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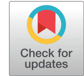
Daptomycin and vancomycin heteroresistance revealed among CC5-SCC*mec*II MRSA clone and in vitro evaluation of treatment alternatives



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ABSTRACT

Objectives: Methicillin-resistant *Staphylococcus aureus* (MRSA) is a threat to the success of clinical treatment. Besides high antimicrobial resistance rates, the presence of heterogeneous vancomycin-intermediate *S. aureus* (hVISA) and heterogeneous daptomycin-non-susceptible *S. aureus* (hDNSSA) in the hospital environment is underestimated and is associated with treatment failure. The aim of this study was to investigate MRSA dissemination in a Brazilian hospital and to evaluate the efficacy of various treatment options in vitro.
Methods: MRSA strains were typed by MLST, PFGE and SCC*mec* typing. Minimum inhibitory concentrations (MICs) to daptomycin, linezolid, quinupristin/dalfopristin, teicoplanin, tetracycline, tigecycline, vancomycin and tedizolid were determined by broth microdilution. The presence of a heterogeneous population was detected by population analysis profile (PAP). Regarding hVISA and hDNSSA strains, the sequences and expression levels of genes involved in resistance to daptomycin and vancomycin were determined as well as cell wall thickness and autolysis.
Results: ST5/ST105-SCC*mec*II lineage was prevalent amongst 27 clinical MRSA characterised in this study. Two hDNSSA strains (one also hVISA) were detected and were confirmed by PAP. Isolate SCMSC29 (hVISA and hDNSSA) showed increased expression of genes involved in cell wall metabolism, slight cell wall thickening, reduction of autolysis, and single nucleotide polymorphisms (SNPs) in the *rpoB* and *mprF* genes compared with the susceptible strain SCMSC31. SCMSC35 (hDNSSA) presented SNPs in the *rpoB* and *mprF* genes as well as a thickened cell wall.
Conclusions: Despite this worrying and hard to detect phenotype, treatment alternatives such as teicoplanin, linezolid, tetracycline, tigecycline, quinupristin/dalfopristin and tedizolid were all active against these isolates.
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1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a widespread pathogen causing both hospital- and community-acquired infections that is largely capable of acquiring resistance to all available classes of antimicrobial agents [1]. Nowadays, sequence type 5/105–staphylococcal cassette chromosome *mec* type II (ST5/ST105-SCC*mec*II) lineages are spread in hospitals worldwide, causing nosocomial infections [2–4]. Besides high antimicrobial resistance rates, the presence of heterogeneous resistant subpopulations is another challenge to clinicians since it is not detected by conventional antimicrobial susceptibility testing methods and can lead to treatment failure. Thus, novel therapeutic alternatives are always needed [5].

Vancomycin and daptomycin are usually used as last-choice antimicrobials as a means to prevent the selection of resistant isolates. Vancomycin is a glycopeptide antibiotic with bactericidal activity due to inhibition of cell wall synthesis. Heterogeneous vancomycin-intermediate *S. aureus* (hVISA) was first described in 1996 and, alarmingly, its rate is still underestimated [5]. Daptomycin acts as a bactericidal antimicrobial by inserting itself

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in the cell membrane and causing depolarisation, resulting in loss of membrane potential and inhibition of DNA, RNA and protein synthesis. Heterogeneous daptomycin-non-susceptible *S. aureus* (hDNSSA) has also been described previously [6].

Moreover, cross-resistance to vancomycin and daptomycin has been reported [6]. A common resistance mechanism for both antimicrobials has been proposed that includes a single nucleotide polymorphism (SNP) in the *rpoB* gene encoding the β subunit of RNA polymerase as well as SNPs in *graRS* and *vraTSR* encoding two- and three-component regulatory systems, respectively, that control metabolic cascades [5,6].

Since daptomycin is a cationic lipopeptide, resistance to this drug has also been associated with a change in the net positive charge of the cell membrane, possibly due to altered expression levels of *mprF* and the *dltABCD* operon [7,8].

The aim of this study was to investigate possible MRSA dissemination in a Southeastern Brazilian hospital from July 2011 to January 2012 and to evaluate the in vitro efficacy of various treatment options against the MRSA strains isolated. Since hVISA and hDNSSA strains were detected during the study, some well described determinants of resistance to daptomycin and vancomycin in *S. aureus* were also searched for in those specific isolates.

2. Materials and methods

2.1. Setting and isolates

A total of 27 *S. aureus* isolates were recovered from infection sites of different patients from the Hospital Santa Casa da Misericórdia de São Carlos (São Carlos, Brazil) between July 2011 and January 2012. The strains were isolated on Mueller–Hinton agar supplemented with 5% sheep blood. Species identification and preliminary antimicrobial susceptibility testing were carried out using a VITEK® system GT and P585 cards (bioMérieux, Marcy-l'Étoile, France). This study was approved by Plataforma Brasil Research Ethics Committee.

2.2. Molecular typing

SCC*mec* typing of all isolates was performed by multiplex PCR as previously described [9]. The following reference strains were used as controls: *S. aureus* RN4220 (SCC*mec*-negative); *S. aureus* 10442 (SCC*mec*I); *S. aureus* N315 (SCC*mec*II); *S. aureus* 85/2082 (SCC*mec*III); *S. aureus* 4744 (SCC*mec*IVa); and *S. aureus* WIS (SCC*mec*V).

