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Research Article

Coagulase-Negative Staphylococci from Hospital-Associated Infections Harboring Virulence Genes

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Abstract

Coagulase-negative staphylococci (CoNS) are frequently isolated from hospital infections associated with biofilm formation on implanted medical devices. However, the toxigenic potential of CoNS is not yet fully understood. Here we report the presence of putative virulence-associated genes usually known for *Staphylococcus aureus* in CoNS isolates from hospital-associated infections in Recife, Pernambuco, Brazil. The presence of the genes *hlg*CB, coding for the staphylococcal gamma-hemolysin toxin; *luk*SF, coding for the Panton-Valentine leukocidin (PVL); and *tst*, coding for the toxic shock syndrome toxin (TSST-1), was assessed by PCR amplification followed by sequencing of the PCR amplicons in 43 CoNS samples. Preliminary identification by conventional biochemical tests was confirmed by automated system and by sequencing the 16S rRNA and *tuf* genes. The absence of the coagulase gene in the strains was confirmed by a PCR test that was negative for the *coa* gene. Eighteen isolates carried at least one of those genes. *hlg*CB and *luk*SF were detected together in five isolates, *hlg*CB occurred alone in 12 isolates, and *hlg*CB and *tst* occurred together in one isolate. Carriage of staphylococcal toxigenic genes indicates the ability of CoNS to incorporate those genes, increasing their toxigenic potential and their ability to invade sterile sites and to cause serious hospital-acquired infections.

Keywords: Coagulase-Negative *Staphylococcus*; Gamma-Hemolysin; Panton-Valentine Leucocidin; TSST-1

Introduction

Coagulase-negative staphylococci (CoNS), habitual components of the skin and mucosa microflora that were long considered saprophytic, are opportunistic organisms responsible for various infections, especially in hospitalized or immunocompromised patients with prolonged use of implanted medical devices [1-3].

Studies on the virulence mechanisms of CoNS are scarce, and their toxigenic potential is not yet fully understood. CoNS pathogenicity is mainly associated with biofilm formation; although reports on CoNS associated with toxigenic genes are increasing, the toxigenic potential of CoNS strains is still controversial [4-8].

Among *Staphylococcus* species, *Staphylococcus aureus* is the best studied. Its association with several virulence factors has been described [7]. Here we report the presence of putative virulence-associated genes usually known for S. aureus in several coagulase negative staphylococcal (CoNS) isolates from nosocomial infections. Detection method was PCR amplification followed by sequencing of the PCR product and the latter confirmed *S. aureus* sequences.

The presence of the staphylococcal gamma-hemolysin, encoded by the cluster *hlg*ACB; the Panton-Valentine leukocidin (PVL), encoded by the locus *luk*SF; and the toxic shock syndrome toxin (TSST-1), encoded by the *tst* gene [9-12], was assessed in methicillin-resistant CoNS strains

involved in hospital infections.

Material and methods

Isolates

The study included 43 clinical CoNS isolates from blood culture (n=39) or catheter tips (n=4) that were obtained from patients at Oswaldo Cruz University Hospital in Recife, Pernambuco state, Brazil, from 2002 to 2010 (Table 1). The isolates were characterized as nosocomial infections according to the Centers for Disease Control and Prevention criteria [13], and were preliminary identified by the conventional bacteriological catalase test, the tube coagulase test, the thermonuclease growth test, and the mannitol salt agar test. All of the samples were methicillin resistant as determined by the presence of the *mec* gene by PCR [14].

16S rRNA [15] and tuf genes [16]. The coagulase gene was assessed in the strains by a PCR test for the *coa* gene [17].

Bacterial DNA was extracted as described [18] and quantified using a NanoDrop 2000 (Thermo Fisher Scientific Inc. 81 Wyman Street, Waltham, MA 02451 USA). The amplifications were carried out in individual reactions prepared in a final volume of 25 µl, containing 5 µl Green Go Taq buffer (Promega Corporation. 2800 Woods Hollow Road, Madison, WI 53711 USA), 2.5 mM of each deoxynucleotide, 25 mM MgCl2, 1 U of Go Taq DNA polymerase (Promega), 20 ng DNA from each sample, and 10 µM of each primer (F-TTAGCCGTGGCTTTCTG and R–GCAGGGTGAGTAACAAGT) for the 16S rRNA [15]; (F-GCCAGTTGAGGACGTATTCT and R-CCATTTCAGTACCTTGTGGTAA for the *tuf* gene [16]; and F–ACCACAAGGTACTGAATCAACG and R–TGCTTTCGATTGTTCGATGC for the *coa* gene (17).

