



Staphylococcus haemolyticus disseminated among neonates with bacteremia in a neonatal intensive care unit in Rio de Janeiro, Brazil[☆]

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ABSTRACT

Oxacillin-resistant *Staphylococcus haemolyticus* (ORSH) was found as the most prevalent (77.5%) species of coagulase-negative staphylococci associated with bacteremia in neonates making use of intravenous catheters in an intensive care unit of a Brazilian teaching hospital. Thirty-one blood isolates were confirmed as *S. haemolyticus* by sequencing of the 16S and clustered in 6 pulsed-field gel electrophoresis types (with 58% of the strains belonging to 2 predominant types B and D). *S. haemolyticus* was mostly oxacillin-resistant (90.3%) displaying multiresistance profiles (70.4%). However, the *mecA* gene was undetected in 22.6% strains. ORSH exhibited slime production on Congo-Red agar (67.7%), adherence to polystyrene (96.7%), and glass (87%) surfaces. Interestingly, *ica*-operon was detected in 58% strains, mostly belonging to the B, D, and F genotypes, which is a significantly higher percentage when compared to other studies conducted at different parts of the globe. Data indicated that *ica* operon and biofilm-forming ORSH are endemic in Brazilian nosocomial environment.

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1. Introduction

Among the coagulase-negative staphylococci (CoNS), *Staphylococcus haemolyticus* plays an important role in hospital-acquired opportunistic infections worldwide (Barros et al., 2012; Botelho et al., 2012; Jain et al., 2004; Monsen et al., 2005; Nystrom et al., 1992; Tuo et al., 1995; Voronina et al., 2011). *S. haemolyticus* is second only to *Staphylococcus epidermidis* in its frequency of isolation from human blood cultures (Falcone et al., 2006; Ing et al., 1997). The pathogen may also cause peritonitis, otitis, urinary tract infections, and septicemia (Kumari et al., 2001). Oxacillin-resistant *S. haemolyticus* (ORSH) strains have been also associated with foreign body infections (Falcone et al., 2006).

S. haemolyticus is also among the CoNS that colonize and cause bacteremia in neonatal intensive care units (NICUs) in many countries (Bjorkqvist et al., 2002; Bradford et al., 2006; Foka et al., 2006; Klingenberg et al., 2007; Neumeister et al., 1995; Tuo et al., 1995; Voronina et al., 2011). In Greece, the analysis of clonality of slime-producing methicillin-resistant CoNS (MR-CoNS) disseminated among pre-term neonates revealed that all MR-CoNS strains were

multiresistant, and 89% produced slime. Ten of 16 *S. haemolyticus* isolates belonged to a single clone (Foka et al., 2006). In Norway, a study of persistent strains of CoNS in a single neonatal unit over a 12-year period showed that only 1 *S. haemolyticus* cluster appeared to selectively colonize and infect the most extreme pre-term infants (Klingenberg et al., 2007). In India, *S. haemolyticus* (36%) and *S. epidermidis* (35%) were indicated as the most frequent CoNS species encountered in a NICU (Jain et al., 2004; Mehta et al., 1991) and as the only pathogens from infants that developed septicemia due to CoNS. Forty-five percent of these CoNS strains were slime producers (Mehta et al., 1991).

2. Objectives

Multiple selective factors, including antibiotic resistance, biofilm formation, and surface proteins with adhesive properties, increase the ability of CoNS to persist in a hospital environment (Klingenberg et al., 2007). Hence, in the present study, *S. haemolyticus* strains were isolated from blood cultures of infants making use of intravenous catheters in the NICU, over a 3-year period. Microorganisms were analyzed to determine their clonal distribution by pulsed-field gel electrophoresis (PFGE), antibiotic resistance patterns, biofilm production, and the prevalence of *mecA* and *icaA* genes associated with oxacillin resistance and biofilm formation, respectively.

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3. Materials and methods

3.1. Patients and origin of CoNS strains

A total of 47 blood culture isolates of CoNS were obtained from 40 neonates admitted to NICU of a teaching hospital, Rio de Janeiro, Brazil, over the period from 2008 to 2010. Thirty-seven of them (92.5%) were diagnosed as bacteremia isolates and 03 (7.5%) as contaminants. Invasive strains were collected from the following clinical groups: a) patients making use of indwelling catheter ($n = 40$); b) patients with clinical evidence of infection and submitted to empiric antibiotic therapy with vancomycin or gentamycin ($n = 37$); c) patients without clinical evidence of infection ($n = 03$); d) patients with fever ($n = 25$); e) patients without fever ($n = 13$); and f) premature patients ($n = 2$).

The assessment of clinical aspects was performed through a review of the medical records. The regional committee for medical research ethics approved the collection and analysis of patient data. Patients with 1 ($n = 33$) or 2 ($n = 07$) positive blood cultures were interpreted as infected when a CoNS was isolated from blood cultures in which there was a suspicion of infection and the physician considered it clinically significant to immediately start an empiric antimicrobial therapy. The diagnosis of infection was staged according to the classification of the Centers for Disease Control and Prevention (CDC, 1992). The isolates categorized as contaminants were not included in this study.

