STANDARDIZATION OF THE PCR TECHNIQUE FOR THE DETECTION OF DELTA TOXIN IN *Staphylococcus* spp.

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ABSTRACT: Coagulase-negative staphylococci (CNS), components of the normal flora of neonates, have emerged as important opportunistic pathogens of nosocomial infections that occur in neonatal intensive care units. Some authors have reported the ability of some CNS strains, particularly *Staphylococcus epidermidis*, to produce a toxin similar to *S. aureus* delta toxin. This toxin is an exoprotein that has a detergent action on the membranes of various cell types resulting in rapid cell lysis. The objectives of the present study were to standardize the Polymerase Chain Reaction (PCR) technique for the detection of the gene responsible for the production of delta toxin (*hld* gene) in staphylococcal species isolated from catheters and blood cultures obtained from neonates, and to compare the results to those obtained with the phenotypic synergistic hemolysis method. Detection of delta toxin by the phenotypic and genotypic method yielded similar results for the *S. aureus* isolates. However, in *S. epidermidis,* a higher positivity was observed for PCR (97.4%) compared to the synergistic hemolysis method (86.8%). Among CNS, *S. epidermidis* was the most frequent isolate and was a delta toxin producer. *Staphylococcus simulans* and *S. warneri* tested positive by the phenotypic method, but their positivity was not confirmed by PCR for the *hld* gene detection. These results indicate that different genes might be responsible for the production of this toxin in different CNS species, requiring highly specific primers for their detection. PCR was found to be a rapid and reliable method for the detection of the *hld* gene in *S. aureus* and *S. epidermidis*.

KEY WORDS: delta toxin, PCR, *Staphylococcus*, coagulase-negative staphylococci

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INTRODUCTION

Coagulase-negative staphylococci (CNS) have emerged over the last few years as important opportunistic pathogens of nosocomial infections that occur in neonatal intensive care units (NICU) (8). The high incidence of infection by CNS in neonates can be explained by the fact that the immunological system of these children, especially premature and low birth weight infants, is not yet completely developed (5). The application of invasive procedures such as mechanical ventilation, parenteral nutrition for more than two weeks and insertion of umbilical catheters, as well as the extensive use of antibiotics and prolonged hospitalization in NICU are factors that contribute to the development of CNS-induced neonatal infections (6, 10, 18, 22).

Some authors have reported the ability of some CNS strains, particularly *Staphylococcus epidermidis*, to produce a toxin similar to *S. aureus* delta toxin. This toxin has a detergent action on cell membranes resulting in cell lysis. It acts on various cell types, including red blood cells, and has also been called delta hemolysin, but since its action is not restricted to blood cells it should better be called cytotoxin. Among the cytotoxins produced by *Staphylococcus*, delta toxin is characterized by its thermostability, neutralization by lectin, and synergism with beta toxin (16). Hemolytic methods, such as synergistic hemolysis of beta hemolysin, have been used for the detection of delta cytotoxin, but its production can be influenced *in vitro* by components present in the culture medium (9).

Based on the above considerations, the importance of developing a genotypic method for the identification of the gene responsible for producing delta toxin in *Staphylococcus* species becomes evident. In *S. aureus*, the *hld* gene responsible for delta toxin production is situated within the RNAIII locus of the accessory gene regulator (*agr* gene), which controls the expression of most exoproteins of *S. aureus*. The *hld* gene of *S. aureus* encodes a 44-amino acid peptide (21). The same activity is mediated in *S. epidermidis* by a peptide highly homologous to *S. aureus* delta toxin, and whose gene is also located within the RNAIII locus of the *agr* gene (4), but the *hld* gene of *S. epidermidis* encodes a 26-amino acid peptide that differs in only three amino acids from the *hld* gene of *S. aureus* (4, 21).

No reports using a specific primer for the detection of the *hld* gene in *S. aureus* and in CNS by Polymerase Chain Reaction (PCR) assay are available in the national and international literature, but only studies such as that of Donvito *et al*. (4), in which the presence of the *hld* gene in *Staphylococcus* species was determined by hybridization

methods. We therefore emphasize the importance of the present study, whose objectives were to standardize the PCR technique for the *hld* gene detection in *Staphylococcus* species, and to compare the results with those obtained with the phenotypic synergistic hemolysis method described by Hébert and Hancock (9).

