLA			
	A	B	C	D	A	B	C	D
RM 6	-	-	-	-	-	-	-	-
RM 10	-	-	-	-	-	-	-	-
RM 11	+	-	-	-	-	-	-	-
RM 15	-	-	-	-	-	-	-	-
RM 16	-	-	-	-	-	-	-	-
RM 21	+	+	+	+	+	-	+	+
RM 30	-	-	-	-	-	-	-	-
RM 31	+	+	+	+	+	-	+	+
RM 38	+	+	+	+	+	-	+	+
RM 39	-	-	-	-	-	-	-	-
RM 60	-	-	+	-	-	-	+	-
RM 63	-	-	-	-	-	-	-	-
RM 69	-	-	-	-	-	-	-	-
RM 75	-	-	-	-	-	-	+	-
RM 76	-	-	-	-	-	-	-	-
RM 77	-	-	+	-	-	-	+	-
RM 79	+	+	+	-	-	+	+	-
RM 81	-	-	-	-	+	-	-	-
RM 82	-	-	+	+	-	-	+	-
RM 83	-	-	-	-	-	-	-	-
RM 84	-	-	+	-	-	-	+	-
RM 85	-	-	-	-	-	-	-	-
RM 91	+	-	+	-	-	-	+	-
RM 92	-	-	-	-	-	-	-	-
RM 93	-	-	-	-	-	-	+	-
RM 95	-	-	+	-	-	-	+	-
RM 99	-	+	+	-	-	-	+	-
RM 103	-	-	+	-	-	-	+	-
RM 107	+	+	+	-	+	-	+	-
RM 124	-	-	+	-	-	-	+	-
RM 128	-	-	-	-	-	-	-	-
RM 131	+	-	+	-	+	-	+	-
RM 137	-	-	+	-	-	-	+	-
RM 143	+	+	+	+	+	+	+	+
RM 146	+	+	+	+	+	+	+	+
RM 147	+	+	+	+	+	+	+	-
RM 148	+	+	+	+	+	+	+	+
RM 151	+	+	+	+	+	+	+	-
RM 152	-	-	+	-	-	-	+	-
RM 158	-	-	+	-	-	-	+	-
RM 161	+	+	+	+	+	+	+	+
RM 163	-	-	+	-	-	-	+	-
RM 164	+	+	+	+	+	+	+	-

RM 168	+	+	+	+	+	-	+	+
RM 174	+	-	+	-	+	-	+	-
RM 184	-	-	-	-	-	-	-	-
RM 186	-	-	-	-	-	-	-	-
RM 188	-	-	+	-	-	-	+	-
RM 191	-	-	+	-	-	-	+	-
RM 193	-	-	+	-	-	-	+	-
RM 195	-	-	+	-	-	-	+	-
RM 197	-	-	-	-	-	-	-	-
RM 198	-	+	+	-	-	-	+	-
RM 199	-	-	-	-	-	-	-	-
RM 200	-	-	-	-	-	-	-	-