The control isolates used for the phenotypic characterization, antimicrobial susceptibility tests, PCRs, and the biofilm assays were *S. haemolyticus* ATCC 29970, *S. epidermidis* ATCC 35984, *S. epidermidis* ATCC 12228, *S. epidermidis* ATCC 14990, *Staphylococcus hominis* ATCC 27844, *Staphylococcus saprophyticus* ATCC 15305, *Staphylococcus warneri* ATCC 10209, *Staphylococcus aureus* ATCC 12600, *S. aureus* ATCC 29213, *S. aureus* ATCC 33591 and *S. aureus* ATCC 25923.

3.2. Phenotypic procedures

3.2.1. Phenotypic characterization

A simplified method using 9 tests was carried out as described by Iorio et al. (2007): 2 susceptibility tests using 5 µg novobiocin and 100 µg desferrioxamine disks; tests that detect the production of clumping factor, PYR, urease, and alkaline phosphatase as well as acid production from D-mannose, D-trehalose and D-xylose.

3.2.2. Antibiotic susceptibility testing

3.2.2.1. Disk diffusion test. Antibiotic susceptibility testing by the disk diffusion method was performed according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2010), except for mupirocin (Fuchs et al., 1990). Sixteen antibiotics (purchased from CECON; São Paulo, Brazil, and Oxoid, Basingstoke, England) were tested: oxacillin (Oxa), cefoxitin (Cfx), penicillin (P), ceftazidime (Caz), gentamycin (Gen), neomycin (Neo), tobramycin (Tob), ertapenem (Etp), meropenem (Mer), ciprofloxacin (Cip), erythromycin (Ery), clindamycin (Clin), chloramphenicol (Clo), mupirocin (Mup), tetracycline (Tet), and vancomycin (Van).

3.2.2.2. MIC of oxacillin and vancomycin. The MICs of oxacillin (Sigma, St. Louis, MO, USA) and vancomycin (Oxoid, Basingstoke, England) were evaluated by the broth microdilution method (CLSI, 2010, 2012). The concentrations ranged from 0.25 to 512 µg/mL for oxacillin and from 0.25 to 256 µg/mL for vancomycin. The plates were incubated at 35 °C for 24 h.

3.3. Biofilm testing

3.3.1. Slime production assays for

Qualitative detection of slime production was studied by culturing the strains on Congo Red Agar plates (CRA; Sigma Chemical Company, St Louis, MO, USA) as described previously (Chaieb et al., 2005;

Freeman et al., 1989). Inoculated CRA plates (0.8 g Congo red mixed with 36 g sucrose in 1 L of brain heart infusion agar) were incubated for 24 h at 37 °C under aerobic conditions and followed overnight at room temperature. Slime-positive variants appeared as reddish-black colonies with a rough, dry, and crystalline consistency on CRA, whereas slime-negative strains developed pinkish-red, smooth colonies with a darkening at the center. *S. epidermidis* strains ATCC 35984 and ATCC 12228 were used as positive and negative controls, respectively.

3.3.2. Glass surface adherence assay

Briefly, microorganisms were inoculated in glass tubes (13 by 100 mm) containing 4 mL of Trypticase Soy Broth (TSB) and incubated for 48 h at 37 °C without shaking. The tubes were gently shaken for 5 s, and the supernatants containing bacterial cells that were non-adherent to the surfaces of the glass tubes were discarded. TSB (4 mL) was then added, and the tubes were re-incubated for 48 h. This procedure was repeated twice. The glass-adherent bacteria created a confluent coat of cells on sides of the tube (Moreira et al., 2003).

3.3.3. Plastic surface adherence assay

Semi-quantitative adherence assay in polystyrene microtiter plates was performed as previously described by Stepanovic et al. (2000). Briefly, CoNS strains were cultivated overnight in TSB medium, and 200 µL of each bacterial suspension was used to inoculate sterile, 96-well polystyrene microtiter plates (Greiner, Frickenhausen, Germany). After incubation for 24 h at 37 °C, the wells were gently washed twice with 200 µL of sterile phosphate-buffered saline. The plates were air dried, and the remaining surface-adsorbed cells of the individual wells were stained with 0.1% crystal violet for 30 s. Absorbance was measured with a Micro-ELISA Autoreader (Titertek Multiscan) at $\lambda = 490$ nm. The well, to which sterile TSB lacking bacterial cells was added, served as control; the value for this well was subtracted from the experimental readings. Each assay was performed in triplicate. The cut-off OD (OD_c) for the microtiter plate test was defined as 3 SDs above the mean OD of the negative control. Based upon the ODs of bacterial films, microorganisms were classified as non-adherent ($OD \leq OD_c$) and adherent ($OD > OD_c$).