Pulsed-field gel electrophoresis (PFGE) was carried out following digestion with the macrorestriction enzyme *Sma*I [10]. Data were analysed using BioNumerics v.7.6 software (Applied Maths NV, Sint-Martens-Latem, Belgium) using the unweighted pair-group method with arithmetic mean (UPGMA) based on Dice coefficients, and optimisation and tolerance were set to 0.7% and 1.25%, respectively. Samples sharing ≥80% similarity were classified as belonging to the same pulsotype [11].

Following PFGE analysis, the sequence type (ST) of at least one representative isolate from each pulsotype was determined by multilocus sequence typing (MLST) as described elsewhere (http://saureus.mlst.net/).

2.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed previously in the hospital using an automated VITEK® system. Minimum inhibitory concentration (MICs) were determined to daptomycin, linezolid, quinupristin/dalfopristin, teicoplanin, tetracycline, tigecycline, vancomycin and tedizolid by the broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [12]. CLSI breakpoints were used for classification of the antimicrobial susceptibility profile, except for tigecycline that followed European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria (http://www.eucast.org/). Reading after 48 h of incubation was also performed for the bactericidal antimicrobials to evaluate the possible presence of a persistent subpopulation [6].

MIC_{50} and MIC_{90} values (MICs at which ≥50% and ≥90% of the isolates in a test population are inhibited, respectively) were calculated according to Schwarz et al. [13]. The efficacy of the antimicrobials tested was calculated as the MIC_{90} in relation to the breakpoint concentration according to CLSI (or EUCAST in the case of tigecycline) [14].

2.4. Screening for heterogeneous vancomycin-intermediate Staphylococcus aureus *(hVISA)*

hVISA screening was performed as described previously [15] on brain–heart infusion (BHI) agar plates containing 4 μg/mL vancomycin. Samples were considered possible hVISA if growth was observed with a countable number of colonies after 48 h of incubation at 37 °C [15]. Confirmation of the hVISA phenotype was performed by population analysis profile (PAP) [15,16].

2.5. Population analysis profile (PAP)

The PAP method for daptomycin or vancomycin was performed to confirm the presence of a heteroresistant subpopulation within the isolates growing on the hVISA screening test or if a change in daptomycin MIC was observed following prolonged incubation. All experiments were performed in triplicate. Analyses were performed as previously described [15,16]. hVISA and VISA control strains (*S. aureus* Mu3 and *S. aureus* Mu50, respectively) were kindly provided by Prof. Keiichi Hiramatsu (Juntendo University, Tokyo, Japan). Since an isogenic pair of the clinical strains evaluated was not available, isolate SCMSC31 was used as a susceptible control strain for both vancomycin and daptomycin PAP. This strain is closely related to SCMSC29 and is of the same clone as SCMSC35 according to PFGE typing.

2.6. Sequencing of graRS, vraRS, mprF *and* rpoB *genes*

To investigate whether the heteroresistant strains isolated during the study possibly shared a known daptomycin or vancomycin resistance mechanism, the nucleotide sequences of the *graR*, *graS*, *vraR*, *vraS*, *mprF* and *rpoB* genes were determined following PCR amplification. Primers were designed based on the *S. aureus* SA16 genome (GenBank accession no. **ASZO00000000.1**) [17] and are presented in Table 1. PCR cycling conditions were as follows: 2 min at 94 °C; followed by 30 cycles of 2 min at 94 °C, 1 min at the specific annealing temperature and 2 min at 72 °C; and a final step for 10 min at 72 °C. The annealing temperature was 55 °C for *mprF*, *graRS* and *vraRS* and 52 °C for *rpoB*. The size and purity of PCR products were checked by agarose gel electrophoresis, and purification of amplicons was performed using a Wizard® SV Gel and PCR Clean-Up System Kit (Promega Corp., Madison, WI). Sanger DNA sequencing was carried out on an Applied Biosystems® 3130 Genetic Analyzer (Life Technologies, Carlsbad, CA). Longer PCR fragments were sequenced with several intermediate primers (Table 1). Contigs were assembled using the module Contig Express from Vector NTI (Thermo Fisher Scientific, Waltham, MA) and the nucleotide sequences were compared using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Since isogenic samples were not available, the nucleotide sequences of heterogeneous resistant isolates were compared with the susceptible isolate from the same pulsotype (SCMSC31).

Table 1
List of PCR, quantitative PCR (qPCR) and sequencing primers for *graRS*, *vraRS*, *rpoB* and *mprF* genes.

