

The antimicrobial susceptibility, biofilm formation and genotypic profiles of *Staphylococcus haemolyticus* from bloodstream infections

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We analysed the antimicrobial susceptibility, biofilm formation and genotypic profiles of 27 isolates of Staphylococcus haemolyticus obtained from the blood of 19 patients admitted to a hospital in Rio de Janeiro, Brazil. Our analysis revealed a clinical significance of 36.8% and a multi-resistance rate of 92.6% among these isolates. All but one isolate carried the mecA gene. The staphylococcal cassette chromosome mec type I was the most prevalent mec element detected (67%). Nevertheless, the isolates showed clonal diversity based on pulsed-field gel electrophoresis analysis. The ability to form biofilms was detected in 66% of the isolates studied. Surprisingly, no icaAD genes were found among the biofilm-producing isolates.

Key words: *Staphylococcus haemolyticus* - SCCmec - biofilm

Staphylococcus haemolyticus isolates are saprophytic bacteria with the ability to colonise human skin and mucosal membranes. However, these coagulase-negative bacteria have recently emerged as hospital-associated pathogens, mainly in immune-compromised patients. Recent studies have identified *S. haemolyticus* as an important cause of primary bloodstream infections associated with the use of central venous catheters (CVC) (Keim et al. 2011, Barros et al. 2012, Bouchami et al. 2012). Notably, *S. haemolyticus* isolates exhibit high resistance profiles and quite frequently display reduced susceptibility to glycopeptide antibiotics (Ahlstrand et al. 2011, Barros et al. 2012). The bacterial ability to form biofilms is thought to be an important mechanism involved in CVC-related infections and in other device-associated diseases. Biofilm formation among staphylococci is mediated by the *ica* operon, which encodes the enzymes involved in the synthesis of poly-N-acetylglucosamine/polysaccharide intercellular adhesin (PNAG/PIA). Nevertheless, PNAG/PIA-independent biofilm matrices have been described in these cocci, including *Staphylococcus epidermidis* (Araujo et al. 2006). However, the biofilms formed by *S. haemolyticus* have not yet been subjected to more detailed studies.

In this paper, 27 consecutive isolates initially identified as methicillin-resistant *S. haemolyticus* (MRSHA)

using the Vitek Automatic System with GPS-105 [BioMérieux Brasil, Rio de Janeiro (RJ), Brazil] were studied. These isolates were obtained from blood samples collected from 17 adult and two paediatric patients who were admitted in different clinical settings to a tertiary hospital in RJ from 2007-2008. Because different isolates recovered from the blood of an individual patient can display different genetic profiles, more than one isolate per patient was included in this study. The exclusion criterion was the presence of isolates from the same patient displaying an identical pulsed-field electrophoresis pattern. In such cases, only one of the repeat isolates was included. The identification of the isolates was confirmed via polymerase chain reaction (PCR) amplification of the *mvaA* gene, as described previously (Barros et al. 2012). *S. haemolyticus* isolates were considered clinically significant based on the criteria described by the Centers for Diseases Control and Prevention (CDC), which includes: (i) a patient with a recognised pathogen cultured from two or more blood cultures collected within a period of two days, (ii) risk factors for bloodstream infection, (iii) classical clinical manifestations and (iv) associated clinical laboratory findings (Horan et al. 2008).

Antibiogram tests were carried out as recommended by the Clinical and Laboratory Standards Institute (CLSI 2011). The minimum inhibitory concentration (MIC) was determined for vancomycin and teicoplanin (TEI) (CLSI 2011). Multi-resistance was defined as resistance to three or more classes of antimicrobials, including β -lactams. High-level multi-resistance was defined as an isolate displaying resistance to six or more drugs. PCR was used to detect *mecA* (Araujo et al. 2006), encoding methicillin resistance and to determine the type of staphylococcal cassette chromosome *mec* (SCCmec) (Boye et al. 2007, Berglund et al. 2008, Zhang et al. 2009). PCR analysis was also employed to assess the *ica* operon (Araujo et al. 2006). The *icaAD* primers exhibited 100%

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correspondence to the *ica* sequence of *S. haemolyticus* (GenBank accession FJ472951.2). Additionally, a PCR probe amplified with specific *icaAD* primers (Araujo et al. 2006) was labelled using the DIG High Prime DNA Labelling and Detection Starter Kit II (Roche Diagnostics, Mannheim, Germany) and was used for dot-blot hybridisation experiments with chromosomal DNA to confirm the PCR-negative results for *icaAD* genes. The *ica*-positive *S. epidermidis* isolate 70D (Araujo et al. 2006) was used as a positive control for the assessment of *ica* via PCR or dot-blot-hybridisation and as a negative control for the experiment involving proteinase K. Biofilm formation was investigated using a microtitre-based system and the biofilm units (BU) was calculated as previously defined (Araujo et al. 2006).

