DETECTION OF *STAPHYLOCOCCUS AUREUS* ENTEROTOXINS AND ENTEROTOXIN PRODUCING STRAINS

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ABBREVIATIONS AND SYMBOLS

WBC ..... White Blood Corpuscles

MHC ..... Major Histocompatibility Complex

TSST ..... Toxic Shock Syndrome Toxin

FAME ..... Fatty Acid Modifying Enzyme

CP..... Capsular Polysaccharide

SE..... Staphylococcal Enterotoxin

NSAR.....National Select Agent Registry

CDC .....Center for Disease Control

SFP.....Staphylococcal Food Poisoning

FDA..... Food and Drug Administration

ELISA .....Enzyme Linked Immunosorbent Assay

CFU.....Colony Forming Unit

kDA.....Kilodaltons

ng.....Nanogram

fg ..... Femtogram

µg .....Microgram

µl ..... Microliter

CHAPTER I

INTRODUCTION

Staphylococcus aureus a Gram-positive bacterium produces enterotoxins, which are causative agents of foodborne intoxications. There are twenty serological types of enterotoxins. They are classified into classical and novel enterotoxins. Enterotoxins are single chain polypeptides and have a molecular weight of about 26-28 kDa and 228-239 amino acid residues (Muller-Alouf et al., 2001). Consumption of foods contaminated with *S. aureus* enterotoxins results in the onset of acute gastroenteritis within 2-6 h. The most common symptoms associated with *S. aureus* food poisoning are nausea, vomiting, abdominal cramps, and headache. The symptoms resolve within 24 h. The organism is a commensal and lives on the anatomical locales of humans and animals. Hence food handlers are the common source of contamination of foods. The organism also enters the food during processing of animal products. Considerable researches have been conducted in the area of detection of enterotoxins from foods. Development of immunoassays has revolutionized the research on detection of enterotoxins. Many commercial assays are available in an easy to use format. However, the need for improving the limit of detection (LOD) is ever present as enterotoxins are potent in very minute quantities. Sensitive detection methodologies are also important because enterotoxins are also potent superantigens and could possibly be used as a biological warfare weapons. Detection of enterotoxigenic strains of *S. aureus* is also important for epidemiological reasons.

Since enterotoxin family encompasses a large number of toxins, it is benificial to identify and characterize the enterotoxigenic strains of *S. aureus.* The characterization of the enterotoxin genes and the enterotoxigenic isolates of *S. aureus* can provide researchers with additional information in an event of an outbreak. Many methods have been developed for detection of the presence of protein toxin molecules in foods. These methods are based on the presence of detectable amounts of toxins. The methods are predominantly immunoassays, as the bioassays were unreliable, cumbersome and expensive. Recently, many methodologies have been developed using biosensor technology. The methods employing signal amplifiying technology can also greatly improve the sensitivity of detection of toxins. The conjugation of antibodies to oligonucleotides has led to signal amplification technologies in which, specificity of antigen antibody interactions and massive amplification potential of polymerase chain recation (PCR) could be put to use to obtain sensitive detection of enterotoxins. Development of DNA dendrimer technology and fluorescent nano particles can be effieciently used to obtained manifold amplification of signals from target molecules. DNA dendrimers, which are developed with multiple layers of DNA molecules, have the potential to amplify signals from very small quantities of target molecules. DNA combs are also signal amplification molecules and could be employed in PCR to obtain manifold amplifications.

The enterotoxin genes of *S. aureus* are present on regions of chromosome known as the staphylococcal pathogenicity islands (SaPIs). The genes could also be plasmids or phage borne. The genes expressed in one environment may not be expressed in another. Since food is a complex environment the organism may produce the toxin in a food and

may not in another. Hence the presence of the gene should always be considered as an indicative of the ability of the organism to produce toxin in a favorable environment. Enterotoxin A is most commonly involved in food poisoning outbreaks (Normanno et al., 2005). Genes for enterotoxin A are also found along with genes like *seg*, *seh* and *sei* (McLauchlin et al., 2000). Hence it is not clear if an outbreak is caused as a result of an individual toxin or is caused due to combination of multiple toxins. Many isolates of *S. aureus* harbor multiple enterotoxin genes. Characterization of enterotoxigenic isolates and enterotoxin genes is necessary to develop indepth knowledge about these food poisoning agents. Enterotoxin genes have been identified by methods like DNA hybridizations and polymerase chain reactions. These methods are reliable alternatives to other cumbersome and expensive methods like microarrays. PCR based methods like multiplex PCR has provided a rapid means to identify the presence or absence of desired genes. However, methods developed should be able to provide more information on enterotoxin genes, so that genes and isolates could be characterized. Methods involving sequencing of genes and methods like single/multi locus toxin sequence typing can provide more information on genes and the enterotoxigenic isolates. Information thus generated can be used by surveillance agencies to identify new strains, link one case of food poisoning to other or identify emerging or re-emerging strains that may have significanceincausinganoutbreak.

CHAPTER II

REVIEW OF LITERATURE

Historical background

Staphylococcus aureus is a Gram-positive bacterium that occurs in grape like clusters. The organism was first discovered in 1880 by a surgeon named Sir Alexander Ogsten in pus from abscesses. In 1884, the two pigmented types of the cocci were described by Rosenbach who also proposed the nomenclature for the bacteria. The bacterium that produced yellow colonies was known as *Staphylococcus aureus* and the one that produced non-pigmented or white colonies was known as *Staphylococcus albus* which is now known as *Staphylococcus epidermidis*. The staphylococci are common inhabitants of the nasal passage, skin and other anatomical locales on humans and other warm blooded mammals. The genus *Staphylococcus* belongs to the bacterial family of *Staphylococcacea.* The closest relatives to the *Staphylococcaceae* family are the *Bacillaceae* and *Listeriaceae.* Other genera that belong to the *Staphylococcaceae* family are *Gamella, Macrococcus* and *Salinococcus. S. aureus* is a very significant bacterium as it causes a number of suppurative infections and toxinoses in humans and animals.

The organism is also notorious for its resistance to antibiotics. The occurrence of Methicillin Resistant *Staphylococcus aureus* (MRSA) and the detection of a vancomycin

resistance gene in organisms like *Enterococcus feacalis*, which could be transferred naturally to *S. aureus* in the gastrointestinal tract, has lead to increased concern regarding this bacteria. There are new reports about the occurrence of vancomycin resistance *S. aureus.* The first report on a strain of *S. aureus* with reduced susceptibility to vancomycin was reported in 1996 (Hiramatsu et al., 1997). The bacterial strain had an intermediate resistance to vancomycin and was known as vancomycin-intermediate *Staphylococcus aureus* (VISA). By 2002 eight documented cases of infection with VISA were reported (Fridkin, 2001; Smith et al., 1999). A Center for Disease Control (CDC) report in 2002 reported on the first documented case of *S. aureus* infection caused by vancomycin resistant *S. aureus* (VRSA).

The common *S. aureus* infections are superficial skin infections like furunculosis, styes, and boils. Infections of organs or deep seated infections like urinary tract infections, osteomyelitis, phlebitis, and mastitis are also caused by *S. aureus*. *S. aureus* can cause infections that may result in life threatening conditions like meningitis, endocarditis, pneumonia, septicemia, and toxic shock syndrome (TSS). *S. aureus* exotoxins such as the exfoliative toxins cause the staphylococcal scalded skin syndrome (SSSS). Enterotoxins produced by the organism are notoriously known as the causative agents of foodborne intoxications.

Food poisoning caused by *S. aureus* is one of the most common causes of foodborne diseases. Foodborne illness similar to staphylococcal food poisoning has been recorded as early as the beginning of the nineteenth century. Olliver (1830) recorded an outbreak of food poisoning due to the consumption of meat pie. He also noted that similar illness symptoms were reported after eating foods like ham, sausages, bacon, and cheese.

The illness reported by the scientist was probably an outbreak of staphylococcal intoxication. In 1872, Selmi said that the toxic compounds which are intrinsic to foods were formed due to proteolysis, and he called the products ptomaines or “animal alkaloids” (Mossel and Netten, 1990). The role of *S. aureus* in food poisoning was demonstrated by Sternberg in 1885 (Mossel and Netten, 1990). Dack et al (1930) isolated a toxin from an organism from cream filled Christmas cake and established its involvement in a food poisoning outbreak in Chicago. There are more than 20 species of *Staphylococcus*, however only *Staphylococcus aureus* and *Staphylococcus epidermidis* interact significantly with humans. *S. aureus* which commonly inhabits the skin and warm nasal passages often gains entry into food systems from food handlers. Foods that require hand preparation such as salads and sandwich spreads are most susceptible to contamination with *S. aureus*. Milk and milk products are also common vehicles for staphylococcal food poisoning. When *Staphylococcus* grows in food it produces the toxins that cause illness. Although cooking kills the bacteria, the toxins produced are heat stable and may not be destroyed. Thus staphylococcal intoxication stands out as one of the main foodborne diseases.

Morphological, cultural, and biochemical characteristics of *S. aureus*

S. aureus appears as Gram-positive cocci in grape like clusters microscopically. They are non-motile, non-spore forming, spherical cells of 1µm in diameter. Since cell division occurs in two planes, they grow in clusters. On a nutrient rich medium, the organism forms large yellow colonies, where as *S. epidermidis* forms relatively small white colonies. The staphylococci are facultative anaerobes and they can grow by aerobic respiration or by fermentation producing lactic acid. The organism is

able to grow at a wide temperature range of 15°C to 45°C, a pH range of 4 to 11 and at NaCl concentration as high as 15%. The ability of the organism to grow in such wide temperature, pH, and salt concentrations makes it a common foodborne pathogen. The bacteria are catalase-positive and oxidase-negative. It is hemolytic on blood agar and almost all strains of *S. aureus* produce the enzyme, coagulase. The organism ferments glucose and mannitol producing lactic acid.

Factors contributing to the pathogenicity of the organism

Staphylococcus aureus produces a number of exoproteins and enzymes that contribute to its ability to invade and colonize tissues. The toxins and virulence factors of the organism can affect immune system of infected individuals directly by lysing white blood cells or indirectly by acting as superantigens. As an example, the leukotoxins can lyse white blood corpuscles (WBCs), where as enterotoxins and Toxic Shock Syndrome Toxin (TSST) can act as superantigens (Mertz et al., 2007).

Some of the most common virulence factors of *S. aureus* are teichoic acid and fibronectin binding protein, which enable the bacteria to gain entry into the host and colonize the tissues. The enzymes, hyaluronidase and coagulase, aid in spreading or localization of infection. The organism also produces leukocidins, catalase, and protein A which help in the multiplication of the organism in host tissues. Damage to the host tissues is caused by hemolysins, exfoliatins, enterotoxins, and toxic shock syndrome toxin produced by the microorganism. These enzymes and exoproteins enable the organism to cause acute and chronic infections (Archer, 1998). Bera et al. (2005) showed that *S. aureus* is completely resistant to lysozyme and the reason for the resistance is the O-acetylation of peptidoglycan at C-6 position of N-acetyl muramic acid (NAM). In

addition the C-6 position of NAM has phosphoester linked wall teichoic acid (Strominger and Ghuysen, 1963). Weidenmaier et al. (2004) showed that Wall Teichoic Acid (WTA) is essential for colonization of nasal epithelial cells by *S. aureus* and it mediates the interaction of the human nasal epithelial cells. The mutants, which were WTA deficient, completely lacked the ability to colonize host tissues. *S. aureus* also expresses fibronectin binding receptors which are very important for adherence of the pathogen to the host cell.

Membrane damaging toxins

S. aureus is able to invade host cells by producing a number of membrane damaging toxins. Alpha hemolysin is the toxin which is important for pathogenesis. Most of *S. aureus* strains produce α-hemolysin which is also known as α-toxin. Alpha toxin can cause lysis of host cell or cause the formation of pores in the cell membrane. The toxin can cause adhesion of polymorphonuclear leukocytes to the surfaces of endothelial cells and thereby can induce inflammatory reactions (Krull et al., 1996). Alpha toxin is also involved in the reduction of the activity of macrophages. The β-toxin of *S. aureus* belongs to the class of enzymes, neutral sphingomyelinase C. The toxin can affect the function of both lymphocytes and neutrophils (Huseby et al., 2007; Marshall et al., 2000), can lyse erythrocytes thereby evading the host immune system and scavenge nutrients (Huseby et al., 2007). The delta toxin belongs to the accessory gene regulator (*agr)* cluster and is responsible for the pathological changes that occur during an infection (Alouf et al., 1988). Delta toxin can lyse many cell types and can be distinguished from other hemolysins by its heat stability. More than 97% of the strains of *S. aureus* produce the toxin (Schmitz et al., 1997). The leukocidin is a protein toxin that plays a major role in necrotic skin infections. Panton-Valentine leukocidin (PVL) is responsible for

leukocyte destruction and tissue necrosis (Genestier et al., 2005). The toxin is commonly associated with community acquired methicillin resistant *S. aureus* (Vandenesch et al., 2003). Studies reveal that even the methicillin susceptible *S. aureus* possess this toxin and can cause necrotizing skin infections (Boubaker et al., 2004; Le Thomas et al., 2001). Leukocidin forms octameric pores on host membranes with *Luk*F and *Luk*S subunits and can also cause hemolysis.

Coagulase and Clumping factor

S. aureus produces another important extracellular protein known as coagulase. The coagulase binds with prothrombin of the host and can form staphylothrombin. The formation of staphylothrombin complex activates the protease activity of thrombin which results in the conversion of fibrinogen to fibrin. Coagulase is secreted by the bacterial cells into the medium (Boden and Flock, 1989; McDevitt et al., 1992). In addition to the free coagulase which is secreted into the medium some coagulase is found attached to the bacterial cell and is known as the bound coagulase (McDevitt et al., 1992). Coagulase probably helps the bacteria to evade the host immune defense by localized clotting. *S. aureus* cell surface also has the clumping factor which plays an important role in its virulence. The clumping factor is different from coagulase (McDevitt et al., 1992, 1994), in that it mediates direct adherence of the bacterial cell with fibrinogen and fibrin. It also mediates platelet aggregation (Bayer et al., 1995). The bacterium also expresses a plasminogen activator known as the staphylokinase and its gene is carried by a phage. Staphylokinase can cause lysis of fibrin and it is believed to enable the spread of the bacteria. Staphylokinase also activates human plasma

fibrinolytic system indirectly (Lack, 1948; Lewis and Ferguson, 1951). It forms a complex with plasmin and activates the plasminogen leading to fibrin specific clot lysis.

Other virulence/pathogenicity associated factors

S. aureus produces proteases, lipases, hyaluronidase, penicillinase, DNAase and fatty acid modifying enzyme (FAME) besides the above-mentioned extracellular proteins. The bacteria also produce a number of factors which interfere with host defenses and enable the bacteria to colonize the host. Examples of these are the capsular polysaccharides, protein A, leukocidin, exotoxins, and superantigens. Most *S. aureus* strains produce at least one of the eleven serotypes of capsular polysaccharides. The capsular polysaccharide serotype CP5 and CP8 are predominantly found in human isolates, implying their importance in pathogenesis. The CP5 and CP8 differ in one position of the O-acetyl groups and linkages between amino sugars (Seaman et al., 2004). The capsular polysaccharides could prevent the bacterium from being phagocytosed by the host macrophages. A great amount of research has been conducted to show the involvement of the capsular polysaccharide in thwarting host defense mechanisms by impeding phagocytosis (O'Riorden and Lee, 2004). Protein A is a 40-60 kDa surface protein. Protein A binds to the Fc portion of the IgG molecule thereby hindering the phagocytosis and opsonization. It acts as an immunological disguise and hence considered as a virulence factor.

Exfoliative toxins

The staphylococcal scalded skin syndrome (SSSS) is caused by the exfoliative toxins, namely ETA and ETB (Arbuthnott et al., 1972). The third type of exfoliative toxin is ETD. The ETA and ETB are the common ones causing SSSS. The exfoliative toxins

have a molecular weight of about 26- 27 kDa. ETA is a very heat stable protein and can withstand extreme heat, whereas ETB is heat labile. Both ETA and ETB have significant amino acid identity and also share biophysical properties (Bailey et al., 1980; Lee et al., 1987). The ETs are virulence factors of *S. aureus* and are encoded on mobile genetic elements (Novick, 2003b). The regulation of the ETs is under the control of the *ag*r locus just like the other virulence factors of *S. aureus* (Novick, 2003a). Dancer et al. (1990) had some indirect evidence suggesting that the exfoliative toxins are serine proteases and that they can cleave desmoglein1 which is a desmosomal cadherin protein in the skin's upper epidermis. X-ray crystal structures of these toxins revealed beyond doubt that these toxins are indeed trypsin-like serine proteases (Cavarelli et al., 1997; Vath et al. 1999). An infection by *S. aureus* capable of producing ETA or ETB could result in SSSS. Though the infection can affect adults, it is infants and very young children that are most commonly affected (Gammell, 1995). The clinical manifestations are due to the cleavage of desmoglein 1 which results in the disruption of the desmosomes. This results in the separation of the epidermis of the skin at the level of the stratum granulosum (Amagai et al., 2000, 2002). The exfoliation of the epidermis occurs at a site which is different from the site of infection. The reason is due to the release of exotoxin by the bacteria into the blood stream and the toxin makes its way into the skin layers thus mediating epidermolysis. Fever and erythema may also accompany the condition.

Superantigens: Toxic shock syndrome toxin and Enterotoxins

The Toxic Shock Syndrome Toxin (TSST) and the enterotoxins produced by *S. aureus* have superantigenic activity. Superantigens stimulate T cells non-specifically without any specific antigen recognition. In a normal antigenic response only one T cell

in a population of about 10,000 is stimulated, where as if a superantigen is involved one in every five T cells is stimulated leading to an enormous release of cytokines. The superantigens bind T cell receptors and major histocompatibility complex (MHC) class II molecules outside the peptide binding groove (Bohach et al., 1990). Even minute quantities of superantigens can lead to massive stimulation of T cells. About 100 pg/ml of superantigen is enough to trigger a response (Bohach et al., 1990). This is one of the reasons for constant research aimed at improving and developing sensitive detection methods for staphylococcal enterotoxins. As a result of non-specific proliferation, large populations of effector CD4 cells begin to stimulate the monocytes resulting in the production of cytokines like IL-1 and TNFα. Since large numbers of cells are involved, instead of localized secretion of these cytokines, a systemic secretion occurs. During an infection only localized secretion occurs, where as the superantigen mediated stimulation results in a massive systemic response (Schlievert, 1993).

Staphylococcal toxic shock syndrome toxin (TSST)

The occurrence of toxic shock syndrome was first reported in 1978 (Todd et al., 1978). Davis et al. (1980) established the association among menstruation, use of tampons, *S. aureus,* and development of TSS. Toxic shock syndrome is characterized by rapid onset of fever, rash, vomiting, diarrhea, hypotension and multiple organ failure. It is fatal if not treated promptly (Bohach. et al., 1990). The toxins causing toxic shock syndrome belong to pyrogenic exotoxins which are secreted by *Staphylococcus aureus* and *Streptococcus pyogenes.* The pyrogenic exotoxin group includes TSST-1(Bergdoll and Schlievert, 1984), exotoxins A, B, enterotoxins A through E of *S. aureus* (Bergdoll et al., 1973; Poindexter and Schlievert, 1985) and streptococcal pyrogenic exotoxins A

through C (Barsumian et al., 1978). TSST-1 and enterotoxins are resistant to proteases and also can withstand temperature of 60°C or higher. TSST-1 is a single chain polypeptide with a molecular mass of 22 kDa (Deresiewicz et al., 1994). It has only 20-30% sequence similarity with other pyrogenic toxin super antigens (PTSAs), but it has some structural similarities like possessing two domains (A and B), one large and one small, respectively. In 1999, annual review of immunology proposed the structure of TSST-1 to contain a large domain that has a β-grasp motif, and a α-helix, which is found along with β-sheets connecting peripheral strands. The small domain has two β-sheets making a β-barrel. However another study by Earhart et al. (1998) proposed that all PTSAs have a bilobed structure and have a domain B, which has five strand mixed β-barrel and the domain A which is present around the central helix lying against the β-sheets. The TSST-1 being a superantigen has a broad specificity for MHC II molecules, but is able to bind only to 1-5 V_β allotypes (Schlievert, 1993). TSST-1 is unable to evoke an emetic response and many researchers suggest that the absence of the cysteineyl residues and the inability to form a disulphide loop may be the reason.

TSS can be divided into two types namely the menstrual TSS and the non-menstrual TSS (Reingold et al., 1982; Shands et al., 1980). TSST-1 is incriminated in 90% of menstrual TSS, and in non-menstrual TSS, 50% is due to TSST-1 and another 50% is due to other exotoxins (Bohach et al., 1990). Most of the menstrual TSS is associated with tampon use. Menstrual TSS occurs during menstruation or two days prior to, or two days after, menstruation. The clinical manifestations are often due to the immune system reacting overtly to the release of massive amounts of cytokines. The release of interleukin 1β is associated with fever, TNF α, β, interleukin 2 and γ-interferon

are all associated with development of rashes and other symptoms of TSS (McCormick et al., 2001). While menstrual TSS affects women between the ages of 12-55, the non-menstrual TSS can affect people of all ages and gender. In a surveillance conducted during 2000-2003, there had been a fourfold increase in the incidence of TSS (Schlievert et al., 2004). Several hypotheses for the increase in the incidence have been proposed. One of the important reasons could be the emergence of new strains of methicillin resistant *S. aureus* which are capable of causing TSS. The CDC has termed the strains as USA1100 (TSST-1 positive), USA 400(SEB/SEC and PVL positive) and USA 300 (PVL and unknown enterotoxin positive). These emerging strains have been associated with an increase in TSS cases. These strains have the potential to produce 10 to 100 times more TSST-1(Schlievert et al., 2004). Other reasons proposed by Schlievert et al. (2004) are that women are menstruating at an earlier age, increased use of tampons, not recognizing the illness promptly, and media advising that TSS is not a problem anymore.

Enterotoxins

Staphylococcal enterotoxins (SEs) are exoproteins which when ingested by humans give rise to symptoms of acute gastroenteritis. Staphylococcal enterotoxins belong to a large family of staphylococcal and streptococcal pyrogenic exotoxins (PT), sharing common phylogenetic relationships, structure, function and sequence homology (Balaban and Rasooly, 2000). There are seventeen known major types of SEs, SEA to SER with no SEF and multiple SEs are commonly found among *S. aureus* strains (Jarraud et al., 2001, 2002). The enterotoxins are single chain polypeptides and have a molecular weight of about 26-28 kDa and 228-239 amino acid residues (Muller-Alouf et al., 2001). The foodborne intoxication caused by *S. aureus* enterotoxins has an incubation

period of about 2-6 h after the consumption of contaminated food. Duration of the illness is usually 6-24 h and prolonged illness or death occurs only in infants, elderly, and severely debilitated persons. Classic symptoms include severe vomiting, abdominal cramps, headache and nausea. In some severe cases transient changes in blood pressure and pulse rate occurs. Fever is generally not a symptom of staphylococcal intoxication. *S. aureus* enterotoxins are also powerful superantigens and are able to stimulate polyclonal proliferation response of murine and human T-lymphocytes possessing Vβ receptors (O'Hehir and Lamb, 1990). Mollick et al. (1988) suggested that, enterotoxins have a greater binding affinity to the MHC than the T lymphocytes thus proposing a ternary complex of MHC-toxin-TCR. Hewitt et al. (1992) showed that SEB was able to stimulate the $V\beta_3$ of human lymphocytes without the interaction with the MHC. The increased stimulation of T cell receptors (TCR) by these superantigens results in massive production of lymphokines leading to a much heightened immunological response compared to a normal immune response. Staphylococcal enterotoxins are potent even in very minute quantities. In dairy products a dose of 0.5 ng/ml or /g has been frequently incriminated in causing staphylococcal food poisoning (Bergdoll, 1991). Foodborne illnesses cause a lot of public health impact. In US alone about 6-80 million people are afflicted by foodborne illness every year (Altekruse et al., 1997). Staphylococcal foodborne intoxication is considered as the second most prevalent cause of foodborne diseases. Apart from being the causative agents of the foodborne intoxications the CDC has listed the staphylococcal enterotoxins as select agents in their National Select Agent Registry (NSAR).

Food poisoning an overview

Food poisoning is a common form of illness, and it could vary in severity from being mild to being fatal. The CDC estimates that, in the United States alone, there are about 76 million food borne illnesses, 325,000 hospitalizations and up to 5,000 deaths each year (Mead et al., 1999). Apart from this, there could be many cases of food poisoning that are not reported because the symptoms could be mild and might resolve quickly. There are more than 200 foodborne diseases identified so far. Food poisoning can be caused due to known causes or unknown causes. The known causes of food poisoning are infectious agents and toxic agents. The infectious agents are organisms such as bacteria, viruses, and parasites which may gain access to the food. Toxic agents includes but not limited to poisonous mushrooms, metals, pesticides, fertilizers, and exotic foods. Food can get contaminated due to improper sanitary conditions and time/temperature abuse during preparation, package, transport or storage.

Food poisoning caused by infectious agents can be divided into two categories, namely foodborne infections and foodborne intoxications. Foodborne infections occur when food consumed is contaminated with pathogens. The pathogen causes inflammation, resulting in poor absorption of water and nutrients. This manifests as diarrhea, vomiting, abdominal pain, fever, flu like symptoms, headache etc. Food intoxications result from ingesting foods contaminated with preformed toxins. Hence even if the microorganisms are destroyed during cooking or processing, some of the toxins which are heat stable may persist in foods and may still cause intoxication. Symptoms of food intoxications usually are severe abdominal cramps, diarrhea, nausea, vomiting, headache, double vision, weak pulse and if left untreated can cause organ

failure and even death. The most common infectious agents that are incriminated in foodborne illness are viruses like noroviruses, rotavirus, and hepatitis A virus. The parasites like *Amoeba, Giardia,* and *Cryptosporidium* are also involved in causing infections. Bacterial agents most commonly associated with infections and intoxications are those belonging to the genus Salmonella, Campylobacter, Shigella and organisms like *Staphylococcus aureus, Bacillus cereus, Escherichia coli, Clostridium botulinum,* and *Vibrio cholera.*

Staphylococcal food poisoning (SFP)

Staphylococcal food poisoning results from the consumption of food contaminated with one or more staphylococcal enterotoxins (SEs). The toxins of *S. aureus* are known as enterotoxins because they are able to promote water loss from the small intestinal mucosa resulting in vomiting and diarrhea (Martin et al., 2003). The CDC estimates that *S. aureus* causes about 185,000 cases of foodborne intoxications in the US (Mead et. al., 1999). Foodborne intoxication caused by the consumption of foods contaminated with SEs is considered as the second most common food borne illness in the world (Di Pinto et al., 2004). The organism is present in the nasal passage, skin, throat, hair and other anatomical locales of humans and animals. Foods requiring much processing by handlers, like salads and sandwiches are commonly contaminated by enterotoxin producing strains of *S. aureus*. Though the organism is heat labile and can be killed during cooking or processing, the toxins are extremely heat stable and can remain after cooking and cause illness. The symptoms of food poisoning by *S. aureus* occur within 2-6 h of consumption of contaminated foods. Patients experience vomiting, diarrhea, abdominal cramps, and nausea. The condition often resolves within a day, in

normal healthy individuals. However the illness may be severe in immune compromised individuals or in the elderly. Vehicles of transmission of the staphylococcal food poisoning are cooked proteinaceous foods such as ham, bakery items that are cream or custard filled, and salads which are rich in protein like tuna or turkey etc (Holmberg and Blake, 1984). The organism present in the anatomical locales of food handlers can gain entry to the food milieu and is able to elaborate toxins if there is any inadequate refrigeration during processing or product storage. In bakery items the cream or custard portions are the ones that are frequently contaminated. Cream filled portions of the product are a very good growth medium for the organism (Bryan, 1976; Stewart et al., 2003). Ham is also one of the most common foods often involved in SFP (Holmberg and Blake, 1984). The salt concentration of 3.5% present in precooked ham offers an ideal growth environment for the bacteria. Other foods that are involved in SFP include milk and milk products such as cheeses, poultry products like turkey salads, eggs, egg rolls, egg salads, and chicken, meat and meat products like beef and pork sausages, fish and fish products like tuna salads, salmon salads and other ready-to-eat (RTE) foods. RTE foods are commonly involved because the organism is able to grow well in the absence of competition from other microorganisms. SFP can be prevented or minimized by following good sanitary practices in food processing, packaging and storage areas. Ensuring that food handlers follow good personal hygiene and are aware of consequences can also minimize SFP. Foods that are to be kept hot should be maintained above 140°F and foods stored cold should be maintained below 40°F. Cooked foods should be stored in shallow containers and should be refrigerated promptly.

