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Biofilm formation of Brazilian meticillin-resistant *Staphylococcus aureus* strains: prevalence of biofilm determinants and clonal profiles

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Biofilms plays an important role in medical-device-related infections. This study aimed to determine the factors that influence adherence and biofilm production, as well as the relationship between strong biofilm production and genetic determinants in clinical isolates of meticillin-resistant *Staphylococcus aureus* (MRSA). Fifteen strains carrying different chromosomal cassettes recovered from hospitalized patients were selected; five SCC*mec*II, five SCC*mec*III and five SCC*mec*IV. The SCC*mec* type, *agr* group and the presence of the virulence genes (*bbp, clfA, icaA, icaD, fnbB, bap, sasC* and IS*256*) were assessed by PCR. PFGE and multilocus sequence typing (MLST) techniques were also performed. The initial adhesion and biofilm formation were examined by quantitative assays. The surface tension and hydrophobicity of the strains were measured by the contact angle technique to evaluate the association between these parameters and adhesion ability. SCC*mec*III and IV strains were less hydrophilic, with a high value for the electron acceptor parameter and higher adhesion in comparison with SCC*mec*II strains. Only SCC*mec*III strains could be characterized as strong biofilm producers. The PFGE showed five major pulsotypes (A–E); however, biofilm production was related to the dissemination of one specific PFGE clone (C) belonging to MLST ST239 (Brazilian epidemic clonal complex). The genes *agr*I, *fnbB* and IS*256* in SCC*mec*III strains were considered as genetic determinants associated with strong biofilm-formation by an *ica*-independent biofilm pathway. This study contributes to the understanding of biofilm production as an aggravating factor potentially involved in the persistence and severity of infections caused by multidrug-resistant MRSA belonging to this genotype.

INTRODUCTION

The epidemiology of meticillin-resistant *Staphylococcus aureus* (MRSA) has been the focus of numerous single and multicentre surveillance studies over the past years (Gales *et al.*, 2009; Sader & Jones, 2009; Sader *et al.*, 2009). MRSA is one of the major human pathogens worldwide. It is responsible for several diseases ranging from superficial to invasive infections such as pneumonia and sepsis. These infections are even more severe if a strain with high biofilm formation ability colonizes an invasive medical device (Gordon & Lowy, 2008; Montanaro *et al.*, 2011).

†In memoriam.

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Abbreviations: BECC, Brazilian epidemic clonal complex; ClfA, clumping factor A; IS, insertion sequence; MLST, multilocus sequence typing; MRSA, meticillin-resistant *Staphylococcus aureus*; PIA, polysaccharide intercellular adhesion; SCC*mec*, staphylococcal cassette chromosome *mec*; ST, sequence type.

Two supplementary figures and one supplementary table are available with the online Supplementary Material.

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The first stage of biofilm formation is cell attachment onto a surface followed by intercellular adhesion, production of an extracellular matrix and then maturation of the biofilm (Otto, 2013). Until recently, the intercellular adhesion of bacteria during biofilm formation was exclusively attributed to the production of polysaccharide intercellular adhesin (PIA), which is encoded by the *ica*ADBC operon. It is now recognized that there are other surface proteins associated with biofilm formation in a PIA-independent manner, which are becoming increasingly more relevant in clinical strains of MRSA (Laverty *et al.*, 2013). In this regard, several studies have reported the participation of cell wall proteins such as biofilm-associated protein (Bap) (Cucarella *et al.*, 2001), *S. aureus* surface protein G (SasG) (Geoghegan *et al.*, 2010), fibronectin-binding proteins A and B (FnBPA and FnBPB) (O'Neill *et al.*, 2008; Vergara-Irigaray *et al.*, 2009; McCourt *et al.*, 2014), the overproduction of protein A (Merino *et al.*, 2009) and *S. aureus* surface protein C (SasC) (Schroeder *et al.*, 2009).

It is well known that a secreted peptide-based communication system, the *agr* quorum sensing system, primarily modulates the virulence of *S. aureus* (George & Muir, 2007). This system seems to have two functions: downregulation of genes that encode proteins associated with the colonization process and upregulation of genes encoding exoproteins associated with cellular damage of the host. The downregulation and upregulation of the genes involved in the described processes promote the establishment and development of MRSA infections. Additionally, it is known that these genes have an important role in MRSA biofilm formation, which in turn results in a more aggressive infection giving the patient a poor prognosis (Vuong *et al.*, 2000; Laverty *et al.*, 2013).