3.4. Genotyping procedures

3.4.1. Multiplex PCR assay (mPCR) to identify methicillin-resistant *S. haemolyticus*

An mPCR to simultaneously identify *S. aureus*, *S. haemolyticus*, and *S. epidermidis* species and to determine methicillin resistance by the presence of gene *mecA* was performed in accordance to previously described methods (Iorio et al., 2011; Potter et al., 2009; Santos et al., 1999; Schuenck et al., 2008). The primers and amplicons used in this study are listed in Table 1.

3.5. Gene amplification and sequencing

S. haemolyticus identification was reconfirmed by sequencing of the 16S rRNA gene of representative strains (SH6, SH8, SH9, SH10, SH12). DNA extraction, primer design, the PCR parameters employed to amplified 16S rRNA gene, and sequencing of amplified PCR products following their purifications were also performed as previously described by Baio et al. (2013). The sequencing reactions were performed with Big Dye Terminator v 3.1 cycle sequencing kit (Applied Biosystems) on an ABI-3730 Automated DNA Sequencer (Applied Biosystems) by standard protocols. The 16S rRNA gene sequences were compared to those available in the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>) using the BLAST algorithm and the Ribosomal Database Project (RDP-II) (<http://rdp.cme.msu.edu/html>).

Table 1
Primers used in this study.

Primers	Sequence of forward and reverse primers 5'→ 3'	Product size (bp)	References
SH1	GGTCGCTTACTCGGAACAAT	285	Schuenck et al., 2008
SH2	CACGAGCAATCTCATCACCT		
SE1	CAGTTAATCGGTATGAGAGC	218	lorio et al., 2011
SE2	CTGTAGAGTGACAGITTCGGT		
MRS1	TAGAAATGACTGAACGTCCG	154	Santos et al., 1999
MRS2	TTGCGATCAATGTTACCTAG		
icaAF	CGATGGGCTCAAGGTGG	287	Potter et al., 2009
icaAR	TTCTTTTCGTAGCGACTGTC		

3.5.1. DNA fingerprinting by PFGE

Chromosomal DNA preparation and PFGE analysis were performed as described previously (Nunes et al., 2005; Vivoni et al., 2006). PFGE banding patterns were analyzed using visual comparison among the strains and with the aid of automated analysis using the Bionumerics program, version 6.0. The Dice similarity coefficients were calculated with a band position tolerance of 1.5%, and the UPGMA method was applied for clusters analysis. Isolates were first assigned to PFGE types, using 80% band-based similarity coefficients as cut-off values. Banding patterns were classified according to the criteria described by Van Belkum et al. (2007). PFGE types were identified by letters, and subtypes were identified by letters followed by a numeric subscript.

3.6. Statistical analysis

The chi-square tests and Fisher exact tests were used to assess the statistical significance for a confidence level of 95%; *P*-value < .05 was considered statistically significant.

4. Results

4.1. Identification of *S. haemolyticus* strains

Phenotypic tests identified CoNS strains as *S. haemolyticus* (77.5%), *S. epidermidis* (15% strains), *S. captis* (5% strains), and *S. warneri* (2.5% strain). The 31 *S. haemolyticus* blood isolates identified by the phenotypic assays were confirmed by mPCR and 16S rRNA sequencing. Genbank accession

numbers for 16S rRNA genes sequences of *S. haemolyticus* strains were deposited under the bank with submitting numbers 1632716, 1632721, 1632727, 1632729, and 1632731 on GenBank database.

4.2. Determination of clonal distribution by PFGE

PFGE analysis showed the presence of 12 restriction profiles clustered in 6 pulse types: A (*n* = 4; 12.9%), B (*n* = 10; 32.2%), C (*n* = 5; 16.1%), D (*n* = 8; 25.8%), E (*n* = 1; 3.2%), and F (*n* = 3; 9.6%) (Fig. 1 and Table 2). Isolates showing a similarity coefficient ≥80% were considered genetically PFGE related. Several clinical isolates were classified as belonging to the same clonal subtype A'1 (*n* = 01), A'2 (*n* = 02), A'3 (*n* = 01), B'1 (*n* = 05), B'2 (*n* = 05), C'1 (*n* = 02), C'2 (*n* = 03), D'1 (*n* = 05), D'2 (*n* = 03), F'1 (*n* = 01), and F'2 (*n* = 02) (Tables 2 and 3). The 16S rRNA sequences from 5 representative PFGE type strains exhibited highest similarity values to the type strain of *S. haemolyticus*, ranges of 99.15% to 99.54%.