Gene	Primer	Sequence (5'→3')	Size (bp)
graR	graR-F1	ATGCAAATACTACTAGTAGAAGATG	675
	graR-R1	AAAAGGATATATGGCTCATGAATAA	
graS	graS-F1	ATGAATAATTTGAAATGGGTAGCTTAT	1040
	graS-R1[a]	CTGTGACAGCCATGAAATTA	
	graS-F2[a]	GAATGGTCTCGTATAAACTCGA	
	graS-R2	GGAAGTGACAAATTTGTCATTTTAA	
vraR	vraR-F1	ATGACGATTAAAGTATTGTTTGTG	630
	vraR-R1	GCATTCCAACATAATTTAATTCAATAG	
vraS	vraR-F1	ATGACGATTAAAGTATTGTTTGTG	1044
	vraR-R1	GCATTCCAACATAATTTAATTCAATAG	
	vraS-F1	ATGAACCACTACATTAGAACAATTG	
	vraS-R1	CAAAGACTAGCTCGAGAACT	
	vraS-F2	CGGCAAGTATGATGCTTT	
	vraS-R2	GGAGGATTCGTATGACGATTAA	
mprF	mprF-F1	ATGAATCAGGAAGTTAAAAACAAAATA	2523
	mprF-R1[a]	ACTTTAGTGTCGTGTGTTGAA	
	mprF-F2[a]	TTGACGCTCATGTATCATTCA	
	mprF-R2[a]	GCTATTATTTTTGTTCTGCTTATTGTA	
	mprF-F3[a]	CATTCTTCACTTACGCTTCATA	
	mprF-R3[a]	ACACATGCCTTTATATCATAATTTC	
	mprF-F4[a]	GAAGCAATTATTGATTTAACGCA	
	mprF-R4	GCGTGTAATACGTCACAAATAA	
rpoB	rpoB-F1	TTGGCAGGTCAAGTTGTCCA	3552
	rpoB-R1[a]	CTCAAGTGACCAAGAAATTGTTGACCTT	
	rpoB-F2[a]	GGCACTGAAAACACTGAACAAGC	
	rpoB-R2[a]	ATCGGTTTATCAAGAATGGAAAGAGTTGTAC	
	rpoB-F3[a]	TTAGGTAACCGTCGTTTACGTTCTGTAG	
	rpoB-R3[a]	ATCGCCGAAGCAAGTTGTTTC	
	rpoB-F4[a]	GCATTGATGGGTGCGAACATG	
	rpoB-R4[a]	TTAGTTGGTAAAGTAACGCCTAAAGGTG	
	rpoB-F5[a]	AATCACAAGAGATATTCCTAATGTTTCTGA	
	rpoB-R5[a]	GCACATGGTTGATGATAAATTACATGCG	
	rpoB-F6[a]	CGTTCAACAGGACCATATTCACTTG	
	rpoB-R6	GCTCCTGAAACACAAAAAGAAGTTACTGATTAA	
qPCR *vraR*	vraR-F	GCTAGTGCATCGCATATTAC	126
	vraR-R	CTATTGAATTAAATTATGTTGGAATGC	
qPCR *graR*	graR-F	GGGATGATGAAGCATTTGTTAG	134
	graR-R	TTATTCATGAGCCTATTATCCTTTT	
qPCR *mprF*	mprF-F	TTGACGCTCATGTATCATTCA	85
	mprF-R	ACTTTAGTGTCGTGTGTTGAA	
qPCR *rpoB*	rpoB-F	ATCGCCGAAGCAAGTTGTTTC	93
	rpoB-R	CATGTTCGCACCCATCAATGC	
qPCR *dltA*	dltA-F	CCTCACAGAGCAGCAAAAGC	82
	dltA-R	ACCGTAGCTTCAGTTGGACC	
qPCR *gmk*	gmk-F	ATCGTTTTATCGGGACCATC	111
	gmk-R	CATTTGACGTGTTGTCATTG	
qPCR *tpi*	tpi-F	TCGTTCATTCTGAACGTCGTGAA	117
	tpi-R	CGTCTGTTTCACCAACACAAAT	
qPCR *pta*	pta-F	GTTAAAATCGTATTACCTGAAGG	95
	pta-R	CCTAACACGATTGGTGTAACAT	

[a] Primers used for Sanger sequencing.

2.7. Relative gene expression analysis of graR, vraR, mprF, rpoB and dltA

Relative gene expression of *graR*, *vraR*, *mprF*, *rpoB* and *dltA* was investigated in strain SCMSC31 (susceptible to both daptomycin and vancomycin), SCMSC29 (hVISA and hDNSSA) and SCMSC35 (hDNSSA). Again, isolate SCMSC31 was used as susceptible control for comparison because of the lack of isogenic pairs. All strains were cultivated in BHI broth at 37 °C under shaking at 130 rpm for 18 h. The following day, 100 μL of the overnight pre-inoculum was diluted into 5 mL of fresh BHI and cultures were grown until an optical density at 600 nm (OD_{600}) of 0.6–1.0 was reached. Cells were harvested by centrifugation for 2 min at 14 000 × *g* at 4 °C and were subjected to lysis with 3 mg/mL lysozyme and 3 mg/mL lysostaphin (both from Sigma-Aldrich, Saint Louis, MO) prepared in TE [Tris–ethylene diamine tetra-acetic acid (EDTA)] buffer (pH 8.0).

RNA was extracted using an SV Total RNA Isolation System (Promega) according to the manufacturer's recommendations. The concentration and purity of total RNA were determined using a NanoDrop™ 2000c Spectrophotometer (Thermo Fisher Scientific). For reverse transcription, a SuperScript™ III First-Strand Kit (Invitrogen, Carlsbad, CA) was used according to the manufacturer's recommendations, using 50 ng of random hexamers and 1 μg of total RNA, generating 20 μL of cDNA (50 ng/μL).