Another key factor leading to MRSA epidemics in hospitals is its remarkable capacity to acquire antibiotic resistance through mobile genetic elements, such as the staphylococcal cassette chromosome *mec* (SCC*mec*). To date, twelve types of SCC*mec* have been assigned to *Staphylococcus aureus* (Hiramatsu *et al.*, 2013; Wu *et al.*, 2015). For the Brazilian MRSA clinical strains the most important SCC*mec* types are SCC*mec*II, SCC*mec*III and SCC*mec*IV (Carvalho *et al.*, 2010). At the beginning of the past decade, the Brazilian epidemic clonal complex (BECC)/SCC*mec* type III/ST239 was the main lineage in Brazilian hospitals. However, over recent years new MRSA strains have been described in community and nosocomial infections (Rodríguez-Noriega & Seas, 2010; Rodríguez-Noriega *et al.*, 2010).

In addition to the protein-coding genes, the role of insertion sequences (IS) in the microbial world is being studied. Recent researches have demonstrated that the *S. aureus* genome contains several copies of insertion sequences, including IS*256*, which seems to be strongly associated with biofilm formation (Kwon *et al.*, 2008).

MRSA typing is commonly done, although few studies have been conducted to associate genotypes with the biofilm production phenotype. In this study, a phenotypic and genotypic approach was used to determine the factors that influence adherence and biofilm production of the most common MRSA SCC*mec* types, and the relationship with antimicrobial resistance, virulence genes and the genetic background of *S. aureus* isolates.

METHODS

Bacterial strains, media and growth conditions. The origin and epidemiological characteristics of 15 MRSA clinical strains used in this study are described in Table 1. *S. aureus* isolates harbouring SCC*mec*II, SCC*mec*III and SCC*mec*IV are responsible for most of the infections worldwide, in the community as well as in the hospital environment. In order to obtain statistical significance, five of each strain (SCC*mec*II, 5; SCC*mec*III, 5; SCC*mec*IV, 5) were randomly chosen from a well characterized collection. These strains were recovered from hospitalized patients at the Clinical Hospital of the Federal University of Uberlândia (UFU), a 533-bed public tertiary care teaching hospital in the south-east of Brazil, during the period 2006–2008. Ethical approval for the collection of isolates was provided by the Ethical Committee of UFU (Protocol 218/06). The identification of the strains and antimicrobial susceptibility tests were done using Vitek II (bioMérieux) and strains analysed according to the Clinical and Laboratory Standards Institute (CLSI, 2013) guidelines. The criteria used for defining multidrug-resistant phenotype was non-susceptible to one or more agent in three or more antimicrobial categories (Magiorakos *et al.*, 2012). Strains were stored at -20 °C and subcultured on trypic soy broth (TSB) and tryptic soy agar (TSA) plates (Becton, Dickinson and Company) for 24 h, at 37 °C. For adhesion and biofilm assays, the strains were grown for 18 ± 2 h, at 37 °C and 120 r.p.m. in 20 ml TSB using bacteria grown on TSA plates for no more than 2 days as inoculum. After cells were harvested by centrifugation (10 500 **g**, 5 min, 4 °C), they were washed twice and resuspended in saline (0.9 % NaCl prepared in distilled water) at approximately 1×10^9 cells ml^{-1}, prior to being used in biofilm and adhesion assays. *S. aureus* ATCC 25923 was used as a control for initial adhesion, biofilm formation, haemagglutination assays and PCRs for *bbp*, *clfA*, *agr*III and *sasC* genes. *S. aureus* N315 was used as a control for PCRs for *icaA*, *icaD* and *agr*II genes; *S. aureus* NCTC 8325 was the control strain for the *fnbB* gene.

Initial adhesion assay. The initial adhesion test was performed according to the method of Cassat *et al.* (2014), with modifications. Briefly, 200 µl of a cell suspension containing 1×10^7 cells ml^{-1} prepared in TSB was added to 96-well unmodified polystyrene plates. Initial adhesion was allowed to occur for 2 h at 37 °C with rotation at 120 r.p.m. Bacteria adhered in 96-well polystyrene plates were washed twice with a 0.9 % NaCl solution and harvested by scraping of wells for 90 s. The cell suspension obtained was plated on TSA for c.f.u. enumeration. All experiments were done in triplicate, in three independent experiments.