4.3. Antimicrobial susceptibility profiles

Results of the disk diffusion tests shown in Fig. 2, Tables 2, and 3 shown multiresistance to varied antimicrobial agents in 87% of *S. haemolyticus* strains isolated from blood of neonates making use of intravenous catheters. Antimicrobial susceptibility profiles were independent of PFGE types and subtypes; 28 out of 31 tested strains (90.3%) were phenotypically resistant to oxacillin (ORSH). Oxacillin-resistant isolates were also resistant to representative drugs from 4 to 6 non-beta-lactam drug classes. The resulting MICs for oxacillin (range: 0.25–512 µg/mL; oxacillin-resistant mean values: 356.07 ± 211.9 µg/mL) were compared to disk diffusion zone diameters and to *mecA* gene detection by PCR (*P* = .028). MICs for oxacillin ranging from >4 to <128 µg/mL were not observed for *S. haemolyticus* strains included in this study. Interestingly, most of the *S. haemolyticus* strains (*n* = 27; 70.4%) showed high resistance levels to oxacillin (MIC values ≥ 128 µg/mL). With the used pair of primers, we could not observe an agreement between high MICs values and *mecA* detection, since the *mecA* gene was not detected for 2 (6.4%) ORSH isolates (SH-27, SH-28 strain) with MICs reported as ≥128 µg/mL. The presence of *mecA* gene was observed in 77.4% (*n* = 24) of *S. haemolyticus* strains belonging to PFGE types A (*n* =

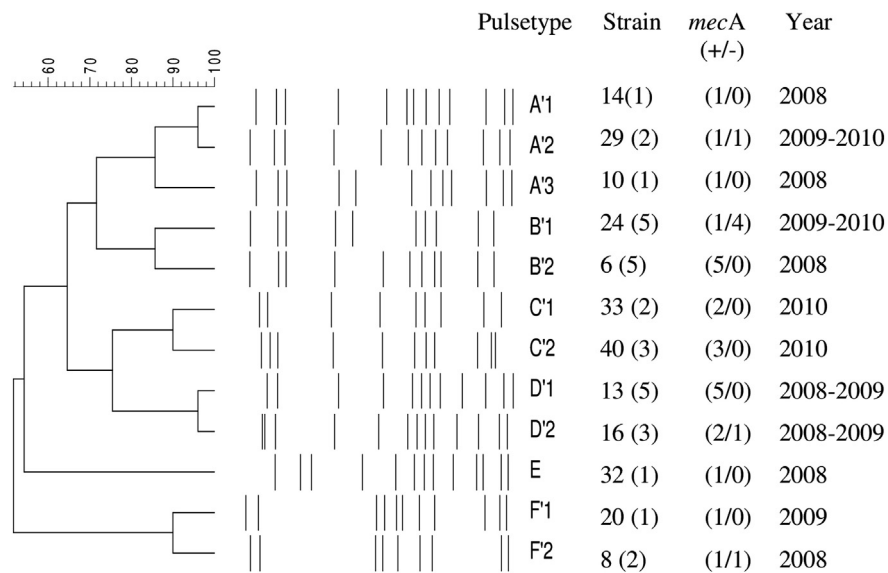


Fig. 1. Dendrogram of the PFGE profile of *Smal*-digested genomic DNA of 31 *Staphylococcus haemolyticus* strains isolated from blood of neonates making use of intravenous catheters. Similarities percentage are identified on the dendrogram derived from the unweighted pair group method using arithmetic averages and based on Dice coefficients. Isolates showing a similarity coefficient ≥80% were considered genetically PFGE related. Pulse types (strain number): A'1 (SH-14), A'2 (SH-21, SH-29), A'3 (SH-10); B'1 (1, 24, 26, 27, 28), B'2(2, 3, 4, 5, 6); C'1 (33, 38), C'2 (16,35, 39); D'1 (7, 12, 13, 18, 11), D'2 (19, 36, 40); E (32); F'1 (20), F'2 (8, 9). PCR reaction: +, positive; −, negative; year, period of isolation of microorganisms.

Table 2
Microbiological and genetic properties of *Staphylococcus haemolyticus* strains isolated from blood of neonates making use of intravenous catheters.