Table 1. Distribution of the CoNS samples by species, source and genes amplified

Number/year	Source	Species identification		Genes		
		Vitek® 2	16S rRNA/*tuf*	*hlgCB*	*lukSF*	*tst*
013/2002	Hemoculture	*S. lugdunensis*	*S. lugdunensis*	+	+	-
039/2002	Hemoculture	*S. epidermidis*	*S. epidermidis*	+	+	-
052/2002	Hemoculture	*S. haemolyticus*	*S. haemolyticus*	+	+	-
081/2002	Hemoculture	*S. epidermidis*	*S. epidermidis*	+	+	-
109/2002	Hemoculture	ND	ND	-	-	-
124/2002	Catheter	*S. epidermidis*	*S. epidermidis*	+	-	-
162/2002	Hemoculture	ND	ND	-	-	-
009/2004	Hemoculture	*S. epidermidis*	*S. epidermidis*	+	-	-
012/2004	Hemoculture	ND	ND	-	-	-
013/2004	Hemoculture	ND	ND	-	-	-
070/2004	Catheter	ND	ND	-	-	-
412/2004	Hemoculture	*S. lugdunensis*	*S. lugdunensis*	+	+	-
522/2004	Catheter	ND	ND	-	-	-
525/2004	Catheter	ND	ND	-	-	-
976/2004	Hemoculture	ND	ND	-	-	-
992/2004	Hemoculture	*S. epidermidis*	*S. epidermidis*	+	-	-
018/2009	Hemoculture	*S. hominis*	*S. epidermidis*	+	-	-
019/2009	Hemoculture	*S. epidermidis*	*S. epidermidis*	+	-	-
021/2009	Hemoculture	ND	ND	-	-	-
022/2009	Hemoculture	*s. epidermidis*	*S. epidermidis*	+	-	-
023/2009	Hemoculture	*S. epidermidis*	*S. epidermidis*	+	-	-
024/2009	Hemoculture	*S. epidermidis*	*S. epidermidis*	+	-	-
026/2009	Hemoculture	ND	ND	-	-	-
027/2009	Hemoculture	ND	ND	-	-	-
028/2009	Hemoculture	ND	ND	-	-	-
029/2009	Hemoculture	*S. xylosus*	*S. epidermidis*	+	-	-
030/2009	Hemoculture	*S. epidermidis*	*S. epidermidis*	+	-	-
032/2009	Hemoculture	*S. epidermidis*	*S. epidermidis*	+	-	-
033/2009	Hemoculture	*S. hominis*	*S. epidermidis*	+	-	-
034/2009	Hemoculture	ND	ND	-	-	-
035/2009	Hemoculture	ND	ND	-	-	-
036/2009	Hemoculture	ND	ND	-	-	-
037/2009	Hemoculture	ND	ND	-	-	-
040/2009	Hemoculture	ND	ND	-	-	-
041/2009	Hemoculture	ND	ND	-	-	-
042/2009	Hemoculture	ND	ND	-	-	-
044/2009	Hemoculture	ND	ND	-	-	-
045/2010	Hemoculture	*S. hominis*	*S. epidermidis*	+	-	+
046/2010	Hemoculture	ND	ND	-	-	-
047/2010	Hemoculture	ND	ND	-	-	-
048/2010	Hemoculture	ND	ND	-	-	-
051/2010	Hemoculture	ND	ND	-	-	-
055/2010	Hemoculture	ND	ND	-	-	-

ND=not determined; +=positive; -=negative; Vitek=VITEK ® 2 microbial identification system (bioMérieux); 16Sr RNA/*tuf*=genes sequencing

Identification of the CoNS isolates at the species-level

Identification of the CoNS isolates at the species level was performed using a VITEK ® 2 microbial identification system (bioMérieux SA. 376, Chemin Orme, Marcy l'Etoile, France 69280) and further confirmed by sequencing the

The thermocycler program was 30 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 7 minutes. Amplification products were electrophoresed in 1.5% agarose gels, stained with GelRed Nucleic Acid Stain (Biotium Inc. 3159 Corporate Place, Hayward, CA 94545

USA), visualized on a UV transilluminator, and digitalized using the L-Pix EX Image equipment (Loccus Biotecnologia, 330 José de Andrade, Cotia, SP 06714-200 Brasil).

