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Expression of superantigens and the *agr* system in *Staphylococcus epidermidis*

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ABSTRACT

Infections with coagulase-negative staphylococci (CoNS) can involve the production of toxins such as superantigens, which contribute to tissue degradation and inflammatory immune responses. The accessory gene regulator (*agr*) quorum sensing system coordinates the expression of most *S. aureus* virulence factors. Therefore, the aim of this study was to investigate the expression of these superantigens and the presence of the *agr* locus in CoNS strains isolated from blood cultures. PCR was used to detect enterotoxin and *agr* genes and expression was analyzed by RT-PCR. Expression of the *sea* gene was observed in one *S. epidermidis* isolate andof *sec-1* in two, *seg* and *sei* were expressed concomitantly in one isolate, and *sei* was expressed in another isolate. The *agr* group I was detected in *S. epidermidis* expressing the *sea*, *seg* and *sei* genes, whereas *agr* group II was detected in isolates expressing the*sec-1* gene. The *agr* groups were only expressed in strains expressing the*sec-1* gene. The results show that enterotoxin genes are highly frequent in CoNS isolated from clinical specimens and confirm the toxin-producing ability of these strains. The *agr* group II may be associated with enterotoxin C production by *S. epidermidis*, increasing the virulence of strains isolated from blood cultures and consequently the severity of sepsis caused by these organisms.

1. Introduction

Most species belonging to the genus *Staphylococcus* are coagulase-negative staphylococci (CoNS), i.e., they cannot produce the coagulase enzyme. Although CoNS are part of the human microbiota, they are considered opportunistic microorganisms because they take advantage of situations such as post-trauma tissue damage and the presence of foreign bodies to proliferate and spread to other tissues, developing a pathogenic behavior [1]. CoNS are the main cause of bacteremia in hospitals and their pathogenesis is complex, involving the production of a variety of virulence factors such as toxins [2,3].

Staphylococcal toxins can contribute to tissue degradation and elicit anti-inflammatory immune responses [4]. Staphylococcal enterotoxins and toxic shock syndrome toxin 1 (TSST-1) are superantigens whose toxic effects can trigger the nonspecific proliferation of T cells through direct binding to major histocompatibility complex class II molecules and to the Vβ region of the T cell antigen receptor. Unlike normal antigen processing, they stimulate many T cells that overproduce cytokines such as interleukin 1 (IL-1), IL-2, interferon gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α) [5].

During infection, toxin production by staphylococci is a multi-stage process that is coordinated by a complex system of communication between bacterial cells. This mechanism, called quorum sensing, allows bacteria to share information about cell density and to establish a phenotypic reaction according to the growth stage of the culture [6,7]. Quorum sensing via the accessory gene regulator (*agr*) system is one of the main systems that coordinate staphylococcal virulence factors [8]. This system comprises promoters P2 and P3, which operate in opposite directions and produce transcripts RNAII and RNAIII, respectively. RNAIII is responsible for the gene transcription of a number of virulence factors, including extracellular toxins and enzymes and surface proteins [9]. The *agr* system is composed of four genes (*agrA*, *agrB*, *agrC*, and *agrD*) arranged in an operon. The products of these genes are proteins AgrA, AgrB, AgrC and AgrD, which are necessary for the function of the system. Proteins AgrB and AgrD combine to produce the autoinducing polypeptide (AIP), which is released into the extracellular medium. When the transmembrane protein AgrC detects AIP in the external environment, it phosphorylates AgrA that induces expression from P2 and P3. The final product of the *agr* locus is RNAIII, an mRNA that induces or inhibits toxin genes [7].

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Three polymorphisms of the *agr* locus (*agr* group I, group II and group III) have been described in *Staphylococcus epidermidis* [10]. These groups show variations in the *agrB*, *agrC* and *agrD* genes and thus in AIP and AgrC, with AIP binding to a specific receptor for each allelic group. When AIP of one allele group binds to the AgrC receptor of another group, it does not produce intrinsic factors and thereby behaves as an antagonist. AIP is the only agonist to its own allele group and bacteria of one *agr* group can therefore affect the regulation of accessory proteins of bacteria from another *agr* group [7].

In view of the increasing incidence of CoNS infection, studies on associated virulence factors are important to better understand the toxigenic potential of these microorganisms. Therefore, the present study evaluated the occurrence and expression of superantigens and the role of the *agr* system in the production of virulence factors by CoNS species isolated from blood cultures of patients hospitalized in the University Hospital of the Botucatu Medical School.

2. Material and methods

2.1. Strains

Three hundred CoNS strains isolated from blood cultures of patients admitted to the University Hospital of the Botucatu Medical School, State University of São Paulo (UNESP), were evaluated. The blood samples were collected between 1990 and 2009 and stored in the Culture Collection of the Department of Microbiology and Immunology, Biosciences Institute of Botucatu (UNESP). An average prevalence of 35%, with a 5% error and 95% confidence interval, were defined as criteria for selection of each CoNS species.

The strains were isolated on blood agar plates as described by Koneman et al. [9] and suspected colonies were submitted to Gram staining. After confirmation of morphology and specific staining, catalase and coagulase tests were used to identify the isolates. Biochemical tests were performed for phenotypic identification of the CoNS species according to the simplified method of Cunha et al. [10]. Genotypic identification was carried out by PCR-based determination of internal transcribed spacer (ITS) regions according to Couto et al. [11].

