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Staphylococcal enterotoxins: Molecular aspects and detection methods

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Members of the *Staphylococcus* genus, especially *Staphylococcus aureus*, are the most common pathogens found in hospitals and in community-acquired infections. Some of their pathogenicity is associated with enzyme and toxin production. Until recently, *S. aureus* was the most studied species in the genus; however, in last few years, the rise of infections caused by coagulase-negative staphylococci has pointed out the need for further studies on virulence factors that have not yet been completely elucidated so as to better characterize the pathogenic potential of this group of microorganisms. Several staphylococcal species produce enterotoxins, a family of related proteins responsible for many diseases, such as the toxic-shock syndrome, septicemia and food poisoning. To this date, 23 different enterotoxin types have been identified besides toxic-shock syndrome toxin-1 (TSST-1), and they can be divided into five phylogenetic groups. The mechanism of action of these toxins includes superantigen activity and emetic properties, which can lead to biological effects of infection. Various methods can detect genes that encode enterotoxins and their production. Molecular methods are the most frequently used at present. This review article has the objective to describe aspects related to the classification, structure and regulation of enterotoxins and toxic-shock syndrome toxin-1 detection methods.

Key words: *Staphylococcus aureus*, coagulase-negative staphylococci, enterotoxins, TSST-1, PCR, molecular detection.

INTRODUCTION

The pathogenic importance of *Staphylococcus aureus* to humans has increased in the past century as a result of the development of bacterial resistance to antimicrobials and of the development in medical care, which has enabled the extensive use of many clinical and surgical procedures, such as the use of intravascular catheters. Therefore, there has also been increasing concern about nosocomial infection, since by representing direct contact of the external environment with intravascular areas, such instruments function as a foreign body, frequently triggering an inflammatory process in the insertion site. The direct contact of catheters with the bloodstream provides potential risk for disseminating bacteria into blood, which may lead to sepsis. These bacteria commonly originate from anatomical sites of the human body, and among them are the coagulase-negative staphylococci (CNS), which have been noted as the most commonly found agents.

Staphylococci can produce a group of toxins called pyrogenic toxic superantigens. Superantigens include the toxic-shock syndrome toxin-1 (TSST-1) and staphylococcal enterotoxins (SEs). Such toxins show at least three biological properties: Pyrogenicity, the capacity to accentuate endotoxicity in rabbits and superantigenicity. The last one is the best characterized property, as it refers to the capacity of such exotoxins to stimulate the proliferation of T lymphocytes, inducing the release of cytokines and, finally, causing cell death. The genes encoding such toxins are transported by plasmids, bacteriophages or pathogenicity islets. Among the virulence factors of staphylococci, enterotoxins are capable of hyperstimulating he host's immune-system

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cells. Such characteristics can benefit bacteria both during the parasite-host interaction stage and in the environment. The enterotoxins are structurally similar and also have similar gene sequences. They are involved with diseases such as rheumatoid arthritis, atopic eczema and the Toxic Shock Syndrome. Some authors also consider enterotoxins to be potential biological weapons (Dinges et al., 2000).

Enterotoxins are also considered to be the major cause of food poisoning. They are associated with a form of gastroenteritis manifesting clinically with vomiting, with or without diarrhea, resulting from the ingestion of one or more staphylococcal enterotoxins contained in food that has been contaminated with these bacteria. Various reports have described the isolation of CNS and the association of these bacteria with clinically significant diseases (Silva et al., 2001; Vandecasteele et al., 2003). The clinical manifestations of infection caused by CNS are different from those caused by *S. aureus.* CNS manifestations are much more subtle and less unspecific, and many times its clinical development is presented in a more subacute or even chronic fashion (Longauerova, 2006). Chronic fatigue syndrome (CFS), has been linked to CNS infection (Tarello, 2001a, b), showing similarities with syndromes caused by enterotoxins in both humans and animals (Tarello, 2003).

Bacteremias caused by *Staphylococcus epidermidis* are rarely severe. However, the septic syndrome may occur as a fatal outcome particularly in immunocompromised patients and/or if the causative bacterial strain has greater virulence. Patients with foreign materials represent the most significant group with sensitivity to CNS-induced infections (O'Gara and Humphreys, 2001). Although, the CNS' virulence factors have not yet been fully elucidated, studies have shown enterotoxin and TSST-1 production by CNS species (Cunha et al., 2007). This review article aimed at describing aspects related to the classification, structure, regulation and detection methods of staphylococcal enterotoxins and TSST-1.

Staphylococcus

According to Euzéby (2010), presently, there are 42 known species and 24 subspecies in the *Staphylococcus* genus. Approximately half of the species are endogenous to human beings, including *S. aureus* (a coagulase-positive species) and coagulase-negative species: *S. epidermidis*, *S. haemolyticus*, *S. saprophyticus*, *S. cohnii*, *S. xylosus* (Schleifer and Kloos 1975), *S. capitis*, *S. warneri*, *S. hominis*, *S. simulans* (Kloos and Schleifer 1975), *S. saccharolyticus* (Kilpper-Balz and Schleifer 1981), *S. auricularis* (Kloos and Schleifer 1983), *S. caprae* (Devriese et al., 1983), *S. lugdunensis*, and *S. schleiferi* (Schleifer and Kloos, 1975). There are also some subspecies that are endogenous to humans and other primates, such as *S. capitis* subsp. *ureolyticus* (Bannerman and Kloos, 1991) and *S. cohnii* subsp. *urealyticum* (Kloos and Wolfshohl, 1991).

The genus *Staphylococcus* is member of the Staphylococcaceae family; they are gram-positive, facultative anaerobic, chemoorganotrophic cocci with a respiratory and fermentative metabolism at an optimal temperature of 37°C. Also, they are non-movable, non-sporulated, catalase positive and found as pathogens or commensal organisms in both humans and animals. These organisms are resistant to adverse environmental conditions and can be recovered from non-physiological environments even months after inoculation. A peculiar characteristic of staphylococci is their capacity to grow in high saline concentrations, and most of them grow in media with 10% of NaCl. The species in the genus are classified based on the production of enzyme coagulase. Coagulase production capacity divides staphylococci into two major groups: coagulase positive, including species *S. aureus*, *S. intermedius*, *S. schleiferi* subsp. *coagulans* and *S. delphini*; and coagulase negative, including more than 30 different species (Cunha et al., 2009a). Species *S. hyicus* is variably coagulase positive and frequently included among coagulase-negative microorganisms. Approximately 35% of the human population in general carries commensal nasal staphylococci, and most newborns become colonized in their first week of life (Dancer and Noble, 1991).