The 27 isolates were analysed through pulsed-field gel electrophoresis (PFGE) following chromosomal DNA digestion with the *Sma*I endonuclease (Teixeira et al. 1995). PFGE types were defined on the basis of DNA-banding patterns according to visual criteria. Isolates exhibiting PFGE patterns that differed from one another by fewer than four bands were assigned a common capital letter, with numerical indices representing subtypes, as described previously (Teixeira et al. 1995). An unpaired Student's *t* test was used for the majority of statistical analyses, except for the determination of correlations between multi-resistance and biofilms and between high-level resistance among clinically significant and non-significant isolates, for which Fisher's exact probability test was chosen. A level of 0.05 was considered significant for both statistical tests.

Based on the CDC criteria, clinically significant *S. haemolyticus* isolates were collected from seven of the 19 patients studied (36.8%). Among these individuals, patient 7 (P7) presented two isolates with dissimilar genetic profiles. In this case, the clinically significant *S. haemolyticus* strain could be identified based on the PFGE type of the isolates compared to those excluded from the study due to showing an identical pattern to the significant isolate. In one other patient (P4), the significant isolate could not be identified on the basis of molecular techniques given that the two isolates recovered showed distinct genetic profiles. A total of 13 isolates recovered from eight patients were considered contaminants. Five isolates collected from four patients could not be classified due to a lack of sufficient patient data (Table). Eight isolates were obtained from 19 patients (42.1%) who presented with malignant tumours, three of whom exhibited community infections (15.8%), while the remainder showed vascular cerebral accident, alcoholic liver cirrhosis, severe hypertension, type II diabetes mellitus and acute pulmonary oedema. Age data are reported for 14 patients. The mean age ($\pm$ standard deviation) of adult patients was 50 ± 22 years (range, 24-93 years). Additionally, two patients were classified as paediatric (> 2 years old).

The gender distribution among the 19 patients tested was close to equal (47% males and 53% females). Despite the limited number of isolates, 85.7% (6/7) of the clinically significant patients were female. In contrast, only 25% (2/8) of the contaminated patients were fe-

males ($p = 0.04$). We were not able to identify any factor to explain the difference between the two gender groups, except that the two oldest patients were females belonging to the group with clinical significance (Table). Due to the small number of studies on *S. haemolyticus* epidemiology available in the scientific literature, we could not comparatively analyse the gender data. Thus, studies with a greater number of clinically significant cases are required to clarify this issue.

The great majority of the tested *S. haemolyticus* isolates displayed multi-resistant profiles (25/27; 92.6%). Among the studied isolates, only two were resistant to all of the drugs tested, except glycopeptides, and these two were therefore defined as high-level resistant isolates (> 8 antimicrobials). These high-level resistant MRSHa isolates were found in the group of clinically significant isolates. Statistical analysis was conducted to compare the high-level resistance detected between clinically significant and non-significant isolates (Table) and the obtained p value was close to the limit of significance ($p = 0.054$). It has been suggested that the intensive use of glycopeptides is associated with increasing resistance to this drug, especially among *S. haemolyticus* isolates (Ahlstrand et al. 2011). In the present study, isolate 05/08 displayed TEI resistance (MIC = 64 $\mu\text{g/mL}$) and was only susceptible to tetracycline, chloramphenicol and rifampicin (Table). During the preparation of this paper, a study was published demonstrating clonal dissemination of linezolid-resistant *S. haemolyticus* isolates exhibiting a G2576T mutation in the 23S *rrna* gene in a hospital in Brazil (de Almeida et al. 2012). Thus, the high levels of multi-resistance observed in this emergent bacterial pathogen, including resistance to new generation antimicrobial drugs, may have serious implications for the therapeutic treatment of the associated infections. It was suggested that the significant number of IS elements distributed within the *S. haemolyticus* genome may represent hotspots for the acquisition of resistance genes and could promote rearrangement of genomic DNA, which accelerates bacterial diversification (Bouchami et al. 2012).