MATERIAL AND METHODS

Isolates

Microorganisms isolated from catheter tips and blood cultures obtained from neonates hospitalized at the Neonatal Unit of the University Hospital, Botucatu Medical School, UNESP - São Paulo State University, from 2001 to June 2003 were studied. Samples were isolated from catheter tips according to the semiquantitative method proposed by Maki *et al.* (15), which consists of seeding a sample by rolling the catheter tip on the surface of a blood agar plate. Microorganisms originating from blood cultures incubated using the automated BACTEC system were isolated as described by Koneman *et al.* (13).

Identification of *Staphylococcus* spp.

Microorganisms grown in culture were Gram stained to verify their purity and to determine their morphology and specific color. After confirmation of these characteristics, catalase and coagulase tests were carried out (13). The genus *Staphylococcus* was differentiated from *Micrococcus* based on the oxidation and fermentation of glucose; on the resistance to bacitracin (0.04 U), indicated by the absence or presence of an inhibition halo measuring up to 9 mm; and on the sensitivity to furazolidone (100 µg), characterized by inhibition halos measuring 15 to 35 mm in diameter (1).

Identification of coagulase-negative staphylococci

Coagulase-negative staphylococci were identified using the criteria proposed by Kloos and Schleifer (11) and Kloos and Bannerman (12) based on a simplified scheme of biochemical tests that determine the utilization of the sugars xylose, arabinose, sucrose, trehalose, mannitol, maltose, lactose, xylitol, ribose, and fructose, as well as the production of hemolysins, nitrate reduction, presence of urease and ornithine decarboxylase, and sensitivity to novobiocin.

Delta toxin production

The production of delta toxin by the isolated strains was determined by using the synergistic hemolysis method described by Hébert and Hancock (9). One beta-hemolytic *S. aureus* strain was seeded vertically onto a 5% sheep blood agar plate and the samples to be tested were seeded perpendicularly at a distance of approximately 1 cm from the *S. aureus* strain. The plates were incubated at 37°C for 20 h under aerobic conditions and kept at room temperature for 4 to 6 h before analysis. Strains showing an increase in their hemolysis area at the extremity close to the beta-hemolytic *S. aureus* were considered to be toxin producers. *Staphylococcus aureus* (ATCC 19095), *S. epidermidis* (ATCC 12228), *S. xylosus* (ATCC 29979), and *S. warneri* (ATCC 10209) were used as positive controls, and *S. saprophyticus* (ATCC 15305) as negative control.

Detection of the delta toxin gene
DNA extraction

Total DNA was extracted from *Staphylococcus* strains cultured on blood agar for 24 h at 37°C, individually inoculated into Brain Heart Infusion broth and incubated at 37°C for 24 h. The GFX kit (Amersham Biosciences) was used for DNA extraction, which consists of initial digestion of the staphylococcal cells with lysozyme (10 mg/ml) and proteinase K (20 mg/ml). Then, 500 µl of the extraction solution was added and the mixture was centrifuged at 5,000 *g* for 1 min. The supernatant was then transferred to a GFX column and centrifuged at 5,000 *g* for 1 min. The collected eluent was discarded and 500 µl of the extraction solution was again added to the column. After centrifugation and disposal of the collected eluent, 500 µl of the wash solution was added to the column, which was centrifuged at 20,817 *g* for 3 min. The column was then transferred to a 1.5-ml tube and 200 µl Milli-Q water heated to 70°C was used for elution under centrifugation at 5,000 *g* for 1 min.