Strain number	Year of isolation	Biofilm formation CRA/G/P	icaA Gene	Vancomycin MIC (µg/mL)	Oxacillin MIC (µg/mL)	Oxacillin susceptibility	mecA gene	PFGE type
SH-14	2008	+/+/+	–	4	512	R	+	A1
SH-21	2009	-/+/-	–	4	512	R	+	A2
SH-29	2010	-/+/+	–	4	4	R	–	A2
SH-10	2008	+/+/+	+	4	512	R	+	A3
SH-1	2009	+/+/+	+	8	512	R	+	B1
SH-24	2009	-/-/+	+	4	0.25	R	–	B1
SH-26	2010	+/+/+	+	2	0.25	S	–	B1
SH-27	2010	+/+/+	+	4	128	R	–	B1
SH-28	2010	+/+/+	+	4	128	R	–	B1
SH-2	2008	+/+/+	+	2	512	R	+	B2
SH-3	2008	+/+/+	+	2	512	R	+	B2
SH-4	2008	+/+/+	+	2	512	R	+	B2
SH-5	2008	+/+/+	+	4	512	R	+	B2
SH-6	2008	+/+/+	+	2	128	R	+	B2
SH-33	2010	+/+/+	–	2	0.25	S	+	C1
SH-38	2010	+/+/+	–	2	256	R	+	C1
SH-16	2009	+/+/+	+	4	512	R	+	C2
SH-35	2010	+/+/+	–	4	1	R	+	C2
SH-39	2010	+/+/+	–	2	4	R	+	C2
SH-7	2008	-/+/+	+	4	4	R	+	D1
SH-12	2008	+/+/+	+	4	512	R	+	D1
SH-13	2008	+/+/+	–	4	512	R	+	D1
SH-18	2009	-/-/+	+	2	512	R	+	D1
SH-11	2008	+/+/+	+	2	512	R	+	D1
SH-19	2009	+/+/+	–	4	512	R	+	D2
SH-36	2010	-/-/+	–	4	0.25	S	–	D2
SH-40	2010	-/+/+	–	4	256	R	+	D2
SH-32	2010	-/+/+	–	2	1	R	–	E
SH-20	2009	-/+/+	–	2	512	R	+	F1
SH-8	2008	+/+/+	+	4	512	R	+	F2
SH-9	2008	-/+/+	+	4	512	R	+	F2

G = Adherence to glass; P = Adherence polystyrene; + = Positive; – = Negative. Experiments were performed in triplicate.

3; 12.5%), B ($n = 6$; 25%), C ($n = 5$; 20.8%), D ($n = 7$; 29.1%), F ($n = 3$; 12.5%), but not E ($n = 0$). Data showed that most of the strains of PFGE types A, B, and D gave positive results for the *mecA* gene. The presence of *mecA* gene was observed in all strains tested of PFGE types C and F. Moreover, results of the disk diffusion test demonstrated 61.2% resistance to gentamicin and 100% susceptibility to vancomycin of all ORSH and oxacillin-sensitive *Staphylococcus haemolyticus* strains. Eighteen *S. haemolyticus* strains (58%) showed MIC of vancomycin of 4 µg/mL, and only the SH-1 strain showed MIC of 8 µg/mL (medium of 3.355 ± 1.284 µg/mL).

4.4. Slime production and adherence to abiotic surfaces

Microbiological and genetic properties of *S. haemolyticus* strains isolated from blood samples of neonates making use of intravenous

catheters are displayed in Tables 2 and 3. Slime production, adherence to hydrophilic (glass), and/or hydrophobic (polystyrene) abiotic surfaces were observed for *S. haemolyticus*, but with different profiles. Twenty-one strains gave (67.7%) simultaneous positive results for these 3 tests. However, we did not observe strains that gave simultaneous negative results for these 3 tests. Slime production evaluated by the CRA method was observed for 67.7% strains; 32.3% *S. haemolyticus* blood isolates were non-slime producers. Most of the strains tested (87%) were adherent to glass surfaces. All *S. haemolyticus* isolates, except the SH-21 strain, adhered to polystyrene surfaces commonly formed biofilms. No correlation among the results of CRA, polystyrene, and glass adherence tests was partially observed ($P = .534$). The SH-21 strain was glass positive but polystyrene negative. Interestingly, we observed only 1 CRA-positive and glass-negative isolate (SH-19 strain), while 7 (SH-

Table 3
PFGE clusters in terms of antibiotic resistance and virulence factors of *Staphylococcus haemolyticus* ($n = 31$) strains isolated from blood of neonates making use of intravenous catheters.

Bacterial characteristics	Total number of strains (%)	Number of strains in PFGE clusters					
		Type A Total = 4	Type B Total = 10	Type C Total = 5	Type D Total = 8	Type E Total = 1	Type F Total = 3
Genes							
<i>mecA</i> -positive	24 (77.4)	3	6	5	7	0	3
<i>icaA</i>	18 (58)	1	10	1	4	0	2
Oxacillin resistance	28 (90.3)	4	9	4	7	1	3
MICs							
Oxacillin (BMD test) ≥ 512 µg/mL	17 (54.8)	3	5	1	5	0	3
Vancomycin (BMD test) ≥ 4 µg/mL	18 (58)	3	5	2	6	0	2
Biofilm formation							
CRA	21 (67.7)	2	9	5	4	0	1
Glass	27 (87)	4	9	5	5	1	3
Polystyrene	30 (96.7)	3 ^a	10	5	8	1	3
Year of isolation	2008, 2009, 2010	2008, 2009, 2010	2008, 2009, 2010	2009, 2010	2008, 2009, 2010	2010	2008, 2009

BMD = Broth microdilution test.

^a SH-21 strain (subtype A2) was polystyrene negative.