Contact angle measurement (bacterial cell hydrophobicity). Hydrophobicity parameters of bacterial cell surface were determined through the sessile drop contact angle technique, using an automated contact angle device (OCA 15 Plus; Dataphysics), as described previously (Sousa *et al.*, 2009), with some modifications. Briefly, a 20 ml suspension of *S. aureus* cells, adjusted to a concentration of approximately 1×10^9 cells ml^{-1} in saline solution (NaCl, 0.9 % w/v), was deposited onto a 0.45 µm cellulose filter (Pall Life Sciences), previously wetted with 10 ml of distilled water. To standardize the moisture content, the filters with the resultant lawn of cells deposited were then left to dry on Petri dishes containing 1 % (w/v) agar (Merck) and 10 % (v/v) glycerol (Sigma-Aldrich), for at least 3 h.

Table 1. Origin and epidemiological characteristics of 15 MRSA strains used in this study

Strain (n=15)	SCC*mec* type	Classification of infection	Source	Ward	Patient outcome
SAII68	II	HAI	BSI	General Medical Unit	Death
SAII207	II	HAI	BSI	Neonatal Intensive Care Unit	Death
SAII376	II	HAI	BSI	Infectious Disease	Discharge
SAII397	II	HAI	BSI	Oncology	Death
SAII483	II	HAI	BSI	Surgery	Death
SAIII06	III	HAI	BSI	General Medical Unit	Death
SAIII29	III	HAI	SSI	Intensive Care Unit	Death
SAIII32	III	HAI	BSI	Burn Unit	Discharge
SAIII108	III	HAI	SSI	Emergency	Discharge
SIII156	III	HAI	SSI	Surgery	Discharge
SAIV70	IV	HAI	PNM	Intensive Care Unit	Discharge
SAIV321	IV	HAI	BSI	Paediatrics	Discharge
SAIV333	IV	HAI	BSI	Neonatal Intensive Care Unit	Discharge
SAIV495	IV	HAI	BSI	Infectious Disease	Discharge
SAIV533	IV	HAI	SSI	Emergency	Discharge

HAI, healthcare associated infection; BSI, bloodstream infection; SSI, surgical site infection; PNM, pneumonia.

At least 25 determinations for each liquid and bacterial strain were performed at room temperature for water, formamide and α-bromonaphthalene, liquids with known surface tension components used as reference for standardized contact angle measurements. Contact angle measurements allowed the calculation of bacterial hydrophobicity parameters, using the van Oss approach (van Oss & Giese, 1995; van Oss, 1997).

Biofilm formation assay. Biofilms were formed as described by O'Toole (2011) and Cassat *et al.* (2014), with modifications. Briefly, 200 µl of a cell suspension containing 1×10^7 cells ml^{-1} prepared in TSB was added to 96-well unmodified polystyrene plates. Biofilm formation was allowed to occur for 24 h at 37 °C with rotation at 120 r.p.m. Bacteria grown in 96-well polystyrene plates were washed twice with a 0.9 % NaCl solution and left to dry in an inverted position. The total biomass was measured by methanol (Merck) fixation, 0.1 % crystal violet (Merck) staining and acetic acid (Merck) elution as previously described. The eluted dye was removed from each well and placed in a new 96-well microtitre plate and its absorbance read on an ELISA plate reader (BioTek Instruments) at 570 nm. The experiments were done with eight replicates for each strain, in three independent experiments. TSB without bacteria was used as a negative control. The interpretation of biofilm production was done according to the criteria of Stepanović *et al.* (2007). The optical density cut-off value (ODc) was established as three SDs above the mean of the OD of the negative control: ODc=mean OD of negative control+3×SD of negative control. For easier interpretation of the results, strains were divided into the following categories according to their optical densities: OD≤ODc or ODc<OD<2×ODc indicates non-biofilm producer/weak biofilm producer; 2×ODc<OD<4×ODc, moderate biofilm producer; 4×ODc<OD, strong biofilm producer.