S. aureus is an extraordinarily versatile pathogen, and it can cause a large spectrum of infections, from mild to severe and fatal. It is important in humans and also economically important when infecting animals. Able to cause superficial lesions and systemic infections, *S. aureus* is responsible for toxin-mediated diseases, such as the Toxic Shock Syndrome (TSS), Kawasaki's Syndrome (Leung et al., 1993) and staphylococcal food poisoning. *S. aureus* is known as one of the most frequent pathogens in both community and nosocomial infections, and it can cause septicemia, endocarditis, osteomyelitis, abscesses, pneumonia, wound infections, impetigo, cutaneous rash, in addition to various toxin-mediated diseases (Howe et al., 1996). The variety of such spectrum of clinical manifestations is mostly dependent on the numerous virulence factors produced by each strain (Le Loir et al., 2003).

In the pre-antibiotic era, bacteremia by *S. aureus* caused a mortality rate of approximately 82% and today despite the availability and efficiency of antibiotics, mortality is still high, at a rate of 25 - 63% (Leung et al., 1993). Approximately 30 - 50% of the human population carries *S. aureus*, and its main habitat is the nasopharynx, a site where strains can persist as transitory or persistent members of the normal microbiota without causing any symptomatology (Vianello, 2006). Originally, of all staphylococcal species, only *S. aureus*

was considered to be pathogenic, and it was distinguished from other species by the production enzyme coagulase, manitol fermentation and the presence of protein A on the cell surface (Pfaller and Herwaldt, 1988). Recently, interest in *Staphylococcus* species, namely the Coagulase-Negative Staphylococci (CNS), has increased due to their increasing importance in hospital infection, particularly in nosocomial bacteremias (Schaberg et al., 1991; Cunha et al., 2004). The CNS group contains the bacteria most frequently isolated in clinical microbiology laboratories (Pfaller and Herwaldt 1988; Patrick, 1990; Cunha et al., 2004); however, distinguishing clinically significant pathogenic strains from those that are only sample contaminants is one of the greatest problems faced by clinical laboratories (Kleeman et al., 1993; Cunha, 2006a). CNS are the major components of the normal bacterial flora in the cutaneous system of the human body, which includes the skin and mucosal membranes (Kloos, 1986; Kloos, 1990; Kloos et al., 1991). Because they are present on the skin, clinical samples are many times contaminated during collection despite antisepsis procedures, since staphylococci present a relatively strong degree of adherence to the epithelial cells of the dermis in addition to their capacity of colonizing catheters and other devices, thus having access to bloodstream and possibly causing sepsis, an infection of great clinical importance (Cunha et al., 2009b).

The increasing importance of CNS is also partly due to the acknowledgement of this group of bacteria as essentially opportunistic as well as to the increased use of transitory or permanent medical devices, such as intravascular catheters and prostheses in severely impaired or immunocompromised patients, for instance those in intensive care units, pre-term newborns, cancer and transplanted patients. In these patients, CNS infections may be severe enough to constitute a death risk. These microorganisms exhibit various virulence factors which are responsible for the successful invasion and infection of their hosts. *S. aureus* strains that are capable of causing diseases express different virulence factors, such as exotoxins, which are molecules on the cell surface associated with adherence and with resistance to various antimicrobials, in addition to enterotoxins, which are extracellular proteins with superantigenic activity (Omoe et al., 2005). All these virulence factors contribute to the pathogenicity of such microorganisms.

The virulence factors of microorganisms in the *Staphylococcus* genus include surface components, such the capsule, peptidoglycans, teichoic acid, protein A, collagen cell attachment protein, enzymes such as lipases, esterases, fatty-acid modifying enzymes, various proteases, hialuronidase, hydrolytic enzymes, desoxirribonucleases, coagulase, catalase, beta-lactamase, staphylokinase, and various toxins, such as exfoliative toxin A and B, leukocidins, enterotoxins, TSST-1 and alpha, beta, gamma and delta hemolysins (Cunha et al., 2006a). At least thirty four (34) different extracellular proteins are produced by pathogenic *Staphylococcus* strains, and several of them already play a definite role in the pathogenesis of recognised staphylococcal disease (Lisa, 2004; Vianello, 2006). Some genes responsible for such factors are frequently transported by genetic elements, such as phages and pathogenicity islets. These are differently sized and potentially movable DNA segments which encode virulence-related genes (Kapper and Kacker, 1999) and are horizontally transferred among the strains (Betley and Mekalanos, 1985; Lindsay et al., 1998; Yamaguchi et al., 2000; Yoshizawa et al., 2000).

According to Peacock et al. (2002), the number of virulence- associated genes carried by a bacterial strain is the product of interaction between the gene's acquisition rates, the cost for biological maintenance and the failure rate of the strain causing the disease. Since most severe infections caused by *Staphylococcus* sp. cannot be explained by the action of a determined virulence factor, the action of several of such factors during the infectious process is imperative.

PHYLOGENETIC GROUPS OF ENTEROTOXINS

Enterotoxins are low-molecular weight proteins (26900 - 29600 Da). To date, 23 different SEs have been described, including SEA to SEIV (Schlievert and Case, 2007; Larkin et al., 2009). All share superantigenic activity, whereas, only few of them (SEA to SEI, SER, SES, and SET) have been proved to be emetic (Le Loir et al., 2003; Ono et al., 2008). The International Nomenclature Committee for Staphylococcal Superantigens proposed that only staphylococcal superantigens inducing emesis after oral administration in an experimental model of primates should be designated as staphylococcal enterotoxins. The committee also recommends that other similar toxins that do not exhibit emetic properties in primate animal models or that have not yet been tested should be designated as staphylococcal enterotoxin-like toxins (SEl) type X (Lisa et al., 2004; Omoe et al., 2005). One toxin involved in the toxic-shock syndrome was initially designated as SEF (Bergdoll et al., 1981; Vianello, 2006). However, it did not show the *in-vivo* biological activity which is characteristic of a true enterotoxin, and it was later designated as TSST-1 (Fueyo et al., 2005). The TSST-1 encoding gene has little gene sequence homology with the genes encoding staphylococcal enterotoxins and with those of streptococcal pyrogenic exotoxins (Marrack and Kappler, 1990). Despite this fact, these toxins are structurally and functionally similar (Klotz et al., 2003).

