**Staphylococcus hominis** subsp. *novobiosepticus* strains causing nosocomial bloodstream infection in Brazil

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**Objectives:** To report the isolation of six *Staphylococcus hominis* subsp. *novobiosepticus* (SHN) strains from hospitalized patients with bloodstream infections in two Brazilian hospitals and to characterize their susceptibility profile to several antimicrobials.

**Methods:** Species identification was performed by biochemical methods and sodA gene sequencing. The MICs of antimicrobials were determined by broth and agar dilution methods and by Etest. Isolates were typed by PFGE and PCR amplification was used to detect the ccr gene complex and the mec class. Morphometric evaluation of cell wall was performed by transmission electron microscopy (TEM).

**Results:** Susceptibility profiles indicated that the majority of isolates (five) were multidrug-resistant. Overlapping and multiplex PCR showed that five out of the six strains harboured SCCmec type III with class A mec and type 3 ccr. The initial vancomycin MIC value of 4 mg/L for these strains increased to 16–32 mg/L after growth for 10 days in BHI broth supplemented with this antimicrobial. TEM indicated that vancomycin resistance was associated with cell wall thickening and to another mechanism not fully elucidated. Only one SHN strain was oxacillin- and vancomycin-susceptible. The nosocomial infections in at least five of the patients from both hospitals were caused by a single clone of SHN.

**Conclusions:** It is very important to consider SHN strains as the cause of nosocomial infections. The clinical implications resulting from the pattern of multidrug resistance in these strains may be complicated by the emergence of vancomycin resistance.

Keywords: staphylococci, mecA, vancomycin, mechanism of resistance

**Introduction**

A new subspecies, described by Kloos *et al.*1 in 1998 and named *Staphylococcus hominis* subsp. *novobiosepticus* (SHN), has been implicated in nosocomial outbreaks.1,2 Multidrug resistance, including resistance to novobiocin and oxacillin, is a particularly important feature of SHN.1,3

The phenomenon of resistance to antimicrobials among staphylococci strains was complicated by the worldwide emergence of vancomycin-intermediate *Staphylococcus aureus* (VISA) and coagulase-negative staphylococci strains resistant to vancomycin. SHN strains causing bloodstream infections are up to now described as vancomycin-susceptible.

Unfortunately, there is not a worldwide consensus over the definition of vancomycin resistance among staphylococci strains. We adopted the term vancomycin-resistant for staphylococci strains that presented an MIC of >8 mg/L.

The main objective of the present study was to report the isolation of six SHN strains from hospitalized patients and also to characterize their susceptibility profile to several antimicrobial agents. The mechanism involved in vancomycin resistance presented by these strains was also investigated.
Materials and methods

Bacterial strains and species identification

The strains described in this study were isolated from the blood of patients admitted to hospitals located in cities 1100 km apart in different states of Brazil (Table 1). Identification of the staphylococcal strains was performed as recommended by Bannerman and Peacock. The strains were also identified by the Autoscan (Dade Behring) and Vitek (bioMérieux) systems. sodA gene sequencing was used for species-level identification. Also, the susceptibility profile to novobiocin and the capacity to produce acid from D-trehalose and N-acetylglucosamine were used to distinguish SHN from S. hominis subsp. hominis. SHN ATCC 700236 was used as a control for species identification.

Antimicrobial susceptibility testing

The susceptibility profile to novobiocin and cefoxitin was determined by the disc diffusion method using Mueller–Hinton agar (Oxoid, UK) supplemented with 2% NaCl. Vancomycin MICs were determined by the disc diffusion method using Mueller–Hinton agar, and interpretative criteria were based on Kloos et al. and on CLSI guidelines, respectively. The MICs of several antimicrobials were determined by the agar dilution method. Oxacillin MICs were determined by the Etest (AB Biodisk, Sweden) with Mueller–Hinton agar (Oxoid, UK) supplemented with 2% NaCl. Vancomycin MICs were determined by the Etest and by the broth dilution method for staphylococcal strains from the initial isolations (parent strains), and also after they were grown for 10 days in BHI broth with 4 mg/L vancomycin (derivative strains). Control strains used in antimicrobial susceptibility testing are described in Table 1.