Biofilm cell number. The biofilm cell number was determined by c.f.u. enumeration. After biofilm formation as described, the biofilms were washed twice with a 0.9 % NaCl solution and harvested after scraping the wells for 90 s. The cell suspension obtained was plated onto TSA plates. All experiments were done in triplicate, on three independent occasions.

Haemagglutination assay. The haemagglutination assay was performed as described previously (Cerca *et al.*, 2005) with some modifications. Human blood collected with EDTA was used to retrieve erythrocytes, by adding 5 ml of blood to 45 ml of saline solution, which was then centrifuged twice at 2500 ***g*** for 10 min. Next, 100 µl of the pellet was added to 10 ml of saline solution, obtaining a 1 % erythrocyte solution to be used in the haemagglutination assays. *S. aureus* cells were grown in fresh TSB overnight (16–18 h) and then resuspended in saline and adjusted to approximately 3×10^9 cells ml^{-1}. Five twofold dilutions of each cell suspension were made (100 µl) in 96-well (U-shaped) microtitre plates. Then, 100 µl of the 1 % erythrocyte solution was added to each well. To ensure thorough mixing of the bacteria and erythrocytes, the total volume of each well was pipetted in and out with a micropipette. Incubation was at room temperature for 2 h, and haemagglutination titres were evaluated macroscopically. Erythrocytes that appeared to be negative for macroscopic haemagglutination were also evaluated microscopically. All experiments were done in duplicate with three repeats.

Biofilm detachment assay. The biofilm detachment assay was carried out as described by Chaignon *et al.* (2007) with some modifications. For this experiment, before treatment with crystal violet (Gram stain solution; NewProv), the biofilm was treated with sodium metaperiodate (10 mM per well in 50 mM sodium acetate buffer, pH 4.5; Sigma) or proteinase K (6 U per well in 20 mM Tris, pH 7.5, 100 mM NaCl, Sigma) at 37 °C for 2 h. After the treatment the biofilms were washed, fixed and stained as previously described.

Genetic techniques. All PCR primers and reaction conditions used in this study are described in Table S1 (available with the online Supplementary Material).

DNA extraction. Genomic DNA was extracted from strains by using the QIAamp DNA Mini kit (Qiagen) according to the manufacturer's instructions. Purified DNA (10 ng) was used as a template in PCR amplification.

SCC*mec* multiplex PCR. SCC*mec* types were determined using a multiplex PCR strategy according to the method described by Kondo *et al.* (2007).

Assessment of the *agr* alleles. The multiplex PCR of *agr* alleles was carried out according to the protocol previously described by Shopsin *et al.* (2003) using the common forward (pan-agr) primer and reverse primers (agrI, agrII, agrIII and agrIV). These primers allowed amplification of 439, 572, 406 and 588 bp DNA fragments of the *agr* groups I to IV, respectively. To distinguish between the similar-sized products, two duplex PCR amplifications were performed for each isolate, the first using the primers pan-agr, agrI and agrII and the second using the primers pan-agr, agrIII and agrIV.

Determination of biofilm-associated genes. The biofilm-associated genes were assessed according to the protocols described by: Martín-López *et al.* (2004) and Arciola *et al.* (2001) for polysaccharide intercellular adhesin genes *icaA* and *icaD*, respectively; Cucarella *et al.* (2001) for biofilm-associated protein (*bap*); Tristan *et al.* (2003) for fibronectin-binding protein B (*fnbB*), clumping factor A (*clfA*) and bone sialoprotein-binding protein (*bbp*); Gu *et al.* (2005) for insertion sequence IS*256*; and Schroeder *et al.* (2009) for *S. aureus* surface protein (*sasC*).

PFGE. Isolates were typed by PFGE according to the protocols described by McDougal *et al.* (2003) and Goering (2010) with some modifications. After digestion of intact genomic DNA with 30 U of *Sma*I (Ludwig Biotecnologia) for 10 h at 25 °C, DNA fragments were separated on 1 % (w/v) agarose gels in 0.5 % TBE (Tris/borate/EDTA) buffer using a CHEF DRIII apparatus (Bio-Rad) with 6 V cm^{-1}, pulsed from 5 s to 40 s, for 21 h at 12 °C. Gels were stained with ethidium bromide and photographed under UV light. Computer-assisted analysis was performed using BioNumerics 5.01 software (Applied Maths). Comparison of the banding patterns was accomplished by the unweighted pair-group method with arithmetic averages (UPGMA) using the Dice similarity coefficient.