Brief overview of enterotoxins and their regulation

The enterotoxigenic strains of *S. aureus* must generally grow to a population of about 10^6 cells or more per g of food, in order to produce toxin. About 100-200 ng of enterotoxin in food is enough to cause an intoxication (Evenson et al., 1988). So far there are eighteen known staphylococcal enterotoxins: SEA-SEE, SEG-SER, and SEU. The SEC is further divided into subtypes such as SEC_1, SEC_2 and SEC_3. Enterotoxin F (SEF) is now known as the toxic shock syndrome toxin and it is not considered as an enterotoxin as it lacks the emetic activity which is the characteristic feature of enterotoxins. Enterotoxin genes are encoded by variable genetic elements such as plasmids, transposons, and phages. The genes for SEB and SEC are present on specific areas on the bacterial chromosome known as pathogenic islands (SaPIs) (Lindsay et al., 1998). SEA enterotoxin is frequently associated with food poisoning outbreaks and accounts for about 75% of the outbreaks in developed countries (Normanno et al., 2005). SED is the second most prevalent toxin causing food poisoning outbreaks followed by the SECs and SEB. The virulence factors of *S. aureus* are controlled by many regulatory loci. Some of most common regulatory loci are the accessory gene regulator (*agr),* staphylococcal accessory regulator *(sar),* sigma-B *(sigB), S. aureus* exoprotein expression *(sae),* and a number of other proteins including *sarA* homologues (Said-Salim et al., 2003). The *Ro*t (repressor of toxins) is another regulator with positive and negative effects on the expression of virulence genes. The *Rot* and *agr* genes have opposite effects on the regulation of *S. aureus* genes (Said-Salim et al., 2003). As an example, in the secretion of the SED, the *agr* control of *sed* promoter was found to be dependent on the presence of a functional *Rot* protein. The increase in the *sed* transcription post-

exponentially is due to *agr* mediated reduction in *Rot* activity and not the direct effect of the *agr* system (Tseng et al., 2004). The *sarA* functions as a transcriptional regulator and binds to a consensus motif in the promoter of its target genes. It also belongs to a large family of transcriptional regulators with a common amino acid motif (Arvidson and Tegmark, 2001; Chien et al., 1999). *S. aureus* produces a number cell-surface associated and secreted proteins which are under the regulation of the *agr* system. *In vitro,* when the cells reach the late stationary growth phase, the *agr* system down regulates the production of the cell wall associated proteins and starts to upregulate the production of the secreted proteins (Novick et al., 1993). The *agr* is a polycistronic locus containing two promoters. One of the promoters directs the synthesis of the transcript RNAIII (Novick et al. 1993). The RNAIII transcript is encoded by the P3 promoter of the two component signal transduction system of the *agr* locus. The P2 promoter (RNAII) contains the open reading frames *agr A, B, C,* and *D* (Balaban and Novick, 1995). The *agr A* expression is essential for RNAIII expression and RNAIII is required for regulation of genes which are under the control of the *agr* system (Janzon and Arvidson, 1990). Enterotoxins SEB, SEC and SED are exoproteins whose expression is under the regulation of *agr* (Tremaine et al., 1993). The variation that is observed in the amount of these enterotoxins produced has been related to the difference in the *agr* expression. Research has shown that there exists a correlation between the level of the RNAIII and the seb mRNA (Compagnone-Post et al., 1991). The enterotoxins share a 50-80% nucleotide identity however they differ in the regulatory mechanisms of the toxin genes, the level of toxin production, and also the factors that may control the toxin production in different environments (Borst and Betley, 1994).

SEA production is not regulated by *agr* (Tremaine et al., 1993). The *sea* gene is carried by a polymorphic family of lysogenic phages (Betley and Mekalanos, 1985). The *seb* gene has been found in the chromosome in strains isolated from food poisoning outbreaks, but could also be plasmid borne. Nonclinical isolates capable of producing SEB had the *seb* gene carried by a 750 kb plasmid (Shalita et al., 1977). As mentioned earlier it is regulated by the *agr* regulatory system (Gaskill and Khan, 1988). Enterotoxin C production has commonly been associated with strains that are methicillin resistant, invasive, and are isolated from animal diseases (Hu et al., 2005; Schlievert, 2001). Some bovine strains of *S. aureus* carry a bovine pathogenic island which encodes three superantigens namely SEC-bovine, TSST-1 and SEL (Fitzgerald et al., 2001). SED which is also commonly associated with the food poisoning outbreaks is encoded by a 27.6 kb penicillinase plasmid pIB485 (Bayles and Iandolo, 1989). The SED encoding plasmid has an open reading frame (ORF) which encodes for another enterotoxin SEJ. The genes encoding SED and SEJ are transcribed in the opposite direction. The two open reading frames are separated by an intergenic region which has 895 nucleotides and has a 21 nucleotide inverted repeat in each arm (Zhang et al., 1998). The similarity in the nucleotide sequences and antibody binding motifs results in the common occurrence of cross reactivity between enterotoxins SEA and SEE and between enterotoxins SEB and SEC (Lee et al., 1978, 1980). SEE is an extracellular protein like the other enterotoxins. SEE is produced as a precursor protein. The *entE* structural gene makes the precursor protein with signal sequence and the signal sequence is cleaved between the lysine and serine residues resulting in a mature extracellular form of enterotoxin E (Couch et al., 1988; Michaelis and Beckwith, 1982). There exists an 84% nucleotide homology

between the *ent E* and the *ent A* genes. The *ent E* gene, like the *ent A* is phage encoded (Couch et al., 1988). SEE is not frequently associated with food poisoning outbreaks.

Jarraud et al. (2001) reported the presence of a cluster known as the enterotoxin gene cluster (*egc*) that encoded five enterotoxin genes. Previous research had shown that the genes *seg* and *sei* of *S. aureus* were present on a 3.5 kb DNA fragment and were present in a tandem orientation (Jarraud et al., 1999). Jarraud et al. (1999) reported that when the *seg-sei* intergenic region and the flanking regions of the DNA fragment were subjected to sequence analysis, it revealed that the 1.9 Kb intergenic region contained open reading frames ORF1, 2, and 3 of 399, 327 and 777 bp respectively. ORF1 was similar to the N-terminal region of SEB, ORF2 had similarities with the C-terminal region of SEC, and ORF3 was similar to SEA. When the flanking regions were analyzed two ORFs (ORF4 of 783 bp and ORF5 of 720 bp) were identified. ORF 4 was homologous to SEJ and ORF5 was similar to SEI. ORF 1 and 2 were shorter than the known sequences of the enterotoxins. The ORF 1 had a Shine-Dalgarno sequence but had a large deletion at its 3' end. The deleted region corresponded to a biologically active site. ORF2 however, did not have any signal peptide or Shine-Dalgarno sequence, but appeared to be like a truncated terminal of SEC. Since these two ORFs did not appear to be like fully active enterotoxin genes they were designated as pseudogenes *ψent1* and *ψent2*. ORFs 3, 4, and 5 had the features of complete active toxins, like the presence of adequate Shine-Dalgarno sequences, translation start site, and were of adequate size. The toxins belonging to the enterotoxin gene cluster, exhibited superantigenicity and each had a specific V_β pattern. Thus the *egc* cluster was reported to encode five enterotoxin genes namely *seg, sei, sem, sen,* and *seo.* (Jarraud et al., 2001). Belkum et al. (2006) showed

that the *S. aureus* isolated from healthy individuals as well as from patients with bacteremia harbored the *egc* cluster. *S. aureus* possessing the genes of the *egc* cluster is prevalent in human isolates. They concluded that the presence of the *egc* probably enhanced the carriage potential of the *S. aureus* strains.

The amount of toxins produced by the *egc* cluster is considerably low when compared to other enterotoxins and are also less immunogenic (Ferry et al., 2005). Though the toxins of *egc* like SEG and SEI, have strong evidence for their superantigenicity, their ability to cause emetic response is not clear (Dinges et al., 2000). Hence their relevance in causing food poisoning must be investigated. However it was also shown that, isolates from food poisoning outbreaks frequently harbored the *sea, seg, seh,* and *sei* genes (McLauchlin et al., 2000). Since these enterotoxins are superantigens they pose a significant threat to the health of consumers, irrespective of their ability to cause food poisoning. Letertre et al. (2003b) reported that strain 382F had an extra ORF and the toxin gene was designated as SEU. Alignments with many other *egc* revealed that the ORF was the result of a 15 bp insertion in the *ψent1* pseudogene region. This insertion leads to the translation of a 261 amino acid putative ORF instead of the two pseudogenes. SEU has a 56% amino acid identity with the SEB, 52% with SEC, and 40% with SEG. The *egc* cluster may or may not harbor the *seu* gene and can be classified as *egc-1* (*seu* negative) and *egc-2* (*seu* positive) (Fueyo et al., 2005). The *sek* gene was described by Orwin et al. (2001) and it was demonstrated that the toxin had many biological activities that are associated with other SEs. The new toxin gene, *sek,* can be genetically related to *seb* as both the genes are present on the SaPI3. But SEK is more related to the SEA, D, and E subfamily than to SEB and SEC. The 5’ end of the *sek* start

site has a Shine-Dalgarno sequence and there is also a -10 and -35 putative promoter sequence (Orwin et al., 2001). Omoe et al. (2003) described a new SE related putative toxin SER. The study showed that SER showed significant T cell stimulating activity and that the amino acid sequence of SER had the highest similarity with SEG. The *ser* gene is carried by two kinds of plasmids, it is either found along with the *sed* and *sej* genes and is carried on the plasmid pIB485, or it is carried along with *sej* on a pF5 related plasmid (Omoe et al., 2004). The toxin producing strains often produce multiple toxins and possess genes encoding multiple enterotoxins. However it is not clear if only one of the toxins or multiple toxins with similar properties are involved in food poisoning or other infections like toxic shock syndrome (Orwin et al., 2001). Hence it is very important that, methods developed to detect the enterotoxins from environments like foods must target not just the presence of toxin molecules, but should also include detection of enterotoxin and enterotoxin like genes from *S. aureus* strains that may be present. The presence of enterotoxin genes could lead to the expression of the genes if the appropriate conditions are presented. A complex environment such as food can lead to the expression of the genes and can cause the organism to elaborate the toxins, which were not produced in another environment. It should also be noted that, most of the enterotoxins are superantigenic and even if they do not possess emetic activity they still pose a threat to the health of consumers.

Detection of staphylococcal enterotoxins in foods

Staphylococcal enterotoxicosis, though a disease of moderate severity can cause considerable economic loss to society (Todd, 1978). The economic loss could be by way of treatment, loss of productivity, intervening measures, and product recalls. The

incidence of the intoxication varies among different countries and it depends on the eating habits of the people of a particular locality and of a country (Todd, 1978). Sensitive methodologies are necessary for detection of staphylococcal enterotoxins because, the staphylococcal enterotoxins are very potent even in minute quantities and can cause an illness, the toxins are superantigens and can pose serious threat to the health of consumers, and enterotoxins like SEB are listed as select agents and can be potentially used as biological weapons. The need for identification of enterotoxins in foods is for two important reasons, one is to identify the toxin and confirm its involvement in food poisoning outbreaks and the other is to confirm the presence or absence of the enterotoxins in a product and hence confirm the safety of a marketable product.

Many programs have been established for developing sensitive and rapid methods for the detection of staphylococcal enterotoxins from foods. In 1947, Division of Microbiology of the Food and Drug Administration established a long range program for the detection of staphylococcal enterotoxins from foods (Casman and Bennett, 1965). The program was successful in encouraging the development of many research endeavors aimed at the detection of enterotoxins and reducing the incidence of staphylococcal food poisoning outbreaks. Since then many methodologies have been developed and tested for sensitive, rapid, and simple detection of enterotoxins.

Biological assays for detection of enterotoxins from foods

Prior to the development of serological assays, biological assays were employed in the detection of staphylococcal enterotoxins. Biological assays involved the administration of samples suspected of containing a toxin to human volunteers or susceptible animals. Biological assays have also involved the administration of the

sample suspected to contain the toxin via a catheter into the stomach of the susceptible animal. The animal is observed for at least five hours, and if it develops any emetic response, then the sample is considered to contain the toxin. Initially, in order to establish a link between the enterotoxicity of foods and organisms isolated from foods, human volunteers were used. Later on, young cats were used in most of the bioassays. Some of the earlier assays were involved with oral administration of the pure toxins to cats and studying the response in the animals (Clark and Page, 1968). One advantage of bioassays is that the biological activity of the toxin can be detected. In serological assays, only the serological activity of the toxin can be detected. If heat treated toxin samples are employed, serological assays can be relied only as long as the test can detect the antigen and antibody interactions. However, some studies show that even if a toxin may not be serologically active, it may still be biologically active and vice versa. In 1953, a monkey challenge test was developed and it tested the effect of administering toxins (culture filtrates) orally to monkeys (Surgalla et al., 1953). However the use of monkeys became limited as these animals were very expensive, difficult to maintain in captivity, and were also not readily available. The bioassays had many disadvantages. The use of animals became difficult as some of the animals did not produce emetic responses when toxins were administered orally. Bayliss (1940) tested the emetic action of enterotoxins on cats and found that emesis did not occur when toxin was administered orally. Cats and dogs showed emetic response only after intravenous inoculation (Bayliss, 1940; Kocandrie et al., 1966). The animal assays were also complicated and the results were not confirmatory. In case of intravenous injection of cats and dogs, the emetic response was also caused due to some nonspecific components. Besides these disadvantages, the cost

of the animals and their maintenance was very high. Hence, these tests became unreliable and the need for more specific and sensitive assays lead to the development of serological assays.

Serological assays employed in the detection of staphylococcal enterotoxins

Gel diffusion/precipitation assays

Serological assays are more sensitive and specific than animal feeding assays (Casman and Bennett, 1964; Hall et al., 1965). Serological assays involve the interaction between antigen and antibody. The enterotoxin antigen has to react with its corresponding antibody resulting in precipitation or agglutination reactions. In single or double diffusion assays the soluble antigen reacts with its specific antibody resulting in the formation of a visible precipitation line. Initially the assays were performed in tubes, where by the melted agar contained the antiserum. The agar was poured into (partially-filled) test tubes and was overlaid with solution containing the antigen, which is the enterotoxin. The interaction between antigen and antibody resulted in the formation of precipitin bands at the interface in the test tubes. In double diffusion, a layer of plain agar separated the antiserum containing molten agar and the enterotoxin solution. The antigen and the antibody migrate into the plain agar and form the precipitin bands. More recently, the tube diffusion assays were replaced by the microslide and plate assays. In microslide assays the enterotoxin was placed in wells cut in antibody-agar mixtures and the interaction between the antigen and antibody resulted in the formation of precipitin rings around the wells. The diameter of the precipitin ring was plotted against the concentration of enterotoxin, and it resulted in a straight line. Crowle (1958) and Wadsworth (1957)

described the serological detection of enterotoxins as a miniaturized version of the gel-double diffusion test of Ouchterlony (1948, 1953). The gel-double diffusion assay was able to detect up to 1 µg/ml of the sample used in the assay. Read et al. (1965) developed an *in vitro* assay for detection of staphylococcal enterotoxins A and B from milk samples using single and double agar gel diffusion. The assay required concentration of test samples and was able to detect about 0.33 and 1.0 µg/ml of toxin A and B respectively in the single-diffusion. In case of double-diffusion assay the minimum detection limit was 0.25 µg/ml for SEA and 0.63 µg/ml for SEB. The micro-slide gel double diffusion assay for the detection of staphylococcal enterotoxins developed by Casman et al. (1969) was approved by the Association of Official Analytical Chemists (AOAC) International and is now used as the current standard for evaluating new methods. The method however requires extensive concentration of food extracts. Another assay which requires minimal laboratory processing and is approved by the AOAC is the semi-solid agar procedure (Casman and Bennett, 1963). The capillary tube immunological assay for staphylococcal enterotoxins A, B, and D was described by Gandhi and Richardson (1971). The assay had a sensitivity of 1 µg/ml and the enterotoxins were detected within an hour. A similar capillary tube assay was described for enterotoxins A, B, and C by Fung and Wagner (1971).

The serological assays required the concentration of toxins from foods and chromatographic purification and concentration by dialysis were often employed prior to performing these tests. Many procedures were developed for the extraction, purification, and concentration of enterotoxin from foods, so that the samples could be subjected to serological procedures. Zehren and Zehren (1968) described a micro-slide gel diffusion

test for detection of enterotoxin A from large quantities of cheese. The procedure described for extraction and purification of enterotoxin enabled the detection of enterotoxin as low as 0.3 µg/100 g of cheese and the recovery was about 16-35%. The enterotoxin was separated from the food by combining the food and 0.2M NaCl at a ratio of 1:5 and mixing in a blender. The pH was maintained at 7.5 with NaOH. The food slurry was mixed, centrifuged, strained through nylon filter and then re extracted. The extract which contained the enterotoxin was purified by overnight dialysis in 50% w/w polyethylene glycol. The dialysis was followed by another extended procedure involving chloroform extraction. The procedure was very labor intensive and time consuming. Dialysis against a solution of polyethylene glycol was used in many of the concentration procedures in earlier experiments in order to reduce the water content of the extract, which was to be used in the serological assays. The purified extracts are often lyophilized as a final concentration procedure (Casman and Bennett, 1965; Hall et al., 1965; Reiser et al., 1974). Since the lyophilization was time consuming, some researchers concentrated the extract using gels. Barber and Diebel (1972) modified the procedure of Zehren for extraction of enterotoxin from fermented sausages. After the chloroform extraction and dialysis the extract was heated at 56°C in a water bath followed by chloroform extraction and elution of toxins from chromatography column. The heating step served to remove any inhibitory substances which may interfere with the serological assay. The enterotoxin was detected by immunodiffusion on slides as described by Casman et al. (1969) with slight modifications which involved the use of hemo-sol, which removed the haze around the agar without affecting the precipitation lines.

Serological assays employing electrophoresis techniques

Electrophoresis assays are one of the early methods involved in the detection of enterotoxins. Many support mediums like paper, parchment, and nylon were used for separation of the toxin, but all had their drawbacks and were impractical. Later, the use of starch as a support medium was employed (Bergdoll, 1956; Casman and Bennett, 1964). Raymond and Weintraub (1959) introduced polyacrylamide gels as a support medium for zone electrophoresis. Baier (1971) employed the polyacrylamide gel electrophoresis for the separation of enterotoxin B. Subsequently, the toxin that had been eluted from the gel band was used for injecting monkeys in order to examine the biological activity of the toxin.

A quantitative electroimmunodiffusion method for detection of enterotoxin A was described by Gasper et al. (1973). The technique which was introduced by Laurell (1966) was initially employed in clinical medicine for analysis of serum proteins. The electroimmuno diffusion was based on the principle that in a constant electric field and a given antibody dilution, the length of the precipitation cone formed is proportional to the concentration of the antigen. The method had a detection limit of 1.5 ng of enterotoxin A, and was rapid, simple, besides being quantitative. The immunofluorescent method for detection of enterotoxin B in food smears and food extracts was another rapid method and could be completed within 4-5 h (Genigeorgis and Sadler, 1966). The antibody was conjugated to fluorescein isothiocyanate and the method was able to detect the toxin as low as 1 to 0.05 µg/ml. The flotation antigen-antibody system was an interesting serological assay (Hopper, 1963). The enterotoxin was separated from the aqueous solution by a froth flotation method. The antiserum labeled with the dye rhodamine, was

added to the separated toxin solution along with an anionic wetting agent. Blowing of compressed air through the mixture resulted in the formation of foam. The dye then reacted with the toxin to form a red colored fraction. When the pressure of the compressed air was increased, the dye toxin foam fraction was separated out from the rest of the solution. Identification of the toxin was done by mixing a drop of the foam fraction with a drop of buffer at a pH of 4.5 and a drop of polystyrene latex suspension. The formation of a thick agglutination reaction was read as positive. Kimble and Anderson (1973) developed a reversed immune-osmophoresis (RIO) method for detection of staphylococcal enterotoxin A. Osmophoresis is defined as "simultaneous electrophoresis of proteins and their homologous antibodies in agar, resulting in the formation of precipitation lines" (Kimble and Anderson, 1973). The method was called "reversed" because the antibody solution was added to the well closest to the cathode instead of the anode. The method was as sensitive as double diffusion method with a detection limit as low as 1 µg/ml. The method could be employed for quick screening of enterotoxins from food. The disadvantages of the method were that it was not quantitative and when samples like cheese and meats were used, the visualization of the precipitation lines became difficult.

Assays employing hemagglutination reactions

Morse and Mah (1967) described a very sensitive microtiter hemagglutination-inhibition assay for staphylococcal enterotoxin B. Hemagglutination inhibition occurs when the enterotoxin antigen and antiserum were allowed to react and when sensitized red blood cells are added subsequently, there is no hemagglutination as long as the antibody has reacted with the antigen. The assay had advantages such as rapid results,

small quantity of reactants, ease of reading, and reproducibility. The gel diffusion methods based on precipitation reactions were time consuming and difficult as it depended on the migration of the precipitation lines, besides being hard to judge. The hemagglutination-inhibition assay was able to detect a concentration of toxin as low as 1.6 µg/ml. A passive hemagglutination assay was described for detection of enterotoxin B from culture filtrates (Johnson et al., 1967). The detection methodologies moved from the application of precipitation reactions to the use of agglutination reactions. The agglutination reactions were rapid when compared to the precipitation reactions. Most of the serological assays employing precipitation reactions had an incubation of 24 h to 1 week in order to read the results. To overcome this bias, agglutination assays were sought. In the passive agglutination assay, bis-diazotized benzidine hemagglutination with formalinized sheep erythrocytes was used for a quick and specific detection of the enterotoxin B from culture filtrates. Silverman et al. (1968) developed a reversed passive hemagglutination assay for detection of enterotoxins in culture filtrates and food samples. The sensitivity of the assay was at least 50-100 times higher than that of the gel diffusion assays and the lowest concentration of enterotoxin B which was detected was 0.0015 µg/ml. The cells were tanned, sensitized with antitoxin globulin, and preserved in formaldehyde or pyruvic aldehyde. The method did not require the concentration of the samples. The presence of other organic components in the samples did not interfere with the reaction.

Radioimmunoassays (RIA)

Radioimmunoassay was first reported by Berson et al. (1956). The RIA for enterotoxins, employed radio labeled enterotoxin which reacted with its specific

antibody. When unlabeled enterotoxin was added, the unlabeled antigen competed with the labeled antigen for combining sites on the antibody. The greater the concentration of the unlabeled antigen in the test sample the greater the probability of the unlabeled antigen to bind to the antibody. After the reaction, the unreacted antigen is separated out from the antibody-antigen complex. The assay began to be employed routinely in detection of staphylococcal enterotoxins from food as it had advantages like rapidity, sensitivity and specificity. A solid-phase radioimmunoassay was employed for detection of enterotoxins A and B from foods such as ham salad, cheddar cheese, salami, custard, and condensed milk (Johnson et al., 1973). The method had two advantages, it was quantitative and it only required a minimal preparation of food extracts. The toxin was extracted from the food samples by just blending the samples followed by centrifugation, pH adjustment, and filtration through a tissue. The assay involved labeling of the enterotoxins A and B with radioactive ^{125}I. Polystyrene tubes coated with specific antibodies were used for the detection and quantitation of the enterotoxins in food. The lowest limit of detection was reported to be 1-10 ng/g of food. A double antibody solid-phase radioimmunoassay (DASP) was described by Lindroth and Niskanen (1977). The method was very sensitive and had a detection limit of up to 200 pg of enterotoxin. The method also detected enterotoxin A as low as 2-5 ng/ml from minced meat and sausages. This sensitive assay involved the precipitation of antigen-antibody complex by anti-rabbit serum, which was adsorbed on to a solid carrier like cellulose. Miller et al. (1978) described a radioimmunoassay for detection of staphylococcal enterotoxins A, B, C, D, and E in food using *S. aureus* cells with protein A as a coprecipitant. The extraction procedure employed was very simple and hence reduced the hands on time. The method

had a detection limit of 0.5-1 ng/g and was better than the micro-slide diffusion assay which is used as the AOAC standard. Protein A was used as an immunoadsorbent in this assay. The protein A attached to the cell wall of the *S. aureus* cells, was able to bind specifically to the Fc region of IgG. This leads to the rapid uptake of the immune complexes, which were then separated by centrifugation. The preparation of protein A cells was easy and also inexpensive. An Affinity radioimmunoassay for enterotoxin B detection was described with detection limits of 1.2 ng/ml in buffer, 2.2 ng/ml in non fat dry milk and 6.3 ng/ml in hamburger (Niyomvit et al., 1978). The method employed sepharose 4B columns which has the covalently bound enterotoxin B. The antibody gel diluted in sephadex G-25 was placed in the column and the sample was sequentially pumped into the column. The ^{125}I- labeled SEB in the appropriate buffer was passed through the column and the amount of the radio labeled iodine removed from the column at an alkaline pH of 10.5 was inversely related to the amount of the toxin present in the sample.

The radioimmunoassay had revolutionized many areas of research and technology, because of its rapidity and sensitivity. However the radioimmunoassay had its draw backs. The primary drawback was the handling and use of radio-labeled components. As the radioactive compounds posed a serious health hazard many researchers and laboratory personnel were unwilling to work with such substances. It also involved training of the researchers and required special lab facilities. The expense involved in working with radioactive components was also higher when compared to other assays. These drawbacks lead to the development of many other immunological

assays which did not involve the use of radioactive compounds but instead employed enzyme labeled reaction components.

Enzyme immunoassay (EIA) and Enzyme linked immunosorbent assay (ELISA)

The need to develop a simple assay, which is non hazardous, requiring very less sample preparation, is cost effective and had other advantages like rapidity, sensitivity, and specificity lead to the development of methodologies involving enzyme labeling of the reaction components. Enzyme immune assays had the sensitivity which was equal to radioimmunoassays and did not have problems like lab safety, waste disposal, equipment expense, and short reagent life, which were encountered when RIA was employed. In 1969 the protocol for conjugation of enzymes to proteins was established (Avrameas, 1969). As the procedure became routinely used, many areas of research and medicine began to employ the ELISA and EIA procedures for rapid and sensitive detection of both antigens and antibodies. Saunders and Bartlett (1977) described a double antibody solid-phase enzyme immune assay for detection of staphylococcal enterotoxin A from foods. The sample preparation required only 15 min and the entire assay was completed within 1-3 h. The lowest detection limit range was 0.4 ng/ml for a 20 h test period and 3.2 ng/ml for a 3h test period. The presence of organic matters and other components did not affect the EIA. The methodology used anti-staphylococcal gamma globulin (ASG) sensitized microplate. Dilutions of toxin containing samples were added to the sensitized plates. The conjugate was added after incubation and washing to remove the unbound reactants. The ASG conjugate (horse radish peroxidase enzyme) reacted quantitatively with the substrate [2,2'-azino-di(3-ethyl-benzthiazoline-6-sulfonate)] ABTS.