Several new types of enterotoxins (SE) and SE-related

toxins were recently described based on the similarity of their gene sequences to those of classic enterotoxins. As regards the homology between SE classes, 15% of the amino acid residues are completely conserved, and most of them are located on the central and C-terminal portions of the sequences (Vianello, 2006). SE and SEl have been classified based on their amino acid sequences. Group 1 comprises the classic SEA, SED and SEE, which have from 53 - 81% of homology in the amino acid sequence (Marrack and Kappler, 1990), as well as the new classes SElJ, SEIN, SElO and SEIP (Larkin et al., 2009). Group 2 comprises the classic SEB, SEC1, SEC2 and SEC3 which have from 66 to 98% of homology in the amino acid sequence (Marrack and Kappler 1990), as well as the new classes SEG, SER and SEIU (Larkin et al., 2009). Gene *sec* is located on a pathogenicity islet, and *Sec-3* is strictly related to gene *Sec-1*, with 98% of homology between the nucleotide sequences. SEC3 differs from enterotoxins SEC1 and SEC2 by four to nine amino acids, respectively (Fitzgerald et al., 2001). Group 3 comprises only the new classes of SE and SEls, such as SEI, SEIK, SEIL, SEIM and SElQ (Omoe et al., 2005; Larkin et al., 2009). Group 4 comprises only TSST-1 and group 5 the SEH enterotoxin (Larkin et al., 2009).

Despite the homology between SE and SEl, the exact role played by staphylococcal enterotoxin-like toxins is still unknown; however, their gene location on pathogenicity islets suggests that they are part of virulence factors and that they play a type of defense role against the host's immune system, thus acting against both adaptive immunity and innate immunity (Proft and Fraser, 2003). Studies characterizing genes *seg* and *sei* provide additional evidence that the family of pyrogenic toxins is large (Munson et al., 1998). The discovery that gene *sei*, present on an operon, has an enterotoxin-like toxin gene on one side and a gene with a partial sequence of an enterotoxin-like toxin on the other supports the hypothesis that there are more toxin genes to be identified and there may be regions in which the re-arrangement of genes encoding enterotoxins in *S. aureus* occurs (Munson et al., 1998). This premise is supported by the fact that the amino acid sequence assumed for the enterotoxin-like toxin located in 59 amino acids from gene *sei* shares 55% of the amino acid sequence assumed for enterotoxin SEI, suggesting that there may be a genetic re-arrangement. It is a fact that two thirds of the C-terminal portion of the protein of this enterotoxin-like toxin has 75% of homology with enterotoxin SEI, whereas, the N-terminal portion has only 36% of homology with the amino acid sequence of SEI. The discovery of new enterotoxin genes is possible, since many of such genes are associated with both movable genetic elements and element-like sequences (Betley et al., 1992; Iandolo et al., 1989).

All toxin encoding genes are located on movable genetic elements, including bacteriophages, pathogenicity islets, genomic islets and plasmids (Novick, 2003; Lindsay and Holden, 2004; Schmidt and Hensel, 2004; Holtfreter and Broker, 2005). The genes of enterotoxins SEB, SEC, SEG, SEI, SEIM, SEIN, SElO, SEIK, SEIL, SElQ and TSST-1 are located on pathogenicity islets (Lindsay et al., 1998; Jarraud et al., 2001; Kuroda et al., 2001; Baba et al., 2002; Becker et al., 2004); those of SEA, SEE and SEIP are located on prophages (Betley and Mekalanos, 1985; Couch et al., 1988; Kuroda et al., 2001), whereas, those of SED, SElJ and SER are found in plasmid pIB485 (Bayles and Iandolo, 1989; Zhang et al., 1998; Omoe et al., 2003).

The association of enterotoxin genes with movable genetic elements results in the horizontal transfer of superantigen genes between staphylococcal lineages, and they constitute an important role in the evolution of *S. aureus* and of CNS as pathogens. For a better understanding of the pathogenicity or the virulence power of staphylococci, it is important to know about the real extent of the diversity of staphylococcal superantigens (Omoe et al., 2003).

Superantigens have received a great deal of attention since the discovery of their action mechanism in 1989. Since then, many studies have been performed, and great knowledge has been developed on their structure and molecular mechanisms. However, little information has been reported on their direct effect in diseases such as food poisoning and the toxic shock syndrome. Nevertheless, there is considerable speculation and evidence that these microorganisms may be related to auto-immune diseases (Proft and Fraser, 2003).

Toxic shock syndrome toxin 1 (TSST-1)

An important superantigen secreted by *S. aureus* is TSST-1, a toxin responsible for the Toxic Shock Syndrome (TSS), a disease that affects the organic system as a whole and may be fatal. It is characterized by rapid fever, arterial hypotension, diffuse cutaneous rash, circulatory failure, vomiting, diarrhea, myalgias, epidermis scaling, hypoalbuminemia and organ failure (multiple organ dysfunction syndrome – MODS) (Reingold et al., 1982; Parrillo, 1993; Chesney, 1997). If it is not properly treated soon after the beginning of symptomatology, a fatal shock may develop 24 h after the onset of symptoms (Deurenberg et al., 2005). It was originally described as a disease occurring in children by Todd et al. (1978), aged 8 - 17 years. In the following years, there was an increase in the number of cases of the toxic shock syndrome, and the disease was associated with young women in their menstrual period (Bergdoll et al., 1981). Later, it became evident that such cases also affected women who were not in their menstrual periods, and it was noted that the disease

could affect any type of individual in the population (Herzer, 2001). In 1980, epidemic TSS clinical conditions were described in women using tampons, particularly those of high absorption. This happened because the toxin is capable of crossing mucosal barriers. The disease was associated with toxigenic strains of *S. aureus* located on the vaginal mucosa or uterine cervix. Studies have shown that more than 99% of menstrual TSS cases are related to the use of absorbing tampons, and more than 45% of all TSS cases are related to menstrual TSS (Herzer, 2001).

There are three criteria to be met for TSS development: (i) The patient must be infected or colonized by a *S. aureus* strain (ii) Such strain must produce TSST-1 or other similar toxins and (iii) The toxin must find a route to enter the bloodstream (Herzer, 2001). The patients with the syndrome do not usually exhibit detectable bacteremia even when clinical findings of the disease are observed, which suggests that TSS results from intoxication with the bacterial toxin only (McCormick et al., 2001). Because it is a superantigen, TSST-1 induces a polyclonal immune response as it directly binds to MHC class-II proteins, T-cell receptors and the MHC of B- and T-cells without being internalized and processed by a normal antigen (Misfeldt, 1990; Herman et al., 1991; Balaban and Rasooly, 2000). This toxin may be involved in the modulation of the host's immune response and can contribute to the evasion of the host's organism's defense and to bacterial persistence (Ferens and Bohach, 2000).