Sequencing the PCR amplicons

The 16S rRNA and *tuf* genes amplicons were purified using ExoSAP-IT® (Affymetrix, 3420 Central Expressway, Santa Clara, CA 95051 USA) and sequenced by the Sanger method [19] using a Big Dye® Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems®. 180 Oyster Point Blvd South, San Francisco, CA 94080-1909 USA). The sequences obtained were confirmed bi-directionally and edited with Lasergene 7.0 (DNASTAR Inc. 3801 Regent Street, Madison, WI 53705 USA) and the sequence consensus was compared with sequences deposited in GenBank. The alignments were used to generate phylogenetic trees using Molecular Evolutionary Genetics Analysis (MEGA) software, version 6.0 [20].

The following genome partial sequences were used for phylogenetic analysis: *Staphylococcus lugdunensis* N920143 (accession number: NC_017353), *Staphylococcus haemolyticus* JCSC1435 (accession number: NR_074994), *Staphylococcus epidermidis* RP62A (accession number: NR_074995), *Staphylococcus epidermidis* ATCC12228 (accession number: NC_004461), *Staphylococcus epidermidis* SeMCV45 (accession number: HM032776) and *Staphylococcus aureus* strain UA-S391_USA300 (accession number: CP007690).

Determination of the presence of toxigenic genes

The presence of toxigenic genes coding for gamma-hemolysin, PVL, and TSST-1 was assessed by PCR using specific primers: hlgCB: GCCAATCCGTTATTAGAAAATGC and CCATAGACGTAGCAACGGAT; lukSF: GCATCAASTGTATTGGATAGCAAAAGC and ATCATTAGGTAAAATGTCTGGACATGATCCA [9]; and tst: AAGCCCTTTGTTGCTTGCG and ATCGAACTTTGGCCCATACTTT [21].

The amplifications were carried out as described above. Amplification products were electrophoresed in 2% agarose gels, stained with GelRed Nucleic Acid Stain (Biotium Inc.), visualized on a UV transilluminator, and digitalized using KODAK 1D software version 3.5.2 (Eastman Kodak Company, 1669 Lake Avenue, Rochester, NY 14652 USA).

Sequencing

The *hlg*CB, *luk*SF and *tst* genes amplicons were purified using ExoSAP-IT® (Affymetrix) and sequenced by the Sanger method [19] using a Big Dye® Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems®). The sequences obtained were processed using SeqMan II from Lasergene 7.0 software package (DNASTAR Inc.) and compared with GenBank sequences using BLAST (http://www.ncbi.nih.gov/BLAST).

Sequences used were: *Staphylococcus aureus* subsp. *aureus* MW2 (accession number: BA000033) for *hlg*CB, *Staphylococcus aureus* (accession number: L01055) for *luk*SF and *Staphylococcus aureus* subsp. *aureus* N315 (accession number: NC_002745) for tst.

Results

Identification of the CoNS isolates at the species-level

Using a VITEK ® 2 microbial identification system (bioMérieux) the 18 CoNS isolates harboring toxigenic genes were identified as *S. epidermidis* (11), *S. hominis* (3), *S. lugdunensis* (2), *S. haemolyticus* (1) and *S. xylosus* (1). Based on the phylogenetic analysis of the 16S rRNA and *tuf* genes they were identified as *S. epidermidis* (15), *S. lugdunensis* (2) and *S. haemolyticus (1)* (Table1). The PCR test was negative for the *coa* gene in all the samples analyzed.

Determination of the presence of toxigenic genes

Out of the 43 CoNS isolates analyzed, 18 (41.86%) has at least one toxigenic gene amplified, and 25 (58.14%) had none.

The 937 base-pair (bp) segment expected for the cluster *hlg*ACB was amplified in 18/43 (41.86%) of the isolates; the segment of 433 bp expected for the cluster *luk*SF was amplified in 5/43 (11.62%) of the isolates; and the 445 bp segment expected for *tst* was amplified in one isolate (2.32%). Figure 1 shows a representative gel of the three segments amplified from either *S. lugdunensis* 412 and *S. epidermidis* 45 (Table 1) with the primers targeting *hlg*CB, *luk*SF and *tst*.

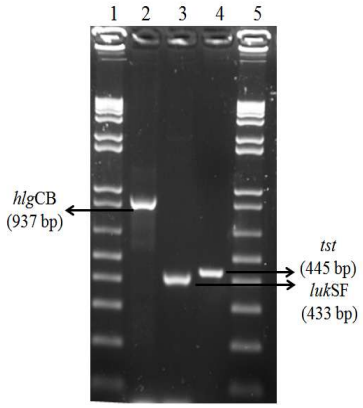

Figure 1. Representative agarose gel showing the amplification products of the expected sizes: 937, 433, and 445 base pairs (bp) with the primers targeting *hlg*CB, *luk*SF and *tst*, respectively. Lanes 1 and 5: DNA 100 bp ladder; Lane 2: *hlg*CB gene cluster (strain 412); Lane 3: *luk*SF gene cluster (strain 412); Lane 4: *tst* gene (strain 45).