Several enzyme-linked immunosorbent assays have been developed for the detection of staphylococcal enterotoxins from food. Among the ELISA techniques three types were reported in earlier studies. The single sandwich method could be performed in microtiter plates, polystyrene tubes, or on spheres. The assay used peroxidase or alkaline phosphatase antibody conjugates (Fey et al., 1984; Freed et al., 1982; Koper et al. 1980; Saunders and Bartlett, 1977). The other two methods are the double-sandwich method and competitive method. In the double-sandwich method the enzyme is coupled to the specific antibody (Notermans et al., 1978; Saunders and Bartlett, 1977). In the competitive method the enzyme is coupled to the toxin molecule (Kauffman, 1980; Koper et al., 1980; Kuo and Silverman, 1980; Morita and Woodburn, 1978; Stiffler-Rosenberg and Fay, 1978). Freed et al. (1982) reported an enzyme–linked immunosorbent assay which employed a solid–phase double antibody sandwich assay. The assay employed polystyrene balls and microtiter plates as solid–phase supports. The assay was rapid and could be completed within a day and detection sensitivity was less than 1 ng/g of various foods that were analyzed. Many commercial detection systems were developed based on the ELISA principle. ELISA became a routine procedure, which is still employed for detection of staphylococcal enterotoxins from foods and clinical samples in many diagnostic laboratories and research institutions.

Commercial kits for detection of staphylococcal enterotoxins from foods

Commercial detection systems that detect staphylococcal enterotoxins are easy to use as most of the reagents come in prepared ready to use form. The ready availability of reagents and buffers reduce the hands on time, thus making the commercial kits a sought after option. The commercial kits also produce results rapidly and require minimal

optimization. The kits are often used in food manufacturing industries in order to monitor the quality of the products and are also in used in public health investigations.

The reversed passive latex agglutination test kits for detection of enterotoxins enable the detection of soluble antigens such as enterotoxins. In an agglutination assay an antibody reacts with the particular antigen, which could be the enterotoxin molecule. However in a reversed passive latex agglutination assay (RPLA) the antibody is attached to particles such as latex beads and reacts with the soluble antigen, whereby crosslinking due to antigen in solution causes agglutination. The commercial SET-RPLA (Unipath, Basingstoke, UK) provided a rapid method for detection of the staphylococcal enterotoxins. Fujikawa and Igarashi (1988) developed a rapid reversed passive agglutination assay, which used high density latex particles for the detection of staphylococcal enterotoxins A to E. The assay required only 3 h incubation which was not as long as the 16 h incubation required for some commercial RPLA kits (SET-RPLA, Denka Seiken Co. Ltd., Tokyo, Japan). The detection limit was comparable to the commercial kit and was about 0.5 ng/ml of the sample employed. The SET- RPLA test kits could be used to detect enterotoxins in wide variety of foods and requires little sample preparation. The results are semi-quantitative. The latex particles are sensitized with antiserum from rabbits and these latex particles agglutinate in the presence of the corresponding enterotoxin. The method was developed to detect the classical enterotoxins A-E.

Many researchers compared various detection methodologies as well as commercial kits that are available. Wieneke and Gilbert (1987) compared the efficiency of four methods in detecting enterotoxins present in foods involved in food poisoning

outbreaks. Wieneke (1991), compared four commercial kits namely the SET-EIA (ELISA-B) from Dr. Bommeli AG (Stationsstrasse 12, CH-3097, Berne, Switzerland), SET-RPLA from Unipath (Basingstoke, UK), ELISA-M (membrane) and ELISA-T (tube) from TRANSIA (8 Rue Saint Jean de Diue, Lyon, France). A number of foods that were incriminated in food poisoning outbreaks in between 1987-1990 were analyzed using the four commercial assays. The ELISA-B and SET-RPLA detected toxins A-D and the ELISA–M and ELISA–T detected enterotoxins A-E. Each kit was able to detect individual enterotoxins. The ELISA-B and ELISA-M were very labor intensive and required extensive extraction procedures. ELISA–T was moderate in labor and extraction. The SET-RPLA was very easy to perform and required only minimal extraction procedures. ELISA-B detected 0.1-1.0 ng/ml of toxin, SET-RPLA and ELISA-M detected 0.5 ng/ml, and ELISA-T had sensitivity down to 0.2 ng/ml. The time required for completion of the assays (excluding the extraction time) varied, the ELISA-B required at least 24 h, followed by the SET-RPLA of 16 h. The ELISA-M could be completed within 4 h and the ELISA-T was the most rapid test and could be completed within 2 h.

Apart from the above mentioned commercial kits many research institutions and industries offer ELISA microtiter plate based kits for research and diagnostic use. Some of them are the SET-VIA kit from TECRA (Australia) and the ELISA kit for detection of staphylococcal enterotoxins from Toxin Technologies Inc. (Sarasota, FL). The SET-VIA from TECRA uses polyvalent capture antibody against SEA-SEE and requires minimal sample preparation. These assays are rapid and could be completed within 4-5 h. The ELISA-M and ELISA-T kits employ monovalent capture antibody system against SEA-

SEE. Another RPLA kit made in Japan also employs a monovalent capture antibody system for detection of enterotoxins A-D.

The RIDASCREEN (R-Biopharm Gmbh, Darmstadt, Germany) commercial enzyme immunoassay kit is another assay which is available for detection of staphylococcal enterotoxins A to E. Park et al. (1994) evaluated the efficiency of this system. The kit employs monovalent capture antibodies against enterotoxins A-E. The advantages of the kit was that there was no cross-reactivity among the reagents, had a high degree of specificity, was simple, required very little processing and extraction procedures, could be completed within 3 h, provided semi-quantitative results and had a sensitive detection limit of 0.2-0.7 ng/ml of food samples used. The VIDAS^{TM} SET and VIDAS^{TM} SET 2 (bioMerieux, Marcy-l'Etoile, France) are two automated detection systems for detection of staphylococcal enterotoxins. These systems enable the simultaneous detection of seven enterotoxins A-E including the subtypes of SEC. These are enzyme-linked fluorescent assays (ELFA) with monoclonal anti-enterotoxin antibodies. The results are read by the VIDAS automated system and are given as relative fluorescence values. The difference between the VIDAS^{TM} SET and VIDAS^{TM} SET 2 is that the later uses two Fab fragments and no Fc fragment for construction of the conjugate. A comparative study conducted by Vernozy-Rozand et al. (2004) reported that the VIDAS^{TM} SET 2 had a sensitivity of less than 0.5 ng/g for toxins A and B and lower than 1 ng/g for toxins C_2 and E and about 1 ng/g for SED. The VIDAS^{TM} SET had the sensitivity of 0.5 ng/g for SEA and SEC_2 and greater than 1 ng/g for SED and SEE.

Other methods currently available for detection of staphylococcal enterotoxins from foods

There are other technologies that enable the detection of enterotoxins from foods. Western immunoblot is one of the sensitive assays (Rasooly and Rasooly, 1998). The immunoblot technique has the advantage of detecting toxin samples which have been heat treated. The drawback of ELISA based techniques is that they are unable to detect toxins which were subjected to heat treatment. This could be due to the conformational changes that might occur when the toxin was subjected to heat treatment. The western immunoblot solublizes the denatured protein toxins and enable the detection of the toxins by its corresponding antibodies. The western immunoblot was reported to detect SEA to a level of 0.1 ng/ml. O'Brien et al. (2000) developed a detection methodology employing bidiffractive grating sensors. The detection limit for a pure sample of SEB was 1 ng/ml. Another method, using the fluorescent based sensors had a detection limit of 4 ng/ml for pure samples of enterotoxins (Rowe-Taitt et al., 2000). The surface Plasmon resonance biosensors detected SEB concentrations as low as 0.5 ng/ml in milk powder samples in 5% water (Homola et al., 2002). An immunomagnetic flow cytometric detection assay was developed for detection of staphylococcal enterotoxins B (Miyamoto et al., 2003). The method had a detection limit of 0.25-0.5 ng/ml in milk samples. The assay was rapid and could be completed within 3 h. A sensitive immunoassay using magnetic beads, for high-through put detection of enterotoxins B was reported (Alefantis et al., 2004). The assay employed paramagnetic beads coated with a primary antibody. The assay also involved the use of monoclonal antibodies which were labeled with Alexa fluor 647. After the capture of the toxin antigen the magnetic beads were exposed to a 625 red laser

and the fluorescence intensity was measured using fiber optic spectrometer. The assay was able to detect SEB concentrations in buffer as low as 100 pg. Since the reagents utilized in this methodology were combined, it eliminated many wash steps involved in many ELISA assays and hence the method used a "time-compressed" format. Since the sensitivity reported was for SEB in culture filtrates and buffer, the sensitivity of the assay in detection of toxins in food samples must be evaluated.

Immuno PCR assays for detection of staphylococcal enterotoxins

The development of methodologies for conjugation of oligonucleotide to antibodies paved a path for sensitive detection of enterotoxins. The reporter/secondary antibodies were not coupled to enzymes but instead were tethered to short oligonucleotide sequences resulting in a specific antibody-DNA conjugate. These oligonucleotides were capable of participating in the polymerase chain reaction (PCR), which had a massive amplification potential. This technology resulted in the amplification of signals from the enterotoxin molecules present in a sample and very minute amounts of toxins present could be detected. The immuno-PCR combines the specificity of the antigen-antibody mediated reaction with the sensitivity of the PCR amplification. The method was developed by Sano et al. (1992) and ever since it has been used in many areas requiring sensitive detection of antigens. Immuno-PCR has been used for the detection of pathogens such as group A streptococcus (Liang et. al., 2003), in the detection of toxins from *Bacillus thuringiensis* (Allen et al., 2006) and *Clostridium botulinum* neurotoxin type A (Wu et al., 2001), and in the detection of viral antigens (Maia et al., 1995).

An immunoquantitative real time PCR for the detection of staphylococcal enterotoxin B was developed by Rajkovic et al. (2006). It was a microtiter plate based assay and employed the antibody-DNA conjugate for the detection of SEB from culture supernatant and foods. The conjugation of the antibody to the reporter DNA was via the biotin-streptavidin complex. The assay was able to detect SEB dilutions in broth at a concentration of less than 10 pg/ml. Fischer et al. (2007) reported the quantitative detection of staphylococcal enterotoxins A and B from culture supernatant. The assay was performed in microtiter plates and the amplification of the DNA conjugate was by real time PCR. The sensitivity of the assay was 100 pg/ml for SEA and 10 pg/ml for SEB in culture supernatant. The covalent conjugation of the antibody to DNA was by using a heterobifunctional cross linking agent such as SMCC (Sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexan-1-carboxylate). Covalent conjugation of the antibody to the reporter DNA eliminates the many wash steps that are involved when biotin-streptavidin conjugates are used and it also prevents the occurrence of any false positive reactions.

Detection of enterotoxin genes

The classical enterotoxins (A-E) have been incriminated as the causative agents of foodborne intoxications. Most of the biological and serological/immunological assays are developed for the detection of these protein toxin molecules in foods. However, the organism *S. aureus* produces other enterotoxins which are now known as the novel enterotoxins. Hence the SE family has expanded and it now includes enterotoxins SEG-SER and SEU besides the classical enterotoxins A-E. The genes for these novel enterotoxins and their variants have been reported (Abe et al., 2000; Jarraud et al., 2001;

Letertre et al., 2003; Marr et al., 1993; Munson et al., 1998; Omoe et al., 2002, 2003; Orwin et al., 2001, 2003; Ren et al., 1994; Zhang et al., 1998). The methods for detection of toxins as well as the toxin genes can be of much use to the food processing industries as it involves consumer health and also economic well being of the industries. As mentioned above most of the immunological assays target only the classical enterotoxins. The reason could be that, aside from the classical SEs being involved in most of the food outbreaks, the availability of the antibodies against the novel enterotoxins is very limited. In most food poisoning outbreaks, the classical enterotoxins are the ones that are responsible, although many of the strains of *S. aureus* that are isolated from suspect foods possess genes such as *seg, seh,* and *sei* along with the classical enterotoxin gene *sea* (McLauchlin et al., 2000). The novel enterotoxins SEG, SEI, and their variants have more evidence towards their superantigenic activity rather than their ability to cause emetic responses (Dinges et al., 2000). So, other enterotoxins may also interfere with the health and well being of consumers. Many authors have noted that for epidemiological reasons, if a toxin gene is present in staphylococci, then the organism should be considered positive for that toxin production since toxin production in food or in vivo cannot be ruled out. The serological methods however, rely on the presence of detectable amounts of toxins. If the method is not sensitive enough to detect the presence of very minute amounts of toxins, it may result in false negative reports. The PCR methods can give information regarding the presence of genes. The DNA-DNA hybridization and PCR techniques are the most reliable methods for the detection of the toxin genotypes and are also dependable alternatives. Quantitative DNA based methods may be useful because

detection of *S. aureus* at $>10^5$ cfu/gm food alone is sufficient to implicate SE foodborne poisoning.

DNA hybridization techniques used in the detection of enterotoxin genes

Methods involving gene identification, like the DNA hybridization has been used for identification of *S. aureus* enterotoxin genes. Notermans et al. (1988) described a DNA-DNA colony hybridization method with three different synthetic probes. The nucleotide probes encoded the amino acid sequences of SEB that were identical with the sequences of the $SEC_{1.}$ The objective was to see if single probe could be used to detect both SEB and SEC producing strains. Neill et al. (1990) prepared 18-base oligonucleotide probes based on the sequences that were specific for the enterotoxins A, B, C, and TSST-1. The probes were able to detect the presence of the enterotoxins genes from reference as well as clinical strains of *S. aureus* by colony blot hybridization. There was a correlation of greater than 93% between the hybridization results and toxin production, which was detected by ELISA. Hence methods developed for genotype identification could be used to validate assays that detect the toxin molecules (phenotype). Bohach et al. (1989) evaluated the production of enterotoxins B and C_1 by *S. aureus* isolated from toxic shock syndrome patients. Internal probes for the enterotoxins were designed and DNA from representative isolates was allowed to hybridize. The sequence homology between B and C_1 genes allowed the detection of both genes under stringent conditions. The toxin levels were determined by immunodiffusion assay.

Detection of enterotoxin genes by PCR

The detection of the enterotoxin genes of *S. aureus* present in foods was done mostly by PCR based methods. The nucleotide sequences of most of the enterotoxin

genes are available and different degree of homology found among the genes lead to the designing of enterotoxin gene specific primers which allowed screening of samples for the presence of these genes. Johnson et al. (1991) designed eight pairs of synthetic primers for detection of enterotoxins A-E, exfoliative toxins A and B, and TSST-1. The primers targeted the internal regions of the toxin genes and the products were analyzed by gel electrophoresis. There are many reports on genotype analysis of enterotoxins by PCR (Gilligan et al., 2000; Mclauchlin et al., 2000; Omoe et al., 2002; Rosec and Giguad, 2002). Some variations and novelties have also been reported using the PCR based techniques. The PCR-ELISA developed by Gilligan et al. (2000) used internal biotin-labeled oligonucleotides as probes that immobilize and detect target sequences on microtiter plates. The PCR-ELISA had a higher sensitivity and was able to detect 250 gene copies when compared to agarose gel analysis which was only able to detect up to 2500 gene copies. The assay was also compared with SE toxin ELISA and found that in the detection of SEB, the sensitivity of SEB PCR-ELISA was much greater, whereas the SEA PCR-ELISA and SEA toxin ELISA were similar. Becker et al. (1998) developed a multiplex PCR enzyme immune assay (PCR-EIAs). This system allowed the simultaneous detection of *sea* to *see, eta, etb, and tst* genes.

Another PCR approach for the detection of enterotoxin genes was the development of the multiplex PCR. Multiplexing results in the simultaneous detection of many genes in a single PCR reaction. Many reports are available on the multiplex PCR detection of MRSA strains (Barski et al., 1996; Brakstad et al., 1993). A considerable research has also been done on the multiplex detection of enterotoxins genes (Becker et al., 1998; Loveseth et al., 2004; Mehrotra et al., 2000; Monday and Bohach, 1999;

Tamarapu et al., 2001). Since the detection of amplicons of multiplex PCR was by agarose gel electrophoresis the method required a lot of post PCR processing which was laborious. In order to overcome this bias, Letertre et al. (2003b) developed a 5' nuclease multiplex PCR for quick detection of nine enterotoxins genes (*sea, seb, sec, sed, see, seg, seh, sei. sej*) of *S. aureus*. The method was fluorescent-based (TaqMan) and resulted in the simultaneous detection of amplicons without involving agarose gel electrophoresis. An oligonucleotide probe was designed which had a complementary region located between the primer binding sites. The probe was dual-labeled with a reporter dye at the 5' end and a quencher at the 3' end. The 5' nuclease activity of the DNA polymerase resulted in the cleaving of the internal probe, allowing fluorescence of the dye that was no longer in close proximity to the quencher molecule. The method is very specific as the probe is specific and no non-specific amplicons were generated. The assay was performed in triplex reactions with probes labeled with different reporter dyes. In each reaction three staphylococcal enterotoxin genes were detected.

Real–time PCR techniques are also gaining popularity among the methods that are currently available for the detection of enterotoxin genes. The advantage of real-time PCR is as the name suggests the amplification of the target could be monitored in real time. It is a quantitative method and it also eliminates the time consuming and laborious post PCR processes of amplicon detection by electrophoresis. Real-time fluorescence PCR for detection of enterotoxins A, B, $C_{1,}$ and D was described by Klotz et al. (2003). The assay is a TaqMan PCR. The TaqMan oligonucleotide probe has a fluorescent reporter dye at its 5'end and a quencher molecule at the 3' end which is hybridized to the internal region which is to be amplified. Toxin production, by the strains used in this

study was evaluated by RPLA. The results indicated that more strains were positive for enterotoxins as detected by TaqMan PCR than were detected by SET-RPLA. Thus PCR was able to identify the presence of the genes, even if detectable amounts of protein toxins were not detected by the SET-RPLA assay. The real-time assays are also very rapid and results could be obtained within 5-6 h. The real-time PCR detection of enterotoxin genes is gaining popularity and is becoming a routine procedure which could be adapted in laboratories (Nakayama et al., 2006).

Microarrays for the detection of enterotoxin genes

Microarrays can be used as a rapid means for the detection of enterotoxin genes. Though microarray based technologies are very expensive, and many laboratories do not opt for it due to cost constraints, the ability of the method to specifically and rapidly detect the presence of the enterotoxins genes is desirable. Sergeev et al. (2004b) reported a microarray-based one-tube assay for simultaneous detection of multiple enterotoxin genes of *S. aureus*. The method involved an initial PCR amplification of the target regions of sixteen enterotoxin genes with degenerate primers. The PCR amplicons thus generated were characterized by microchip hybridization with oligonucleotide probes specific for the enterotoxin genes. The disadvantage was that, since it was a multiplex reaction, the presence of different copy numbers of the genes resulted in varying signal intensities in the arrays. Sergeev et al. (2004a) also reported the development of a microarray based technology for detection of many pathogens and their virulence factors such as the *S. aureus* enterotoxin genes, *C. botulinum* toxin genes and pathogens including *Listeria spp* and *Campylobacter spp*. The reliability of the system was enhanced by incorporating three elements, redundancy of genes, redundancy of

oligonucleotide probes for each gene and quality control oligonucleotide probes which monitors array spotting and target DNA hybridization. The microarray based technologies could potentially be used for very sensitive detection of *S. aureus* enterotoxin genes.

S. aureus is an opportunistic pathogen capable of causing many superficial as well as deep seated and systemic infections. Staphylococcal enterotoxins pose a serious threat to the health of consumers. The organism also produces many exoproteins which enhances its pathogenic and virulence potential. The enterotoxins, besides causing foodborne illness, are also a civilian threat as they can potentially be used as bioweapons by terrorists. Many detection technologies have been developed for the detection of the toxins as well as the genes. The detection of enterotoxins and enterotoxin genes has been reviewed in this article. Apart from the above mentioned methodologies, new technologies which have the potential for sensitive detection are currently being explored. The assays must be cost effective, easy to use and must be easily adaptable for routine use in laboratories. Since the enterotoxins are causative agents of foodborne intoxications, the detection methodologies must be efficient to detect the toxins or its genes in complex environments such as foods. Many detection technologies have constraints due to the interference from the food particles and other organic materials that may be present in food. Technologies that eliminate or overcome these biases should be developed and must be employed for much easier and sensitive detection of these agents. The use of immuno-paramagnetic beads for detection of enterotoxins in food is being explored. The method is able to detect femtogram quantities of toxins, is rapid, and very easy to use. The use of signal amplification molecules like the fluorescent nanoparticles,

DNA dendrimers, and DNA combs could also be employed to detect even miniscule amounts of enterotoxins from complex environments. The DNA dendrimers have a massive potential to amplify signals obtained from any target molecule. The multiple layers of DNA dendrimers or DNA combs may offer manyfold amplification of signals from the target by the way of PCR amplification. These are some of the many technologies that are currently available that could be used for detection of enterotoxins of the pathogen *S. aureus.* The methods described can be adapted for detection of other pathogenic microorganisms and their toxins as well.

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CHAPTER III

AN IMMUNOMAGNETIC PCR SIGNAL AMPLIFICATION (IPCR-SA) ASSAY FOR SENSITIVE DETECTION OF *STAPHYLOCOCCUS AUREUS* ENTEROTOXINS IN FOODS.

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INTRODUCTION

Staphylococcus aureus ranks as one of the most important bacteria that cause outbreaks of foodborne poisoning (Aycicek et al., 2005; Balaban and Rasooly, 2000; Le Loir et al., 2003). The organism's pathogenicity is due to its ability to produce a wide array of extracellular protein toxins and other virulence factors (Akineden et al., 2001). The organism has gained a lot of notoriety in clinical venues for occurrences of methicillin resistant *S. aureus* (MRSA). However, it is also well known for foodborne intoxications that result from the consumption of foods contaminated with preformed enterotoxins. The presence of staphylococcal enterotoxins (SEs) in food is a great concern to the food industry and regulatory agencies and is a leading cause of worldwide foodborne illness (Di Pinto et al., 2004) contributing to health problems in consumers and economic losses from contaminated products. The intoxication or food poisoning illnesses, caused by SEs are characterized by enteric responses such as diarrhea, abdominal cramps, and vomiting within 1-6 hrs of consumption of contaminated food (Bergdoll, 1991). *S. aureus* has been known to produce at least twenty different types of enterotoxins, the most common ones being SEA-SEE (Nema et al., 2007). The toxins are protein molecules having a molecular weight of 26,900-29,600 kDa (Holeckova et al., 2002). In 1989, four outbreaks were associated with canned mushrooms, presumably resulting from heat-stable enterotoxins produced before canning (Levine et al., 1996). Besides causing gastroenteritis, the toxins are also capable of activating the immune

system by possessing super antigenic activity. They interact with antigen presenting cells and T-cells, causing nonspecific activation of T lymphocytes (Akineden et al., 2001; Balaban and Rasooly, 2000). This causes massive and uncontrolled release of lymphokines which can have fatal consequences. Some of the enterotoxins produced by *S. aureus* are also classified as biological 'select agents', possession of which is highly regulated as they have been designated as a potential biological threat agent (Anonymous, 2005).

Dose levels of staph enterotoxins involved with foodborne illness have varied, and levels of SEA as low as 0.5-0.75 ng/ml in chocolate milk resulted in 850 cases of foodborne illness in school children (Evenson et al., 1988). Although the presence of bacterial levels of *S. aureus* organisms alone $>10^5$ cfu/gm are sufficient cause to implicate staph enterotoxin as the causative agent of a foodborne disease outbreak (Anonymous, 1996), it is imperative to be able to have sensitive detection of SE in food given the consequences of staphylococcal foodborne poisoning and its current designation and potential use as a biological threat. Furthermore, the currently established intoxication dose may be underestimated due to limits of detection methodologies (Rajkovic et al., 2006). The breakthrough in SE testing in food has been attributed to the development and availability of polyclonal and monoclonal anti-SE antibodies that have lead to the development of many immunological assays (Bergdoll, 1991). The use of magnetic beads for recovery of biologicals has further provided the means to capture and concentrate organisms and molecules (Safarik et al., 1995; Olsvik et al., 1994). The incorporation of antibody-coated magnetic beads has resulted in enhanced detection sensitivities capable of detecting as little as 0.1 ng/ml of SEB and (Alefantis et al., 2004;

Kijek et al., 2000). Signal amplification of reaction specificities can be accommodated by multiple binding kinetics of fluorescent-labelled polyclonal secondary antibodies (Alefantis et al., 2004) or PCR amplification of oligonucleotides tethered to secondary antibodies (Sano et al., 1992).

The present study is involved with improving the limits of detection (LOD) of SEs in food systems, by using magnetic bead-based immunological capture followed by real-time amplification of the capture signal (i.e., signal amplification). The method described herein appears to be robust considering the complex foodstuffs examined and could be applied to other toxins of either food safety concern or for those targeted as potential biological threat agents.

MATERIALS AND METHODS

Organisms used in the study and conditions employed.

The *Staphylococcus aureus* strains were grown in Tryptic Soy Broth (TSB, EMD Chemicals Inc., Gibbstown, NJ) by incubation for 24-36 hr at 37^{o}C. For experiments, a freshly transferred overnight culture of *S. aureus* was used. Reference stock cultures were maintained at -80°C in TSB with 10% glycerol. Baird Parker agar (Himedia Labs., India) with egg yolk and tellurite and Mannitol Salt Agar (MSA)(Becton, Dickson & Co., Cockeysville, MD) were used for confirming colony morphology, coagulase production and mannitol fermentation, respectively. Staining reactions, biochemical tests, and PCR reactions with *S. aureus* 16S rRNA-specific primers were also employed to confirm the identity of the strains obtained from the stock cultures. The *S. aureus* strains used in this study are listed in Table 1.

Antigens, antibodies, and commercial kits.

Highly purified staphylococcal enterotoxins SEA, SEB, and affinity purified sheep polyclonal antibodies for the corresponding toxin antigens were obtained from Toxin Technology, Inc., (Sarasota, FL). Two commercial kit systems were also obtained for comparison with assays developed in this study: Staphylococcal Enterotoxin Visual Immunoassay (SET VIA; TECRA, Australia) and ELISA Kit (Toxin Technology, Inc.).

Coating of paramagnetic beads with anti-SE polyclonal antibodies.

Hydrophilic COOH-modified 2.8µm magnetic beads (M-270 Carboxylic Acid; Dynal/Invitrogen, Carlsbad, CA) were coupled with sheep polyclonal anti-SE antibodies

as described elsewhere (Alefantis et al., 2004). Briefly, 125 µl of beads (2 x 10^9 beads/ml) were separated from their suspension media and were washed and suspended in 0.1 M 2-(N-morpholino)ethanesulfonic acid, pH 5.5 (MES buffer). Finally, 100 µl of EDAC (1- ethyl-3-(3-dimethyl amino propyl) carbodiimide (125 mg/ml; Sigma, St Louis, MO) was added to the beads in MES and incubated at 25^{o}C on a rotary shaker (100 RPM) for 30 min. The beads were washed thrice and suspended in coupling buffer, 0.1 M phosphate buffered saline (1X PBS, pH 7.2). Sheep polyclonal antibodies 55 µl of 4 x 10^{12} molecules/µl were added to the bead preparation and incubated for 3 hr at 25^{o}C with gentle rotation. The beads were washed and then incubated overnight with constant rotation in 1.25 ml of quenching buffer (35 mM glycine, 1.0% gelatin, pH 7.4; Sigma). Finally the beads were washed thrice and stored for future use at 4^{o}C in 1.25 ml of storage buffer (1.0% gelatin, 2.25% Tween 20, 0.01% sodium azide, pH 8)(Sigma). Coating of the anti-SE antibodies to the beads, as stated above, was performed using a semi-automated Bead Retriever^{Tm} (Dynal Biotech/Invitrogen). The concentration of capture antibodies (1^o antibody) coated onto the beads was ~4 x 10^{12} molecules/µl.