Evaluation of gene expression by quantitative PCR (qPCR) for the genes *vraR*, *graR*, *mprF*, *rpoB* and *dltA* (reference for primer design: *S. aureus* SA16, GenBank accession no. **ASZO00000000.1**) was performed using the following genes as references for normalisation of expression: *gmk* (gene ID BAB42304); *tpi* (gene ID BAB41962); and *pta* (gene ID BAB41777). Primers used for qPCR amplification are listed at Table 1.

A cDNA pool was used for optimisation of annealing temperatures; this pool was also used in a standard curve for determination of primer efficiency, which was used to correct expression values [18]. The same pool was used to evaluate the stability of the reference genes (*gmk*, *tpi* and *pta*) with RefFinder [19]. All three genes were considered stable for normalisation of relative expression.

For qPCR amplification, the fluorescent DNA-binding dye PowerUp™ SYBR® Green PCR Master Mix (Thermo Fisher Scientific) was used according to the manufacturer's recommendations, with 5 ng of cDNA per reaction. A CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) was used with the following cycling conditions: 2 min at 50 °C; followed by one hold for 2 min at 95 °C; then 40 cycles for 15 s at 95 °C and 1 min at the optimal annealing temperature (varying from 56–60 °C depending on the primers). A melting curve was performed after each run (raising 0.5 °C/s from 65 °C to 95 °C) to certify amplicon specificity in each reaction. The qPCR reaction was carried out in technical triplicates and in two independent experiments. Relative expression was calculated using the ratios of the three reference genes by the comparative ΔΔCt method with PCR efficiency correction [20].

2.8. Evaluation of cell wall thickness by transmission electron microscopy (TEM)

Cells of SCMSC29, SCMSC31 and SCMSC35 were prepared for TEM as described previously [21]. Briefly, cell cultures were grown in BHI broth at 37 °C until an OD_{600} of 0.3, were washed with phosphate-buffered saline (PBS) and were treated with a 3% glutaraldehyde solution for 2 h. After washing, cells were fixed using osmium solution and were then washed again and were dehydrated with increasing concentrations of ethanol (30% to 100%). Cells were then washed with propylene oxide and were transferred to an araldite block. Slices of 1 μm were used for observation on a JEOL 100CX-II electronic microscope (JEOL USA Inc., Peabody, MA). Thirty cells of each strain were used to measure cell wall thickness using magnified photographic images up to 100 000 times in Image J software [22]. The mean ± standard deviation values of cell wall thickness were determined for each isolate. The experiment was performed in duplicate.

2.9. Autolysis assay

Autolysis assays were performed as described previously [23] with 0.05% Triton X–100 v/v. Briefly, cells were grown to an OD_{600} of 0.3 and cultures were then washed and were re-suspended to an OD_{600} of 1.0 in a 50 mM glycine/0.05% Triton X-100 buffer (pH 8.0). Autolysis was followed by measuring the OD_{600} during incubation at 37 °C on a SpectraMax® M5 spectrophotometer (Molecular Devices, Sunnyvale, CA). Experiments were performed in technical and biological triplicates.

2.10. Statistical analysis

The statistical significance of the data was evaluated using Student's *t*-test.

3. Results

3.1. Molecular typing

During a 6-month period (July 2011 to January 2012), 27 MRSA isolates were recovered from infection sites of inpatients of Hospital Santa Casa de Misericórdia de São Carlos. Similarity analysis by PFGE clustered the isolates into only three pulsotypes. Pulsotype A was the most prevalent (24/27; 88.9%) and was split into seven subtypes (A1–A7). MLST analysis of representative isolates from each pulsotype revealed the presence of ST5 and ST105 in pulsotypes A and B, and one ST8 isolate in pulsotype C. All isolates possessed SCC*mec*II, except the ST8 isolate that had SCC*mec*IV (Fig. 1).

3.2. Antimicrobial susceptibility profile

Among the 19 antimicrobials tested using the VITEK® system, the isolates were considered susceptible only to vancomycin and trimethoprim/sulfamethoxazole (Supplementary Table S1). However, by broth microdilution all isolates were considered susceptible to daptomycin, linezolid, quinupristin/dalfopristin, teicoplanin, tetracycline, tigecycline, vancomycin and tedizolid after 20 h of incubation (Fig. 1).

Teicoplanin presented the best efficacy since its MIC_{90} was only 12.5% of the breakpoint, followed by tedizolid (MIC_{90} = 50% of breakpoint). The other antimicrobials tested, although effective in vitro, did not showed good efficacy since their MIC_{90} values reached the border concentration for susceptibility (MIC_{90} = 100% of breakpoint) (Table 2).

Curiously, the daptomycin MIC of isolates SCMSC29 and SCMSC35 changed from 1 μg/mL to 2 μg/mL after 48 h of incubation, reaching a non-susceptible status. In a similar way, the vancomycin MIC of SCMSC29 changed from 2 μg/mL to 4 μg/mL after 48 h and this isolate was also considered positive on hVISA screening test. PAP was carried out to confirm the presence of heterogeneous resistant subpopulations in both cases (Fig. 2).