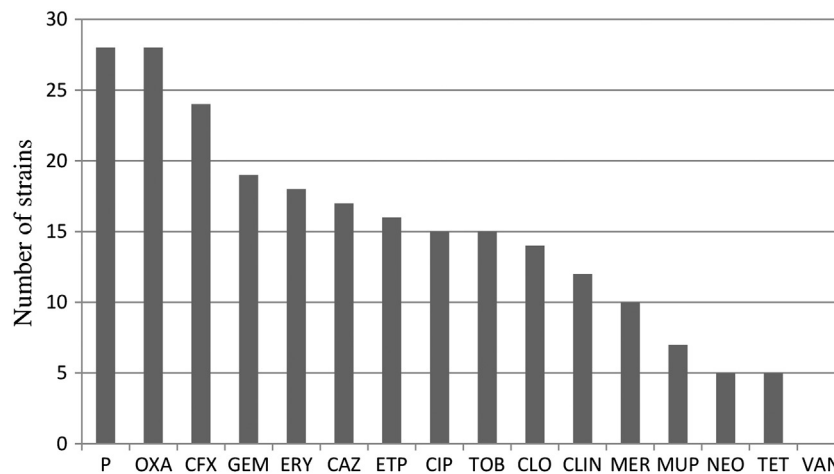


Fig. 2. Antimicrobial susceptibility profiles evaluated by the disk diffusion method of *Staphylococcus haemolyticus* blood isolates ($n = 31$) of neonates making use of intravenous catheters: 90.3% strains were resistant to penicillin (P) and oxacillin (Oxa), 77.4% strains to cefoxitin (Cfx), 61.2% strains to gentamycin (Gen), 58% strains to erythromycin (Ery); 54.8% strains to ceftazidime (Caz), 51.6% strains to ertapenem (Etp), 48.3% strains to ciprofloxacin (Cip), and tobramycin (Tob); 45.1% strains to chloramphenicol (Clo), while 38.7% strains were resistant to clindamycin (Clin); 32.2% strains to meropenem (Mer), 16.1% strains to tetracycline (Tet) and neomycin (Neo); 22.6% strains were resistant to mupirocin (Mup) and 100% strains were susceptible to vancomycin (Van).

21, SH-29, SH-07, SH-09, SH-32, SH-40, SH-20 strains) isolates were CRA negative and glass positive.

Microorganisms from PFGE types B and C showed the highest proportion of strains with simultaneous ability to produce slime and/or biofilm in all 3 abiotic surfaces tested (CRA, glass, and polystyrene). The presence of *icaA* gene positive was observed in 58% of *S. haemolyticus* blood isolates. No correlation was observed among biofilm formation on abiotic surfaces, oxacillin phenotypic resistance (glass $P = .422$; polystyrene $P = .822$), and the presence of *mecA* gene (glass $P = .120$; polystyrene $P = .754$) and *icaA* gene (glass $P = .195$; polystyrene $P = .654$).

5. Discussion

In many countries, *S. haemolyticus* is second to *S. epidermidis* in its frequency of isolation from newborns (Bjorkqvist et al., 2002). The number of infections caused by CoNS has dramatically increased as the management of patients in the NICUS has become more dependent on invasive procedures and indwelling devices (Brodie et al., 2000). Some studies of the clonality of ORSH responsible of epidemic infections in NICU suggested that ORSH neonatal infections are caused by a limited number of clones (Kazembe et al., 1993; Low et al., 1992).

PFGE typing showed that the *S. epidermidis* isolates regarded as inducers of sepsis were more homogeneous than isolates considered contaminants (Bjorkqvist et al., 2002). The occurrence of a dominating genotypic group among the sepsis isolates of *S. epidermidis* may represent strains with higher invasive capacity (Chu et al., 2011). Recently, cluster and phylogenetic analysis of CoNS strains isolated in hospitals emphasized the high adaptive ability of some nosocomial strains of *S. haemolyticus* that circulate in hospitals in the Russian Federation and threaten both neonates and hospitalized adult patients (Voronina et al., 2011).

In the present study, *S. haemolyticus* was the most prevalent species (77.5%) isolated from infants with bacteremia and subjected to therapy with vancomycin and/or gentamicin in a Brazilian NICU, as previously observed in India (Jain et al., 2004; Mehta et al., 1991). PFGE results revealed 6 pulse types (A, B, C, D, E, and F) indicating a genetic diversity among these *S. haemolyticus* strains during the period of 2008–2010. Pulse types A, B, D, and E were observed during the 3 years of the study. Most of the types were mainly observed in 2008, while type C was observed mainly in 2010 ($P < .358$). Among the *S. haemolyticus* isolates, 18 (58%) strains belonged to the 2 predominant pulse types, B ($n = 10$) and D ($n = 08$). Similar to

data described in the Russian Federation (Voronina et al., 2011), the presence of 2 predominant PFGE types (B and D) indicated the adaptive ability of some nosocomial strains of *S. haemolyticus* to circulate in this hospital located in Rio de Janeiro and to infect neonates hospitalized patients making use of intravenous catheters.