Generation of amino modified *reporter DNA*.

DNA template encoding the luciferase gene (Promega, Madison, WI) was used for the generation of the amino-modified reporter DNA (Wu et al., 2001). In brief, the forward primer was synthesized with an amino-modified 5'-end whereas the reverse primer was unmodified. The primer sequence used was 5'-(amino) GTTCGTCACATCTCATCTAC-3' and the reverse primer was 5'-TCGGGTGTAATCAGAATAGC-3'. Each 50-µl reaction contained 2 µl of the concentrated template containing DNA (80 ng), 0.2 µM of each of the primers, 2.5 mM

$MgCl_2$, 1X thermophilic buffer, 0.2 mM DNTP mix, 1.5 units of Taq DNA polymerase (Promega) and 30.8 µl of nuclease-free water. Clear PCR tube strips (Bio-Rad, Hercules, CA) were used for containing the reaction mix and the reaction was performed in a PTC-200 DNA Engine (MJ Research/Bio-Rad, San Francisco, CA). The reaction conditions were: initial denaturation for 1 min at 95^{o}C, followed by 30 cycles of denaturation at 95^{o}C for 45 sec, annealing at 54^{o}C for 30 sec, extension at 72^{o}C for 1 min and final extension at 72^{o}C for 10 min, followed by continuous hold at 4^{o}C. The reaction volumes from multiple tubes were pooled and the PCR product was purified using the Montage PCR clean up kit (Millipore, Bedford, MA). The presence of the expected 563-bp amino modified product was confirmed by electrophoresis on a 1% agarose gel. The DNA samples were quantified using NanoDrop (Fischer Scientific, Willmington, DE).

Covalent coupling of the 563-bp oligonucleotide to the anti-SE antibodies.

The conjugation of the reporter DNA to the anti-SE sheep polyclonal antibodies was performed as previously described along with required modifications (Hendrickson et al., 1995; Joerger et al., 1995; Allen et al., 2006). Briefly, the amino-modified reporter DNA (60 µl of 20 ng/µl) was activated with succinimidyl-4-[N-maleimidomethyl]-cyclohexane-1-carboxylate (SMCC; 540 µl of 10 mg/ml; Geno Tech, St. Louis, MO). SMCC was prepared fresh at 10 mg/ml in 50 mM sodium phosphate (pH 7.6). The primary amine group on the reporter DNA covalently binds to the carbonyl group on the SMCC molecule via an ester reaction. Sheep polyclonal antibody (300 µl of 4 x 10^9 molecules/µl) was reduced with 40 mM (300 µl) dithiothreitol (Promega). The removal of excess reagents was performed by sephadex G-50 gel filtration. The activated DNA and the reduced antibody were mixed and allowed to react while incubating at 25^{o}C with

gentle and constant rotation for 2 hr in the dark. This enabled the formation of the covalent bond between the maleimide group of the SMCC molecule and the reduced sulfhydryl groups on the antibody. The conjugate was then stored at 4^{o}C for future use. The approximate concentration of DNA for generation of the antibody-DNA conjugate (2^{o} antibody) was 20 ng/µl and the antibody concentration was 4×10^{9} molecules/µl.

Detection of pure toxins in broth media by iPCR.

The iPCR signal amplification assay for SE toxin detection is a 3-phase assay consisting of a) immunomagnetic bead capture, b) secondary antibody-oligo DNA application, and c) real-time PCR amplification of the reporter DNA. The reporter DNA is based on a eukaryotic luciferase gene and should reduce the risk of non-specific amplification. The primers for the real-time amplification of a 159-bp internal segment of the reporter DNA were designed using the PrimerQuest^{SM} software (Integrated DNA Technologies, Coralville, IA). The sequence of the forward primer was 5'GTTCGTCACATCTCATCTAC-3' and the reverse primer was 5'-AATCTGACGCAGGCAGTTCT-3'. The COOH-modified beads (50 µl), coated with either anti-SE (A or B) antibodies, were incubated with dilutions of pure SE (A or B, depending on the antibody combinations) in TSB. The starting concentration of the pure toxin was either 750 ng/ml or 75 ng/ml and tenfold serial dilutions were carried out until the concentration was 7.5 fg/ml. The mixture containing the antibody-coated beads and the antigen were incubated at 25^{o}C for 1 hr with gentle agitation. The beads were then washed 3 x with 300 µl of PBS in each wash and collected in 300 µl of distilled water. Washing of the beads was performed using the semi-automated Bead Retriever^{Tm}. Gelatin (100 µl of 1 mg/ml) was added as a blocking agent. The mixture was incubated at

25°C with rotational agitation for 15 min. The particles were washed 3 x with wash buffer and collected in 200 µl of distilled water. The secondary antibody-oligo conjugate (50 µl; DNA concentration ≤ 15 ng/µl) was added and samples were incubated at 25°C for 1 hr with constant rotational agitation. Finally the particles were retrieved, washed 3 x with wash buffer (1x PBS), 3 x with distilled water, and then collected in 200 µl of double-distilled water. The washed beads were then incubated at 85°C for 5 min and centrifuged at 5000 x g for 5 min (Eppendorf Model 5417-R, Brinkmann Instruments, Inc., Westbury, NY) with a standard fixed angle rotor (F 45-30-11).Heating at this step is required to release the complimentary strand of the double-stranded reporter nucleotide, and/or release of the 2^0antibody itself from the capture complex. Following centrifugation, 5 µl of the supernatant was added to a PCR reaction mixture containing 25 ul of SYBR green PCR Master Mix 1x (Abgene, Rochester, NY), 0.1 µM of each of the forward primer and reverse primer, 18 µl of distilled water in a total reaction volume of 50 µl and subjected to PCR in a real-time thermal cycler (Opticon 2; MJ Research/Bio-Rad, Hercules, CA). The thermal cycling conditions were 95°C for 15 min (enzyme activation), followed by 40 PCR cycles of denaturation at 95°C for 15 sec, annealing at 56°C for 1 min, and extension at 72°C for 1 min.The Taq polymerase used was Thermo-start™(ABgene, UK) which requires heat activation to release bound adduet that prevents premature amplification. Melting curve analysis was performed for every run to confirm the presence of a single amplified product. A negative PCR reaction control had all of the reagents except the supernatant containing the reporter DNA. Other controls were samples without the antigen (toxin), without the conjugate, without the capture (primary) antibody, without the reporter (secondary) antibody, and samples without beads. The data

generated was analyzed using the Opticon 2 data analysis software (MJ Research/Bio-Rad).

Comparison of iPCR with commercial ELISA kits.

Two microtitre plate-based commercial kits were tested along with iPCR-SA in order to compare the sensitivity of the methods. SET VIA (TECRA, Australia) and ELISA kit for SEB (Toxin Technologies, Inc., Sarasota, FL.) were used according to the manufacturer's protocol for performing the tests. Two-fold serial dilutions of the pure toxins, encompassing the range detected by the iPCR were tested in broth as well as in food samples. The optical density of the colorimetric reactions were measured at 405 nm using an ELISA plate reader (TECAN, GENios, Research Triangle Park, NC).

Detection of pure toxins (SEA and SEB) spiked into milk, tuna salad, lemon meringue pie, and raw ground turkey.

Detection of pure SEA and SEB toxins spiked in food samples and their limit of detection using iPCR-SA was investigated. We examined simple foods such as milk, foods incriminated in prior food poisoning outbreaks like tuna salad, foods which can be contaminated during handling like lemon meringue pie with cream and meringue toppings, as well as complex, potentially difficult products such as raw ground turkey (high fat, protein, and background microorganisms) to demonstrate the robustness of the magnetic bead extraction and PCR-detection procedure. The food items were obtained at a local supermarket. Toxin was added directly to liquid food samples (milk) or after initial mixing (lemon meringue cream pie). Semi-solid food samples (tuna salad, ground turkey) were partially diluted/mixed with sterile water (20-40%) prior to spiking with toxin-containing dilutions in order to render them usable and insure satisfactory mixing.

Pure SEA, or SEB, (starting concentration of 75 ng/gm and 10-fold dilutions down to a final concentration of 7.5 fg/gm) were spiked into the respective partially-diluted food sample(s). The food samples were mixed well in sterile/filtered stomacher bags (Whirl-Pak®, Nasco, Modesto, CA) using a stomacher blender (Stomacher 400-C Lab blender, Seward Medical Ltd., London, UK) prior to, and during, addition of the toxin. The food/toxin mixtures were allowed to stabilize for 30 min after the addition and mixing of the toxin in order to standardize treatment time before further testing. The toxin extraction method was modified from Rajkovic et al. (2006) and was recommended by the manufacturers of the commercial ELISA test kits. After 30 min, a 1:1 ratio of extraction buffer (0.25 M Tris buffer, pH 8.0) was added to the food samples, mixed with the stomacher, and allowed to sit (standardize) for 20 min. After this final incubation period, liquid samples were retrieved from the filtered side of the Whirl-Pak bags, centrifuged at 3000 x g for 15 min at 23^{o}C, and the supernatant was further filtered through a syringe with prewetted cotton in its cylinder (Rajkovic et al., 2006). The filtered supernatant was then analyzed by immunomagnetic bead recovery and PCR signal amplification and compared to commercial ELISA kits.

Detection of enterotoxins produced by enterotoxigenic strains by iPCR.

Enterotoxin production was first tested in growth media by iPCR and with the commercial ELISA test kits. Supernatant fractions from 10 ml of overnight cultures of enterotoxigenic strains of *Staphylococcus aureus* were collected by centrifugation and analyzed by iPCR and ELISA. Enterotoxigenic strains: ATCC 13565 (SEA), ATCC 14458 (SEB), ATCC 51740 (SEB) were used as positive control strains and non-enterotoxigenic strains, or those that did not produce SEA or SEB (ATCC 51650, ATCC

19095, ATCC 27664) were used as negative controls. All assays were repeated in duplicate. The foods (milk, meringue cream pie, tuna salad, and RTE deli turkey) were also inoculated with the same enterotoxigenic strains of *S. aureus* and tested for detection of enterotoxin by iPCR. The pH and water activity of all the tested foods were above 5.8 and 0.94, respectively. 20-gm portions of each of the food items were inoculated with ~100 cfu/ml of washed cells of enterotoxigenic strains of *S. aureus* as positive controls and non-enterotoxigenic strain as negative controls. Prior to inoculation the foods were blended in a stomacher to homogenize the mixture, and further mixed after inoculation, and the sample pouches were incubated at 37^{o}C for 24 hr. Extractions of the inoculated foods were carried out as described previously (Rajkovic et al. 2006). The extracts were analyzed immediately for the presence of toxin by both iPCR and ELISA kits.

Detection of toxins in heat-treated food samples.

The ability of the iPCR to detect enterotoxin in heat-treated food samples was examined. Whole fat milk (3.3% fat) and ground turkey were used as food matrices in these experiments. Various dilutions of pure SEA and SEB were spiked into 20-gm (or ml) portions of milk and ground turkey at the same levels tested earlier, ranging from 7.5 ng/ml (or /gm) to 7.5 fg/ml (or /gm) in the various samples. An equivalent amount of spiked food was held at room temperature (controls) while another portion was heated (66^{o}C for 30 min in the case of milk and 77^{o}C for 30 min in case of ground turkey). After heat treatment, the enterotoxin was extracted from the ground turkey samples with extraction buffer as described earlier, or directly extracted from milk. The heat-treated and control food samples were analyzed by iPCR and the results were compared with the results from the commercial kits.

Detection of heat inactivated toxins by iPCR.

The ability of the iPCR assay to detect enterotoxin after it has been inactivated by autoclaving was evaluated. Pure toxin (SEA & SEB) dilutions in 10 ml of milk were used for conducting the experiments. The starting concentrations were 7.5 ng ml^{-1} and the final concentration was 7.5 fg ml^{-1}. The experiment was performed in duplicate. The toxin dilutions in pre-autoclaved milk (toxin was added to autoclaved milk) served as controls. The toxin dilutions in milk were autoclaved at two different time and temperature combinations (100^{o}C/15 psi for 10 min and 121^{o}C/15 psi for 2 h 45 min) and detection was examined by iPCR.

RESULTS AND DISCUSSION

Preliminary considerations.

The use of magnetic beads has helped increase the sensitivity of detection assays because of their ability to capture and concentrate target antigens from large volumes (Olsvik et al., 1994; Safarik et al., 1995). In 1999, USDA-FSIS adopted the use of immunomagnetic separation (IMS) for detection of *E. coli* O157:H7 because of improved sensitivity in detecting low levels of O157:H7 from meat products. Methods for targets even smaller than bacterial cells have also found IMS to enhance detection capabilities. Kijek et al. (2000) used immunomagnetic bead separation coupled with chemiluminescent detection to detect SEB toxin in clinical samples. In our assay, we chose sheep polyclonal anti-SE antibodies since monoclonal antibodies were reported to have lower sensitivity (Rajkovic et al., 2006; Fischer et al., 2007). The combination of these two methods whereby the reporter 'signal' of an immunomagnetic PCR assay ('iPCR') is amplified ('signal amplification', SA) should lead to very sensitive detection levels (Fig. 1).

Enterotoxin detection in broth and foods by iPCR-SA.

Common vehicles of transmission resulting from post-process (i.e., post-cook) contamination have included proteinaceous (ham, turkey, chicken) and manually-prepared foods such as deli salads and cream filled pies and desserts (Holmberg and

Blake, 1984; Stewart et al., 2003; Soriano et al., 2002). We assessed this assay against various complex food components that are either typical food matrices that have been involved in enterotoxin foodborne illness or which may represent a robust challenge for a molecular diagnostic assay. Our initial test solution was TS broth and iPCR-SA allowed detection of both SEA and SEB down to 7.5 fg/ml (Fig. 2). Detection of SEA or SEB in milk, lemon meringue cream pie, tuna salad, and even raw ground turkey was detected in serial 10-fold dilutions corresponding to 7.5 fg/gm (or /ml)(Fig. 2). In iPCR-SA positive samples, the threshold cycle (C_t) for detection ranged from 18-28 cycles. No signal was derived from controls until near, or after, 40 PCR cycles. The appearance of slight signal amplification from controls could be attributed to insufficient washing of beads after the 2^oantibody with DNA oligo conjugate was added. The appearance of residual antibody conjugate would show up as a slight signal in later cycles, or not at all within 40 cycles if washed well (Fig. 2). Controls that gave negative reactions included samples without added antigen (i.e., non-spiked "no Ag"), samples without added 1^o- or 2^o-antibody ("no Ab"), samples with opposing/different enterotoxins than the Ab used in the reaction ("Negative Ctrl"), and blank wells (Fig. 2).

Comparison of iPCR-SA with commercial ELISA assays for staphylococcal enterotoxin.

The iPCR-SA assay was tested in comparison with two commercial ELISA test kits. The iPCR-SA assays were capable of detecting 7.5 fg/ml of the appropriate enterotoxin (SEA or SEB) for which they were designed. However, both the SET VIA toxin kit (for SEA, SEB, SEC,SED, SEE toxins) and the ELISA SEB kit (for SEB only) were designed for a low limit of 1 ng/ml and the 0.75 ng/ml assay was below the

acceptable signal for detection as stipulated in the manufacturer's instructions (Table 2). Recently, a new version of the ELISA SEB kit is reportedly capable of detecting 0.1 ng/ml. An obvious reason why the iPCR-SA assay is more sensitive is that even if a semi-solid food sample requires dilution to render it workable, a large volume of the extracted liquid may be treated with the immunomagnetic capture beads, concentrating the antigen captured by those beads into a much smaller volume; sensitivity is further enhanced by amplification of the reporter via the PCR reaction. Traditional ELISA assays, however, take the extracts obtained from diluted food samples and use them directly in the reaction without concentration (Schotte et al., 2002; Khan et al., 2003). Although traditional ELISA methods are not as sensitive as the iPCR-SA method, it still provides quantitative determination where such may be required or needed. The iPCR-SA could theoretically be made quantitative if monoclonal rather than polyclonal antibodies were used and if only a single oligonucleotide could be tethered to the 2^{o}-antibody molecules to provide a 1:1 ratio of tethered oligonucleotide to captured toxin Ag. The corresponding C_T of the ensuing PCR reaction would be related to the amount of bound enterotoxins based on similar iPCR-SA reactions with a standard set of toxin solutions.

Detection of toxin in foods produced by enterotoxigenic strains of *S. aureus*.

In addition to detection of toxin spiked directly into foods, we also tested the same broth media and foods by inoculating with SEA/SEB enterotoxigenic strains (Table 1) to insure that our assay was capable of detecting microorganism-produced enterotoxins as well as commercially purified toxins. The iPCR-SA SEA assay readily detected toxin produced by *S. aureus* ATCC 13565 (SEA) and the iPCR-SA SEB assay detected toxin produced by *S. aureus* ATCC 14458 and 51740 (both produce SEB). The assays detected

toxin after low levels of these organisms (washed) were inoculated and incubated in microbial media, milk, meringue pie, tuna salad, and in minced ready-to-eat (RTE) deli turkey (Fig. 3). No positive assays were observed for the various negative control samples processed along with the positive samples. Toxin was not detected without incubation for growth of the organisms (data not shown). We felt it was important to insure that the assay could detect pure toxin available commercially and that which is produced by organisms in foods and considered 'crude' enterotoxins. It is conceivable that toxin produced by the latter could be complexed with additional adducts that are not present on commercially purified toxin preparations that would also be used for production of antibodies. When the strains were inoculated into growth media, we noticed that the detection of SEA occurred much earlier than SEB (data not shown). The iPCR-SA was able to detect SEA within 10-12 h of inoculation of the organism in the growth media. This could be attributed to the fact that the production of SEA is not *agr* dependent and the maximal levels of toxin production for SEA-producing strains occurs during the exponential growth phase of the organism (Tremaine et al., 1993; Swarup et al., 2000).

Determination of the optimal temperature conditions for the assay.

The incubation of the primary antibody coated beads with the toxin antigen was initially carried out at room temperature (23^{o}C). To test if there is any improvement in the detection, the antibody coated beads and toxin antigens were allowed to interact at a warmer temperature of 37^{o}C. The incubation of the toxin and the conjugate was also performed at 37^{o}C. A comparison of the procedure performed at room temperature and at a warmer temperature of 37^{o}C indicated that the antigen-antibody interactions at a

temperature of 37°C and at 23°C were similar and no significant improvements in the results were obtained.

Detection of toxin after heating in foods or upon heat inactivation.

The detection of SEA and SEB toxins were also tested in liquid and solid foods (ground turkey and in milk) at cooking or pasteurization temperatures and at extreme (inactivation) temperatures in milk. When both enterotoxins were seperately mixed into (diluted) ground turkey and heated to 77°C for 30 min, detection was as sensitive (detection to 7.5 fg/gm) as that obtained without heating (Table 3). *S. aureus* is also quite common in raw milk and dairy products. One outbreak of staph foodborne poisoning in Japan involved more than 13,000 people whereby SEA was detected at 0.05-1.6 ng/ml (Asao et al., 2003; Rajkovic et al., 2006). In that outbreak no live cells of *S. aureus* were recovered from milk suggesting that viable cells were destroyed during pasteurization, but the heat-stable enterotoxin survived processing. We obtained similar results with milk autoclaved for 10 min at 100°C (15 psi) as we did in ground turkey to which SE was added before heating (Table 3). However, when both enterotoxins were subjected to heat treatments that are considered sufficient for inactivation (121°C, 15 psi, 2.75 hr), SEA and SEB were still detected, but only at 7.5 pg/ml and 0.75 ng/ml, respectively (Table 3). The results indicate that extreme heating, reduces, but does not completely prevent detection of toxin. It is not clear if the remaining toxin that is immunologically-detected has viable enterotoxic capabilities since an *in vivo* bioassay was not performed in this study. Studies on heat inactivation of staph enterotoxin A indicate that endpoints for inactivation are dependent on toxin concentration (i.e., 27 vs. 40 min for 5 vs. 60 ug/ml, respectively, in beef bouillon at 121.1°C) and suspension medium (i.e., 5 vs. 27 min for

phosphate buffer vs. beef bouillon, respectively, at 5 ug/ml and 121.1^{o}C) (Denny et al., 1971). The possibility that enterotoxins survive autoclaving is likely the reason why the Centers for Disease Control and Prevention (CDC) regulatory disposal measures require that autoclaved samples of staphylococcal enterotoxins also be incinerated (CDC, 2007). The extended heating implemented in this study reduced detection sensitivity, but did not eliminate it completely as we were still able to detect both SEA and SEB toxin to sub-nanogram levels. It is likely the foods tested herein were even more 'protective' to the low toxin levels than phosphate buffer or bouillon broth (Denny et al., 1971). Any protective food components were adequately removed during our semi-automated magnetic recovery process using the Bead Retriever^{Tm}. The data suggests that we should be able to detect SE's even if present in ingredients before cooking with equal sensitivity of detection as post-process contamination. The iPCR-SA assay was able to detect significantly lower levels of toxin from heated foods than levels tested in prior studies due to the sensitivity of detection. The current iPCR-SA assay was capable of detecting SEA/SEB toxins as low as 7.5 fg/ml from food samples which is 10^{3}-fold greater than reported in microtiter-based iqPCR assays (Rajkovic et al., 2006) and nearly 10^{6}-fold greater than the commercial ELISA kits used in this study. DNA melting curve analysis for the signal amplifications of the captured toxins revealed specific and discrete melting profiles for the expected amplimers, even on toxins recovered from heating trials (Fig. 4). This indicates that PCR amplification of the conjugated antibody-DNA oligo is occurring with specificity and that non-specific priming or PCR artifacts were not observed with our assay (Fig. 4). The same specific melting profile was observed for all iPCR-SA reactions in this study (data not shown).

The iPCR-SA assay for SEA and SEB was specific for these two toxins. SEA and SEB iPCR-SA assays did not react with culture extracts from strains known to produce other enterotoxins that were tested (data not shown) and provided levels of sensitivity beyond many current testing regimens. Using antibodies for other staph enterotoxins, or for toxins from other pathogens, we believe sensitive detection could easily be achieved for food safety purposes or for detection of potential biological threat agents such as ricin or botulinum toxin. The latter may even be easier to detect than detection from foods because of the additional complexity that food testing adds to diagnostic methods. The method described herein could readily be commercialized for a wide array of biological agents for which antibodies are available.

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Table 1. Strains of *S. aureus* and PCR primers used in this study.

Strain	SE Produced	Source
ATCC 13565	SEA	S.E. Gilliland, OSU
ATCC 14458	SEB	S.E. Gilliland, OSU
ATCC 51740	SEB	S.E. Gilliland, OSU
ATCC 51650	TSST	S.E. Gilliland, OSU
ATCC 19095	SEC	S.E. Gilliland, OSU
ATCC 27664	SEE	S.E. Gilliland, OSU
PCR Primer	**Product Size**	**Source**
For: Amino-5'GTTCGTCACATCTCATCTAC-3' Rev: 5'-TCGGGTGTAATCAGAATAGC-3'	563 bp	Wu et al. 2001
For: 5'GTTCGTCACATCTCATCTAC-3' Rev: 5'-AATCTGACGCAGGCAGTTCT-3'	159 bp	This study

Table 2. Comparison of iPCR-SA assay sensitivity to SEA or SEB toxin diluted into TS broth with several commercial kits.

Spiked level or Condition	SEA iPCR-SA Assay	SEB iPCR-SA Assay	SET VIA (Tecra)		ELISA SEB (Toxin Tech)
			SEA	SEB	
7.5 ng/ml (or /gm)	+	+	+	+	+
0.75 ng/ml	+	+	-	-	-
0.075 ng/ml	+	+	-	-	-
0.0075 ng/ml (7.5 pg/ml)	+	+	-	-	-
0.00075 ng/ml	+	+	-	-	-
0.000075 ng/ml	+	+	-	-	-
0.0000075 ng/ml (7.5 fg/ml)	+	+	-	-	-

Table 3. Detection of toxin by the iPCR assay in heated food samples

Sample	SEA iPCR Assay*			SEB iPCR Assay*		
Spiked level or condition	**Ground Turkey (77°C, 30 min)**	**Milk (100°C, 15 psi, 10 min)**	**Milk (121°C, 15 psi, 2 hr, 45 min)**	**Ground Turkey (77°C, 30 min)**	**Milk (100°C, 15 psi, 10 min)**	**Milk (121°C, 15 psi, 2 hr, 45 min)**
7.5 ng/ml (or /gm)	+	+	+	+	+	+
0.75 ng/ml	+	+	+	+	+	+
0.075 ng/ml	+	+	+	+	+	-
0.0075 ng/ml	+	+	+	+	+	-
0.00075 ng/ml	+	+	-	+	+	-
0.000075 ng/ml	+	+	-	+	+	-
0.0000075 ng/ml (7.5 fg/ml)	+	+	-	+	+	-
No toxin	-	-	-	-	-	-
No 1° antibody	-	-	-	-	-	-
No 2° antibody	-	-	-	-	-	-
Negative Control	-	-	-	-	-	-
Blank	-	-	-	-	-	-

*Note: The appropriate SE toxin (SEA or SEB) was added in the spiked samples.

Table 4. Detection of SEA by SEA iPCR-SA assay in spiked samples. Detection of SEA from broth media , milk, meringue pie, and ground turkey.

SEA Level	TS Broth		Milk		Meringue Pie		Ground Turkey	
	SEA	C_T	SEA	C_T	SEA	C_T	SEA	C_T
7.5 ng/ml (or /gm)	**+**	**22**	**+**	**26**	**+**	**28**	**+**	**27**
0.75 ng/ml	**d.n.a.**		**+**	**27**	**d.n.a.**		**+**	**27**
0.075 ng/ml	**+**	**23**	**+**	**28**	**+**	**29**	**+**	**28**
0.0075 ng/ml (7.5 pg/ml)	**d.n.a.**		**+**	**29**	**d.n.a.**		**+**	**29**
0.00075 ng/ml	**+**	**25**	**+**	**30**	**+**	**30**	**+**	**30**
0.000075 ng/ml	**d.n.a.**		**+**	**30**	**d.n.a.**		**+**	**31**
0.0000075 ng/ml (7.5 fg/ml)	**+**	**30**	**+**	**31**	**+**	**31**	**+**	**32**

d.n.a., did not assay.

Table 5. Detection of SEB by SEB iPCR-SA assay in spiked samples. Detection of SEB from broth media, milk, tuna salad, and ground turkey.

SEB Level	TS Broth		Milk		Tuna Salad		Ground Turkey	
	SEB	C_T	SEB	C_T	SEB	C_T	SEB	C_T
7.5 ng/ml (or /gm)	+	**24**	+	**23**	+	**26**	+	**22**
0.75 ng/ml	**d.n.a.**		+	**24**	**d.n.a.**		+	**21**
0.075 ng/ml	+	**25**	+	**25**	+	**27**	+	**23**
0.0075 ng/ml (7.5 pg/ml)	**d.n.a.**		+	**26**	**d.n.a.**		+	**24**
0.00075 ng/ml	+	**26**	+	**26**	+	**30**	+	**25**
0.000075 ng/ml	**d.n.a.**		+	**27**	**d.n.a.**		+	**26**
0.0000075 ng/ml (7.5 fg/ml)	+	**28**	+	**28**	+	**32**	+	**27**

d.n.a., did not assay.