2.2. Extraction of DNA

Total DNA was extracted from the CoNS strains cultured on blood agar, individually inoculated into BHI broth, and incubated for 24 h at 37 °C. In brief, staphylococcal cells were digested with lysozyme (10 mg/mL) and proteinase K(20 mg/mL). Next, 500 μL of the extraction solution was added and the mixture was centrifuged at 5000 × *g* for 1 min. The supernatant was transferred to a GFX column and centrifuged at 5000 × *g* for 1 min. The fluid collected was discarded and 500 μL of the extraction solution was again added to the column. After centrifugation and disposal of the collected fluid, 500 μL of the washing solution was added and the column was centrifuged at 14,000 rpm for 3 min. The column was transferred to a 1.5-ml tube and 200 μL Milli-Q water heated to70°Cwas used for elution. The samples were centrifuged at 5000 × *g* for 1 min and the GFX column was discarded. The extracted DNA was stored in a freezer at 4 °C.

2.3. Detection of enterotoxin and TSST-1 genes

PCR was carried out in 0.5-ml centrifuge tubes containing10 pmol of each primer (Table 2), 2.5 U Taq DNA polymerase, 200 μmol/L deoxyribonucleotide triphosphates (dNTP), 20 mmol/L Tris-HCl, pH 8.4, 0.75 mmol/L $MgCl_2$, and 3 μL of the sample in a final volume of 25 μL. A negative control in which the nucleic acid was replaced with water was included in all reactions. Amplification was carried out in an MJ Research PTC-100 thermocycler using the following parameters: one cycle at 94 °C for 4 min, denaturation at 94 °C for 2 min, primer annealing at 55 °C and extension at 72 °C for 1 min 30 s, followed by a second cycle of denaturation at 94 °C for 2 min, annealing at 53 °C and extension at 72 °C for 1 min 30 s. In the third cycle, the annealing temperature was reduced to 51 °C and 37 cycles were carried out using the last parameters. After completing40 cycles, the tubes were incubated at 72 °C for 7 min and then cooled to 4 °C.

2.4. Extraction of RNA and cDNA synthesis

Total RNA was extracted using the Illustra RNA spin Mini kit according to manufacturer recommendations. After treatment with DNAse, the mRNA samples were converted into cDNA. For that purpose, 12 μL mRNA treated with DNase was added to 1 μL of random primer(75 ng/μL), 6 μL nuclease-free water, and 1 μL dNTP (200 μM). The mixture was heated for 5 min at 65 °C for RNA denaturation and primer binding and 4 μL reverse transcription buffer, 1 μL dithiothreitol and 1 μL SuperScript™ III (200 U/μL) were added. cDNA was synthesized in a PTC-100 thermocycler using one cycles at 25 °C for 5 min, 50 °C for 60 min, and 70 °C for 15 min, followed by cooling at 4 °C. As internal control, the expression of 16S rRNA using 16S1 and 16S2 primers (Table 2) was analyzed, which correspond to gene regions that are conserved in staphylococci and specific to the genus. The cDNA obtained was amplified by PCR and the resulting products were visualized by electrophoresis.

2.5. Determination of the agr group

The CoNS strains that tested positive for superantigen mRNA by the reverse transcription polymerase chain reaction (RT-PCR) were subjected to *agr* group typing by PCR as described by Li et al. [12]. The reactions were performed with primers targeting *agrA*, *agrB* and *agrC* (Table 1).

2.6. Staphylococcus epidermidis typing by PFGE

The modified protocol of McDougal et al. [13] was used to determine the clonal profile of the *S. epidermidis* spp. isolates. The strains were inoculated into BHI broth and incubated for 24 h at37 °C.The isolates were centrifuged in microtubes at 15,294 × *g* for 1 min. The supernatant was discarded and 300 μLTE solution (10 mM Tris, 1 mM EDTA, pH 8.0) was added. The strains were incubated in a water bath for 10 min at 37 °C. The cells were lysed by adding 5 μL lysostaphin and vortexed, and 300 μL of 1.8% low-melt agarose was added at 37 °C. Plugs were prepared from the strains and the agarose was allowed to solidify. The plugs were then transferred to a 24-well plate containing 2 mL EC solution (6 mM Tris-HCl, 1 M NaCl, 100 mM EDTA, 0.5% Brij-58, 0.2% sodium deoxycholate, 0.5% sodium lauroyl sarcosinate) and incubated for 4 h at 37 °C. The EC solution was removed and the plugs were washed four times in 2 mL TE solution for 30 min at 21 °C.

One-third of the plug and 2 μL SmaI were used for the restriction of genomic DNA. For restriction, buffer without the enzyme (45 μL Milli-Q water and 5 μL of the enzyme buffer) was added to a 96-well plate and the plate was stored in a refrigerator for 30 min at 4 °C. The buffer without the enzyme was removed and buffer containing the enzyme (43 μL Milli-Q water, 5 μL enzyme buffer, and 2 μL of the enzyme) was added. The plate was incubated in an oven for 6 min at 37 °C. Electrophoresis was carried out in a CHEF-DR III System using 1% agarose gel prepared in 0.5 M TBE (0.1 M Tris, 0.08 M boric acid, 1 mM EDTA) under the following conditions: pulse times of 5–40 s for 21 h on a linear ramp; 6 V/cm; angle of 120°; 14 °C; 0.5 M TBE as running buffer. The Lambda Ladder PFG Marker was used as a molecular marker. The gels were stained with GelRed (400 mL distilled water and 30 μL GelRed) for 1 h and photographed under UV transillumination.

The BioNumerics 6.1 software was used for analysis of similarity, calculation of the Dice correlation coefficient, and construction of the dendrogram by the UPGMA method. Band position tolerance and optimization were set at 1.25 and 0.5%, respectively. A similarity