TSST-1 was the first TSS-related toxin, and today it is accepted as being responsible for 75% of clinical cases and approximately all cases of menstrual TSS; however, non-menstrual TSS is also attributed to staphylococcal enterotoxins SEB and SEC. There are cases in which non-menstrual TSS may occur in association with other infections, as for instance, post-surgery TSS, influenza-associated TSS, erythematous syndrome and TSS associated with the use of diaphragms as a contraceptive method (McCormick et al., 2001). TSST-1 is produced by approximately 100% of *S. aureus* strains isolated from the cervical or vaginal mucosa of women with TSS, and in approximately 50% of the isolates from other bodily sites with non-menstrual TSS (Schliviert et al., 2000).

Panton valentine leukocidin (PVL)

In the last few years, there was a significant increase of the clinical isolations of methicillin-resistant *S. aureus* (MRSA) strains. To combat the life-threatening infections, there is a need to better understand the bacteria-host interaction and virulence factors involved. Strains that produce Panton Valentine Leukocidin (PVL) were strongly associated with human primary necrotizing cutaneous infections such as pneumonia and furuncles (Couppié et al., 1994; Löffler et al., 2010) and 2% of *S. aureus* strains produce PVL (Prévost et al., 1995). This bicomponent toxin has been shown to induce lysis of host defense cells such as human polymorphonuclear neutrophils (PMN), monocytes, and macrophages (Gladstone et al., 1957). This toxin, having a cytolytic effect on only human and rabbit PMNs (Finck-Barbançon et al., 1991), has been described as a crucial marker of virulence (Cribier et al., 1992), since PVL is found in almost all MRSA strains that cause community associated MRSA infections (Lina et al.,1999; Gillet et al., 2002).

Molecular tests as Polymerase Chain Reaction (PCR) are the most efficient and rapid to detect positive PVL *S. aureus* strains (Oliveira and Lencastre, 2002; Zhang et al., 2008). Al-Talib et al. (2009) developed a combined molecular test for the rapid identification and discrimination of the *Staphylococcus* genus from others, with simultaneous discrimination of methicillin-resistant from susceptible staphylococcal strains, *S. aureus* from CNS, and concomitant detection of PVL genes. Multiplex PCR assay is proved to be reliable for direct detection of community-acquired methicillin-resistant *S. aureus* (Moussa and Shibl, 2009).

ACTION MECHANISM OF STAPHYLOCOCCAL TOXINS

Various staphylococcal toxins exhibit superantigenic properties, including enterotoxins, exfoliative toxins and the Toxic-Shock Syndrome toxin. The first bacterial superantigen was isolated in 1959 by Bergdoll et al. They isolated a toxin secreted by a *S. aureus* strain denominated staphylococcal enterotoxin A (SEA) due to its potent enterotoxigenic properties, which included superantigenic and emetic activity. Such activities are located on different protein domains (Dinges et al., 2000) and although they are two distinct functions, there is a great correlation between these activities. In most cases, decreased superantigenicity (due to a genetic mutation, for instance) results in decreased enterotoxic activity (Harris et al., 1993). However, how these activities are inter-connected is uncertain (Balaban and Rasooly, 2000). The most accepted old hypothesis concerning the activity of staphylococcal enterotoxins (SE) was that they caused food poisoning and induced vomiting and diarrhea from one to two hours after ingestion, and little was known about how SE caused food poisoning symptoms. The proposed mechanism was that they triggered an effect directly on the intestinal epithelium and on the vagus nerve, causing stimulation of the emetic center and increasing intestinal motility (Vianello, 2006). However, at present, the most accepted hypothesis is that enterotoxin activity may facilitate transcytosis, enabling the toxin to enter the bloodstream, thus allowing the interaction with T-cells and leading to superantigenic activity (Balaban and Rasooly, 2000). With the

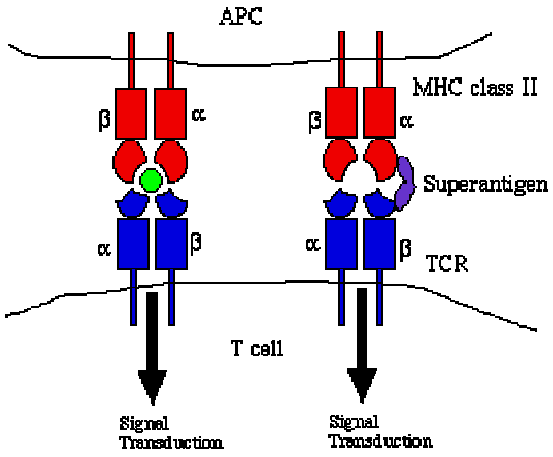

Figure 1. Comparison between the binding of a normal antigen and the binding of a superantigen with the T-cell receptor (TCR in blue) and with the class-II MHC complex (in red). Figure adapted from Janeway et al. (1999) and Schlievert (1993).

exaggerated induction in T-cell production, cytokine release occurs, causing systemic toxicity and suppression of the adaptive immune response (Marrack and Kappler, 1990; Balaban and Rasooly, 2000).

The mitogenic activity of SE was only discovered years after its isolation, but the term superantigen was only used in 1989, when Marrack et al. observed that mitogenic activity resulted from a massive expansion of T-cells (Marrack and Kappler, 1990). Superantigens are the most mitogenic T-cell substances ever found (Proft and Fraser, 2003). Concentration smaller than 0.1 pg/ml of a bacterial superantigen are enough to stimulate T-lymphocytes in an uncontrolled fashion, resulting in fever, shock and possibly death (Bohach et al., 1990; Miethke et al., 1992). Superantigens are microbial antigens which have in common an extremely potent activating effect on T-cells, presenting a specific variable region. Superantigens make a cross bind between the variable regions and class-II MHC proteins regardless of the peptide bind to the T-cell receptor. The result is a transitory expansion, subsequent death and lack of response from T-cells with appropriate variable regions. This is called anergy, thus the disappearance of the organism's capacity to react to a substance or pathogenic agent relatively to which it had been previously sensitized. Superatingens affect the immune system by binding to the proteins of the class-II major histocompatibility complex (MHC II) and the activation of specific T-cell types through the variable regions of the T-cell receptors beta chains. As a consequence of the activation of such T-cells, cytokine release occurs, such as that of the Tumor Necrosis Factor (TNF), of various interleukins and of interferon-gamma (Vianello, 2006).