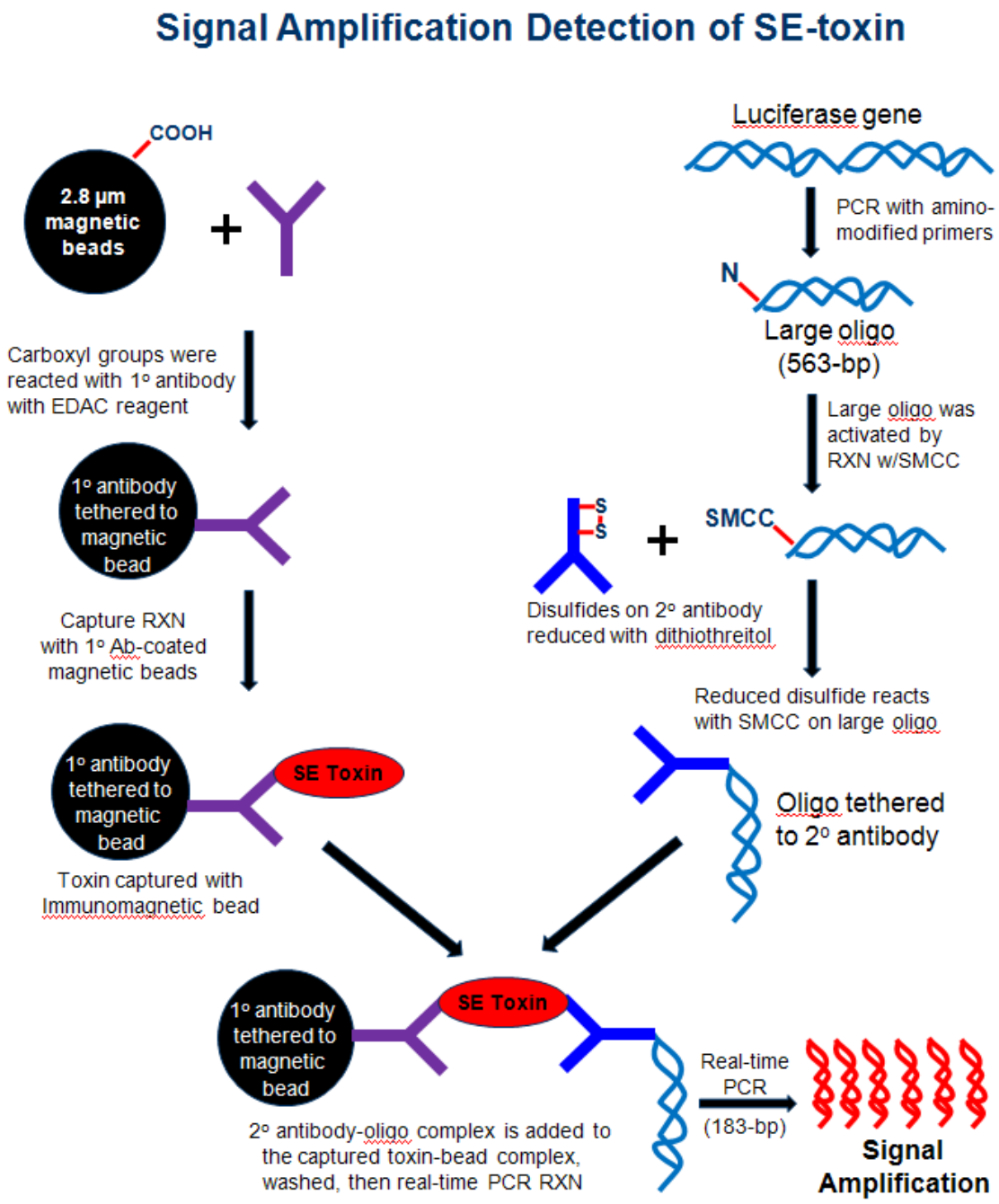

Figure 1. Schematic representation of the flow of reactions involved in the immuno-PCR signal amplification detection of staphylococcal enterotoxins.

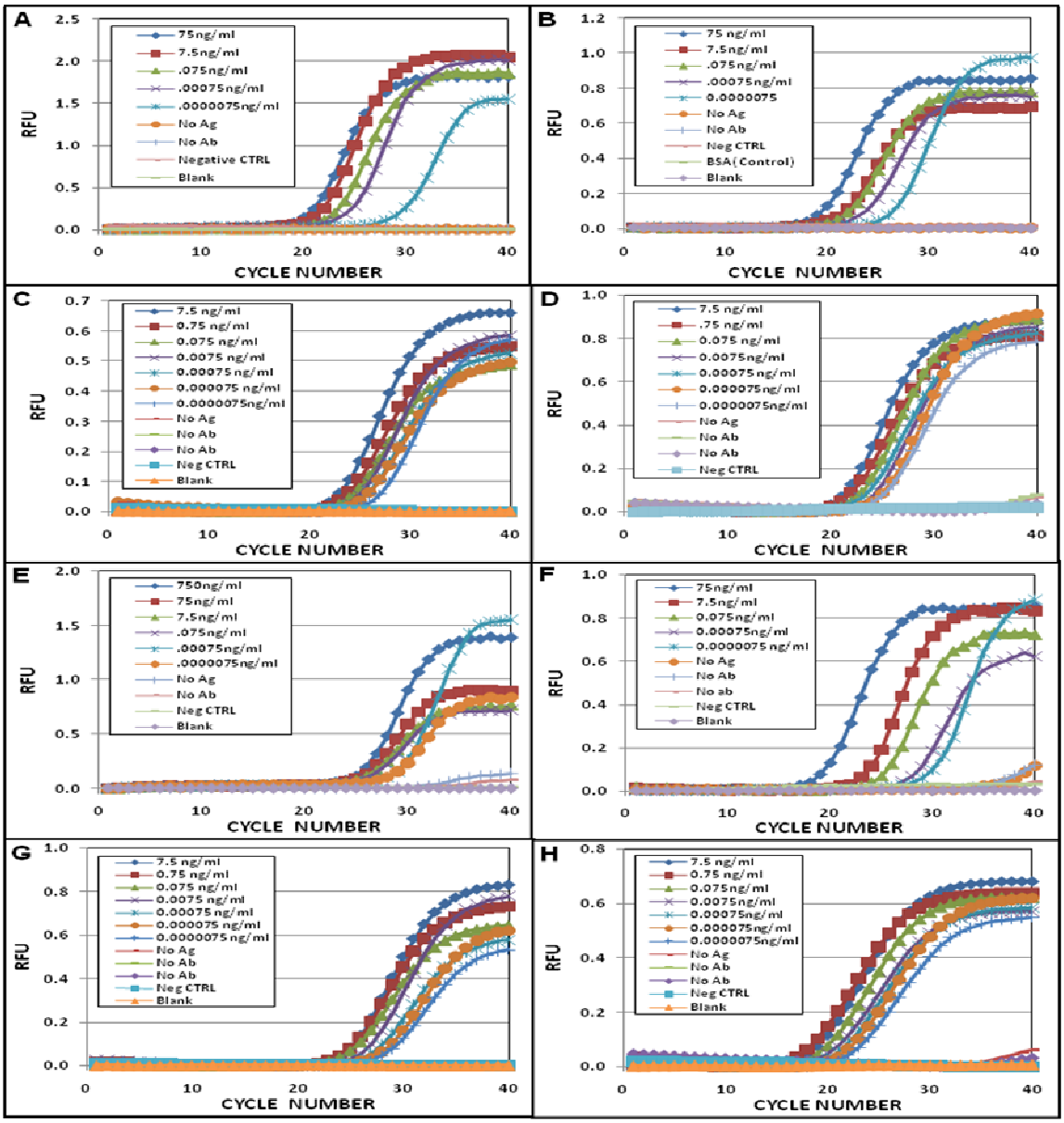

Figure 2. Detection of SEA and SEB in spiked samples. Detection of SEA from broth media (panel A), milk (C), meringue pie (E), and ground turkey (G). Detection of SEB from broth (B), milk (D), tuna salad (F), and ground turkey (H).

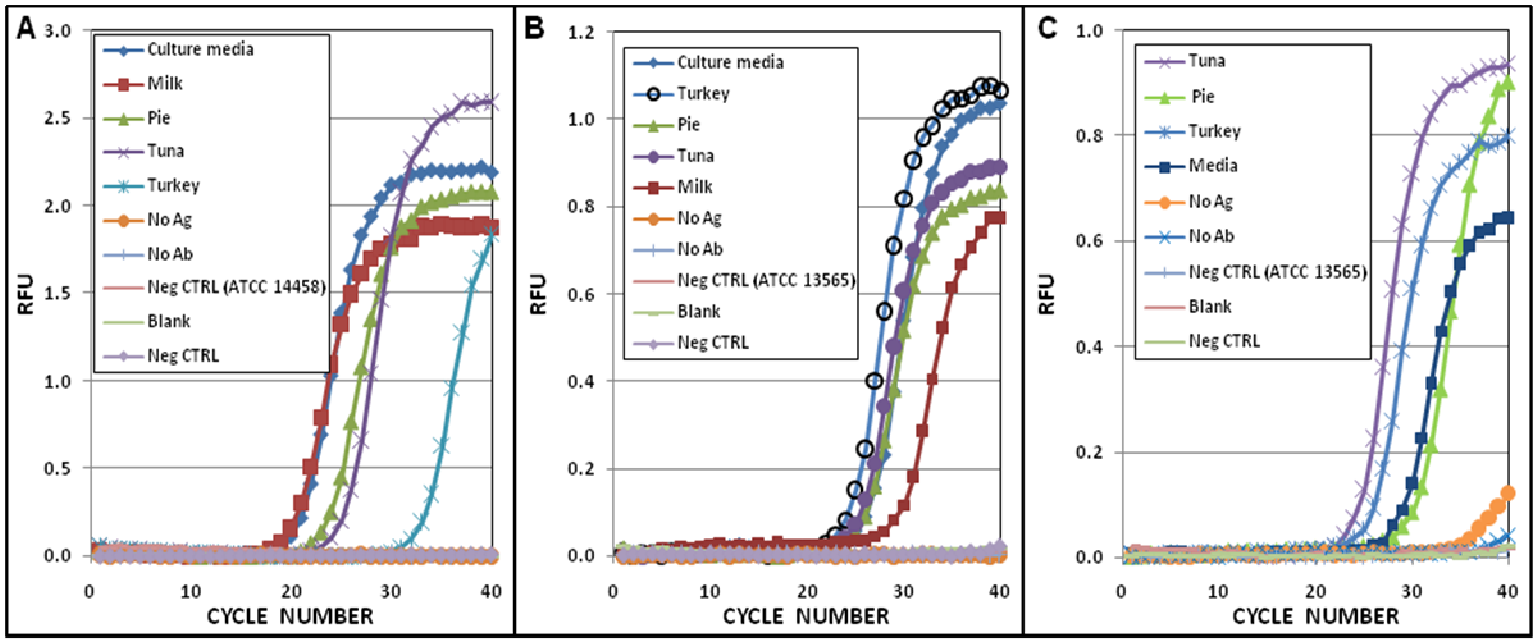

Figure 3. Specific detection of staphylococcal enterotoxins SEA (panel A) or SEB (panels B & C) using the immuno-PCR assay in various foodstuffs after incubation with toxin-producing strains. Panel A, foodstuffs incubated with *S. aureus* ATCC 13565 (SEA+). Panel B, foodstuffs incubated with *S. aureus* ATCC 14458 (SEB+). Panel C, foodstuffs incubated with *S. aureus* ATCC 51740 (SEB+).

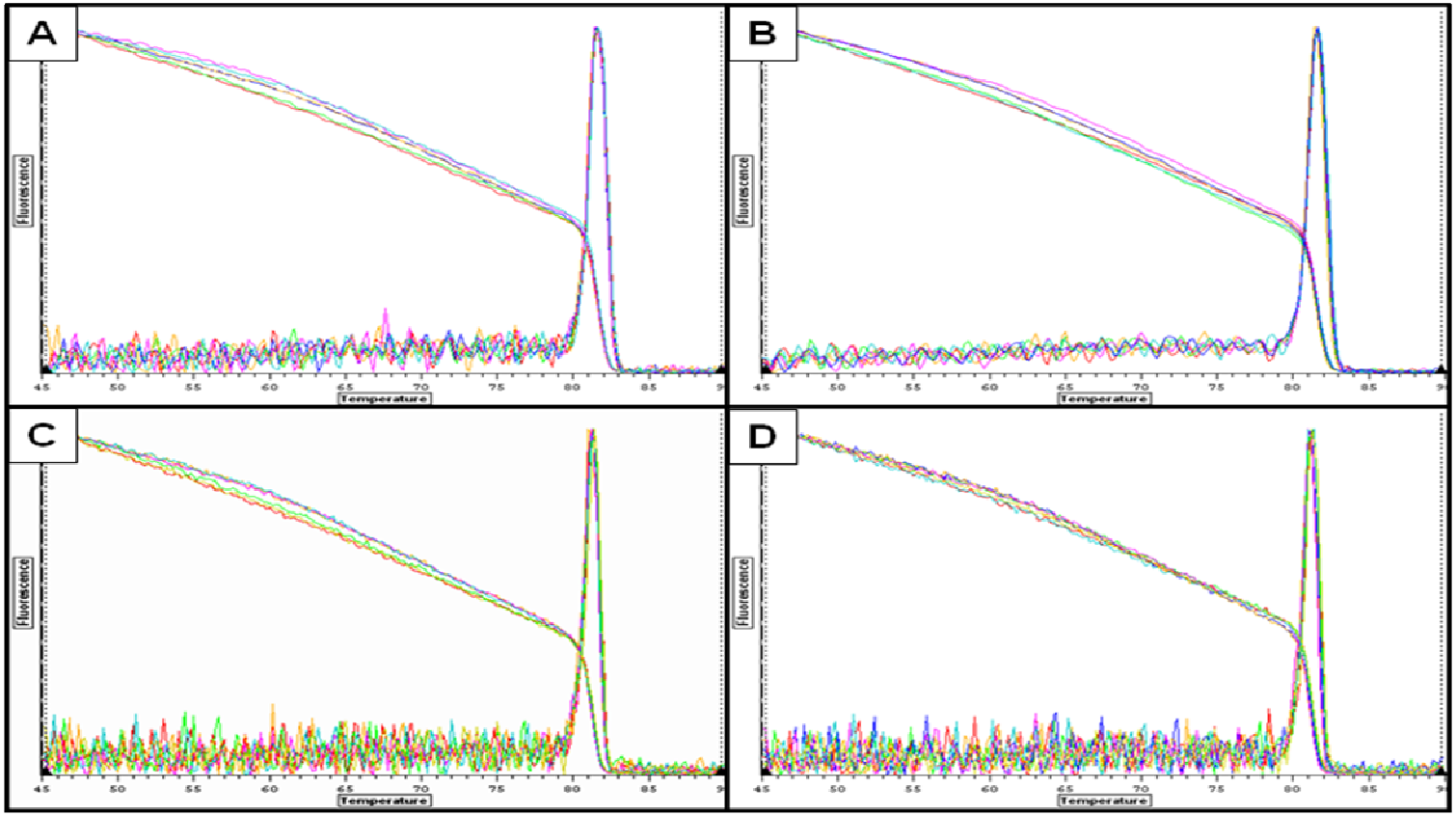

Figure 4. Melting curves of PCR products derived from detection of SEA and SEB toxins using the immuno-PCR assay. Melting curves from immuno-PCR of SEA (panel A) and SEB (panel B) autoclaved in milk. Melting curves of amplimers from detection of SEA (panel C) and SEB (panel D) after heating in ground turkey.

CHAPTER IV

A "PCR PRIMER ARRAY" FOR RAPID DETECTION AND TYPING OF *STAPHYLOCOCCUS AUREUS* ENTEROTOXIN GENES

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INTRODUCTION

Staphylococcus aureus is an opportunistic pathogen that is capable of producing a variety of virulence factors that enable it to invade and colonize a susceptible host. The CDC estimates that *S. aureus* causes about 185,000 cases of food poisoning annually in the US (Mead et al., 1999). Humans are the most common reservoirs of staphylococcal food poisoning bacteria. The organism is commonly found in the nasal passage, skin and throat and gains access to food during processing and food handling. Animals are also heavily colonized with the organism and contamination of foods during processing of animals and their products is also a common occurrence. The ability of this pathogen to invade the host involves the production of a vast array of extracellular proteins. Though the organism produces many metabolites such as enzymes and cytotoxins which contribute to its pathogenicity and virulence, the enterotoxins present the greatest risk of foodborne illness to consumers. The enterotoxins are heat resistant proteins which have similar structure and molecular masses of about 22-30 kDa (Bi-Su et al., 2005). The staphylococcal enterotoxins (SEs) are potent gastrointestinal toxins, possessing superantigenic activity and are capable of nonspecific stimulation of T cells (Balaban and Rasooly, 2000). The enterotoxin genes of staphylococcus show high nucleotide sequence homology (Betley et al., 1992). The enterotoxin genes could be present in the

chromosome or they could be carried by mobile genetic elements such as plasmids, bacteriophage, or on genetic areas known as staphylococcal (*S. aureus*) pathogenic islands (SaPIs) which could be transferred horizontally between strains (Mlynarczyk et al., 1998; Orwin et al., 2001). The classical antigenic types of enterotoxins are SEA-SEE (Dinges et al., 2000). Besides the classical SEs, other SEs (SEG-SEJ) and their genes have been reported (Munson et al., 1998; Ren et al., 1994; Zhang et al., 1998). More recently many new SE genes such as *sek, sem, sen, seo, seq, ser, seu* encoding homologue enterotoxins have also been reported (Jarraud et al., 2001; Letertre et al., 2003b; Omoe et al., 2003; Orwin et al., 2003). The most common enterotoxin implicated in staphylococcal food poisoning is SEA (Holmberg and Blake, 1984). The organism produces SEA in the mid exponential phase of growth. The gene *entA* is also carried by a temperate bacteriophage. The production of SEA is not regulated by the accessory gene regulator (*agr)* (Tremaine et al., 1993). The expression of the *entB* gene, however, is *agr* dependent as are the *sec* and the *sed* genes. Isolates from food poisoning outbreaks have been shown to carry the *entB* in their chromosome whereas isolates from non-outbreak events often carry the gene on a 0.76 MDa plasmid (Shalita et al., 1977). Enterotoxin C is classified into three antigenically distinct subtypes (SEC_1, SEC_2, and SEC_3) that are highly conserved and have immunological cross reactivity (Balaban and Rasooly, 2000). There also exist sequence similarities between *entC3, entC1* and *entB* genes (Couch and Betley, 1989). The second most common enterotoxin causing food poisoning outbreaks is SED (Chang and Bergdoll, 1979). The *entD* gene is carried on a 27.6-kb plasmid pIB485 (Bayles and Iandolo, 1989). SED forms a Zn^{2+} homodimer and is able to interact with the

MHC class II molecules causing nonspecific activation of T-lymphocytes, and therefore superantigenic activity of SED is Zn^{2+}-dependent (Sundstrom et al., 1996). SEE shares 84% sequence homology with the SEA and is also one of the classical enterotoxins, but it is infrequently involved in food poisoning outbreaks.

Apart from the classical enterotoxins SEA-SEE, the novel enterotoxins SEG-SER and SEU have been investigated although not much is known about their significance to public health and food safety. The novel enterotoxin genes *seg, seh* and *sei* are also frequently detected (Omoe et al., 2002). *S. aureus* isolates from food poisoning outbreaks frequently harbor the *sea, seg, seh* and/or *sei* genes (McLauchlin et al., 2000). In case of new SEs like SEG and SEI there is evidence confirming their superantigenic activity but not much regarding their emetic activity (Dinges et al., 2000). This could mean that isolates producing enterotoxins SEG-SER will still have relevance to consumer health, even if they may not cause food poisoning. The *seg* and *sei* genes belong to an operon of the *egc* (enterotoxin gene cluster) which contains three more enterotoxin genes, *sem, sen,* and *seo* besides *seg* and *sei.* The *seg* and *sei* genes are present in a tandem orientation (Jarraud et al., 2001). The *egc* can harbor the *seu* gene and be classified as *egc-1* (*seu* positive) or it may not harbor the *seu* gene and be classified as *egc-2* (*seu* negative) (Fueyo et al., 2005b). In the past decade there has been much research on the classical enterotoxins and their relevance to food safety and consumer health. The presence of a variety of enterotoxins and their genes calls for rapid and simple methodology for their identification and characterization. The use of polymerase chain reaction (PCR) for detecting enterotoxin genes and the use of serological techniques for detection of the protein toxins have been common methods for identification and characterization of

enterotoxigenic isolates and their enterotoxins. The presence of enterotoxin genes does not necessarily mean that the particular toxin is produced. However, in most instances the presence of the toxin gene correlates with the production of the toxin and the ability of the organism to produce the toxin in a favorable food environment cannot be overlooked. Hence the detection of toxin genes and characterization of enterotoxigenic strains of *S. aureus* is as important as the detection of the toxin itself. Many methods have been employed so far for detection of the enterotoxin genes. Assays involving restriction fragment analysis and probe hybridization are multistep processes and they could be time consuming and cumbersome. Microarrays for detection of enterotoxin genes have been described (Sergeev et al. 2004). However the use of microarrays for identification and characterization of the enterotoxin genes could be an expensive endeavor. The immunological methods always depend on the ability of the organism to produce detectable amounts of toxins in a given medium. The organism may not produce the toxin in a food, and that does not mean, it will not produce the toxin in another food environment which is more favorable for toxin production. Hence there is always a need for rapid detection of the enterotoxin genes of *S.aureus* for epidemiological and diagnostic purposes. The simplest methods would be PCR-based methods. If more information could be obtained regarding the target genes, like the sequence information rather than just the presence or absence of the genes, it will aid researchers in understanding the organism, the enterotoxins and their significance in public health. The putative significance of the newly discovered enterotoxins like the SEG-SER and SEU in public health and safety calls for greater knowledge and information regarding the

occurrence of these toxins and so efficient means for screening their genes is necessary (Chen et al., 2004).

We have designed a “PCR Primer Array” for rapid identification and sequencing of seventeen enterotoxin genes. The sequences of all *S. aureus* strains currently available in NCBI database were retrieved. An ~500-bp coding region was chosen as the target region and primers flanking the 5’ and 3’ ends of this region were designed with Vector NTI-10 software (Invitrogen Corp). The DNA from enterotoxigenic *S. aureus* ATCC strains (used as control strains) and also from new isolates were subjected to PCR amplification with seventeen enterotoxin-specific “Primer Array” in individual reactions. The amplification was followed by sequencing which gave additional information about the toxin genes present, as well as the strain which carried the genes. Sequencing of the amplicon, followed by analysis of the sequence enabled to determine if the identity of the sequences were preexistent with others currently available in the GenBank or were new sequences. Sequencing followed by procedures like Multi Locus Sequence Typing (MLST) of the isolates will also enable the identification of any difference between the sequences present in the new isolates versus the sequences present in the GenBank database. This methodology allows for rapid screening and typing of the enterotoxin genes and also the enterotoxin producing isolates of *S. aureus.*

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are described in Table 1. The enterotoxigenic strains of *S. aureus* (ATCC strains used as control) were grown in Tryptic Soy Broth (TSB; EMD Chemicals Inc., Gibbstown, NJ) by incubation for 24-48 h at 37^{o}C. For experiments, a freshly-transferred overnight culture of *S. aureus* was used. Reference stock cultures were maintained at -80^{o}C in TSB with 10% glycerol. Colony morphology was examined on Baird Parker agar (Himedia Labs, India) with egg yolk and tellurite. Mannitol Salt Agar (MSA) (Becton, Dickson & Co., MD) was to confirm mannitol fermentation. Gram-stain reactions, biochemical tests, and PCR with *S. aureus* 16S rRNA-specific primers were also used to confirm the identity of all strains used.

Isolation of enterotoxigenic strains of *S. aureus*. Samples were collected from human nasal passages and from farm dairy cattle, as the microbial flora from the anatomical sites of humans and animals harbor the bacteria. Samples of foods commonly known to harbor *S. aureus*, such as raw milk from dairy cows, were also collected for isolating the organism. Foods were sampled within 24 h. Samples were aseptically weighed into sterile stomacher bags, diluted 1:10 with M-Staph broth (Difco Laboratories, Detroit, MI), stomached, and incubated for 24 h at 37^{o}C for enrichment. Following enrichment, dilutions of the samples were plated on MSA, which is a selective and differential medium for staphylococcus. Mannitol fermenting colonies were selected and streaked onto the same medium thrice. Colonies were also streaked onto Baird-Parker agar to

study colony morphology, potassium tellurite reduction, and lecithinase production. A single colony was picked and grown in TSB and was used for performing the staining reactions and biochemical tests such as catalase and coagulase. A Polymerase Chain Reaction (PCR) with *S.aureus* 16S rRNA-specific primers was performed to confirm the identity as *S. aureus.* Swab samples collected from human nasal passages, from anatomical sites of animals, and from processing environments were enriched in test tubes containing 9 ml of M-Staph broth for 24 h at 37^{o}C. Following enrichment the swab suspensions were streaked on MSA to confirm mannitol fermentation and on Baird-Parker agar for colony morphology, potassium tellurite reduction and lecithinase production. Gram-stain reactions, biochemical tests (catalase, oxidase, tube and slide coagulase, Mannitol fermentation, lecithinase production, tellurite reduction) and PCR with *S. aureus* 16S rRNA-specific primers confirmed the identity of the organisms.

Strategy for designing the "Primer Array" for enterotoxin genes. The sequences of all known *S. aureus* enterotoxin genes currently available in the GenBank database were retrieved. The sequences of the enterotoxin genes *sea-see, seg, seh, sei, sej, sek ,sem, sen, seo, seq, ser* and *seu* were aligned using the Clustal W program provided by the software Vector NTI 10 (Invitrogen Corp). Our initial aim was to design a universal primer which could amplify all the enterotoxin genes in a single PCR reaction. However, no region had 100% homology for all enterotoxins genes that would allow this approach. Our next approach was to develop PCR primers for each different enterotoxins correlating to the same region of an alignment to that a sequence alignment could be made of all partial toxin gene sequences. Using a 'similarity index' for the multiple sequence alignment of

all known staph enterotoxin genes, we were able to determine a 500-600 nucleotide region that could span several regions of dissimilarity and be obtained within a single PCR reaction (Fig. 1). We then focused on regions of high homology spanning 60-bp flanking both sides of the 500-600-bp region targeted for PCR analysis (Fig. 2). By performing individual multiple sequence alignments only on genes for the same enterotoxin, we were able to confirm that the selected 60-bp region demonstrated high homology between same-toxin sequences obtained from different strains (via GenBank) for use in obtaining PCR primers (Fig. 3). The alignment showed regions of consensus and also regions of heterogeneity. A 500-bp region was chosen as the target region. The primers were chosen in such a way that, among individual groups of enterotoxin genes, the sequence of the primer was in a consensus 60-80 bp region and the amplified region was the region which had the heterogeneity. By following this strategy, a set of primers could be used to amplify a specific enterotoxin gene sequence. The primers flanked the 5' and the 3' ends of the chosen ~500 bp region of the target enterotoxin gene (Table 2). Any variations in the sequence of the amplimer would enable us to easily identify and characterize the target enterotoxin. The primers were designed using the Vector NTI v.10 software. The criteria for the primer design included an optimum length of 25 bp and melting temperature (T_m) of $50^oC - 55^oC$. A total of 17 pairs of primers were designed (Table 2).