Only 3.7% (1/27) of the *S. haemolyticus* isolates tested did not carry the *mecA* gene based on the absence of the expected 164-base pairs (bp) *mecA*-fragment. However, in DNA obtained from the *mecA*-negative isolate 220/08, a 415-bp fragment was amplified corresponding to the IS1272 insertion sequence-like element, which is present in some *mec* islands, including type I, IV and VI (Boye et al. 2007, Berglund et al. 2008, Zhang et al. 2009). Remarkably, isolate 220/08 was resistant to oxacillin (OXA), but was susceptible to cefoxitin in disk tests. In addition, the MIC value recorded for OXA was 1 $\mu\text{g/mL}$. It is well known that the OXA MIC interpretative criteria can lead to overestimation of resistance for certain coagulase-negative staphylococci other than *S. epidermidis* isolates, for which the OXA MICs are between 0.5-2 $\mu\text{g/mL}$ (CLSI 2011).

SCC*mec* type I was identified among 67% (18/27) of the tested isolates (Table). This SCC*mec* type has been described as the most common type found among nosocomial isolates of MRSHa in the south of Brazil (Mom-

TABLE
Antimicrobial resistance, pulsed-field gel electrophoresis (PFGE) patterns, staphylococcal cassette chromosome *mec* (*SCC_{mec}*) types and biofilm formation of 27 *Staphylococcus haemolyticus* isolated from blood

Isolate (patient number, sex, age)	Collection date	Hospital unit	Antimicrobial resistance	PFGE	<i>SCC_{mec}</i>	Biofilm ^e
05/08 (P1, M, 41)	6 Jan 2008	Intensive care	PEN, OXA, CEF, CLI, ERY, GEN, CIP, SXT, TEI	K	VII	NP
33/08 (P2, F, 37)	4 Nov 2007	Internal medicine	PEN, OXA, CEF, CLI, ERY, TET, GEN, CIP, SXT	K	VII	0.56 ± 0.17
37/08 (P2)	4 Nov 2007	Neurosurgery	PEN, OXA, CEF, CLI, TET, GEN, CIP, SXT	I	VII	0.27 ± 0.12
27/08 (P3, M, 24)	31 Oct 2007	Neurosurgery	PEN, OXA, CEF, CLI, ERY, RIF, GEN, CIP, SXT	H ₂	I	NP
47/08 (P3)	31 Oct 2007	Neurosurgery	PEN, OXA, CEF, CLI, ERY, RIF, GEN, CIP, SXT	H ₁	I	1.32 ± 0.54
32/08 (P4, F, 82)	29 Oct 2007	Internal medicine	PEN, OXA, CEF, CLI, ERY, CHL, GEN, CIP, SXT	L	VIII	0.57 ± 0.09
43/08 (P4)	29 Oct 2007	Internal medicine	PEN, OXA, CEF, CLI, ERY, GEN, CIP, SXT	ND	I	0.89 ± 0.17
34/08 (P5, F, 34) ^a	25 Oct 2007	Internal medicine	PEN, OXA, CEF, CLI, ERY, CHL, CIP	J	VIII	NP
56/08 (P6, F, 93) ^a	25 Oct 2007	Internal medicine	PEN, OXA, CEF, CLI, ERY, GEN, CIP, SXT	G	VII	NP
65/08 (P7, F, 25) ^a	4 Feb 2008	Intensive care	PEN, OXA, CEF, CLI, ERY, CHL, TET, GEN, CIP, SXT	A	I	1.45 ± 0.38
69/08 (P7)	4 Feb 2008	Intensive care	PEN, OXA, CEF, CLI, ERY, CHL, GEN, CIP, SXT	F	I	0.25 ± 0.10
74/08 (P8, M, 57)	28 Jan 2008	Neurosurgery	PEN, OXA, CEF, CLI, ERY, CHL, GEN, CIP, SXT	A	I	3.38 ± 0.94
136/08 (P8)	28 Jan 2008	Neurosurgery	PEN, OXA, CEF, CLI, ERY, CHL, GEN, CIP, SXT	B	I	1.60 ± 0.66
85/08 (P9, M, NR) ^b	4 Jan 2008	Intensive care	PEN, OXA, CEF, CHL	O	VII	NP
86/08 (P9)	4 Jan 2008	Intensive care	PEN, OXA, CEF, CLI, ERY, CHL, TET, GEN, CIP, SXT	A	I	2.60 ± 0.46
140/08 (P10, F, NR) ^a	25 Feb 2008	Internal medicine	PEN, OXA, CEF, CLI, ERY, CHL, TET, RIF, GEN, CIP, SXT	G	VII	NP
142/08 (P11, M, NR)	28 Feb 2008	Coronary	PEN, OXA, CEF, CLI, ERY, TET, GEN, CIP, SXT	C ₁	I	NP
153/08 (P11)	28 Feb 2008	Coronary	PEN, OXA, CEF, CLI, ERY, GEN, CIP, SXT	C ₂	I	0.19 ± 0.07
161/08 (P11)	28 Feb 2008	Coronary	PEN, OXA, CEF, CLI, ERY, GEN, CIP, SXT	D	I	0.19 ± 0.1
155/08 (P12, M, 26) ^a	26 Feb 2008	Intensive care	PEN, OXA, CEF, CLI, ERY, CHL, TET, RIF, GEN, CIP, SXT	C ₁	I	0.48 ± 0.19
177/08 (P13, F, 59) ^b	26 Mar 2008	Trauma	PEN, OXA, CEF, CLI, ERY, CHL, GEN, CIP, SXT	D	I	0.90 ± 0.07
202/08 (P14, F, 14m)	1 Apr 2008	Paediatric unit	PEN, OXA, CEF, ERY, GEN, CIP, SXT	C ₁	I	NP
207/08 (P15, M, 56)	7 Apr 2008	Neurology	PEN, OXA, CEF, CLI, ERY, RIF, GEN, CIP, SXT	ND	I	0.44 ± 0.11
220/08 (P16, F, NB) ^b	26 Apr 2008	Intensive care	PEN, OXA, ERY	M	I	NP
283/08 (P17, F, NR) ^a	8 Jul 2008	Dialysis	PEN, OXA, CEF, CLI, ERY, GEN, CIP, SXT	E	I	0.20 ± 0.06
285/08 (P18, M, NR) ^b	27 Jun 2008	Surgery	PEN, OXA, CEF, ERY, CHL, TET, RIF, GEN, CIP, SXT	N	VII	0.38 ± 0.15
286/08 (P19, M, 62)	27 Jun 2008	Intensive care	PEN, OXA, CEF, ERY, CHL, RIF, GEN, CIP, SXT	E	I	0.24 ± 0.09