It is known that in the cellular immune response, the first event is the antigen recognition by the T-cell antigen receptor (TCR) located on the membrane of T-lymphocytes. The antigen is presented to TCR in the form of peptides bound to molecules of the class-I and class-II major histocompatibility complex (MHC), whose constituent molecules are proteins bound to membranes on the surface of antigen-presenting cells (APC). This type of recognition is key to the high specificity of the immune response, where only a few T-cells can recognize a specific antigen. Because staphylococcal enterotoxins present a superantigenic behavior, they interact with various T-cells in an unspecific fashion and act as potent activators of those cells. For such activation, enterotoxins require only the recognition of portion V of the TCR beta chain, thus promoting a bond between the TCR and MHC II of APCs as is shown in the Figure 1. Conventional antigens, when presented with the MHC, on APCs, require recognition by all five variable elements of TCR (V-beta, D-beta, J-beta, V-alpha and J-alpha) (Vianello, 2006). In contrast, superantigens are mainly recognized by the V-beta portion of TCR. Hence, they can stimulate approximately 20% of all T-lymphocytes, whereas, conventional antigens stimulate approximately 0.01% of T-lymphocytes (McCormick et al., 2001). Such superstimulation of T-cells causes an excessive release of various cytokines and lymphokines, resulting in the immunosuppression of T- and B-cells with impairment of the immune response to the invading agent (Jarraud et al., 2001). It is believed that such massive synthesis and release of cytokines are responsible for most severe outcomes of superantigens, which act as capillary vasodilators, leading to hypotension, shock, failure of various organs and possible death (McCormick et al., 2001).

Enterotoxin studies began from the analyses of various *S. aureus* strains involved in staphylococcal food poisoning (Le Loir et al., 2003); however, not only *S. aureus*, but various other *Staphylococcus* species also produce enterotoxins (Jay, 1992; Cunha et al., 2006a, b, 2007). Among the coagulase-negative species, *S. cohnii*, *S. epidermidis*, *S. xylosus* and *S. haemolyticus* were isolated from sheep milk, and in 1988, Bautista et al. reported the production of one or more enterotoxins in the isolates. Other authors have also reported the presence of toxigenic genes in CNS isolated from foods, such as in a study conducted by Cunha et al. (2006b), in which they describe the presence of enterotoxigenic CNS isolated from food where, of the twenty CNS samples isolated, three showed gene *sea*, and one showed gene *sec-1*. Gene *sea* was detected in one sample of *S. epidermidis,* in one sample of *S. xylosus* and in one of *S. hominis*, whereas, gene *sec-1* was detected in one sample of *S. xylosus*.

When studying the virulence factors of CNS isolated from newborns, Cunha et al. (2006a) found by the reverse passive latex agglutination (RPLA) method, a

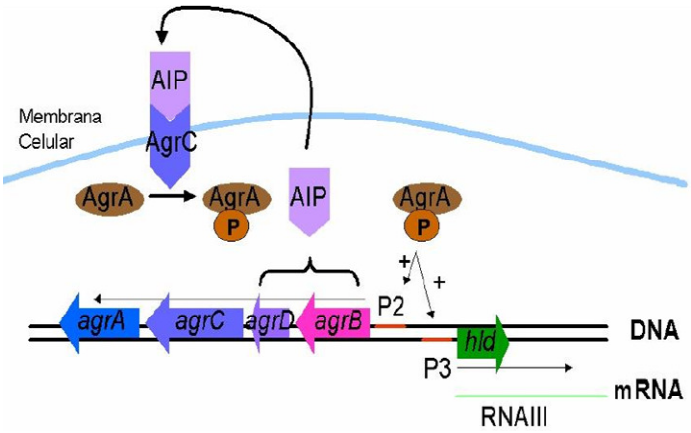

Figure 2. Structure and functioning of locus *agr* (McCulloch, 2006).

percentage of 37.6% of CNS producing SEA, SEB or SEC. Species *S. epidermidis,* and *S. lugdunensis* produced SEA, SEB and SEC, whereas, *S. haemolyticus*, *S. hominis* and *S. simulans* produced only SEC. Cunha et al. (2007) used the PCR technique to detect the genes responsible for enterotoxins SEA, SEB, SEC, SED and TSST-1 in lineages of staphylococcus isolated from newborns. The results obtained for PCR were compared to those by the RPLA method. From the total number of 120 *S. aureus* samples isolated, 38.3% were enterotoxin producers, according to RPLA, whereas, PCR detected 46.6% of positive samples. Coagulase-negative staphylococcus presented 40.0% of positive lineages by PCR as compared to 26.7% by the RPLA method. Gaebler and Cunha (2008) studied 90 CNS samples and found the presence of toxin genes in various CNS species, including *S. epidermidis, S. warneri, S. lugdunensis, S. hominis, S. haemolyticus, S. simulans, S. xylosus* and *S. saprophyticus.* Most of these CNS were isolated from animals showing pathological signs and immunological anomalies highly evocative of Chronic Fatigue Syndrome (Tarello, 2001a, b; Tarello, 2003). When evaluating CNS samples for the presence of toxigenic genes, Schmitz et al. (1998) also found two positive samples for gene *sec-1* in a total of 50 samples studied.

REGULATORY MECHANISMS

The best-known staphylococcal regulatory systems are *agr* (accessory gene regulator) (Peng et al., 1988), *sar* (staphylococcal accessory regulator, divided into *sar*A, *sar*S, *sar*T and *sar*R) (Cheung et al., 1992; McCulloch, 2006) and *rot* (repressor of toxins) (McNamara et al., 2000), which can directly affect staphylococcal enterotoxin production. There are also regulatory systems *sae*RS, σ^B (sigma-B), *arl*RS (McCulloch, 2006) and *srr*AB (Throup et al., 2001; Yarwood et al., 2001).

The *agr* is the best known and studied locus, and it is a group of genes with quorum sensing activity which regulate the expression of various virulence factors. Quorum sensing is the name given to the mechanism of "communication" among bacteria by means of which a bacterium can "perceive" the population density in the medium. This mechanism is important in *Staphylococcus* since some accessory proteins (such as virulence factors) are only expressed in certain growth phases (McCulloch, 2006).

Similarly to most staphylococcal virulence factors, enterotoxin production is also coordinated by the *agr* locus (Shopsin et al., 2003; Novick 2003). Locus *agr* has been sequenced and cloned, and it is composed of two different operons (Figure 2). It comprises at least five genes: *agrA, agrB, agrC, agrD* and the gene for delta-hemolysin (*hld*) (Peng et al., 1988), and it is controlled by two promoters, P_2 and P_3. The operon whose promoter is P_2 contains four genes: *agrA, agrB, agrC, agrD*, and the operon whose promoter is é o P_3 contains only gene *hld* (McCulloch, 2006). This locus is a regulatory system of two components. Mack et al. (2007) explain that *agrD* originates a signaling component, and *agrA* encodes a transition activator. The two main transcripts produced are designated as RNA II (*agrA, agrB, agrC, agrD*) and RNA III (*hld*). RNA III acts firstly in order to initiate the transcription of virulence factor. The *agrC* is the signal receptor, and *agrA* is the response regulator. Such genes are part of quorum sensing and responsible for the production of RNA III, the main effector of the *agr* response (Balaban and Novick 1995).