Antibiotic resistance in CoNS has increased over the years and is driven by the acquisition of the *mecA* gene. The *mecA* gene is present in more than 80% of the CoNS late-onset sepsis isolates. Given the large number of methicillin-resistant CoNS, inclusion of vancomycin in empiric therapy for neonates with late-onset septicemia may be justified (Jain et al., 2004). *S. haemolyticus* has the highest level of antimicrobial resistance among all CoNS species, and heteroresistance to glycopeptides is common (Brodie et al., 2000; Chiew et al., 2007; Froggatt et al., 1989; Sieradzki et al., 1998; Tabe et al., 2001).

In the present study, the disk diffusion tests revealed 90.3% of the clinical isolates as ORSH. However, the presence of *mecA* gene was detected only in 77.4% of *S. haemolyticus* strains independently of oxacillin susceptibility and pulse types. Similar to data described in the literature (Marshall et al., 1998; Zafar Hussain et al., 2000), a categorical agreement between high MICs values and *mecA* detection was not verified. OSSH strains presenting *mecA* gene as well as ORSH isolates without *mecA* gene were observed, among these Brazilian *S. haemolyticus* nosocomial isolates.

A high proportion (87%) *S. haemolyticus* strains isolated from neonates showed multiresistance to antimicrobial agents, including gentamicin used as initial empiric therapy. Microorganisms exhibited different multiresistance profiles independent of pulse types and subtypes; 22.6% were also resistant to mupirocin. The diversity of high-level mupirocin resistance plasmids [Mup(R)] was also demonstrated in *S. haemolyticus* strains belonging to different PFGE types or subtypes. (do Carmo Ferreira et al., 2011).

The mechanism of reduced vancomycin susceptibility in CoNS is unclear but may be related to the selection of resistant subpopulations under pressure of antimicrobial exposure (Dunne et al., 2001). In a study made in Japan, the results indicated that methicillin resistance may not be related to reduced susceptibility to glycopeptide in *S. haemolyticus* and that a multiresistance profile is more associated with a decreasing susceptibility to glycopeptides than with resistance to oxacillin. These facts limit the therapeutic options available and make an ORSH infection a serious threat (Tabé et al., 2001). A reduction in the efficacy of vancomycin has been described in studies of methicillin-resistant *S. aureus* infections treated with this antibiotic, and it has been suggested that slight increases in vancomycin MICs of between 1 and

2 µg/mL, which are within the susceptible range, may be related to suboptimal clinical outcomes. Some of these studies have used the broth microdilution method for determining vancomycin MICs. Despite the fact that *S. haemolyticus* are usually more resistant to glycopeptides (Ashtiani et al., 2008; Bannerman and Peacock, 2007), the results of our study showed most MICs to vancomycin ≤4 µg/mL (18 strains). Only 1 strain showed intermediate resistance with MIC = 8 µg/mL.

Takeuchi et al. (2005) observed that the highly glycopeptide-resistant *S. haemolyticus* JCSC1435 strain frequently generated mutants that lost antibiotic resistance during passage in drug-free medium. JCSC1435 strain may provide an ideal tool for elucidating the genetic mechanisms for acquisition of glycopeptide and β-lactam antibiotic resistance that poses such a difficult medical problem in modern hospitals. Comparative analysis of the genomes of *S. haemolyticus* JCSC 1435 strain, *S. epidermidis*, and *S. aureus* detected an average sequence identity of 78% in genes found as orthologues. In that opportunity, complete genome sequencing of *S. haemolyticus* JCSC1435 uncovered a wide range of open reading frames encoding putative virulence factors. The virulence genes in *S. haemolyticus* and *S. epidermidis* were rather “benign” in nature, as indicated by surface adhesins, secretory antigens, serine proteases, and exonucleases (Takeuchi et al., 2005).

Slime production and biofilm formation are important virulence factors of CoNS, allowing them to attach to smooth surfaces of biomaterials; it has been associated with infections of implanted medical devices (Bernardi et al., 2005; Flahaut et al., 2008). Some materials such as glass, hair, and nylon tend to give up electrons and become positively charged. Other materials such as polypropylene, polystyrene, vinyl (PVC), silicon, Teflon, and silicone tend to collect electrons and become negatively charged. Sialic acid may be a constituent molecule of slime and involved in bacterial adherence to inert surfaces, as demonstrated for *Corynebacterium diphtheriae* (Mattos-Guaraldi et al., 1999) and CoNS (Sakarya et al., 2004). Krepsky et al. (2003) investigated cell surface hydrophobicity and slime production of *S. epidermidis*. Data suggested involvement of protein components in adherence to polystyrene, but not in auto-aggregation properties and adherence to glass surfaces. Previous studies also gave an insight into the mechanism of slime production and adherence of slime-forming CoNS to polystyrene plates and polypropylene tubes (Sakarya et al., 2004). Biofilm formation and adherence to acrylic by *S. haemolyticus* were found significantly reduced compared to those of *S. epidermidis* (Cerca et al., 2004, 2005). Nowadays, biofilm formation is also a common phenotype in clinical *S. haemolyticus* isolates (Fredheim et al., 2009). In contrast to *S. epidermidis*, the molecular basis of the virulence of *S. haemolyticus* in general and in the context of foreign material-associated infections is largely unknown. Confocal laser scanning microscopy indicated that the biofilm structure of *S. haemolyticus* clearly differed from that of *S. epidermidis* (Fredheim et al., 2009).