PCR amplification

The protocol used for DNA extraction was based on the mechanical disruption of cell walls described previously.

Multiplex PCR amplifications with a set of primers were carried out to detect the type of recombinase system (ccrA1, ccrA2, ccrA3, ccrA4, ccrB and ccrC gene complexes) among the strains in this study.

Overlapping PCR was performed to characterize the mec class in the strains and the eight selected loci were included on the basis of previously described mec element sequences.

S. aureus 10442 (SCCmec type I), S. aureus N315 (SCCmec type II), S. aureus 85/2082 (SCCmec type III), S. aureus 4744 (SCCmec type IVa) and S. aureus WIS (SCCmec type V) were used as control strains in PCR amplification.

PFGE

Molecular epidemiology analysis of strains was performed after bacterial lysis, Smal digestion of chromosomal DNA and characterization of the DNA fragments by PFGE as described previously.

Transmission electron microscopy (TEM)

Cells suspended in growth medium were processed for TEM according to a protocol described previously. Statistical significance of data was evaluated by Student’s t-test.

Results

Species identification and antibiotic resistance patterns

The staphylococcal strains included in the study were initially identified at species level as S. hominis, based on automated systems, classical biochemical methods and sodA gene sequencing (GenBank accession no. AJ 34911, AJ 34912). All SHN strains studied were distinguished from S. hominis subsp. hominis by failing to produce acid aerobically from D-trehalose, N-acetylglucosamine and by resistance to novobiocin.

Antimicrobial MICs for the staphylococcal strains determined by all described methods are summarized in Table 1. The cefoxitin disc diffusion method predicted oxacillin resistance in SHN strains studied (Table 1) and five SHN strains showed patterns of multidrug resistance, but maintained susceptibility to quinupristin/dalfopristin. Vancomycin MICs (4 mg/L) for parent SHN strains suggested that resistance to vancomycin should be investigated. Five out of six of the derivative SHN strains showed MIC values rising to 16–32 mg/L after cultivation for 10 days in BHI broth containing 4 mg/L vancomycin. Only one strain, SHN 667, maintained the same initial vancomycin MIC value (4 mg/L) after exposure to the antibiotic for 10 days. The relationship between parent and derivative strains was verified by PFGE, excluding the possibility of culture contamination during subculture. Instability in the vancomycin resistance phenotype was suggested by reversion to the susceptibility profile after 12 days of cultivation in vancomycin-free BHI broth.

PCR for detection of ccr genes encoding recombinases A, B and C and typing of SCCmec elements

Overlapping PCR combined with PCR determination of the ccr gene complex showed that five SHN strains harboured SCCmec type III with class A mec (mecA gene and complete mec regulatory, mecl–mecR genes) and type 3 ccr. Strain SHN 667 does not harbour the SCCmec element.

PFGE profile

According to epidemiological data, four SHN strains showed the same PFGE profile (type A). SHN 667, the strain susceptible to the majority of antimicrobial agents, including oxacillin and vancomycin, showed a related PFGE profile (A1) differing from the predominant one in three bands. Only one strain (SHN 225) had an unrelated PFGE profile (type B) (Table 1).

Morphometric evaluation

All strains in the study and two controls, S. aureus N315 and S. aureus Mu50, were subjected to a morphometric study by TEM. The mean values ± SDs obtained for cell wall measurements are given in Table 1. This showed that the SHN strains, except SHN 688 and SHN 667 strains, had significantly thicker cell walls than the VSSA control. The majority of staphylococcal strains showed thicker cell walls when cultivated in vancomycin-containing BHI medium. No effect was observed in S. aureus N315 and SHN 667, which are vancomycin-susceptible. Vancomycin-resistant SHN 688 did not show cell wall thickening when cultivated in the BHI medium containing the antibiotic, but displayed an interesting structure on the cell surface when observed by TEM (Figure 1).