The signaling system promoted by locus *agr* is auto-induced by means of an auto-inducing peptide (AIP) encoded by *agrD*. *S. aureus* isolates can be divided into four predominant *agr* groups based on the specificity between the auto-inducing peptide and the signal receptor (*agrC*) (Mayville et al., 1999). Staphylococci belonging to an *agr* group are capable of activating response only in bacteria from their same group. Nevertheless, these microorganisms can usually inhibit response in members from other groups (Ji et al., 1997). In the *agr* locus (Figure 2), two promoters with opposite orientation, P_2 and P_3, produce two transcripts, RNAII and RNAIII, respectively. RNAIII comprises 510 nucleotides that are responsible for transcription of genes for many virulence factors, such as extracellular toxins, enzymes and cell-surface proteins present in *S. aureus* (Huy, 1994). RNAIII synthesis is induced when the concentration of the medium-specific auto-inducing polypeptide (AIP) reaches certain levels that are usually detected in transition from the exponential growth phase to the stationary phase.

RNAII, in turn, comprises four genes *agr* (*agrB*, *agrD*, *agrC* and *agrA*, arranged in one operon (*agr*). They work together to induce RNAIII synthesis. In addition to its regulatory role, RNAIII is also an mRNA which encodes

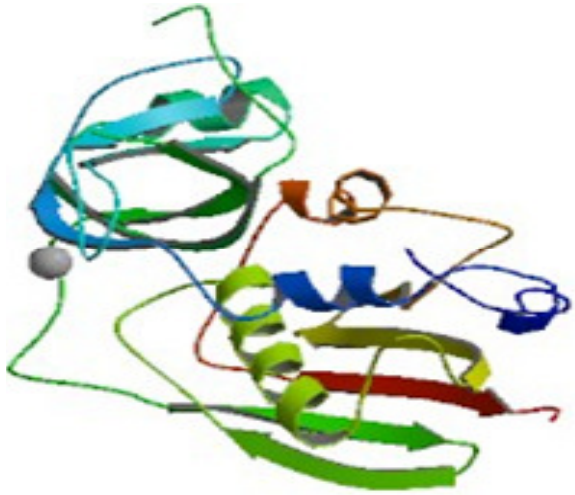

Figure 3. Crystallographic structure of staphylococcal enterotoxin C2 (Kumaran et al., 2001).

delta-hemolysin in staphylococci. Such hemolysin has a property to form pores in membranes and to lyse erythrocytes (Tegmark et al., 1998). In *S. aureus*, it is a polypeptide formed by 44 amino acids, whereas in *S. epidermidis*, it consists of 26 amino acids.

One *agr* operon has also been shown in some coagulase-negative staphylococcal species. In a study (Vandenesch et al., 1993), RNAIII was detected in *S. lugdunensis* species; however, the mRNA detected did not encode the delta-toxin, and its role in gene regulation is not well-known. Other authors (Van Wamel et al., 1998) described an *agr* locus in *S. epidermidis* with one homology of 68% when compared with the *agr* locus of *S. aureus.* The RNAII of *S. epidermidis*, similarly to that of *S. aureus*, also encodes proteins AgrA, B, C, and D involved in the regulation system of the genes responsible for toxin production. The same authors also investigated the production of delta-toxin in 23 samples of *S. epidermidis* and showed its presence in 21 samples, which was produced during the post-exponential phase (Van Wamel et al., 1998). The s*arA* is a necessary transcription factor for RNA III expression and also stimulates some genes expression without the *agr* interference (Dunman et al., 2001). *Rot* is the major transcription factor for *hla* and other exoprotein genes (McNamara et al., 2000).

The interaction of all regulatory systems shows the complexity of regulation of accessory genes in *Staphylococcus*. Positive- and negative-feedback events occur among them, amplifying or inhibiting signs, and a competition takes place between such systems to regulate a gene. For instance, the *agr* system increases the expression of alpha-hemolysin, whereas, the *arl*RS system decreases the expression of the same gene (McCulloch, 2006). Since enterotoxin-encoding genes have different genetic supports and most of them are movable, not all are controlled by *agr*. Hence, *seb*, *sec* and *sed* are dependent on *agr*, whereas, genes *sea* and *sej* are not (Le Loir et al., 2003).

MOLECULAR STRUCTURE OF ENTEROTOXINS

Enterotoxin molecules are rich in lysine, aspartic acid, glutamic acid and tyrosine residues. Many enterotoxins have a cystein arch that is probably involved in the molecule's emetic activity (Hovde et al., 1994; Le Loir et al., 2003; Orwin et al., 2003). Crystallography-based studies have shown that the molecules of various classes of superantigens have a similar three-dimension structure (Dinges et al., 2000; McCormick et al., 2001). Figure 3 shows an example of a crystallographic structure of a staphylococcal enterotoxin, they have a small N-terminal α-helix connected to a β-folded sheet known as domain B or oligosaccharide-binding fold (O/B). Such O/B fold is connected to a wall of β-folded sheets by a central diagonal α-helix forming domain A. All superantigens have this characteristic; however, some differ slightly as they feature small additional folds. The most notable of such folds is the cysteine fold, which is found in many enterotoxins as well as in streptococcal pyrogenic exotoxin A (SPE A) (Hovde et al., 1994; Dinges et al., 2000). High homology levels between nucleic acids and sequences deduced from amino acids of staphylococcal gene *seg* and from streptococcal toxin *ssa* (streptococcal superantigen) and *speA* (streptococcal pyrogenic toxin A) support the hypothesis that toxins from *S. aureus* and from *S. pyogenes* may have evolved from a common ancestral toxin gene or that there has been an exchange of genetic material between the two microorganisms so that this family of related toxins could exist (Goshorn and Schlievert, 1988; Van Den Bussche et al., 1993).

Recently, toxins SEI, SEIK, SEIL and SEIQ, have been identified with the cysteine fold. They were characterized as superantigens, but emetic activity is significantly reduced in magnitude in SEI and lacks in SEK and SEQ (Munson et al., 1998; Orwin et al., 2001; Fitzgerald et al., 2001; Orwin et al., 2003). TSST-1, which does not have cysteine residues, is considered to be non-emetic (Schlievert et al., 2000). Toxins SEA, SEB, SEC, SED, SEE and SEH have clearly been shown to present a greater or smaller emetic potential, depending on the molecule. Superantigenicity and emetic activity of SEs are two separate functions located in different domains of the protein (Hovde et al., 1994; Dinges et al., 2000). Despite this, there is a strong relationship between these functions since, in most cases, genetic mutations resulting in superantigenic activity loss also result in emetic activity loss (Harris et al., 1993). The emetic function of enterotoxins has not been characterized as superantigenic activity. Enterotoxins are classified as

such only due to their ability to cause emetic responses when orally administered to primates, whereas, other superantigens are not emetic (Dinges et al., 2000).