Based on methodologies already described for PAP of vancomycin [15,16,24], SCMSC29 was classified as hVISA (Fig. 2A). PAP for daptomycin confirmed the presence of a heterogeneous non-susceptible population for isolates SCMSC29 and SCMSC35 using adapted criteria of Hiramatsu et al. [15] and El-Halfawy and Valvano [16]. Therefore, isolates SMSC29 and SCMSM35 were classified as hDNSSA (Fig. 2B).

Table 2
MIC_{50} and MIC_{90} values based on broth microdilution results for methicillin-resistant *Staphylococcus aureus* isolates (n = 27).

Antimicrobial agent	MIC (μg/mL)		
	MIC_{50}	MIC_{90}	Range
Daptomycin	1.0	1.0	0.5–1.0
Linezolid	2.0	4.0	2.0–4.0
Quinupristin/dalfopristin	1.0	1.0	0.5–1.0
Teicoplanin	0.5	1.0	0.5–2.0
Tedizolid	0.25	0.25	0.125–0.5
Tigecycline	0.25	0.5	0.125–0.5
Tetracycline	1.0	4.0	0.5–4.0
Vancomycin	2.0	2.0	1.0–2.0

MIC, minimum inhibitory concentration; $MIC_{50/90}$, MICs at which ≥50% and ≥90% of the isolates in a test population are inhibited, respectively.

Isolates	Date (dd/md/yy)	Pulsotype	ST	SCC*mec*	DPC	VAN	TCP	LZD	TET	TGC	QDA	TDZ
SCMSC7	07/08/2011	A3	105	II	0.5	2	0.5	2	1	0.5	1	0.25
SCMSC18	10/09/2011	A3		II	0.5	1	0.5	4	2	0.25	1	0.25
SCMSC20	17/09/2011	A3		II	1	2	0.5	2	1	0.25	1	0.25
SCMSC27	14/10/2011	A3		II	0.5	2	0.5	2	1	0.25	1	0.5
SCMSC30	21/10/2011	A3		II	0.5	2	0.5	2	2	0.25	1	0.25
SCMSC31	31/10/2011	A3	105	II	0.5	2	0.5	2	1	0.25	1	0.25
SCMSC32	17/11/2011	A3	105	II	0.5	2	0.5	2	1	0.25	1	0.25
SCMSC35	01/12/2011	A3	105	II	1*	2	0.5	2	0.5	0.25	1	0.25
SCMSC39	18/12/2011	A3	105	II	1	2	0.5	2	1	0.25	1	0.25
SCMSC28	08/10/2011	A4	105	II	1	2	0.5	2	4	0.5	1	0.25
SCMSC4	26/07/2011	A1	105	II	1	2	0.5	2	1	0.25	1	0.25
SCMSC11	15/08/2011	A1		II	1	2	0.5	4	1	0.5	1	0.25
SCMSC14	19/08/2011	A1		II	1	2	0.5	2	1	0.125	1	0.25
SCMSC25	09/10/2011	A1		II	1	2	0.5	2	1	0.25	1	0.25
SCMSC34	25/11/2011	A1	105	II	0.5	2	0.5	2	1	0.125	1	0.25
SCMSC36	30/11/2011	A1		II	0.5	4	0.5	2	1	0.25	1	0.25
SCMSC43	29/12/2011	A1		II	0.5	2	0.5	2	0.5	0.25	1	0.25
SCMSC42	29/12/2011	A2	105	II	1	2	0.5	4	0.5	0.25	1	0.25
SCMSC21	23/09/2011	A5	5	II	0.5	2	1	2	4	0.125	1	0.25
SCMSC22	26/09/2011	A5		II	1	1	0.5	2	4	0.25	1	0.25
SCMSC24	04/10/2011	A5		II	1	1	0.5	2	2	0.25	1	0.25
SCMSC49	05/01/2012	A5	105	II	1	2	0.5	2	1	0.125	1	0.25
SCMSC6	05/08/2011	A6	105	II	0.5	1	0.5	2	0.5	0.25	1	0.25
SCMSC29	21/10/2011	A7	5	II	1*	2*	1	2	2	0.25	1	0.25
SCMSC9	10/08/2011	B1	5	II	1	2	2	4	1	0.25	1	0.25
SCMSC17	02/09/2011	B2	5	II	1	2	1	2	2	0.125	1	0.25
SCMSC44	02/01/2012	C1	8	IV	0.5	2	0.5	2	0.5	0.25	0.5	0.125

Fig. 1. Clonality of methicillin-resistant *Staphylococcus aureus* isolates. The dendrogram was generated using BioNumerics v.6.5 software using the unweighted pair-group method with arithmetic mean (UPGMA) based on Dice coefficients, and optimisation and tolerance were set to 0.7% and 1.25%, respectively. Samples sharing ≥80% similarity were classified as belonging to the same pulsotype. ST, sequence type; SCC*mec*, staphylococcal cassette chromosome *mec*. Minimum inhibitory concentrations (MICs; in μg/mL) for various antimicrobial agents are shown: DPC, daptomycin; VAN, vancomycin, TCP, teicoplanin; LZD, linezolid; TET, tetracycline; TGC, tigecycline; QDA, quinupristin/dalfopristin; TDZ, tedizolid. * Resistance after 48 h.