PCR

Polymerase Chain Reaction was carried out in 0.5-ml microcentrifuge tubes in a total volume of 25 µl containing 20 pmol of each primer (Table 1), 2.5 U Taq DNA polymerase, 200 µM dNTPs, 20 mM Tris-HCl, pH 8.4, 2.0 mM $MgCl_2$, and 3 µl of the sample. A negative control, in which DNA was replaced with water, was run in parallel with all reactions. *Staphylococcus aureus* (ATCC 19095) and *S. epidermidis* (ATCC 12228) were

used as positive controls. Amplification of the *hld* gene of *S. aureus* was performed in MJ Research PTC-100 Thermocycler, and the cycle conditions used were: one cycle at $94^{o}C$ for 5 min, denaturation at $94^{o}C$ for 2 min, annealing of the primers at $50^{o}C$, and extension at $72^{o}C$ for 1 min, followed by 4 cycles during which the annealing temperature was reduced by $1^{o}C$ per cycle. During the sixth cycle, the annealing temperature was reduced to $45^{o}C$, followed by 25 cycles of denaturation at $94^{o}C$ for 2 min, annealing at $45^{o}C$, and extension at $72^{o}C$ for 1 min. At the end of the 30 cycles, the tubes were incubated at $72^{o}C$ for 5 min before being stored at $4^{o}C$. The same parameters were used for the amplification of the *S. epidermidis* gene, except for the annealing temperature, which, during the first 6 cycles, ranged from 45 to $40^{o}C$, with a reduction of $1^{o}C$ per cycle, and was then maintained at $40^{o}C$ during the following 24 cycles.

The primers were designed based on the *S. aureus* delta toxin sequence described by Takeuchi *et al.* (19) and the *S. epidermidis* sequence published by Tegmark *et al.* (20) using the GeneRunner program.

Visualization of the amplified products

Amplification efficiency was determined by electrophoresis on 2% agarose gels in 1X TBE buffer stained with ethidium bromide. The size of the amplified products was compared with a 50-kb standard and the gels were photographed under UV transillumination.

RESULTS

Strains

Forty-four strains belonging to the genus *Staphylococcus* were isolated. Twenty samples were isolated from catheter tips, 18 from blood, and 6 were simultaneously isolated from catheters and blood obtained from neonates hospitalized at the NICU of the University Hospital, Botucatu Medical School, UNESP - São Paulo State University, from September 2001 to June 2003.

Identification of *Staphylococcus* spp.

Coagulase-negative staphylococci were the most frequent microorganisms, accounting for 41 isolates (93.2%) compared to three (6.8%) *S. aureus* isolates. Among CNS, *S.*

epidermidis was the most predominant species with 38 strains (86.4%), followed by two *S. simulans* (4.5%) isolates and one *S. warneri* (2.3%) isolate.

Table 2 shows the frequency of *Staphylococcus* isolates according to clinical material. *Staphylococcus epidermidis* was the predominant species in blood and catheters.

Delta toxin detection

Synergistic hemolysis of beta hemolysin (Figure 1) revealed production of delta toxin in 39 (88.6%) of all staphylococcal strains, including 36 (87.8%) CNS and three (100%) *S. aureus* isolates. *Staphylococcus epidermidis* was the toxin producer among 33 (86.8%) of the 38 strains studied. The two *S. simulans* and one *S. warneri* isolates were also positive for toxin production.

PCR for detection of the gene responsible for production of delta toxin demonstrated the presence of the *hld* gene in all three *S. aureus* strains studied and in 37 (97.4%) of the *S. epidermidis* isolates (Figure 2). On the other hand, PCR amplification did not show the presence of the *hld* gene in the two *S. simulans* samples and in the only *S. warneri* isolate (Table 3).

Comparison of delta toxin production determined by the synergistic hemolysis method and by PCR for the gene detection showed that, although four *S. epidermidis* strains had the *hld* gene, toxin production could not be confirmed by the phenotypic method.

Table 1. Primers used for the detection of the delta toxin gene.

Gene	5'- 3' nucleotide sequence	Target	Size of the amplified product
hld 1	GAA TTT GTT CAC TGT GTC G	*S. aureus* delta toxin	357 bp
hld 2	TTT ACA CCA CTC TCC TCA C	*S. aureus* delta toxin	357 bp
hld 1	ATG GCA GCA GAT ATC ATT TC	*S. epidermidis* delta toxin	444 bp
hld 2	CGT GAG CTT GGG AGA GAC	*S. epidermidis* delta toxin	444 bp

Table 2. Frequency of *Staphylococcus* species according to clinical material.