Discussion

Five out of the six SHN strains characterized in this study showed the multiresistant pattern common to this species/
Table 1. Susceptibility profiles of the isolated staphylococcal strains to several antimicrobials agents (determined by agar dilution and disc diffusion methods and by Etest), PFGE profiles and cell wall thicknesses measured in SHN strains by TEM

<table>
<thead>
<tr>
<th>Strains</th>
<th>MIC (mg/L)a</th>
<th>Etest</th>
<th>Means of cell walls measured in nanometres ± SDs</th>
<th>P valued</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VANc</td>
<td>agar dilution</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DDb</td>
<td></td>
<td>FOX (mm)</td>
<td>PFGE profile</td>
</tr>
<tr>
<td>SHN 667e</td>
<td>4 4 0.25</td>
<td>≥256 ≥256 ≥256</td>
<td>256 32 16 64 1 64 32 0 1 1 4</td>
<td>28 A1</td>
</tr>
<tr>
<td>SHN 646e</td>
<td>4 32 1/C21</td>
<td>≥256 ≥256 ≥256</td>
<td>256 32 16 64 0.5 128 64 0 1 1 4</td>
<td>28 A1</td>
</tr>
<tr>
<td>SHN 688e</td>
<td>4 32 2/C21</td>
<td>≥256 ≥256 ≥256</td>
<td>256 32 16 64 0.5 128 64 0 1 1 4</td>
<td>28 A1</td>
</tr>
<tr>
<td>SHN 225f</td>
<td>4 32 1/C21</td>
<td>≥256 ≥256 ≥256</td>
<td>256 32 16 64 0.5 128 32 0 1 1 4</td>
<td>28 A1</td>
</tr>
<tr>
<td>SHN 499f</td>
<td>4 32 1/C21</td>
<td>≥256 ≥256 ≥256</td>
<td>256 32 16 64 0.5 128 32 0 1 1 4</td>
<td>28 A1</td>
</tr>
<tr>
<td>SHN 943g</td>
<td>4 16 1/C256</td>
<td>≥256 ≥256 ≥256</td>
<td>256 32 16 ≥256 0.5 128 32 0 1 1 4</td>
<td>28 A1</td>
</tr>
<tr>
<td>S. aureus N315 (VSSA)</td>
<td>1 2 ND</td>
<td>ND ND ND ND ND ND ND ND ND ND ND ND ND ND ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. aureus Mu 50 (VISA)</td>
<td>8 8 ND</td>
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<tr>
<td>S. aureus ATCC 29213</td>
<td>ND ND 0.125</td>
<td>ND ND ND ND ND ND ND ND ND ND ND ND ND ND</td>
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</tr>
<tr>
<td>S. aureus ATCC 25923</td>
<td>ND ND ND</td>
<td>ND ND ND ND ND ND ND ND ND ND ND ND ND</td>
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</tr>
</tbody>
</table>

ND, not determined; VSSA, vancomycin-susceptible S. aureus; VISA, vancomycin-intermediate S. aureus; VAN, vancomycin; OXA, oxacillin; CLI, clindamycin; GEN, gentamicin; OFX, ofloxacin; PEN, penicillin; Q/D, quinupristin/dalfopristin; CIP, ciprofloxacin; TEC, teicoplanin; FOX, cefoxitin.

a,bThe agar dilution and disc diffusion (DD) methods were performed and interpreted according to the CLSI guidelines.

cThe Etests for vancomycin were performed both for the parent strains (P) and derivative strains (D) with Mueller–Hinton agar medium. Broth dilutions were performed as standardized by CLSI and confirmed with Etest results (data not shown).

dThe agar dilution and disc diffusion (DD) methods were performed and interpreted according to the CLSI guidelines.

eStrains isolated from patients hospitalized at Complexo Hospitalar Santa Casa, Rio Grande do Sul State.

fThere is a statistically significant difference between the input groups; power of performed test with α = 0.05.

gStrains isolated from patients hospitalized at Hospital Nove de Julho, São Paulo State.
subspecies. In contrast, strain SHN 667 did not show this multi-
resistant pattern, being susceptible to oxacillin and resistant only
to penicillin and novobiocin. This suggests that resistance to
novobiocin is intrinsic to this subspecies and oxacillin resistance
was acquired from a methicillin-resistant strain.