MOLECULAR METHODS FOR ENTEROTOXIN AND TSST-1 DETECTION

The detection of staphylococcal enterotoxins has been performed by biological methods consisting in the toxin administration in monkeys through the intragastric route, but since their purification, detection techniques have been based on the use of antibodies prepared against them. Great efforts have been made so as to develop methods for detecting enterotoxins and related genes. Methods based on immunological assays were the first methods to be used for such detections. Specific reactions of agarose precipitation were used and developed, among which the microslide immunodiffusion assay and the Optimal Sensitivity Plate (OSP) assay, which attains detection levels of 0.5 µg/g of enterotoxins on culture supernatant (Pereira et al., 2001), are noteworthy.

The traditional method for enterotoxin identification based on serological typing with antibodies is a useful technique as an epidemiological tool in the investigation of various infectious diseases. However, serological typing is less sensitive to small variations between SEs than DNA-based methods, in addition to being relatively more complex, consuming more time and not being practical for detecting and identifying a large group of inter-related SEs with significant antigenic similarities (Lee et al., 1978; Lee et al., 1980). Staphylococcal enterotoxin detection on culture supernatant by classic immunodiffusion, agglutination and ELISA methods is lengthy and does not always detect low toxin concentrations. Also, anti-serum is commercially available only for SEA, SEB, SEC, SED and SEE. Experimental tests have been developed for some of the new toxins (SEG, SEH and SEI), but they are not commercialized due to difficulties in purification and preparation of specific antibodies (Cremonesi et al., 2005). Among the immunologic tests for enterotoxin detection are the immunodiffusion, radioimmunoassay, immunoenzymatic (ELISA) assays (Cunha et al., 1996; Oliveira and Hirooka, 1999), and a wide variety of ELISA have been described for SE detection (Poli et al., 2002). At present commercial kits (VIDAS Staph enterotoxin SET kit, BioMerieux; ELISA test Kit, 3M Tecra; Staphylococcal Enterotoxin Test Kit SET-RPLA, Oxoid) are available for direct SE detection; however, their high cost prevents and makes routine laboratory use unfeasible.

Polymerase Chain Reaction (PCR), a recent technique, has proven to be a more useful and reliable tool for detecting such genes (Johnson et al., 1991; Becker et al., 1998; Tamaparu et al., 2001; Cunha et al., 2006b, 2007); however, the fact that PCR is only capable of showing that a gene is present or not in the sample without indicating whether enterotoxins are produced is noteworthy. Nevertheless, with Reverse Transcriptase PCR (RT-PCR), sequences of mRNA responsible for enterotoxin production can be detected, thus, proving gene activity. The presence of toxin mRNA sequences does not leave any doubts about the microorganism's toxigenic potential. There are several PCR-based methods available for staphylococcal enterotoxin typing (Johnson et al., 1991; Schmitz et al., 1998). These methods are laborious because many reactions are required for distinguishing different enterotoxin genes (*se*). Some techniques used to identify toxigenic genotypes, including DNA-DNA hybridization and PCR, are protocols that have been developed to detect only one or few toxin genes (Johnson et al., 1991; Schmitz et al., 1998). More recently, the Multiplex PCR method was described for typing *S. aureus* toxins (Becker et al., 1998; Monday and Bohach, 1999; Mehrotra et al., 2000). This PCR type is based on combinations of specific primers for gene *se* or on combinations of universal forward primers with specific reverse primers (Sharma et al., 2000), and it is a technique in which various enterotoxin genes are detected in a single procedure (Becker et al., 1998; Monday and Bohach, 1999; Mehrotra et al., 2000; Sharma et al., 2000; Najera-Sanchez et al., 2003). However, restriction endonuclease assays or some additional steps are necessary to ensure a non-ambiguous identification of enterotoxin-specific amplicons. Consequently, a fast and specific method that can simultaneously detect and identify enterotoxins both for diagnostic and epidemiological purposes is still lacking, although most wanted.

A PCR-microarray assay combination for detection and identification of enterotoxin genes was described by Sergeev et al. (2004). The analysis is based on PCR amplification of a variable region found in almost all known enterotoxin genes by using a single set of degenerated primers whose sequences correspond to highly preserved flanked regions. One important problem arising with all PCR methods presented is that new and unexpected toxin genes may lead to false-positive or false-negative results. For instance, every specific primer for *sea* described in the literature can also successfully amplify gene *sep* (Sergeev et al., 2004). Such ambiguity may lead to mistaken conclusions about a sample's presenting gene *sea* as well as about the distribution of enterotoxin genes in microorganisms. Various *Staphylococcus* strains carry more than one *se* gene. The evolution of pathogenic strains of *Staphylococcus* sp. has been due to the accumulation of mobile genetic elements (Lindsay, 2009). In general, approximately 77% of the *S. aureus* isolates are positive for one or more enterotoxin genes. Genes s*eg* and *sei* commonly co-exist

in the same *S. aureus* strain (Omoe et al., 2002), and the co-existence of genes *sej* and *sed* has been reported by many researchers (Zhang et al., 1998; Becker et al., 2003; Nashev et al., 2004; Mendoza and Martin, 2005).

Recently Gaebler and Cunha (2008) studied 90 samples of *S. aureus* and 90 samples of CNS by PCR and RT-PCR for the detection of genes and mRNA for the enterotoxins E, G, H and I. The results showed that of 45 (50.0%) samples of *S. aureus* positive for *seg* and *sei* by PCR, 32 (71.1%) were positive to the enterotoxins G and/or I by RT-PCR, while of the 21 samples of CNS positive by PCR for these genes, 9 (42.8%) were positive to the expression of these. *S. epidermidis* was the most toxigenic species between the CNS, six of its samples were positive to the enterotoxins G and I mRNA expression. About the other CNS species, only *S. warneri* and *S. lugdunensis* showed positive result by RT-PCR method, being the first positive for the enterotoxin I and the second for the enterotoxins G and I. The analysis of these results allows saying that PCR method was efficient in confirming the toxigen capacity of *S. aureus* and coagulase-negative staphylococci and the RT-PCR method was also rapid and efficient and proved the capacity of CNS in expressing the enterotoxin codifying mRNA, what confirms the toxigen capacity of these organisms.