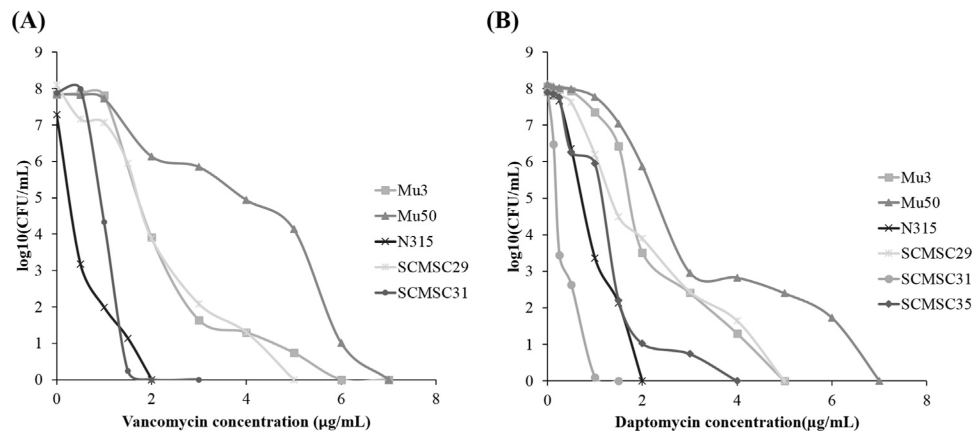

Fig. 2. Population analysis profile (PAP) of methicillin-resistant *Staphylococcus aureus* isolates to (A) vancomycin and (B) daptomycin. An isolate was considered heteroresistant if growth was observed after 48 h at two-fold breakpoint antimicrobial concentrations. A representative example of experiments performed in triplicate is shown.

3.3. Sequencing of the graRS, vraRS, mprF *and* rpoB *genes*

Analysis of the *mprF* gene revealed mutations leading to Met347Leu and Leu770Phe substitutions in SCMSC29, and Leu770Phe in SCMSC35 (Table 3). Regarding the *rpoB* gene, SNPs were found both in SCMSC29 and SCMSC35, leading to Thr622Ala substitution. Only synonymous substitutions were found in *graR*, *graS*, *vraR* and *vraS* of isolates SCMSC29 and SCMSC35 compared with SCMSC31.

Table 3
List of single nucleotide polymorphisms found in the *rpoB* and *mprF* genes of methicillin-resistant *Staphylococcus aureus* strains SCMSC29 and SCMSC35 compared with SCMSC31.

Gene	Mutation	Amino acid change	Sample
rpoB	A1864G	T622A	SCMSC29 (hVISA/hDNSSA) SCMSC35 (hDNSSA)
mprF	A1039T	M347L	SCMSC29 (hVISA/hDNSSA)
	A2310T	L770F	SCMSC29 (hVISA/hDNSSA) SCMSC35 (hDNSSA)

hVISA, heterogeneous vancomycin-intermediate *S. aureus*; hDNSSA, heterogeneous daptomycin-non-susceptible *S. aureus*.

3.4. Relative gene expression analysis of graR, vraR, mprF, rpoB *and* dltA

Gene expression levels of *graR*, *vraR*, *mprF*, *rpoB* and *dltA* were normalised against validated endogenous controls (*gmk*, *tpi* and *pta*) and these analyses are presented in Fig. 3 and Supplementary Table S2. The data show that all five genes tested were overexpressed only in SCMSC29 (hVISA and hDNSSA) compared with SCMSC31 with statistical significance ($P < 0.05$); *rpoB* and *dltA* were three-fold overexpressed, whilst *vraR*, *graR* and *mprF* were two-fold overexpressed.

3.5. Cell wall thickness and autolysis

Although no visible differences in cell wall thickness were visually noticed in TEM images (Fig. 4), measurements revealed a statistically significant ($P < 0.01$) thicker cell wall in SCMSC29 and SCMSC35 compared with SCMSC31.

Following 3 h of exposure to the non-ionic surfactant Triton X-100, SCMSC29 exhibited lower cell lysis than SCMSC31 ($P < 0.05$) (Fig. 5). Although there was a clear tendency of greater autolysis in SCMSC35 compared with SCMSC31, the difference was not statistically significant (Fig. 5).

4. Discussion

Molecular typing analysis showed there was dissemination of MRSA ST5/ST105-SCC*mec*II lineages among patients in Hospital Santa Casa de Misericórdia de São Carlos during the study period.