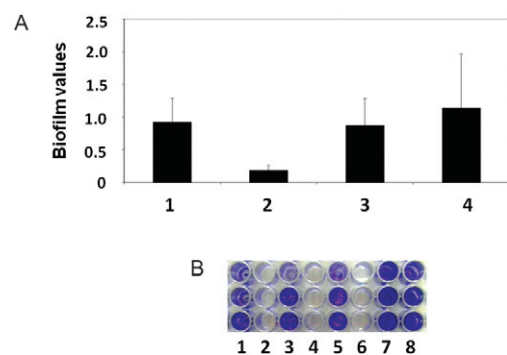
a: clinically significant isolates (for patient P4 the clinically significant isolate could not be assigned on the basis of the genotype, since the two isolates recovered had different genetic profiles); *b*: for these patients, clinical significance could not be evaluated due to lack of sufficient data; *c*: biofilm values were determined using a microtitre-based method; CEF: cefoxitin (30 µg); CHL: chloramphenicol (30 µg); CIP: ciprofloxacin (5 µg); CLI: clindamycin (2 µg); ERY: erythromycin (15 µg); F: female; GEN: gentamicin (10 µg); M: male; NB: newborn; ND: not-done; NP: non-producer; NR: not reported; OXA: oxacillin (1 µg); RIF: rifampicin (5 µg); SXT: sulphamethoxazole-trimethoprim (1.25 µg/23.75 µg); TEI: teicoplanin (minimal inhibitory concentration ≥ 64 µg/mL); TET: tetracycline (30 µg); P4, P5, P6, P7, P10, P12, P17 were patients with significant bacteraemia.

bach et al. 2007). The remaining isolates analysed in the present study were distributed among SCCmec type VII (7/27; 26%) and type VIII (2/27; 7%) strains. The prevalence of SCCmec V in MRSHa isolates had been reported in previous studies (Barros et al. 2012, Bouchami et al. 2012). However, Zong et al. (2011) found a variety of SCCmec types among 19 MRSHa isolates recovered from a hospital in China. Despite the prevalence of SCCmec I, we observed that these isolates displayed considerable genetic diversity. The 27 analysed isolates were classified into 15 PFGE types and two subtypes (Table). A similar clonal diversity was reported in Brazil for *S. haemolyticus* isolates collected from another hospital (Barros et al. 2012). In contrast, Bouchami et al. (2012) investigated 36 MRSHa isolates from Tunisia and found that 77% of the isolates clustered in only four PFGE types. A collection of 72 isolates of *S. haemolyticus* from Norway could also be grouped into only seven different clusters, the largest of which consisted of 16 isolates obtained from neonates treated in a neonatal intensive care unit at the same hospital (Fredheim et al. 2009). Thus, it is possible that this difference may be because the patients included in the present study came from different hospital units (Table), while those studied by the cited authors (Fredheim et al. 2009, Bouchami et al. 2012) came from a more limited number of hospital settings. Similar to what has been observed previously (Bouchami et al. 2012), we found that MRSHa isolates displaying different PFGE types could carry the same SCCmec type (Table), suggesting that horizontal transfer of SCCmec is a relatively common event among MRSHa strains.