Multilocus sequence typing (MLST). MLST was carried out according to the protocol described by Enright *et al.* (2000). Briefly, the housekeeping genes carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glp*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*) and acetyl coenzyme A acetyltransferase (*yqiL*) were amplified by PCR. Amplified PCR products were purified with the Wizard SV Gel and PCR Clean Up System (Promega) and the gel-eluted PCR products were sequenced with an automated DNA sequencer (ABI-PRISM 3100 Genetic Analyzer; Applied Biosystems). The sequences were submitted to http://saureus.mlst.net, the allelic profile was generated and the sequence type (ST) assigned.

Statistical analysis. Statistical analyses were performed using GraphPad Prism v.5 (GraphPad Software). Quantitative assays were compared using the Kruskal–Wallis, applying Dunn's multiple comparison, test. All tests were performed with a confidence level of 95 % and statistical significance was defined as $P<0.05$.

RESULTS

Hydrophobicity and adhesion of MRSA strains

To evaluate the association between the hydrophobicity and the ability of MRSA cell to adhere to an unmodified polystyrene surface, two parameters were measured, namely surface tension and hydrophobicity. As shown in Table 2, the 15 MRSA clinical strains studied showed similar values of water contact angles, lower than 65°, ranging from 16.59° (strain SAIII29) to 29.55° (strain SAIV70), which is indicative of a hyphophilic surface. These values are quite similar to those obtained for formamide, which is also a polar solvent. The contact angles determined using an apolar liquid, α-bromonaphthalene, showed variation between strains ranging from 48.48° (strain SAII376) to 89.06° (strain SAIV333). In addition, all strains showed positive values of ΔGiwi and so they can be considered hydrophilic. Regarding the surface tension components, all strains predominantly showed electron donation, with higher values of electron donor parameter (γ^-) compared with the low values of the electron acceptor parameter (γ^+). It is also possible to estimate the hydrophobic or hydrophilic nature of surfaces using the surface tension components values. The electron donor surface free energy component (γ^-) can be a semiquantitative indicator of hydrophobicity. Values of $\gamma^- \leq 25.5$ mJ m^{-2} are indicative of hydrophobic surfaces (Azeredo & Oliveira, 2000). Again, according to these results, all tested strains are hydrophilic, presenting γ^- values above this limit.

All MRSA strains assessed were able to adhere to an unmodified polystyrene surface (Fig. 1a). When individually analysed, no association could be established between the degree of hydrophobicity and the ability of initial adhesion. No difference was observed in the adhesion ability between the most hydrophilic MRSA (SAII483) and the least hydrophilic strain (SAIV333), as can be seen in Table 2 and Fig. 1a. When the evaluation was carried out according to the SCC*mec* type, an association between the degree of hydrophobicity (ΔGiwi) and adhesion ability was observed, since strains harbouring SCC*mec* types III and IV were more weakly hydrophilic (Table 2) and adhered better than SCC*mec*II strains ($P<0.0001$; Fig. 1b). Although differences were observed when comparing the strains carrying SCC*mec* types III and IV ($P<0.001$), both showed better adhesion capacity compared with the SCC*mec*II strains (Fig. 1b). Also from this analytical perspective, there was an association between the values of the interfacial tension of the electron acceptor component (γ^+) and the ability of initial adhesion. The higher the component values, the higher were the numbers of adhered cells.

Association of SCC*mec* type, biofilm production and multidrug resistance

MRSA strains carrying SCC*mec* type III showed a significantly increased ability to form biofilms compared with the strains harbouring SCC*mec* type II and type IV. The strains with the SCC*mec* type II and type IV genotype produced less biomass, this value being below the cut-off established using the Stepanović *et al.* (2007) criteria (Fig. 2a, b). Regarding the number of cells present on each biofilm, once again the SCC*mec* type III strains had the highest number of bacterial cells (Fig. 2c, d).

The classification of isolates as strong biofilm producers occurred in 33.3 % of the strains, all harbouring SCC*mec* type III. The profile of non-producer/weak producer was observed in all other strains (Table 3).