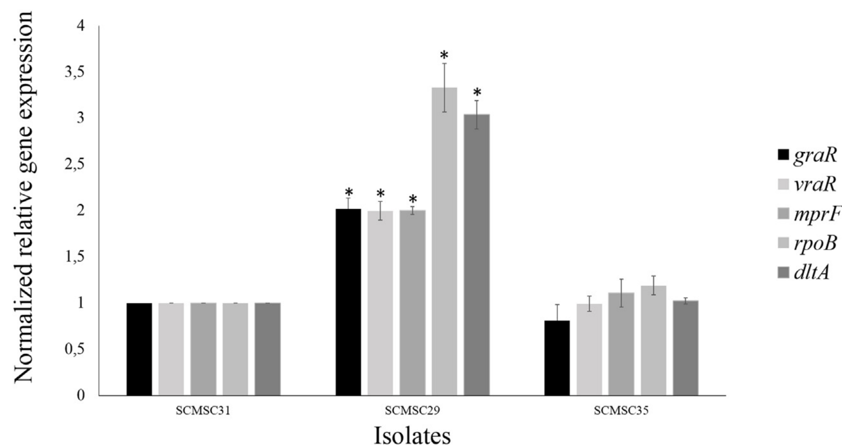

Fig. 3. Relative gene expression of *graR*, *vraR*, *mprF*, *rpoB* and *dltA* for methicillin-resistant *Staphylococcus aureus* isolates SCMSC31, SCMSC29 and SCMSC35. Expression of all five genes was plotted relative to the reference genes *gmk*, *tpi* and *pta*. Relative expression was normalised to SCMSC31 ($P < 0.05$ as determined by Student's *t*-test) and statistically significantly overexpressing strains are marked with *. Level of transcription, standard deviation and statistical significance are listed in Supplementary Table S2.

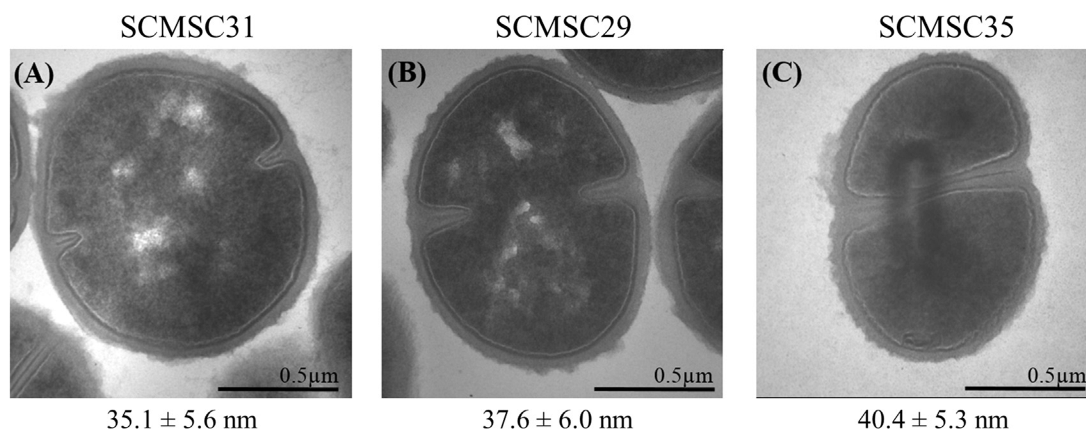

Fig. 4. Transmission electron microscopy of susceptible and heterogeneous resistant *Staphylococcus aureus* strains: (A) SCMSC31, susceptible to both vancomycin and daptomycin; (B) SCMSC29 (hVISA and hDNSSA); and (C) SCMSC35 (hDNSSA). hVISA, heterogeneous vancomycin-intermediate *S. aureus*; hDNSSA, heterogeneous daptomycin-non-susceptible *S. aureus*.

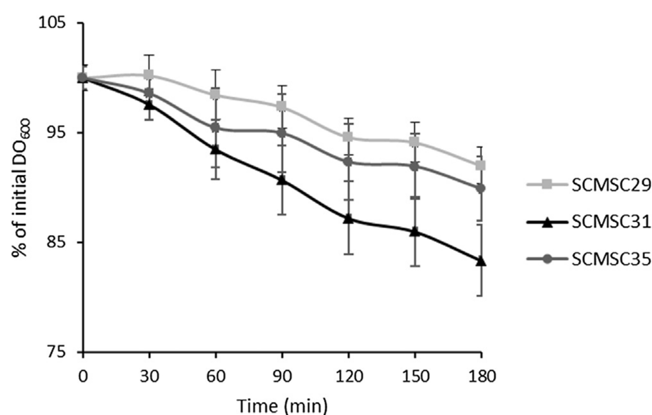

Fig. 5. Autolysis assay with 0.05% Triton X-100 v/v of methicillin-resistant *Staphylococcus aureus* isolates. Lower autolysis was observed for isolate SCMSC29 compared with the susceptible control SCMSC31 ($P < 0.05$). No significant difference between SCMSC35 and SCMSC31 was observed. OD_{600}, optical density at 600 nm.

The fact that two isolates from the most disseminated pulsotype were classified as hDNSSA, and one of them was also classified as hVISA, is of great epidemiological concern since the heterogeneous phenotype cannot be detected by routine methods and can lead to treatment failure.

In fact, heteroresistance was related to a higher number of bacteraemia episodes in a study conducted in Argentina [25]. Other authors observed that patients with infectious endocarditis caused by hVISA had more positive blood cultures and a trend towards longer duration of bacteraemia than patients infected with MRSA. Also, the daptomycin MIC was higher in hVISA isolates and there were two cases of DNSSA only among the hVISA isolates [26].