Extraction of the DNA. The bacterial strains were resuscitated by inoculating into TSB and transferred again before use. An overnight growth culture was used for extraction of the DNA. The DNA was extracted using a DNA extraction kit according to the manufacturer's instructions (QIAGEN Inc, Valencia, CA).

Amplification of the enterotoxin genes using the array. The enterotoxin genes of the *S. aureus* strains were amplified using the array of primers previously determined and synthesized (IDT Labs, Coralville, IA). The PCR reaction mix consisted of the following: 5 µl of 200 ng of template DNA extracted from the *S.aureus* strains, 12.5 µl (1x concentration) of SYBR Green PCR mix (Sigma-Aldrich, St. Louis, MO; contains PCR buffer, $MgCl_2$, dNTPs, and DNA polymerase), and 100 nM of the seventeen individual primers in seventeen different reactions. The final reaction volume was 25 µl. The reaction was contained in 0.2 ml white, low profile, unskirted, PCR reaction tubes (MJ Research, Hercules, CA). PCR was performed in a real-time thermal cycler (Opticon 2; MJ Research, Bio-Rad). The reaction conditions were as follows: initial denaturation at 95°C for 2 min (initial denaturation), followed by 40 cycles of 95°C for 15 s (denaturation), 55°C for 1 min (annealing), 72°C for 1 min 20 s (extension), followed by a final hold at 4°C. All reactions included a non-template control (no DNA) and a blank control consisting of PCR-grade water. The reactions also had positive controls which were ATCC strains known to harbor the enterotoxin genes. Each run also had a negative control, which had the template DNA from staphylococcus, which did not give amplification with any of the seventeen enterotoxin gene specific primers employed in this assay. At end of each run a melting curve analysis was performed from 45°C to 90°C at a ramp rate of 0.2°C/s to confirm the specificity of the PCR reaction. The amplicon obtained was purified using Montage PCR clean up kit (Millipore, Burlington, MA). The presence of the amplicon was also confirmed by electrophoresis on 1.5% agarose gel. The DNA sequencing of the PCR amplicons was performed at the Department of

Biochemistry and Molecular Biology Recombinant DNA/Protein Resource Facility (Oklahoma State University 'Core Facility') using an automated DNA sequencer via "Big Dye™"1.1- terminated reactions analyzed on an ABI model 3730 DNA Analyzer.

Sequence analysis. The sequence information obtained from the DNA/Protein Resource Facility OSU was imported into MEGA 4.1 (Molecular Evolutionary Genetics Analysis 4.1) software http://www.megasoftware.net/index.html. The ability to conduct sequence alignments, editing DNA sequences trace files, mask regions on the sequence traces, and direct BLAST search are a few of the features of the software. The software was used for the cleanup of the obtained enterotoxin gene sequences from PCR amplimers. The gene sequences were then analyzed by using the BLAST (Basic Local Alignment Search Tool) algorithm, through the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/blast).

RESULTS AND DISCUSSION

S. aureus is one of the common causes of food poisoning (Letertre et al., 2003a). *S.aureus* is a common inhabitant of human nasal passages (Kluytmans et al., 1997) and as many as 30% of the healthy human population carry the organism in their anterior nares (Nilsson and Ripa, 2006). Since these organisms are prevalent inhabitants of human nasal passages, there is ample opportunity for the organism to contaminate food when handled during processing. Over the past decade, many methods have been developed for detection of the organism itself, the toxins it elaborates, or the various toxin genes they contain. The most common methods for detecting toxins are the immunological assays such as the Enzyme Linked Immunosorbent Assay (ELISA), Reverse Passive Latex Agglutination Assay (RPLA), Radioimmunoassay (RIA), Enzyme Immuno Assay(EIA) etc. These assays are usually designed to detect the classical enterotoxins SEA-SEE. In epidemiological analysis of outbreaks, the identification of levels of *S. aureus* at $>10^5$ CFU/gm is sufficient cause to implicate enterotoxins foodborne poisoning, and therefore methods that can detect (and quantitate) the presence of *S. aureus* in food samples may help support such efforts. The use of PCR for detection of specific enterotoxin genes has previously been reported (Becker et al.,1998; Sharma et al., 2000). Many investigators have noted that for epidemiological reasons, if a toxin gene is present in staphylococci, then that organism should be considered positive for that toxin production since the toxin production in food cannot be ruled out. PCR methods can give more information than simply the presence of genes. Current techniques allow the easy cleanup, submission, recovery and comparison of sequence information such that it allows 'molecular typing'

of toxin genes (and therefore the strains themselves). The method is capable of detecting small differences in sequences. The aim of our study was to develop a strategy in which ample of information could be obtained regarding the enterotoxin genes, with inexpensive, rapid and easy to use PCR methods followed by automated sequencing.

The sequences for all currently available *S. aureus* enterotoxin genes were retrieved from the GenBank and grouped according to the toxins they produced. When the sequences were examined, we were able to locate regions of homology (similarity) and heterogeneity (dissimilarity) of the aligned toxin genes (Fig. 1). Regions on the alignment which showed similarity among the individual toxin groups were selected for designing of the forward and reverse primers for each toxin gene group (Fig. 2). The primers flanked the 5' and the 3' ends of a ~ 500-bp coding region were chosen as the target region for the generation of the amplicon (Fig. 3). By choosing PCR primers for the same region based on multiple sequence alignment, we would be able to subsequently align sequence information obtained from newly generated amplicons for Clustal W and dendrographic analysis that would allow typing of the various toxin genes identified. Melting curve analysis confirmed whether PCR reactions were specific (i.e., presence of a single product) to rule out the formation of primer-dimers. Isolates from human nasal passages, animal anatomical sites, and foods were subjected to PCR using the array of primers for the seventeen toxin genes.

In most of the isolates we processed against our SA-enterotoxin primer array, most harbored multiple enterotoxin genes. In samples obtained from raw milk strain Milk-8 possessed two genes *sem* and *sen* (data not shown), Milk-10 possessed 3 enterotoxin genes (*sej, sed, ser*)(Fig. 4), and Milk-159 possessed 4 enterotoxin genes

(*sem, sei, seg, sen*)(Fig. 5). Of the four strains isolated from raw milk, three of the strains had multiple enterotoxin genes, whereas strain Milk-11 possessed only one, *sen* (Fig. 6). Strain Milk-159 had the *seg, sei, sem* and *sen* the combination belonging to the egc-1 enterotoxin gene cluster lacking *seo.* The egc1 cluster is carried by the pathogenic island vSaβ type 1. In our isolate, the *seo* gene was missing. The relationship between the novel enterotoxins and food poisoning is not clear, though they can still lead to nonspecific activation of the immune system (Dinges et al., 2000). Strain Milk-159 had both *seg* and *sei.* Among the newly identified enterotoxin genes, *seg*, *she,* and *sei* were reported to be the most frequently distributed genes from a variety of sources (Omoe et al., 2002). Other researchers reported that *sea, seg, she,* and *sei* were commonly present along with other SE genes in food poisoning outbreaks (Mclauchlin et al., 2000). Jarraud et al. (2001) also demonstrated that the *seg* and *sei* have a high rate of coexistence in many isolates and they are often in tandem orientation. Earlier research showed that twelve isolates involved in three food poisoning outbreaks had only *seg* and *sei* genes and none of the other enterotoxin genes including the classical enterotoxin genes were detected (Omoe et al., 2002). This may suggest the possible involvement of the genes *seg* and *sei* in causing foodborne poisoning. In earlier studies, the strains having the *seg* and *sei* genes have been analyzed for the production of the toxins SEG and SEI. Research with animal models have suggested that these two toxins have the capability to induce emetic response but their involvement in causing food poisoning in humans in not clear. Omoe et al. (2002) also showed that the strains having the *seg* and *sei* genes produced very low amounts of the respective toxins and it is not clear if production of low amounts of these toxins have any significance in food poisoning. Although they did not demonstrate high levels of

toxin production, they were able to demonstrate significant levels of transcription of the mRNAs of the *seg* and *sei* genes among the strains harboring these genes (Omoe et al., 2002). The toxins SEM, SEN, and SEO have not shown any emetic activities in animal models but there is a possibility that the toxins may be translated from mRNA in larger amounts than SEG and SEI, and as a result even these enterotoxins may cause food poisoning outbreaks (Omoe et al., 2002). Among the four raw milk isolates none had the *seo* or *seu* genes. One raw milk isolate (Milk-10; Fig. 4) did not possess the *egc* cluster but had a different combination of enterotoxin genes namely the *sed, sej* and *ser.* The *sed* gene encodes for the classical enterotoxin SED which is one of the most commonly associated enterotoxins in food poisoning outbreaks (Smyth et al., 2005). Hence the strain might have greater significance in food poisoning than the other isolates from raw milk. The gene *sed* is present on the plasmid pIB485 (Bayles and Iandolo, 1989). Later research revealed that the plasmid also carried the genes *sej* and *ser* (Omoe et al., 2003; Zhang et al., 1998). Fueyo et al. (2005a) reported the presence three plasmids which are associated with the *sed, sej* and *ser* genes. The pUO-Sa- SED1 is the commonly found plasmid among SED producing strains, the pUO-Sa-SED2 is present in certain human isolates and had similar restriction-hybridization patterns like the pIB485 plasmid. A third plasmid pUO-Sa-SED3 had a different restriction pattern from the pIB485 plasmid, carried the genes sed and *sej*, and had one or more *ser*-like genes. Our results show that isolate Milk-10 has all the three genes *sed, sej* and *ser* and the significant differences in threshold cycle (C_T) obtained for the different enterotoxins genes suggest the template DNA is present at different copy numbers within this strain (Fig. 4). The lowest C_T was obtained with *sed* suggesting it may be plasmid-borne in strain Milk-10 (Fig. 4). It is

possible that the 3 toxin genes could be distributed among the chromosome and 2 plasmids of different copy number considering the drastically different C_T of the 3 toxin genes whereas raw milk isolate Milk-159 shows close similarity in C_T value for the 4 toxin genes detected, *sem*, *sei*, *seg*, and *sen* (Fig. 5). Raw milk isolate Milk-11 was the only milk isolate that demonstrated a single staph enterotoxins gene, *sen* (Fig. 6). When a BLAST search comparing the enterotoxin genes from our raw milk isolates against the GenBank data base was performed, we were able to obtain a 99% sequence homology with the partial sequence of the *sed*, *sej*, and *ser* genes identified in strain Milk-10. The *sed* partial sequence had two bases that were different from the *sed* gene sequences present in the database and *ser* partial sequence had one base that was different. Similarly strain Milk-159 had a 99% homology with a one-base difference in the (partial) *sem* gene.

We also isolated *S. aureus* strains from the udders of cows. The strains C1, C2, C3, C4, C5, C6, C7, C8, and C9 were identified as *S. aureus* based on their Gram-stain characteristics, biochemical reactions, morphology, colony characteristics, and amplification with *S. aureu*-specific 16s rRNA primers. However the strains did not show amplification with any of the seventeen enterotoxin gene specific primers (data not shown). Another bovine strain C-A isolated from cow hide was positive for *sed* and *sej* genes, but it lacked the *ser* gene (Fig. 7). The presence of *sed* and *sej* together indicates the possible presence of the plasmid pIB485 as previous studies have indicated that these two genes are carried on the plasmid in opposite orientations and are separated by 895 nucleotides (Jarraud et al., 2001). The demonstration of different C_T for the real-time PCR plots also suggests their template is present in different copy numbers (Fig. 7). The

sequence of the *sed* gene from the C-A isolate had 99% homology with the *sed* sequences in the data base and had two base which were different from the rest. All five isolates of bovine origin either possessed the genes of *egc* cluster namely *seg, sei, sem, sen* or the plasmid borne genes like *sed, sej* or *ser* (Figs 4-7). This is in accordance with previous research which indicates that strains with genes *seg* and *sei* or *sed* and *sej* are most frequently associated with bovine *S.aureus* isolates (Lammler et al., 2000; Omoe et al., 2002).

A strain isolated from spoiled (fermented) apple had enterotoxin genes *sea, sed* and *sej* of nearly identical C_T values suggesting similar template copy number (Fig. 8). This strain has genes for two classical enterotoxins SEA and SED which are most frequently isolated from samples causing food poisoning (Isigidi et al., 1992; Lindqvist et al., 2002). The *sea* and *sej* partial sequences had 100% homology with the sequences in the database but the *sed* gene had 99% with two nucleotide bases that were different.

We also examined human isolates since food handlers themselves are often common vehicles for contamination of food products (Figs. 9-14). *S. aureus* strain JG-1 was isolated from human nasal passages carried genes for eight enterotoxins namely *sec, sed, seg, sei, sej, sem, sen,* and *ser* (Fig. 9). The genes *seg, sei, sem* and *sen* of the *egc*-1 were present and also the genes of plasmid pIB485 *sed, sej* and *ser.* In addition to these enterotoxins genes, the strain also had the gene *sec.* The genes *sec, ser, sem ,sei* and *sed* of the isolate JG-1 had 99% homology with those in the GenBank database, with one to two nucleotide base differences. The strain had a combination of classical and novel enterotoxin genes. The presence of *sec* and *sed genes* is significant as they can produce the enterotoxins SEC and SED, respectively. *S. aureus* strain MK-1from human nasal

passage also had the enterotoxin genes of the *egc-1* cluster except for *seg* (Fig. 10). When the genes from the isolate MK-1 were subjected to sequencing and subsequent BLAST analysis we were able to obtain a homology of 99 % with the *sei* and *sen* partial gene sequences. However the *sem* and seo partial sequences had only 95% homology with those in the data base suggesting that the strain MK-1 harbors a variant of the *sem* and *seo* genes. The strain JB-1 had the *seg, sei, sei(v) sem, sen* and *seu* genes and hence can be grouped in *egc-2* (Fig. 11). The strain however lacked the *seo* gene of the *egc-2* cluster. Strains having the genes *seg, sei, sem, sen* and *seo* and two pseudo genes *Ψent1* and *Ψent2* belong to the *egc-1* and if a strain has the *seu* gene it is classified as belonging to the *egc-2* cluster. The *egc* operon is believed to code for the *seu* gene if there is any sequence divergence in the region of the pseudo genes *Ψent1* and *Ψent2* (Letertre et al., 2003b). The strains MK-1 and JB-1 also gave amplification with primers for *sei(v)* gene. Strain J-306 had the *seg* and *sei* coexisting along with the *sem* gene of the *egc-1* (Fig. 12). The strain lacked *sen* and *seo* genes of the cluster. The *sem* gene had two bases that were different, whereas the *seg* and *sei* had 100% homology with the database. Strain SH-1 had only the *sei* and the *sem* genes of the *egc-1* (Fig. 13). The strain also had a positive amplification with the primers for *sei(v)* gene. Strain SM-306 had the *sej* and *ser* genes (Fig. 14). Most of the genes of the human nasal isolates had gene sequences that had 99% homology with those in the database and some of them had 100% homology during the BLAST search. The interesting feature of most of the isolates from human nasal passage is that they all had indication of the presence of the enterotoxin gene cluster. However the *egc* did not possess all the genes of the cluster and variants of *sei* was present in three of the strains. The prevalence of the *egc,* in the isolates from human nasal passage have

been reported in earlier studies (Bania et al. 2006). The strain MK-1 had *sem* and *seo* genes which had only 95% homology with the already existing *sem/seo* genes of the database. Except for JG-1 which had the *sec* and *sed* genes none of the human nasal isolates had genes for the classical enterotoxins. We were also not able to detect the *seb* or *see* genes, the reason could be that these genes are considered as the least frequent among different sources (Bania et al., 2006; Feuyo et al., 2005b; Omoe et al., 2005).

The use of "Primer arrays" is a rapid method for identification of enterotoxin genes present in different *S.aureus* isolates. The strategy employed here for designing of the primers will enable the quick identification of any variations present in the DNA sequences. Most of the *S.aureus* strains in this study had multiple enterotoxin genes. Amplification using the primer array enabled the simultaneous detection of all seventeen of the enterotoxin genes present in the strains. Based on the presence of the enterotoxin genes, the *S.aureus* isolates could also be typed. PCR followed by sequencing gives information not only on the presence and absence of the toxin genes, but also gives additional information regarding the (partial) sequence of the gene amplified. In a limited amount of time, we were able to obtain information regarding the presence of specific genes and through sequence alignment with existing enterotoxins sequences can use this information to 'type' enterotoxigenic strains either by single-locus sequence typing (Fig. 15) or combining partial sequences from multiple enterotoxin genes for multi-locus toxin sequence typing. This kind of molecular typing capability may prove most fruitful to epidemiological investigations where quick and rapid analysis of what potential enterotoxins may be afflicting individuals involved in unsolved foodborne poisoning

outbreaks showing symptoms typical of enterotoxicosis. The method can detect toxin variants and be employed for rapid identification of new strains or new enterotoxin genes.

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Table 1. Strains of *Staphylococcus aureus* used in this study.

Strain	Enterotoxin (Genes detected)	Source
ATCC 13565	SEA (*sea, sed, sej)*	S.E.Gilliland, OSU
ATCC 14458	SEB (*seb)*	S.E.Gilliland, OSU
ATCC 19095	SEC (*sec, seg, seh, sei)*	S.E.Gilliland, OSU
ATCC 27664	SEE (*see)*	S.E.Gilliland, OSU
J-306	*seg, sei, sem*	Human nasal isolate
JB-1	seg, *sei, sei(v), sem, sen, seu*	Human nasal isolate
MK-1	*sei, sei(v), sem, sen,seo*	Human nasal isolate
JG-1	*sec, sed, seg, sei, sej, sem, sen, ser*	Human nasal isolate
Milk-159	*seg, sei, sem, sen*	Raw milk; Private dairy
Milk -8	*sem, sen*	Raw milk; OSU dairy barn
Milk -10	*sed, sej, ser*	Raw milk; OSU dairy barn
Milk -11	*Sen*	Raw milk; OSU dairy barn
SH-1	*sei, sei(v), sem*	Human nasal isolate
SM-306	*sej, ser*	Human nasal isolate
Cow A (C-A)	*sed, sej*	Cow hide; OSU dairy barn
Apple(Ap-1)	*sea,sed,sej*	Apple
C1-C9	No toxin gene detected	Cow udders; OSU dairy

The identity of *Staphylococcus aureus* isolates was based on initial recovery with typical reactions on Baird Parker agar followed by 16S rRNA-based PCR specific for S. aureus.

Table 2. Primers used in this study for the staphylococcal enterotoxin 'primer array'.

Toxin (accession #)	Forward Primer	For Tm (℃)	Reverse Primer	Rev Tm (℃)	Amplimer (bp)
SEA (STATOXAA)	5'-TTTTTACAGATCATTCGTGGTATAA-3'	50.0	5'-GCATGTTTTCAGAGTTAATCGTT-3'	50.0	504
SEB (STAENTB)	5'-TCATGTATCAGCAATAAACGTTAAA-3'	50.9	5'-ACCATTTTATTGTCATTGTACATCA-3'	50.2	576
SEC (STAENTTXC)	5'-AGATAAATTTTTGGCACATGATTT-3'	51.1	5'-CGTTTTATTGTCGTTGTACATCAT-3'	50.7	544
SED (IB4ENTTOXD)	5'-GCGCTAAATAATATGAAACATTCTT-3'	50.8	5'-TCGTAATTGTTTTTCGGGAA-3'	50.1	570
SEE (STAENTE)	5'-AACAAAGAGAGTGATGATCAGTTTT-3'	50.2	5'- AAATCAATATGGAGGTTCTCTGAA	50.9	566
SEI (AF064774)	5'-AACCTACCTATTGCAAATCAACTC-3'	50.7	5'-AAAAAACTTACAGGCAGTCCATC-3'	51.3	494
SEIv (AY158703)	5'-AAAGGCGTCACAGATAAAAACTT-3'	51.2	5'-AAAACTTACAGGCAGTCCATCTC-3'	51.2	510
SEK (U93688)	5'-AGGAATTGATAATCTCAGGAATTTT-3'	51.0	5'-AACTTTTTGGTAACCCATCATCT-3'	50.7	563
SEM (AF285760)	5'-TCATCTTTCGCATCAATTAGTTT-3'	50.5	5'-AAATGGAATTTTTCAGTTTCGAC-3'	51.3	540
SEJ (AB075606J)	5'-CACGATTAGTCCTTTCTGAATTTT-3'	50.9	5'-CTTTAGTTTACAGCGATAGCAAAA-3'	50.4	569
SEN (AF285760)	5'-GGCAATTAGACGAGTCAAATAAA-3'	50.2	5'-AAAAACTCTGCTCCCACTGAA-3'	50.8	523
SEG (AY291449)	5'-CATGATTTAATTTTTCCAATTGAGT-3'	50.9	5'-GAATCAACAACTTTATTATCTCCGT-3'	50.1	521
SEO (AF285760)	5'-TCGATTTACGACAGTAAAATCAATT-3'	51.4	5'-ATAAATTTGCAAATATTGATCTGGT-3'	50.6	547
SEQ (U93688)	5'-CAAGGAGTTAGTTCTGGAAATTTT-3'	50.2	5'-CCAAATGAAAATTCTCTGCATC-3'	50.8	562
SER (AB075606R)	5'-CAATTCTTAGGACATGATTTGATTT-3'	50.8	5'-TTCTATCTTAACACTCGAAGCATCT-3'	50.8	552
SEU (AY205305)	5'-CATTAAAGCCCAAGAGAAGTTTT-3'	51.0	5'-TCATAAGGCGAACTATTAAATTCA-3'	50.2	453
SHE (AJ937548)	5'-GGTCAATATAATCACCCATTCATTA-3'	51.2	5'-TTCTCCTTTTAAATCATAAATGTCG-3'	51.2	498

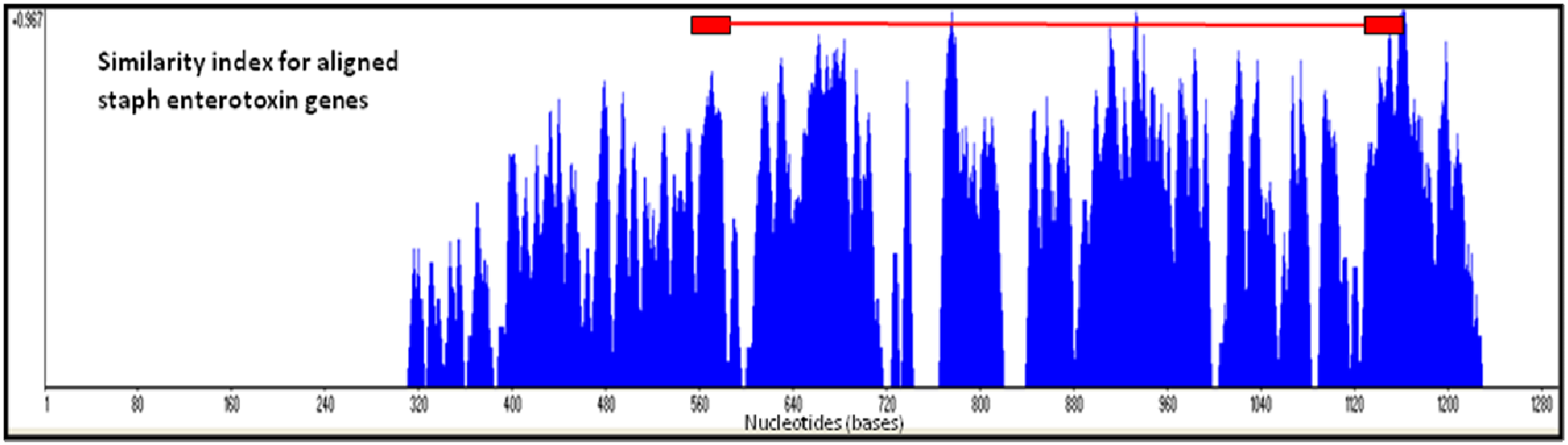

Figure 1. Similarity index for aligned staphylococcal enterotoxin genes. Gaps represent regions of most dissimilarity. Red boxes represent regions targeted for primer design spanning the areas between them.

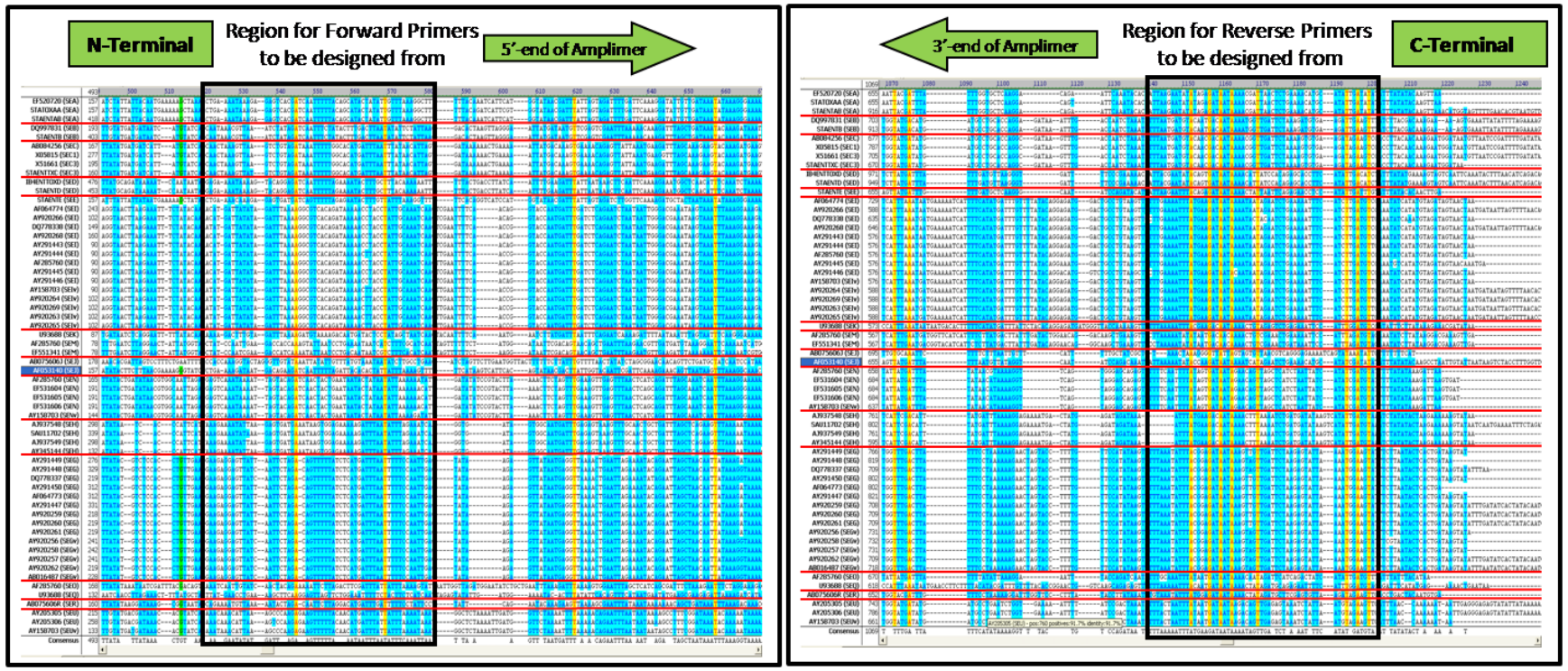

Figure 2. Partial region of multiple sequence alignment targeted for design of forward and reverse enterotoxin-specific primers based on the similarity index.