Table 2. Water (θ_W), formamide (θ_F) and α-bromonaphthalene ($\theta_{\alpha\text{-}B}$) contact angles (in degrees), surface tension components and hydrophobicity (in mJ m^{-2}) of the surface of MRSA clinical strains and SCC*mec* groups

Strain	Contact angle ± SD (°)			Surface tension components (mJ m^{-2})			
	θ_W	θ_F	$\theta_{\alpha\text{-}B}$	γ^{LW}	γ^+	γ^-	ΔGiwi
SAII68	20.43 ± 1.90	18.48 ± 2.76	51.70 ± 4.36	29.12	3.74	49.60	23.78
SAII207	25.39 ± 4.91	19.95 ± 2.34	48.49 ± 2.15	30.69	3.29	46.07	20.97
SAII376	23.14 ± 3.86	18.31 ± 1.65	48.48 ± 2.59	30.69	3.36	47.38	22.07
SAII397	21.47 ± 2.81	22.19 ± 3.07	52.88 ± 2.86	28.54	3.49	50.59	25.36
SAII483	21.33 ± 2.28	29.63 ± 4.93	55.5 ± 4.00	27.24	2.71	55.56	32.13
SAIII06	20.85 ± 4.76	17.31 ± 3.99	64.31 ± 4.38	22.81	6.80	47.95	18.30
SAIII29	16.59 ± 3.48	17.32 ± 1.55	56.64 ± 4.32	26.66	4.66	51.64	24.21
SAIII32	20.41 ± 4.74	17.65 ± 2.4	57.53 ± 3.83	26.22	5.02	48.89	21.42
SAIII108	16.65 ± 2.72	16.37 ± 2.78	59.93 ± 4.39	25.01	5.54	50.97	22.32
SAIII156	18.58 ± 4.82	18.43 ± 3.35	61.32 ± 4.17	24.31	5.68	50.44	21.75
SAIV70	29.55 ± 4.67	19.97 ± 2.89	64.22 ± 4.87	22.85	7.07	40.99	12.91
SAIV321	24.83 ± 4.91	25.33 ± 4.10	66.74 ± 5.97	21.60	6.20	48.53	19.62
SAIV333	20.39 ± 2.53	20.89 ± 3.38	89.06 ± 4.86	11.47	15.10	48.32	5.57
SAIV495	23.84 ± 3.57	17.65 ± 2.21	63.83 ± 4.87	23.05	6.84	45.56	16.52
SAIV533	21.58 ± 3.98	21.55 ± 2.65	63.58 ± 4.79	23.18	5.90	49.47	20.75
SCC*mec* groups							
II	22.12 ± 3.58	21.25 ± 4.88	50.12 ± 3.83	29.90	3.19	49.67	24.80
III	18.31 ± 4.40	17.4 ± 2.94	60.38 ± 4.96	24.78	5.59	50.22	21.68
IV	23.77 ± 5.04	20.92 ± 3.99	66.58 ± 7.88	21.67	7.01	47.00	17.35

γ^{LW}, Apolar Lifshitz–van der Waals surface free energy component; γ^+, electron acceptor surface free energy component; γ^-, electron donor surface free energy component; Δ*G*iwi, degree of hydrophobicity.

Based on antibiotic susceptibility tests performed with these isolates, four antibiotypes (R1–R4) were identified among the meticillin-resistant strains. Five strains were in the R1 antibiotype and six strains in the R2 antibiotype, all of which were classified as multiresistant MRSA (mrMRSA). The other antibiotypes (R3 and R4) were categorized as non-multiresistant MRSA (nmMRSA). No relationship between multidrug resistance and biofilm production was observed (Tables 3 and 4).