Several genes have been identified to play roles in the biofilm formation. The *ica* gene locus (*icaADBC*) encodes the biosynthesis of polysaccharide intercellular adhesion (PIA), involved in the accumulation process (Heilmann et al., 1996). Fredheim et al. (2009) demonstrated the presence of the *ica* gene in 3 of 72 *S. haemolyticus* strains. Hence, a low prevalence compared with the prevalence in *S. epidermidis*. In concurrence with previously reported sequences, a high degree of similarity between the *ica* operons was found in different staphylococcal species. Phylogenetic analyses showed that the *ica* operon of *S. haemolyticus* is closely related to that of *S. epidermidis*. However, it has become clear that PIA is neither an essential nor necessarily the major component of CoNS biofilms (Fitzpatrick et al., 2005; Kogan et al., 2006; Oliveira and Cunha, 2010). The presence of an *ica* operon in *S. haemolyticus* has been reported, but to date, its contribution to biofilm formation is unclear. The biofilm-associated *ica* locus, present in *S. epidermidis* and *S. aureus*, may be absent from clinical isolates of *S. haemolyticus* (Cerca et al., 2005; De Silva et al., 2002; Fredheim et al., 2009). One of the most novel parts of

this present study is the description of a high prevalence (58%) of the *ica*-operon in the collection. This separates from other studies where a max prevalence of 1–2% has been reported. Comparative genome analyses and the possibility to detect genes with higher variation are limited by the fact that there is currently still only 1 fully sequenced genome published (Takeuchi et al., 2005).

Other cell wall structures, such teichoic acid and protein, have been shown to mediate staphylococcal biofilm formation in strains lacking or not expressing the *ica* locus. A capsular polysaccharide was proposed to be an important virulence factor in *S. haemolyticus*. However, there was no correlation among the clinical *S. haemolyticus* isolates between capsular polysaccharide production and biofilm production (Flahaut et al., 2008). Although capsules produced by staphylococci have been detected by changes in colonial appearance, this approach has not been found suitable for determining slime production. The production of slime by CoNS is routinely detected using the Christensen method, although this method may not always be successful for detecting weak slime production. An alternative method for detecting slime that uses a specially prepared solid medium was described by Freeman et al. (1989). CRA was chosen because it has been used as a stain for showing the presence of the exopolysaccharide of bacteria. The exact mechanism of the Congo red phenomenon is as yet unknown.

In the present study, the subtype B2 of *S. haemolyticus* seemed to exhibit a more expressive virulence potential than the other pulse types since it comprised 5 strains with identical phenotypical and genotypical profiles with involvement of all the virulence factors considered in this study. Therefore, all B2 samples were ORSH and exhibited a reduced sensitivity to vancomycin in addition to being carriers of methicillin resistance, adhesion, and biofilm production (*mecA* and *icaA*) genes besides producing biofilm on CRA, polystyrene, and glass. Among the other pulse types, only 2 strains of subtypes B1 and F2 exhibited identical profiles of B2 subtype. Qualitative and quantitative diversity in the ability of biofilm production indicated the involvement of other bacterial and environmental features that need further investigation.

In conclusion, we were able to describe the clonality of ORSH within the NICU in Brazilian hospital and that some clones are endemic in the hospital environment. Measures have to be taken to reduce the risk of hospital-acquired *S. haemolyticus* infections.

The data further suggested that intraspecies differences in virulence occur for *S. haemolyticus*, as previously described for *S. epidermidis* (Gunn, 1989). The occurrence of prevalent genotypic groups among the bacteremia isolates of *S. haemolyticus* may represent strains with higher invasive capacity. The ability to produce biofilm and the notoriously multi-resistance to antimicrobial agents, including glycopeptides, favor *S. haemolyticus* as an emerging cause of nosocomial infections (Cavanagh et al., 2012). Although *S. haemolyticus* biofilm formation *in vitro* has been reported, the molecular mechanisms involved remain partially elucidated. Additional studies of the presence and expression of *ica* genes may clarify the different adhesion mechanisms in the pathogenesis of *S. haemolyticus* infections, including those cases associated with medical devices.

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