There are few reports of hVISA in South America. In 2010, one MRSA isolate from Brazil and one from Venezuela were detected as hVISA, but both were susceptible to daptomycin [27]. Later, in 2015, three *S. aureus* (ST5-SCC*mec*I or ST100-SCC*mec*IVNv), two of which were methicillin-resistant, were detected as hVISA in Argentina [25], and one DNSSA (MIC = 2 mg/L) MRSA belonging to clonal complex 5 (CC5) was isolated in Chile [28].

On the other hand, all of the isolates were susceptible to the last-resort antimicrobials, with teicoplanin and tedizolid being the most effective. The fact that the new oxazolidinone tedizolid demonstrated excellent activity even against the hVISA and hDNSSA isolates reveals that new drugs for treating infections caused by multidrug-resistant MRSA are viable.

We wondered whether mechanisms of heterogeneous resistance in SCMSC29 and SCMSC35 were based on what is already known by the scientific community regarding vancomycin and daptomycin resistance, therefore we began a series of preliminary investigations.

Several genes regulating cell wall structures have been reported as being involved in mechanisms of resistance of drugs targeting this site [5]. Based on this, expression levels of genes related to cell wall charge (*mprF, dltA*), cell wall thickness (*rpoB*) and regulatory mechanisms (*graR*, *vraR*) were quantified in the heterogeneous isolates SCMSC29 (hDNSSA and hVISA) and SCMSC35 (hDNSSA) and were compared with those of the susceptible isolate SCMSC31 to verify whether they might be also correlated with the heteroresistant phenotype [5,29,30].

SCMSC29 showed elevated expression of all of the evaluated genes, whilst SCMSC35 did not show any overexpression compared with SCMSC31, thus the results for SCMSC29 might be due to an hVISA phenotype.

A weak point of this study is the fact SCMSC31 and SCMSC29 are not isogenic (different PFGE subtypes and single-locus variants by MLST), and in such cases observing two- or three-fold variations the gene expression may not be relevant. Nevertheless, we observed an agreement of increased *rpoB* and *vraR* gene expression and thickened cell wall, as well as increased *graR* gene expression and diminished autolysis, the last probably caused by down-regulation of the WalKR operon [29].

These genes have been associated with hVISA and VISA phenotypes many times [5–7]. *vraR* is part of the three-component system *vraTSR* that regulates synthesis of the vancomycin target, the cell wall. *graR* is also a member of a two-component system, namely *graSR*, known to control the expression of more than 200 genes, including the *walKR* operon regulating autolysis [29]. *rpoB* encodes the β subunit of RNA polymerase and a mutation in this gene might cause a change in the transcriptional profile of the cell. Indeed, others have already demonstrated that mutations in *rpoB* caused cell wall thickening [30].

In addition, increased expression of *dltA* could cause an elevation in cellular net positive charge and collaborate in the hVISA phenotype in SCMSC29.

Regarding the hDNSSA phenotype of SCMSC29 and SCMSC35, it is most likely due SNPs in the MprF protein synthase domain, which might lead to changes in cell wall charge and repulsion of the daptomycin molecule, as well as in RpoB, which might lead to a

thickened cell wall and difficulty in the drug reaching its target, the cell membrane.

The *mprF* gene of isolate SCMSC29 presented two SNPs. The first, which resulted in a Met347Leu substitution, is located in a region described as a 'hot spot' for mutations [29]. It is part of the biofunction domain of MprF, which has already been associated with daptomycin-non-susceptibility [8]. The second *mprF* SNP found in SCMSC29, which was also observed in SCMSC35, resulted in Leu770Phe substitution compared with SCMSC31. It corresponds to the protein synthase domain and might be an explanation to the hDNSSA phenotype of both isolates.

The substitution Thr622Ala in RpoB occurred both in SCMSC29 (hDNSSA and hVISA) and SCMSC35 (hDNSSA). Interestingly, both SCMSC29 and SCMSC35 had a thicker cell wall compared with SCMSC31.

The sequences containing these SNPs were blasted against the NCBI's database and none was found with 100% identity, increasing its relevance.

Future studies are required to confirm whether the point mutations found in this study are always present in the process of heterogeneous resistance, or maybe only one step to gain full resistance.

Nevertheless, much remains to be studied in order to confirm all of these hypotheses, particularly due to the lack of isogenic pairs for comparisons.

It is also important to bear in mind that trying to elucidate the mechanisms involved in heteroresistance is not a trivial task, since the determinants are present only in a small subpopulation of the isolates being studied.

In conclusion, ST5/ST105-SCC*mec*II is the prevalent MRSA lineage in this Southeastern Brazilian hospital, where hVISA and hDNSSA were identified among the isolates. Preliminary results showed that genes involved with cell wall metabolism might have contributed to the hVISA and hDNSSA phenotypes. Luckily, last-resort antibiotics such as linezolid, tedizolid and tigecycline were all active in vitro against these isolates, with tedizolid showing even higher efficacy.

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Competing interests

None declared.

Ethical approval

Plataforma Brasil Research Ethics Committee [CAAE 55310916.7.0000.55.04, protocol n. 1.556.620].

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jgar.2018.05.001.

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