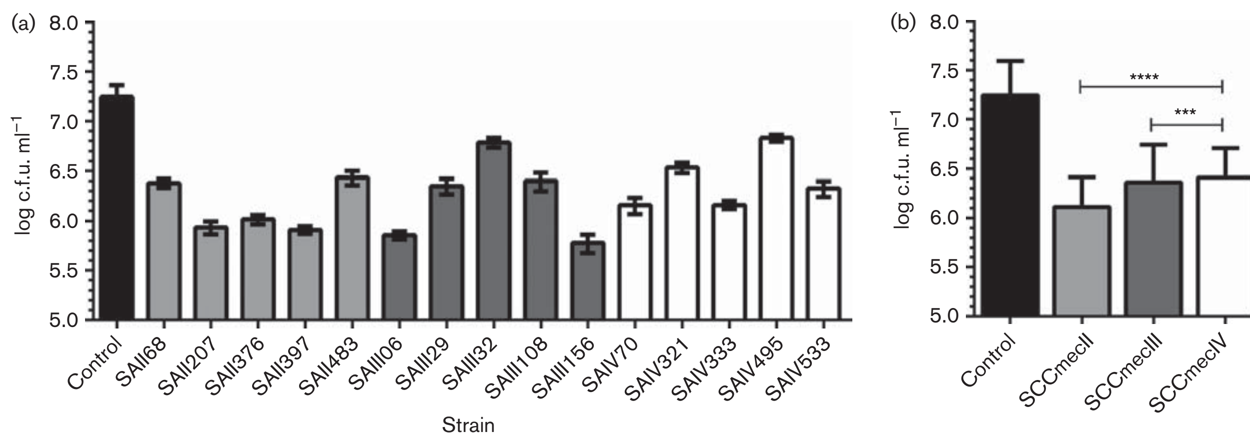

Fig. 1. Number of cells adhered onto polystyrene plates after a 2 h period of contact, expressed as log c.f.u. ml^{-1} for 15 clinical MRSA strains (a) and according to the SCC*mec* type (b). *S. aureus* ATCC 25923 was used as a control. The culture medium without bacterial inoculum was used as negative control (data not shown). The strains are colour coded according to the SCC*mec* type: SCC*mec*II, light grey; SCC*mec*III, dark grey; SCC*mec*IV, white. Results represent means+SD (error bars) of three independent experiments. ***$P \leq 0.001$; ****$P \leq 0.0001$ using Kruskal–Wallis, Dunn's multiple comparison test. The positive control served as proof that the experiment could produce a positive result; this analysis did not aim to compare the groups with this control.