Species	Clinical material			
	Catheter N (%)	Blood N (%)	Catheter + Blood[*] N (%)	Total N (%)
S. epidermidis	19 (95.0)	16 (88.9)	3 (50.0)	38 (86.4)
S. simulans	0	1 (5.6)	1 (16.7)	2 (4.5)
S. warneri	0	1 (5.6)	0	1 (2.3)
All CNS	19 (95.0)	18 (100)	4 (66.7)	41 (93.2)
S. aureus	1 (5.0)	0	2 (33.3)	3 (6.8)
Total	20 (45.4)	18 (40.9)	6 (13.6)	44 (100.0)

[*]: Strains isolated simultaneously from catheters and blood showing the same drug sensitivity profile.

Table 3. Frequency of delta toxin-positive *Staphylococcus* strains according to detection method and species.

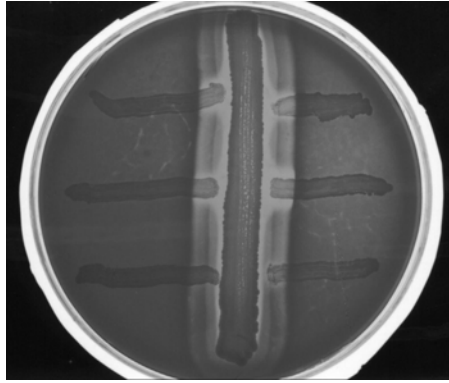| Species	Method	
	Synergistic hemolysis N (%)	PCR N (%)
S. epidermidis (n=38)	33 (86.8)	37 (97.4)
S. simulans (n=2)	2 (100)	-
S. warneri (n=1)	1 (100)	-
All CNS (n=41)	36 (87.8)	37 (90.2)
S. aureus (n=3)	3 (100)	3 (100)
Total (n=44)	39 (88.6)	40 (90.9)

Figure 1. Synergistic hemolysis between a beta-hemolytic *S. aureus* strain (vertical) and six *S. epidermidis* strains. The first two strains on the left and right showed an increase in *S. aureus* beta hemolysis, while the last two yielded a negative result.
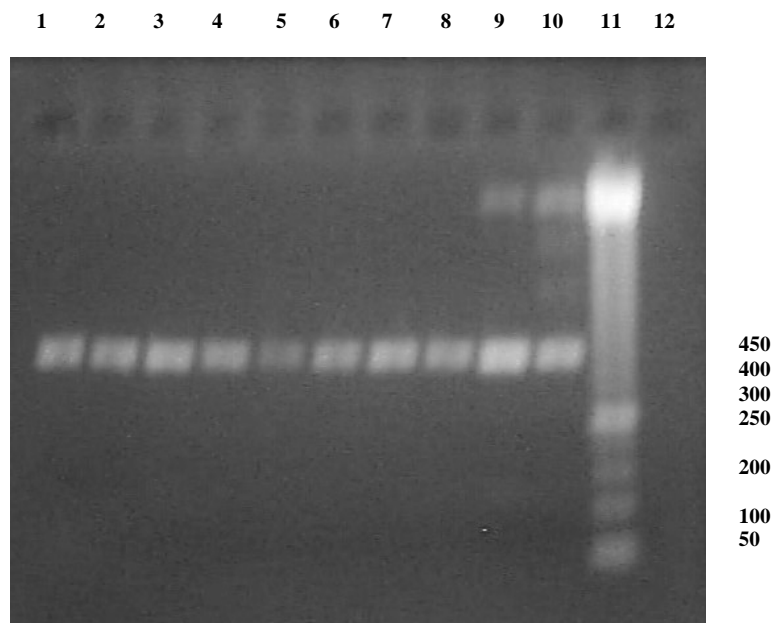

Figure 2. Agarose gel electrophoresis showing the PCR amplified products of the delta toxin gene (*hld*) in *S. epidermidis* (444 bp). Lanes 1-9, *S. epidermidis* strains isolated from neonates and positive for the *hld* gene; lane 10, *S. epidermidis* ATCC 12228; lane 11, molecular weight marker (50 bp); lane 12, negative control.