Analysis of the sequences revealed 99% identity to the sequences deposited in GenBank: accession number BA000033 for hlgCB, L01055 for lukSF and NC_002745 for tst.

The primers targeting *hlg*CB and *luk*SF amplified a product in five isolates (2 *S. epidermidis*, 2 *S. lugdunensis* and 1 *S. haemolyticus*); *hlg*CB was found alone in 12 *S. epidermidis* isolates; and *hlg*CB and *tst* were found together in one *S. epidermidis* isolate.

*hlg*CB was the most prevalent of the three amplicons and was identified in 18/43 (41.86%) isolates. It occurred alone in 12 (66.67%) isolates, combined with *luk*SF in five (27.77%) and combined with *tst* in one (5.55%). *luk*SF occurred only in combination with *hlg*CB, as did *tst*.

Discussion

Although CoNS are often responsible for serious infections, resulting mainly from the increasing frequency of invasive procedures [22], their toxigenic potential is not yet clear.

In this work, 18/43 (41.86%) CoNS isolates from blood cultures harbored the *hlg*CB genes, which code for the staphylococcal gamma-hemolysin. Until now, among the staphylococcal hemolysins, only delta-hemolysin was reported in CoNS [7, 23]. Hence, this is the first time staphylococcal gamma-hemolysin genes were detected in CoNS. The occurrence of this toxin represents an important increase in toxigenic potential, contributing to the emergence of CoNS as a pathogen in the hospital environment.

The presence of *luk*SF genes, coding for PVL, was detected in 5/43 (11.62%) of the isolates, and they were always present with *hlg*CB. The presence of *luk*SF was previously described in CoNS strains of animal origin only [24]. To our knowledge, it has not been detected among human isolates. Thus, this is the first report of *luk*SF genes in hospital-associated CoNS isolates from blood cultures.

Staphylococcal gamma-hemolysin and PVL act synergistically [9]. In our study, five CoNS strains harbored both *hlg*CB and *luk*SF. This association, common in *S. aureus*, possibly enhances the bacterial invasive and cytotoxic capacity [9, 25].

The *tst* gene, coding for TSST-1, was detected in only one CoNS isolate. The ability of CoNS to produce TSST-1 has been questioned, and it was speculated that it could be *S. aureus* that does not express the coagulase enzyme [26-28]. In our work, DNA sequencing of the *tst* amplicon revealed a *tst* sequence that was 99% identical to the published S. aureus *tst* sequence. The isolate was identified as *S. epidermidis* by 16S rRNA and *tuf* genes sequencing and its lack of a coagulase gene was confirmed by PCR.

Furthermore, to avoid misdiagnosis and to unambiguously confirm their identification, the 18 CoNS harboring *S. aureus* toxin genes were typed by sequencing the 16S rRNA and *tuf* genes. Commercial microbial identification systems may provide ambiguous results or fail to identify isolates with atypical biochemical characteristics [15, 29]. In this work, concordant results were obtained in 14 of 18 CoNS isolates by VITEK ® 2 and 16S rRNA and *tuf* genes phylogenetic analysis. However, one isolate identified as *S. xylosus* and three as *S. hominis*, by VITEK ® 2, revealed *S. epidermidis* by the 16S rRNA and tuf genes sequencing. Considering molecular identification-based approaches more reliable than the phenotypic-identification methods, the isolates from this study were categorized according to the results of the phylogenetic analysis (Table 1).

All the samples of this study were methicillin resistant [14], as determined by the presence of the *mec*A gene by PCR. The treatment of infections by methicillin-resistant strains requires greater attention than other infections because, besides the therapeutic limitations, empirical beta-lactam antibiotic administration can increase the expression of virulence factors such as PVL [25] and induce a high frequency of horizontal transfer of pathogenicity islands carrying genes coding for virulence factors.

In conclusion, our results reveal the toxigenic potential of the CoNS population isolated from blood culture and catheter tips. Although generally considered a simple contaminant, CoNS was found to be an important nosocomial infectious agent acting as a reservoir of virulence genes in the hospital environment. Carriage of staphylococcal toxigenic genes indicates the ability of CoNS to incorporate toxigenic genes, increasing their toxigenic potential and their ability to invade sterile sites and to cause serious hospital-acquired infections.

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