Jarraud et al. (1999; 2001) showed that genes *se* of *egc* (enterotoxin gene cluster) are arranged with a tandem orientation and are co-expressed. Jarraud et al. (1999) and McLauchlin et al. (2000) observed in the *S. aureus* A900322 standard strain, that genes s*eg* and *sei* belong to an *egc* operon in tandem orientation. The sequencing of the intergene region between *seg* and *sei* and adjacent regions has shown three enterotoxin-like toxins related to genes *seg* and *sei*, which have been designated as *sen*, *seo* and *sem*, in addition to two pseudogenes (genomic sequences that are similar to normal genes, but not functional), namely φ *ent1* and φ *ent2* (Jarraud et al. 2001). The *egc*-encoded staphylococcal superantigens are neutralized by human serum with less efficiency than classic staphylococcal enterotoxins and the Toxic Shock Syndrome toxin (Holtfreter et al., 2004). Additionally, enterotoxins SEG and SEI interact differently with the class-II major histocompatibility complex and stimulate completely different sub-sets from human and rats' T-cells. These characteristics indicate complementary superantigenic activity and suggest an important advantage to staphylococcal lineages that produce enterotoxins SEG and SEI at the same time (Jarraud et al., 2001).

The role of the newly described staphylococcal superantigens in human diseases is still unclear, although, the *egc*-related genes were reported as the most prevalent enterotoxin genes among *S. aureus* clinical and carriage isolates (Jarraud et al., 2001; Becker et al., 2003; Nashev et al., 2004). There are reports for the involvement of *egc*-encoded toxins SEG and SEI in food poisoning, toxic shock syndrome, staphylococcal scarlet fever and neonatal infections (Jarraud et al., 1999; Martin et al., 2004; Gaebler and Cunha, 2008).

Hence, by analyzing the diversity of staphylococcal superantigens and their properties, knowledge on the pathogenicity and the development of both *S. aureus* and coagulase-negative species has been dramatically increased, facilitating the enhancement of detection techniques and even the development of new methods, thus improving diagnoses and epidemiological studies.

Very recently, some authors (Dupuis et al., 2008; Hennekinne et al., 2009) developed proteomics approaches, a method of mass spectrometry (MS) for detection and absolute quantification of SEA in foods. The development of new analytical complementary approach including physicochemical methods using specific SE trypsic peptides is a new perspective to properly characterize and investigate staphylococcal food poisoning.

Conclusions

Microbial pathogens have great versatility to colonize various ecological niches and to cause infection in different anatomical sites of their hosts. The molecular process responsible for disease in specific hosts is not widely known. However, in this context, great importance is attributed to genetic variation among strains.

Species in the *Staphylococcus* genus, either commensal or pathogenic, have a wide range of virulence factors that may be expressed or not, depending on the microorganism's moment of life and its environment. These factors, which are responsible for direct tissue invasion or toxigenicity due to the action of toxins released, are very important in the high prevalence and success of such microorganisms in infections both in the community and in hospitals. Mechanisms that facilitate the bacterium's evasion from the host's immune system defense strategies favor their survival and colonization; hence, the strains that can escape more efficiently will be the most prevailing types to be found.

Further studies are required for a complete understanding of the biological regulation of organisms in the *Staphylococcus* genus and of the biological and clinical effects of the various enterotoxins. For such studies, it is important to develop a universal and reliable method that is not ambiguous in identifying similar gene sequences of different enterotoxins for the accurate characterization of those that have already been described and for facilitating the identification of new enterotoxins. The importance of such accurate characterization and understanding of the pathogenicity of each species in the *Staphylococcus* genus is due to the great pathogenic potential of this group of microorganisms

and indicates that more attention must be given to the toxigenic capacity of *Staphylococcus* sp., particularly to that of coagulase-negative species. Wider knowledge on the epidemiology of staphylococci can contribute to more adequate control, prevention and treatment of infections by this group of microorganisms. Molecular techniques are important tools in the investigation of outbreaks and epidemiology of pathogenic microorganisms. They can also help to understand the complex genetics of such microorganisms' virulence mechanisms, in addition to being able to determine the pathogenic potential of bacterial isolates by observing both the presence of virulence factors encoding genes and their expression. However, it is important to apply an appropriate methodology for the success of the study and for production of reliable results.

Abbreviations: ***agr*,** Accessory gene regulator; **AGRP,** accessory gene regulator protein; **AIP,** auto-inducing peptide; **APC,** antigen-presenting cells; **CNS,** coagulase-negative staphylococci; **DNA,** deoxyribonucleic acid; ***egc,*** enterotoxin gene cluster; **ELISA,** Enzyme linked immunosorbent Assay; ***hld*,** delta-hemolysin; **MHC,** major histocompatibility complex; **MODS,** multiple organ dysfunction syndrome; **mRNA,** messenger ribonucleic acid; **NaCl,** sodium chloride; **O/B,** oligosaccharide-binding fold; **OSP,** optimal sensitivity plate; **P,** promoter; **PCR,** polymerase chain reaction; **RNA**, ribonucleic acid; ***rot*,** repressor of toxins; **RT-PCR,** reverse transcript polymerase chain reaction; ***sar,*** staphylococcal accessory regulator; **SE,** staphylococcal enterotoxin; **SEI,** staphylococcal enterotoxin like; **SEA,** staphylococcal enterotoxin A; **SEB,** staphylococcal enterotoxin B; **SEC_1,** staphylococcal enterotoxin C_1; **SEC_2**, staphylococcal enterotoxin C_2; **SEC_3,** staphylococcal enterotoxin C_3; **SED,** staphylococcal enterotoxin D; **SEE**, staphylococcal enterotoxin E; **SEG,** staphylococcal enterotoxin G; **SHE,** staphylococcal enterotoxin H; **SEI,** staphylococcal enterotoxin I; **SEIJ,** staphylococcal enterotoxin like J; **SEIK,** staphylococcal enterotoxin like K; **SEIL**, staphylococcal enterotoxin like L; **SEIM,** staphylococcal enterotoxin like M; **SEIN,** staphylococcal enterotoxin like N; **SEIO**, Staphylococcal enterotoxin like O; **SEIP**, staphylococcal enterotoxin like P; **SEIQ,** staphylococcal enterotoxin like Q; **SER,** staphylococcal enterotoxin R; **SES,** staphylococcal enterotoxin S; **SET,** staphylococcal enterotoxin T; **SEIU,** staphylococcal enterotoxin like U; **SEIV,** staphylococcal enterotoxin like V; **SPEA,** streptococcal pyrogenic exotoxin A; ***speA*,** streptococcal pyrogenic toxin A; **SSA,** streptococcal superantigen; **TCR,** T-cell antigen receptor; **TNF,** tumor necrosis factor; **TSS**, Toxic-shock syndrome; **TSST-1,** toxic-shock syndrome toxin-1; **φ *ent*,** enterotoxin pseudogene.

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