DISCUSSION

The advances made in neonatology over the last few decades have led to a significant improvement in the survival of premature and low birth weight infants; however, as a consequence, a progressive increase has been observed in the diagnosis of nosocomial infections in NICU (7). This increase might be explained by the larger number of immunocompromised patients and the longer duration of hospitalization in these units, in addition to invasive procedures to which these patients are often submitted (12). Coagulase-negative staphylococci, the main components of the skin and mucosal flora, are the most frequent etiological agents involved in these infections in neonates (18).

In the present study, CNS accounted for 93.2% of all staphylococcal isolates. *Staphylococcus epidermidis* was the most frequent species isolated from both catheter tips (95.0%) and blood (88.9%), followed by *S. aureus*, which was identified in 5.0% of cultured catheters, confirming the data reported by Hudome and Fisher (10) and Li-Yin *et al.* (14).

Six strains were simultaneously isolated from catheter tips and blood, including three *S. epidermidis* isolates (50.0%), two *S. aureus* strains (33.3%), and one *S. simulans* isolate (16.7%), a finding emphasizing the importance of CNS of the newborn's normal flora in the colonization of catheters (5). According to D'Angio *et al.* (3), *S. epidermidis* becomes the predominant species in the microbiota of neonates about the fourth day of life. This predominance in the colonization of individuals and the high pathogenicity of some strains might explain the fact that *S. epidermidis* is the species most commonly associated with infectious processes in neonates, as reported by Cunha *et al.* (2).

In the present study, detection of delta toxin in *S. aureus* isolates by the phenotypic and genotypic method yielded similar results, while in the case of *S. epidermidis* a higher frequency of positive isolates was obtained by PCR (97.4%) compared to the synergistic hemolysis method (86.8%). The frequency of isolates positive for the production of delta toxin obtained in the present study was similar to those reported by others (4, 17).

Comparison of the methods revealed the presence of the *hld* gene in four *S. epidermidis* isolates, which did not show toxin production by the phenotypic method. In these cases, *in vitro* toxin production was either insufficient to be detected by the method used or the genes responsible for toxin production were inactive. In clinical practice, *Staphylococcus* isolates testing positive for the toxin gene can be considered to have the potential for producing these toxins, since toxin production *in vivo* cannot be excluded.

Furthermore, our results showed the production of delta toxin in two *S. simulans* samples and in the only *S. warneri* isolate included in the study, which were not confirmed to be positive when submitted to PCR for the *hld* gene detection. These results demonstrate differences in the gene responsible for delta toxin production in such species or the occurrence of gene insertions and deletions. Complementary studies and highly specific primers will be necessary to detect the gene responsible for delta toxin production in these species. Donvito *et al.* (4), identifying the *hld* gene by hybridization, also observed the absence of hybridization in *S. simulans*, which was shown to be a delta toxin producer by the phenotypic method.

Although Tegmark *et al.* (20) have demonstrated the existence of homology between the *hld* gene of *S. epidermidis*, *S. warneri*, and *S. simulans*, the primers designed in the present study for *S. epidermidis* were unable to detect the *hld* gene in these species.

According to Donvito *et al.* (4), the existence of at least two distinct molecular supports for a common synergistic hemolysis phenotype suggests that this characteristic is important in the interaction between staphylococci and their hosts. The detailed distribution and regulation of the different loci involved might clinically influence important facts such as virulence and affinity for host tissue, especially in CNS.

The results of the present study confirm that PCR is a rapid and reliable method for detecting the *hld* gene in *S. aureus* and *S. epidermidis*. Although *S. simulans* and *S. warneri* produced a delta toxin-like exotoxin, further studies regarding the genes responsible for its production are necessary.

ACKNOWLEDGEMENTS

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