Our data showed that 18 of the isolates (67%) were able to form biofilms (mean BU: 0.89 ± 0.86), eight (44%) of which displayed a high ability to accumulate biofilms (BU ≥ 0.89) (Table). Likewise, Fredheim et al. (2009) found that 74% of the *S. haemolyticus* isolates analysed could produce biofilms. Despite the limited number of isolates tested in this study, 11 contaminants, four clinically significant MRSHa isolates and three other isolates that could not be classified were able to form a biofilm on an inert polystyrene surface (mean BU: 0.91 ± 1.06 , 0.76 ± 0.54 and 1.27 ± 1.19) (Table). Student's *t* test showed that there was no significant difference between these populations ($p > 0.5$). Indeed, some contaminants (isolates 47/08, 136/08 and 74/08) showed a high capability to accumulate biofilms (Table). Notably, the only four isolates that were comparatively more susceptible to most of the antimicrobials tested (isolates 34/08, 85/08, 202/08 and 220/08) did not produce biofilms in this study and all of the biofilm producers were classified as showing high-level multi-resistance ($p < 0.01$) (Table). A relationship between multi-resistance and biofilm formation has also been observed by others (Araujo et al. 2006). Phase variation is a well-known common mechanism of biofilm formation for both *ica*-positive and negative staphylococci. In this study, we observed that some isolates clustering within a same PFGE type could exhibit quite different biofilm values (Table). It is possible that surface proteins involved in the formation of an *ica*-negative biofilm matrix might have been influenced by switching transcriptional regulators on or off.

Amplification of the *icaAD* genes was not detected in any of the 27 isolates analysed. The absence of the *icaAD* genes was confirmed through hybridisation experiments. Similar results have recently been reported by Fredheim et al. (2009), who detected the *ica* operon in only two of 53 biofilm-producing *S. haemolyticus* isolates. In the present study, the proteinaceous nature of the *ica*-independent biofilms was indicated by the significant disruption of the mature biofilms generated by the *S. haemolyticus* isolates following treatment with 6 U/well proteinase K (Figure). Thus, after treatment with proteinase K, the mean biofilm values for 10 representative MRSHa isolates tested (displaying different biofilm phenotypes) decreased from 0.927 ± 0.358 to 0.19 ± 0.077 ($p = 0.002$). As expected, the *ica*-dependent biofilm produced by *S. epidermidis* 70D (BU = 2.283 ± 0.204) was only slightly affected (1.648 ± 0.169 ; $p = 0.0408$) by the proteinase K treatment (B in Figure). Similar results have been found by others (Fredheim et al. 2009). As expected, treatment of mature biofilms with the carbohydrate-oxidant sodium metaperiodate (10 mM/well) did not significantly affect pre-formed biofilms (1.146 ± 0.824 ; $p = 0.26$). Previous studies have suggested that extracellular DNA is one of the components of the biofilm matrix formed by *S. haemolyticus* (Fredheim et al. 2009). However, the incorporation of DNase I (28U/well) into the microtitre-based system did not significantly impair biofilm formation among these isolates (0.878 ± 0.405 ; $p = 0.30$).

In conclusion, our analyses demonstrated that *S. haemolyticus* isolates collected from human blood, representing both clinically significant and contaminant isolates, showed clonal diversity, high-level antimicrobial multi-resistance and an ability to form *ica*-independent, proteinaceous biofilms. The increasing importance of these multi-resistant isolates in hospital infections in different countries confirms the fitness of these bacteria as opportunistic nosocomial pathogens. Additional studies are necessary to clarify the primary molecules involved in the composition and modulation of the *ica*-independent biofilms formed by *S. haemolyticus*.



A: biofilms of representative isolates of *Staphylococcus haemolyticus* (1: untreated; 2: proteinase K; 3: DNase; 4: sodium metaperiodate); B: microtitre based biofilm of representative isolates (1, 2: 33/08; 3, 4: 37/08; 5, 6: 69/08; 7, 8: *ica*-dependent biofilm producer *Staphylococcus epidermidis* 70D); even numbers: proteinase K treatment; odd numbers: untreated.

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