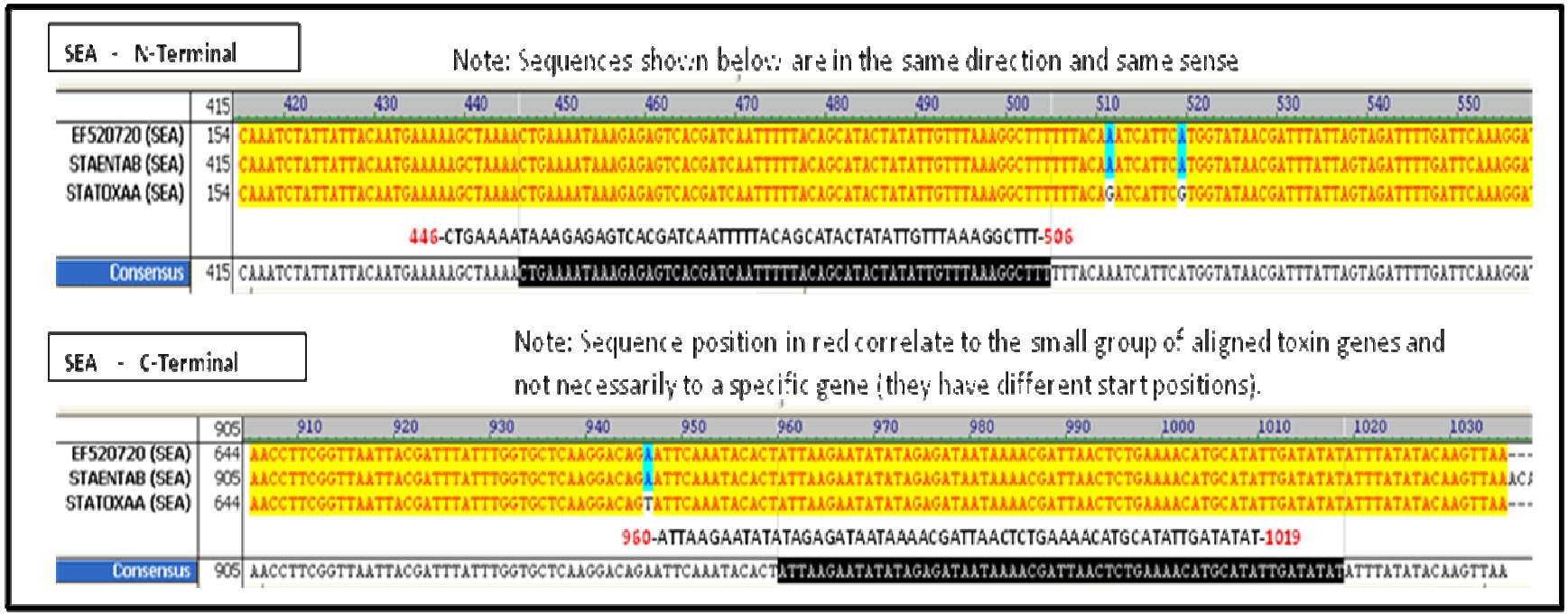

Figure 3. In order to select primers for specific enterotoxins, we examined the respective regions identified from the large multiple sequence alignment on a smaller multiple sequence alignment of only the sequences for the same toxin for both the N- and C-terminal regions. The sequence region selected here (as indicated by the black high-lighted area) is based on the boxed regions on the large multiple sequence alignment (Fig. 2). The sequence position numbers are now based on the 3 sequences and not the 20-30 shown earlier. The multiple sequence alignments are examined here to make sure there is homogeneity within the selected region and primers can be selected within these regions.

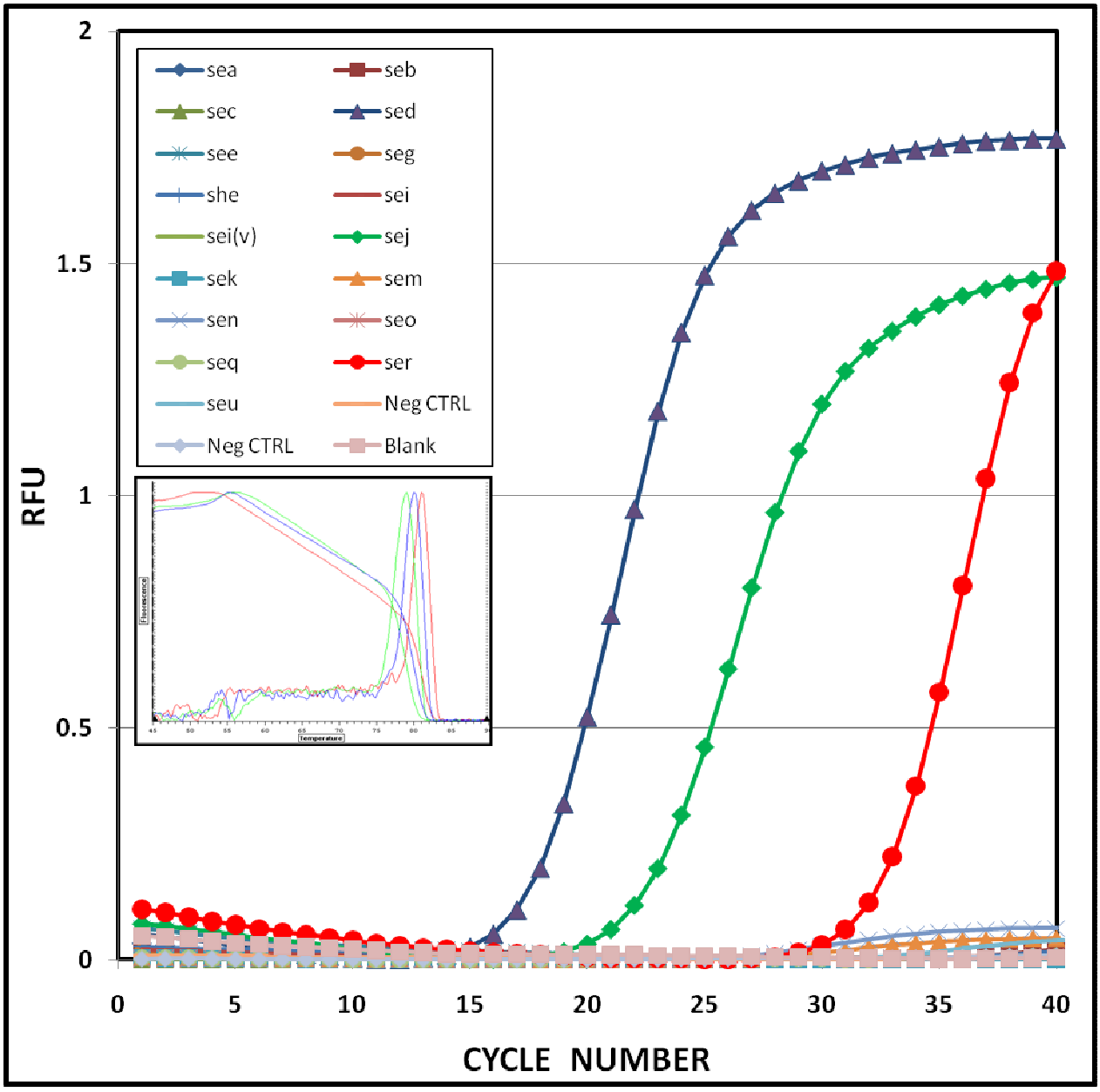

Figure 4. SA enterotoxin primer array real-time PCR of the enterotoxin genes of a *S.aureus* isolate from raw milk (Milk-10) in individual PCR reactions with seventeen pairs of primers. The enterotoxin genes *sej* (Green), *sed* (Blue), and *ser* (Red) were amplified. Bottom inset shows the melting curve of the amplified products obtained from individual PCR reactions.

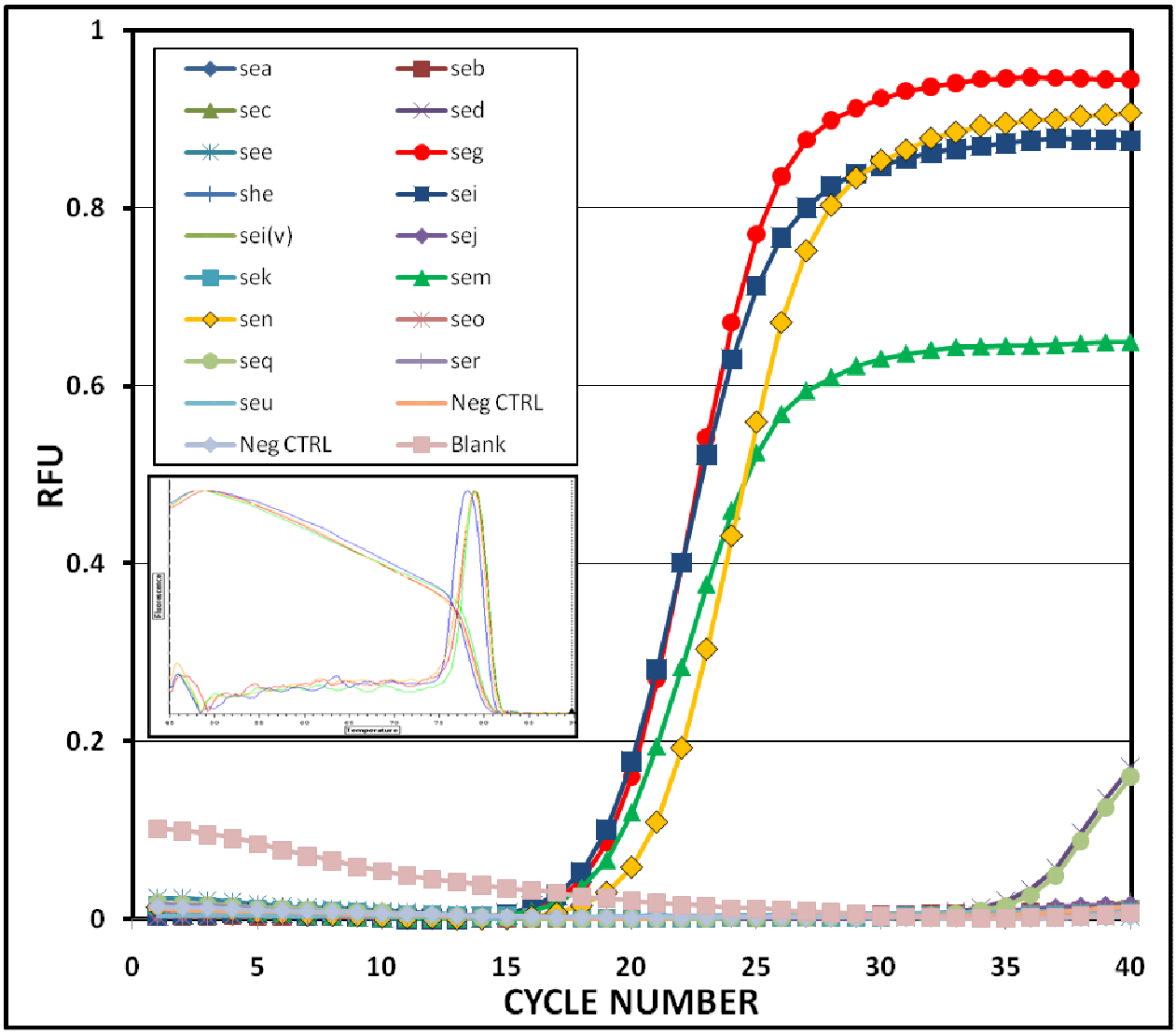

Figure 5. SA enterotoxin primer array real-time PCR of the enterotoxin genes of a *S.aureus* isolate from raw milk (Milk-159) in individual PCR reactions with seventeen pairs of primers. The enterotoxin genes *sem* (Green), *sei* (Blue), *seg* (Red) and *sen* (Yellow) were amplified. The lower inset shows the melting curve of the amplified products obtained from individual PCR reactions.

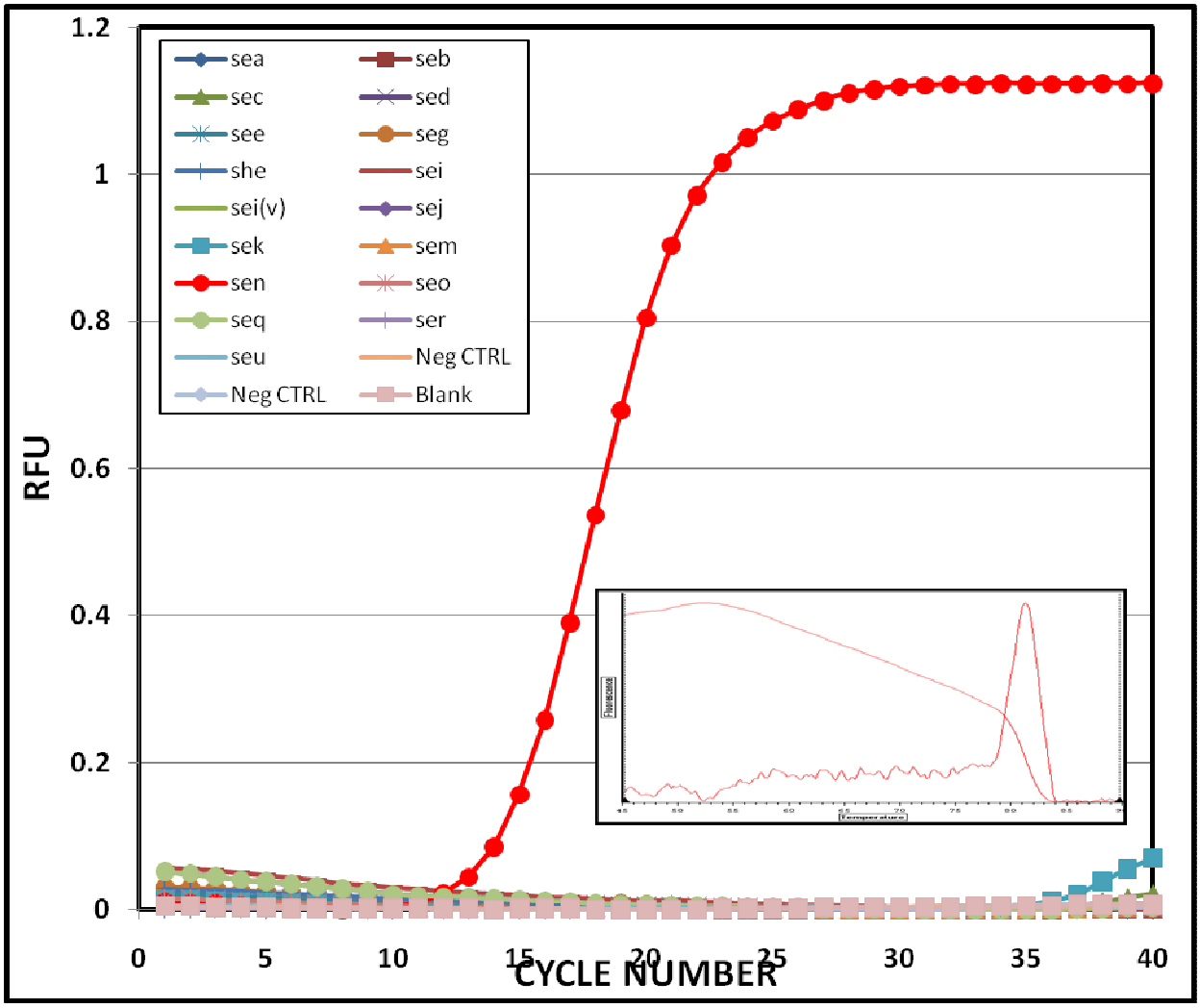

Figure 6. SA enterotoxin primer array real-time PCR of the enterotoxin genes of a *S.aureus* isolate from raw milk (Milk-11) in individual PCR reactions with seventeen pairs of primers. The enterotoxin gene *sen* (Red) was amplified. The lower inset shows the melting curve of the amplified product obtained from individual PCR reaction.

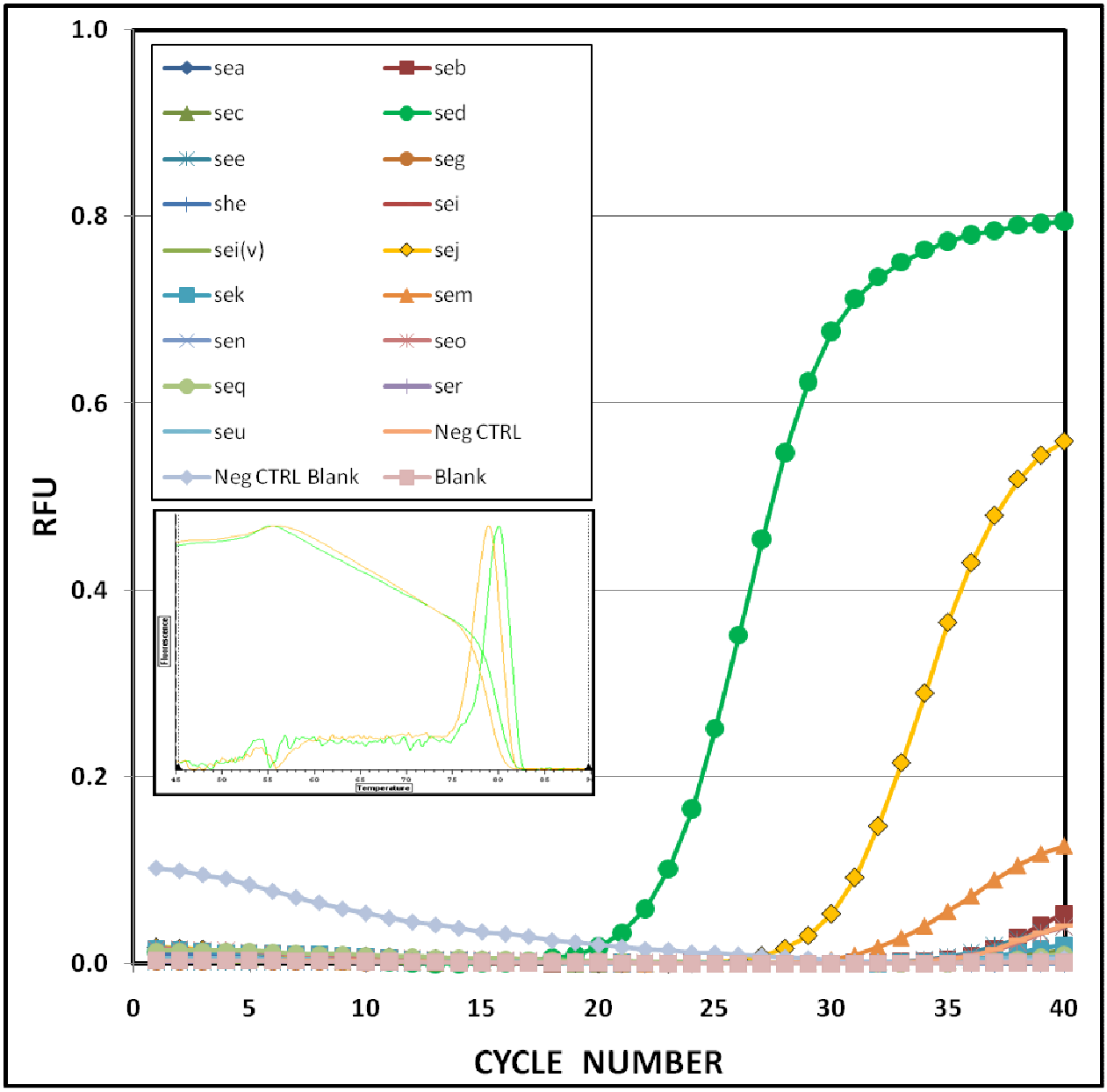

Figure 7. SA enterotoxin primer array real-time PCR of the enterotoxin genes of a *S.aureus* isolate from from cow hide (C-A) in individual PCR reactions with seventeen pairs of primers. The enterotoxin genes *sed* (Green) and *sej* (Yellow) were amplified. The lower inset shows a melting curve of the amplified products obtained from individual PCR reactions.

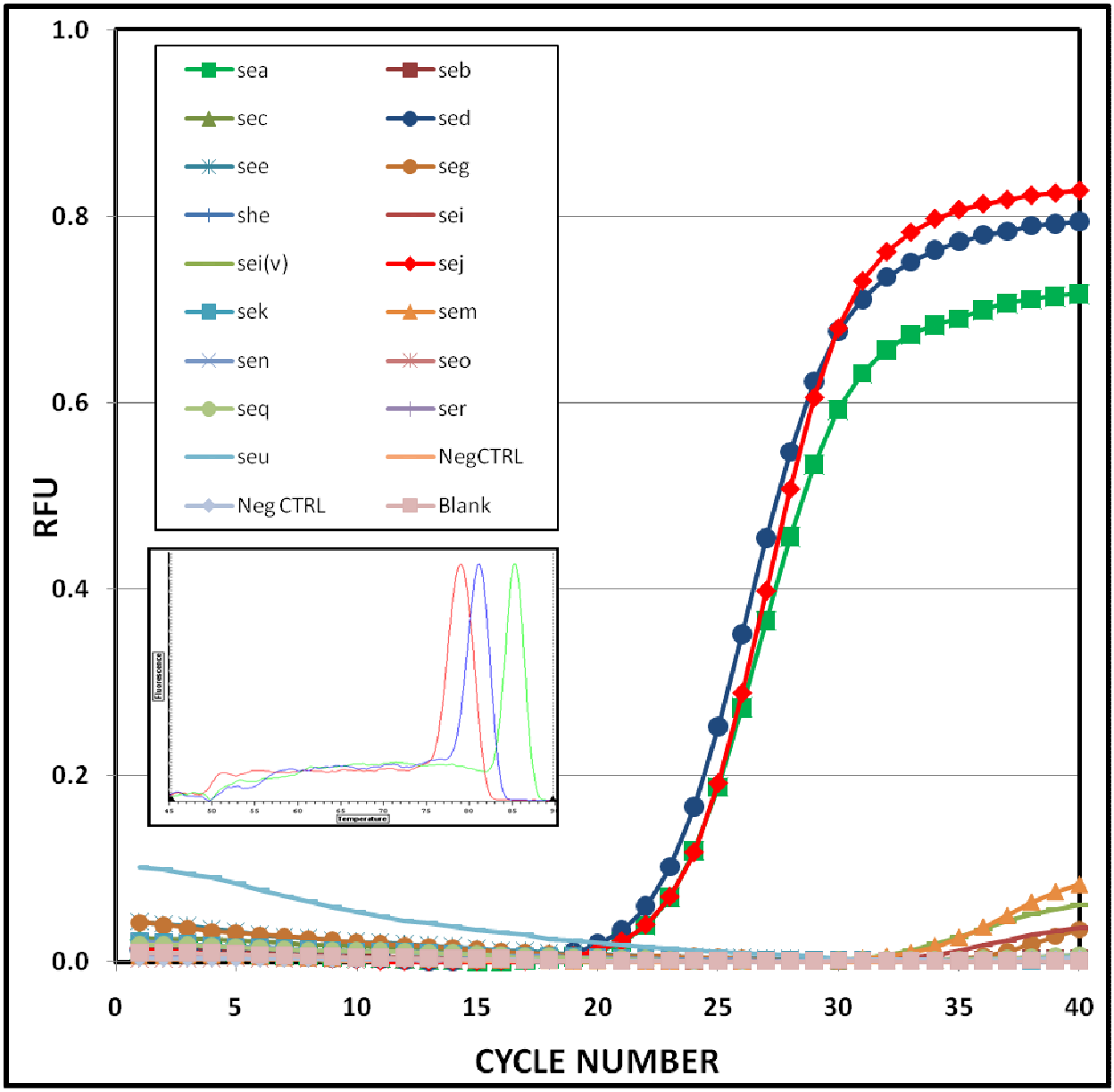

Figure 8. SA enterotoxin primer array real-time PCR of the enterotoxin genes of a *S.aureus* isolate from from a partially-fermented apple in individual PCR reactions with seventeen pairs of primers. The enterotoxin genes *sea* (Green), *sed* (Blue), and *sej* (Red) were amplified. The lower inset shows melting curves of the amplified products obtained from individual PCR reactions.

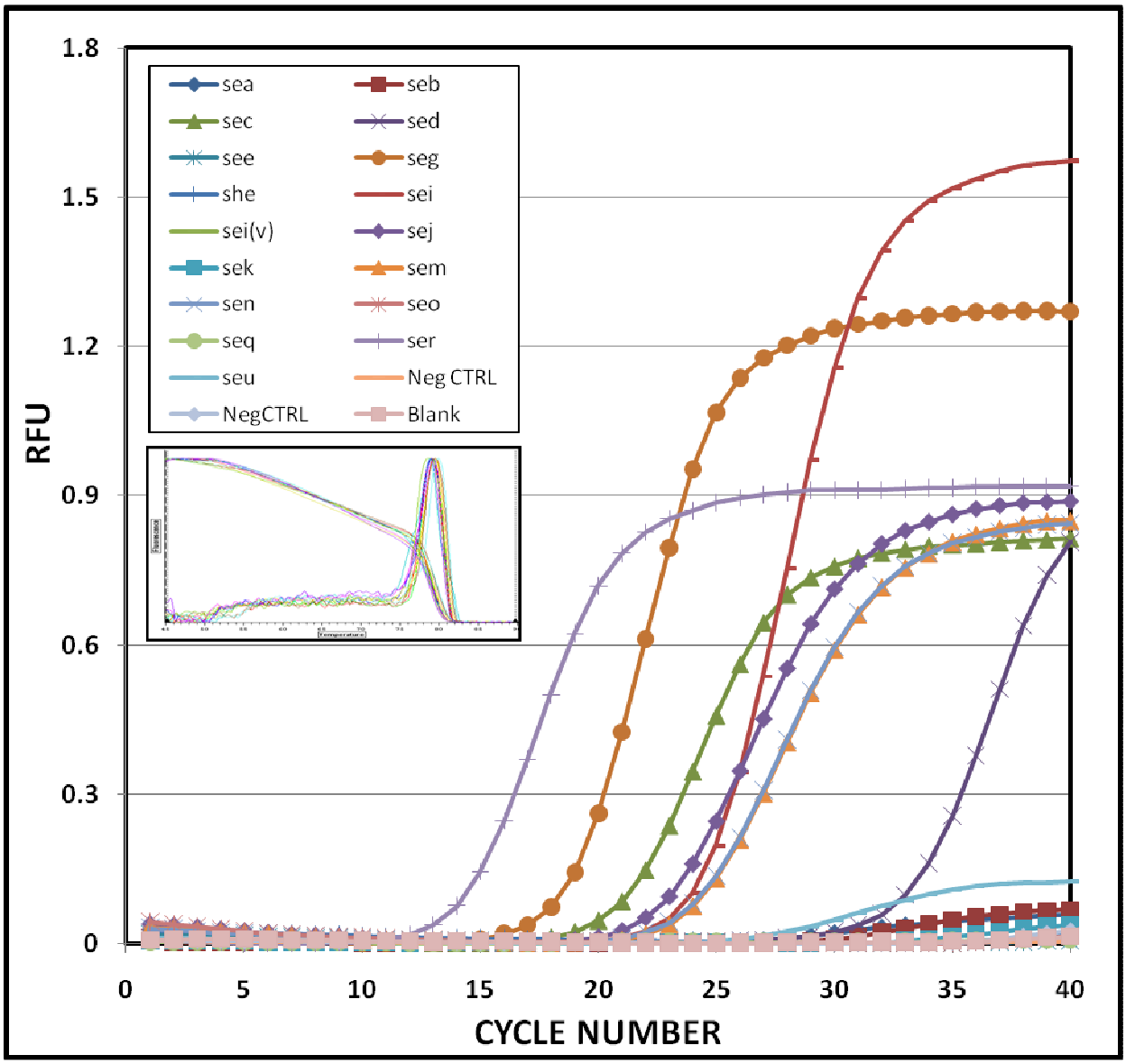

Figure 9. Primer SA enterotoxin primer array real-time PCR of the enterotoxin genes of a *S.aureus* isolate from from human nasal passage (JG-1) in individual PCR reactions with seventeen pairs of primers. The enterotoxin genes *sec, sed, seg, sei, sej, sem, sen, ser* were amplified. The lower inset shows the melting curve of the amplified products obtained from individual PCR reactions.