Kloos et al.\(^1\) suggested that this subspecies could have
emerged as a consequence of the acquisition of antibiotic resis-
tance genes. Although the multiresistance pattern in SHN strains
seems to be related to the presence of SCC\(\text{mec}\), the biochemical
characteristics expressed by this subspecies could not be a con-
sequence of SCC\(\text{mec}\) or antibiotic gene acquisitions. In addition,
it should be considered that the data refer to only a single strain
(SHN 667) and also that this could have lost this element of
resistance.

Molecular epidemiology showed that a predominant SHN
cloned caused sepsis in at least five patients at both hospitals
during the stated period. The predominant type A PFGE profile
and the subtype A1 were present in five SHN strains. Although

\[ \text{S. hominis in bloodstream infection} \]

Figure 1. SHN 688 cell walls compared by TEM after cultivation in BHI medium only or BHI medium supplemented with \(\gamma\)-glutamine and vancomycin. The staphylococcal strain (SHN 688) was cultivated in BHI broth at 37°C for 4 h and cells analysed by TEM (a and b). At the same time, SHN 688 was cultivated in BHI broth supplemented with 30 mM \(\gamma\)-glutamine at 37°C for 2 h, followed by vancomycin addition to a final concentration of 6 mg/L, reincubation for 2 h and then analysed by TEM (c and d). The photographic images were obtained at final magnifications of \(\times 50\ 000\) (a–c) and \(\times 80\ 000\) (d).

SHN 225 showed an unrelated PFGE profile, the SCC\(\text{mec}\) type
III was present in this strain and it could emphasize the hori-
zontal transfer of this resistance element.

The MIC values (4 mg/L) obtained for SHN strains immedi-
ately after isolation suggested that resistance to vancomycin
should be investigated and it was confirmed in five strains after
culturing them in the presence of this antibiotic for 10 days. The
presence of \(\text{van}\) genes was investigated by PCR including low
stringency conditions in the assay (data not shown), and \(\text{vanA}\),
\(\text{vanB}\) and \(\text{vanC}\) genes were not found.

Comparing mean cell wall thicknesses between VSSA, VISA
and the SHN strains studied (Table 1), including vancomycin-
susceptible SHN 667 strain, it was possible to observe a ten-
dency of SHN strains to show thickened cell walls even in the
absence of vancomycin. This tendency may be the consequence
of a genetic background allowing vancomycin resistance.

The SHN 688 strain also showed a cell wall thickness that
even in the absence of vancomycin was significantly different.
(P < 0.001) when compared with both VSSA and VISA strains (data not shown). However, the MIC value (32 mg/L) showed by this strain after being cultured in a vancomycin-supplemented medium is probably not related to increased cell wall synthesis.

The data obtained in this study do not provide a fully satisfying and clear-cut proposition for the mechanism of vancomycin resistance in SHN 688. The structure present on the surface of SHN 688 cells shown in Figure 1 could be related to a vancomycin-trapping mechanism. The entrapping could be due to decreased peptidoglycan cross-linking, reflecting larger amounts of teichoic acid produced by this strain or structural changes in the cell wall that resulted in surface extrusions. Thus, further studies are necessary to evaluate the surface alterations shown by this strain in the presence of vancomycin.

In summary, the acquisition of resistance genes by SHN strains does not appear to be the cause of the origin of this new subspecies, which may act as a reservoir of antimicrobial resistance determinants. SHN strains studied showed a thickened cell wall even in the absence of vancomycin indicating that these strains have genetic backgrounds that support the resistance to this antibiotic.

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Transparency declarations

None to declare.

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