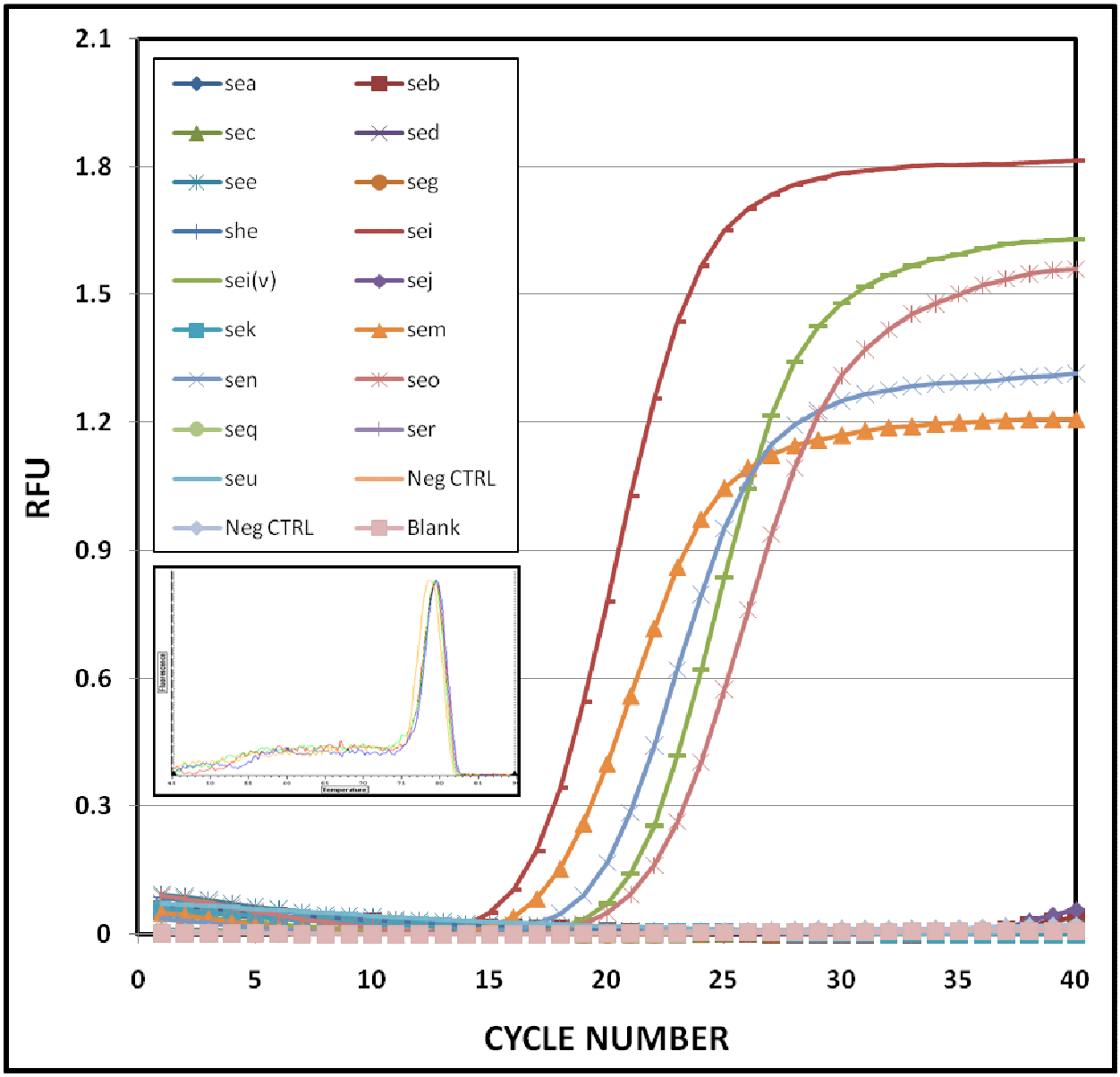

Figure 10. SA enterotoxin primer array real-time PCR of the enterotoxin genes of a *S.aureus* isolate from human nasal passage (MK-1) in individual PCR reactions with seventeen pairs of primers. The enterotoxin genes *sei, sei(v), sem, sen, seo* were amplified. The lower inset shows the melting curves of the amplified products obtained from individual PCR reactions.

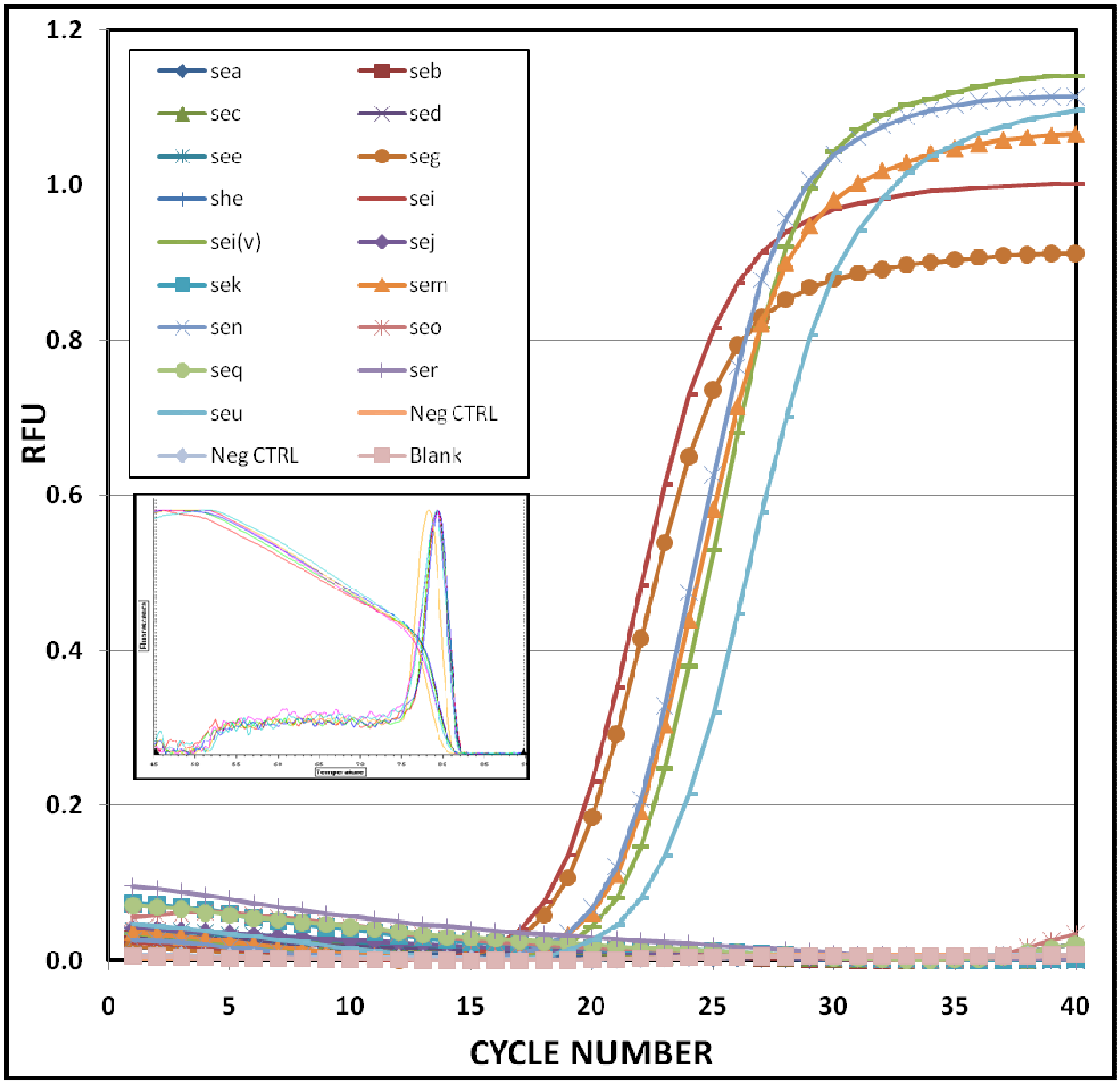

Figure 11. SA enterotoxin primer array real-time PCR of the enterotoxin genes of a *S.aureus* isolate from human nasal passage (JB-1) in individual PCR reactions with seventeen pairs of primers. The enterotoxin genes *seg, sei, sei(v), sem, sen, seu* were amplified. The lower inset shows melting curves of the amplified products obtained from individual PCR reactions.

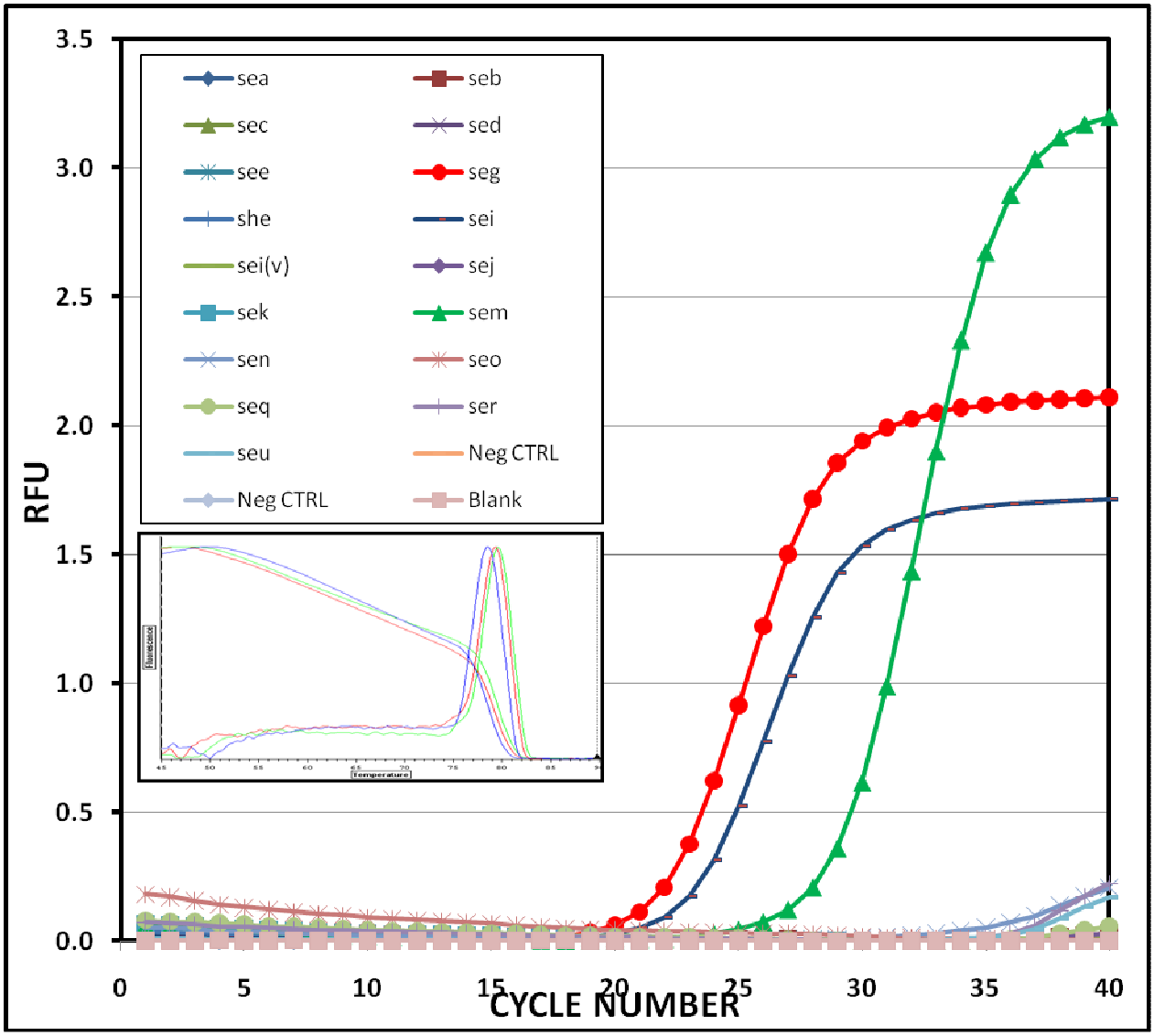

Figure 12. SA enterotoxin primer array real-time PCR of the enterotoxin genes of a *S.aureus* isolate from human nasal passage (J-306) in individual PCR reactions with seventeen pairs of primers. The enterotoxin genes *sem* (Green), *sei* (Blue), and *seg* (Red) were amplified. The low inset shows melting curves of the amplified products obtained from individual PCR reactions.

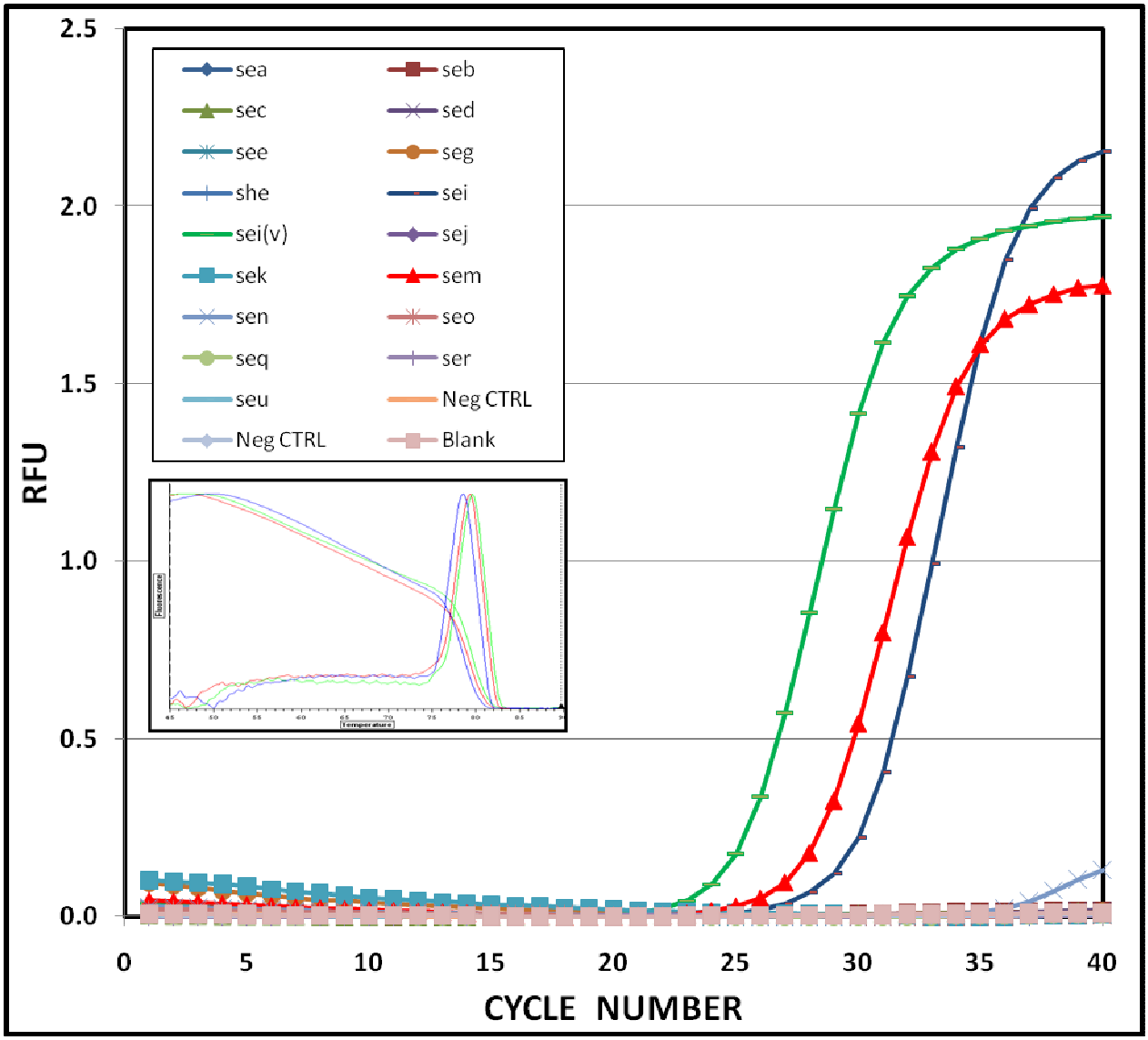

Figure 13. SA enterotoxin primer array real-time PCR of the enterotoxin genes of a *S.aureus* isolate from human nasal passage (SH-1) in individual PCR reactions with seventeen pairs of primers. The enterotoxin genes *sei* (Blue*)*, *sei(v)* (Green), and *sem* (Red) were amplified. The lower inset shows the melting curves of the amplified products obtained from individual PCR reactions.

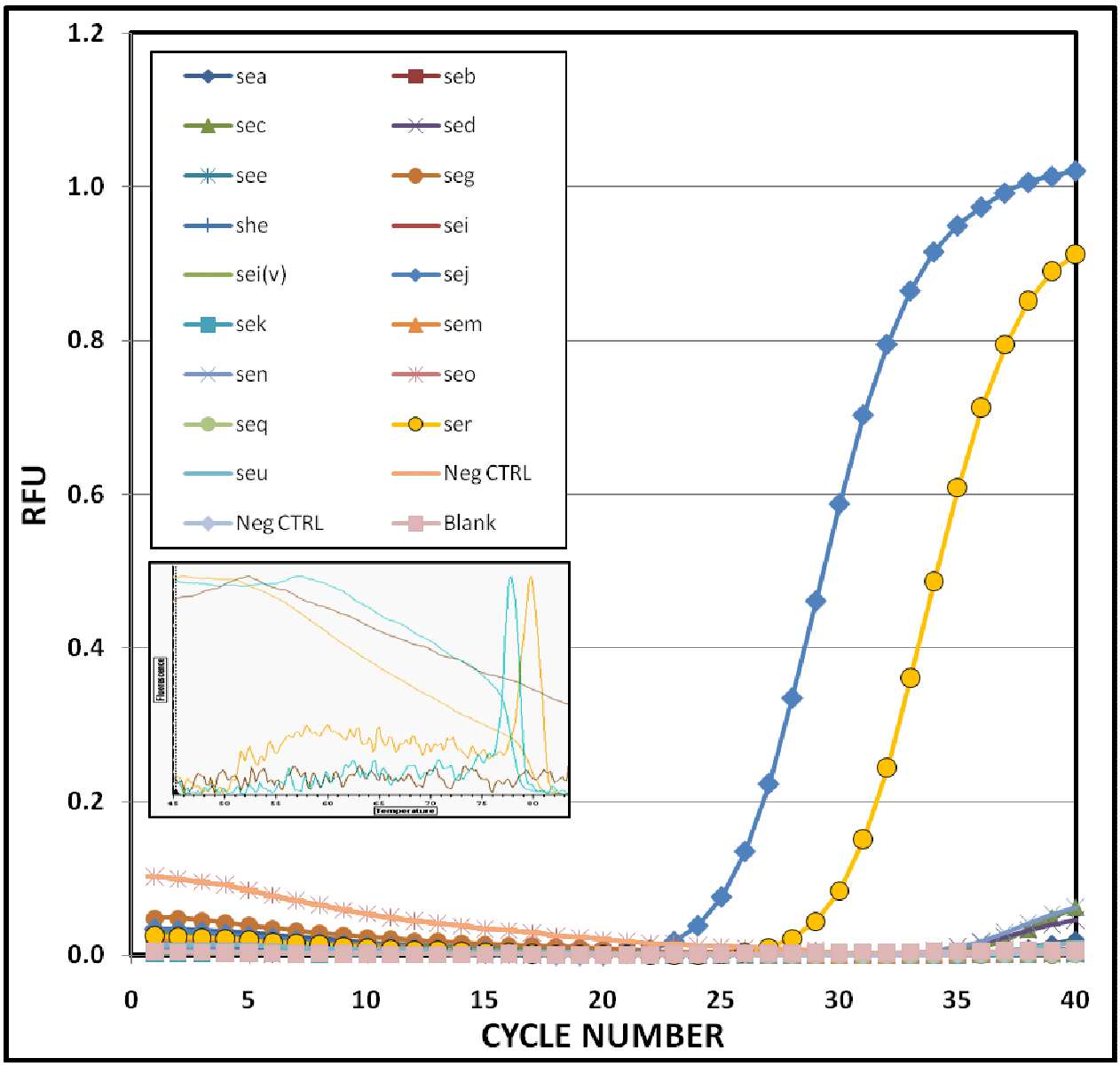

Figure 14. SA enterotoxin primer array real-time PCR of the enterotoxin genes of a *S.aureus* isolate from human nasal passage (SM-306) in individual PCR reactions with seventeen pairs of primers. The enterotoxin genes *sej* (Blue), and *ser* (Yellow) were amplified. The lower inset shows the melting curves of the amplified products obtained from individual PCR reactions.

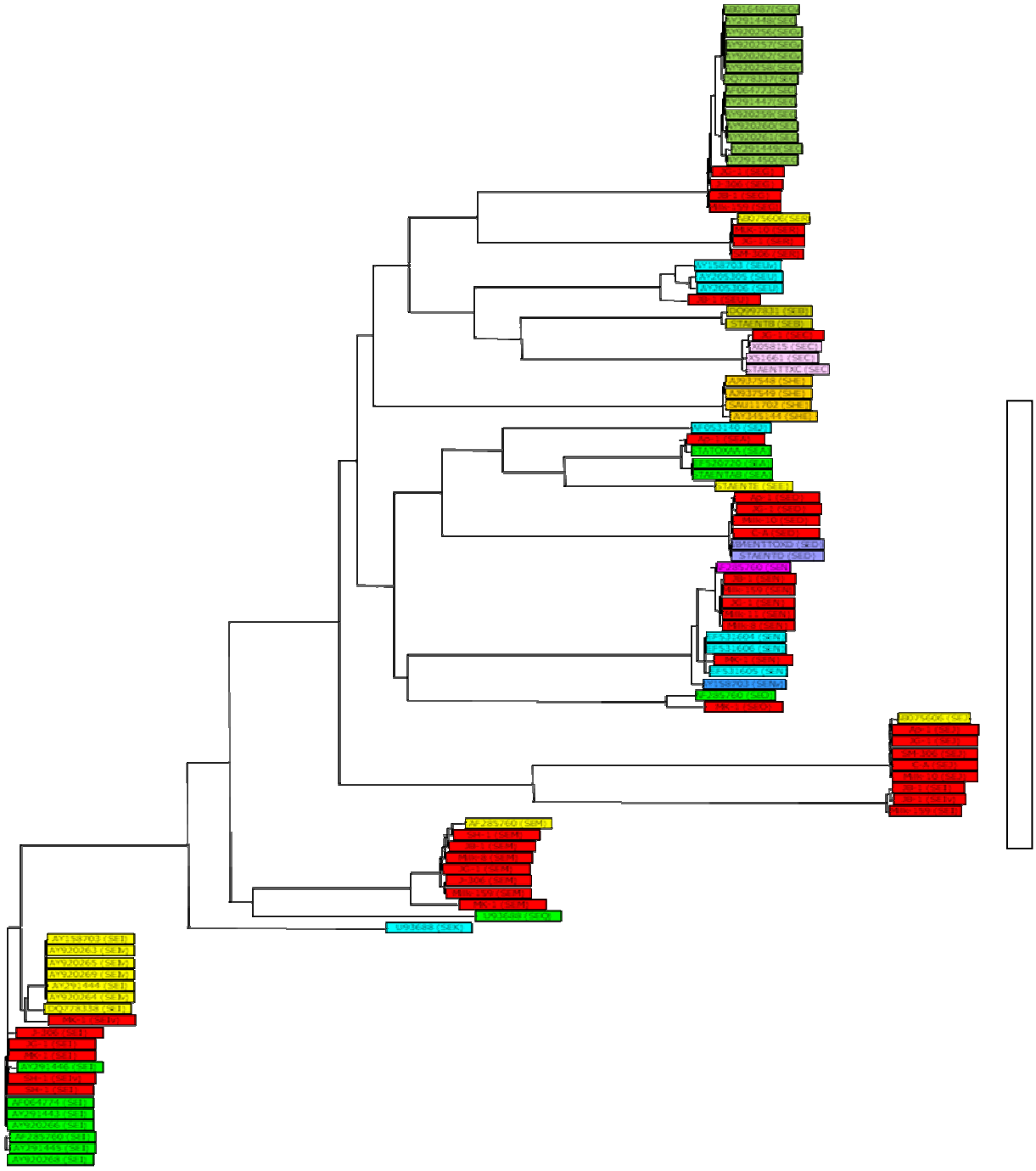

Figure 15. Dendrogram displaying Clustal W analysis of sequence alignments ofportionsof pre-existing enterotoxin genes from the NCBI Database and sequences ofSE toxin genes from isolates obtained in this study (highlighted in dark red with white text).

VITA

LEENALITHA PANNEERSEELAN

Candidate for the Degree of

Doctor of Philosophy

Dissertation: DETECTION OF *STAPHYLOCOCCUS AUREUS* ENTEROTOXINS AND ENTEROTOXIN PRODUCING STRAINS.

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Name: Leenalitha Panneerseelan Date of Degree: December, 2008

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: DETECTION OF *STAPHYLOCOCCUS AUREUS* ENTEROTOXINS AND ENTEROTOXIN PRODUCING STRAINS.

Pages in Study: 150 Candidate for the Degree of Doctor of Philosophy

Major Field: Food Science

Scope and Method of Study: This study is focused on developing methodologies for rapid and sensitive detection of *Staphylococcus aureus* enterotoxins and enterotoxin genes. *S. aureus* produces twenty serological types of enterotoxins. Enterotoxins are heat resistant proteins that may cause food poisoning outbreaks. Besides being the causative agents of foodborne intoxications, enterotoxins are also superantigens and select agents. Many methodologies have been developed in the past for detection of enterotoxins from foods. Sensitivity in detection methods is very important as these toxins are potent in minute quantities. The existing methods have many constraints, as it is not easy to detect toxins from complex environments such as foods due to interference from organic matter. We have developed a very sensitive and rapid immunomagnetic PCR signal amplification (iPCR-SA) assay for detection of enterotoxins in foods. The assay involves, capture of toxin antigens in foods by primary antibody coated paramagnetic beads. A conjugate consisting of secondary antibody covalently linked to reporter DNA was amplified using real-time PCR. Our next objective was to develop a rapid method for detection of enterotoxin genes from *S. aureus,* which may be present in foods as the result of post-process contamination. Seventeen sets of primers were designed for this purpose. All known coding sequences of enterotoxin genes were aligned using ClustalW program provided by Vector NTI 10, software. Primers were selected in consensus regions of each toxin group, and the resulting amplicon was in a region of heterogeneity. A ~500 bp region thus amplified was sequenced and analyzed by the BLAST algorithm. Isolates of *S. aureus* from foods, humans, and cows were analyzed using the primer array

Findings and Conclusions: The limit of detection of iPCR-SA assay was less than 7.5 fg/ml or g of various foods that were analyzed. The assay was not inhibited by the presence of other organic matters that are present in foods, and could be completed within 5-6 h. Enterotoxin genes were amplified using primer array in seventeen individual PCR reactions. Amplification followed by sequencing and analysis enabled quick identification of enterotoxin genes. Some strains harboured as many as 7 different toxin genes and differences in sequences of toxin genes from our isolates were identified.

ADVISER'S APPROVAL: Dr. Peter Muriana