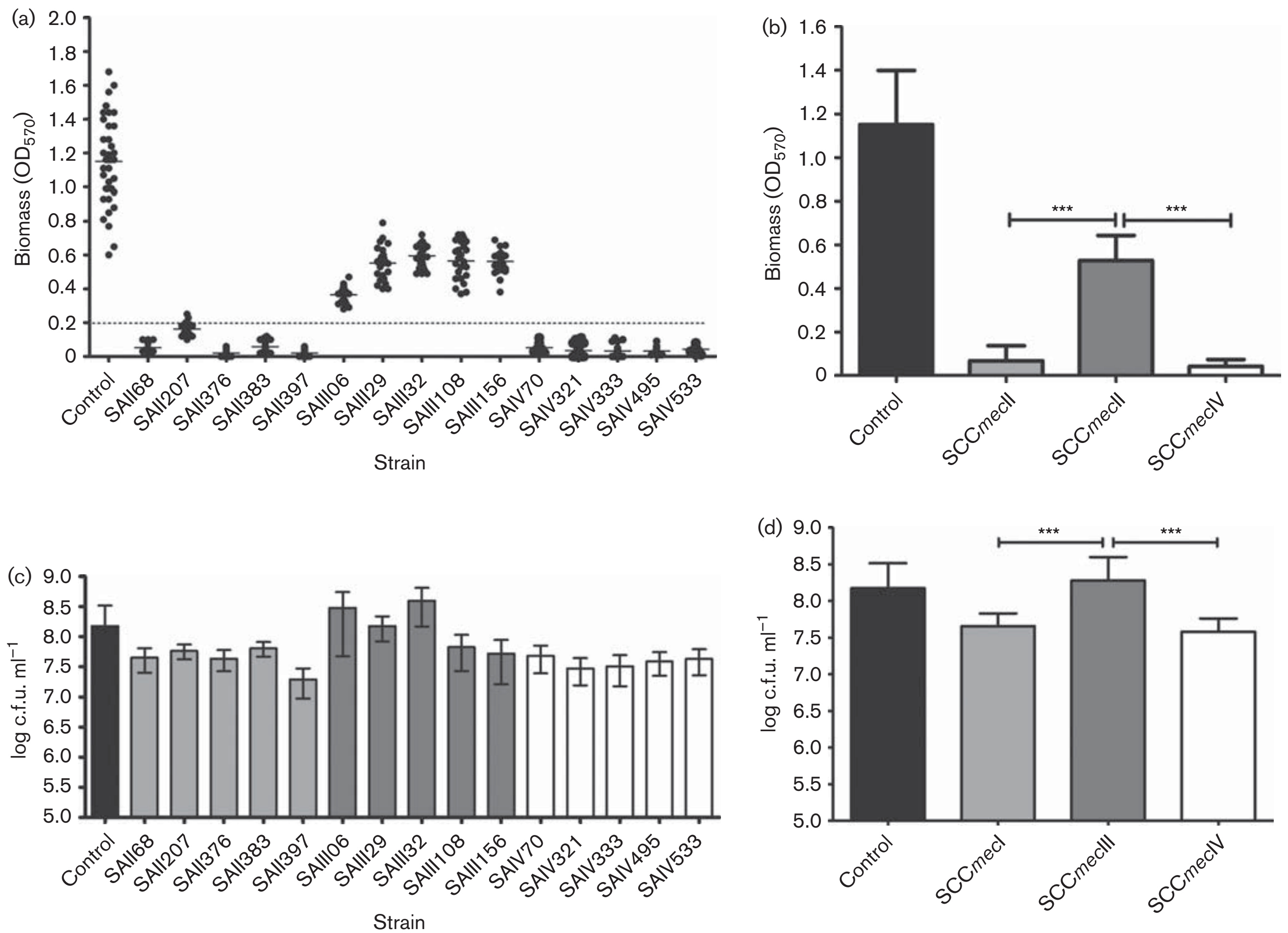

Fig. 2. Biofilm biomass expressed as crystal violet OD_{570} for 15 clinical MRSA strains (a) and according to the SCC*mec* type (b). Biofilm cellular number expressed by log c.f.u. ml^{-1} of 15 clinical MRSA strains (c) and according to the SCC*mec* type (d). *S. aureus* ATCC 25923 was used as a control. The culture medium without bacterial inoculum was used as negative control (data not shown). The strains are colour coded according to the SCC*mec* type: SCC*mec*II, light grey; SCC*mec*III, dark grey; SCC*mec*IV, white. Results represent means+SD (error bars) of three independent experiments. The dashed line represents the cut-off calculated according to the criteria established by Stepanović *et al.* (2007). ***$P \leq 0.001$, using Kruskal–Wallis, Dunn's multiple comparison test. The positive control served as proof that the experiment could produce a positive result; this analysis did not aim to compare the groups with this control.

Proteinaceous nature of the biofilm

In order to investigate the nature of biofilms produced by the MRSA strains, the haemagglutination assay was used for indirect inference of expression levels of the polysaccharide intercellular adhesin. None of the strains tested was able to cause haemagglutination. To validate this result, the biofilms produced by strong biofilm producers were treated with proteinase K and sodium metaperiodate. The treatment with proteinase K virtually disrupted preformed biofilms for five isolates tested. However, the carbohydrate oxidant metaperiodate almost did not affect the biofilm accumulated by these isolates (Fig. 3).

Association among *icaA*, *icaD*, *bap*, *fnbB*, *clfA*, *bbp*, *sasC* genes, insertion sequence IS*256*, *agr* group and biofilm formation

To analyse the genetic determinants associated with biofilm formation in MRSA clinical strains, the presence of genes associated with *ica*-dependent and *ica*-independent biofilm pathways were evaluated.

Through PCRs, four genetic determinants were found to be predominant in the strains classified as strong biofilm producers and not in the non-producer/weak biofilm producers: *fnbB*, IS*256*, *agr*I and SCC*mec*III. All isolates, biofilm producers and non-biofilm producers, were positive for *clfA*, *sasC* and *icaD*, and only one strain

Table 3. Association of biofilm production of isolates and multiresistant phenotype

Strain	Mean OD	Biofilm production	Resistance
Negative control*	0.05	–	–
SAII68	0.05	Non/weak	mrMRSA
SAII207	0.16	Moderate	mrMRSA
SAII376	0.02	Non/weak	mrMRSA
SAII397	0.06	Non/weak	mrMRSA
SAII483	0.06	Non/weak	mrMRSA
SAIII06	0.37	Strong	mrMRSA
SAIII29	0.55	Strong	mrMRSA
SAIII32	0.60	Strong	mrMRSA
SAIII108	0.56	Strong	mrMRSA
SAIII156	0.53	Strong	mrMRSA
SAIV70	0.05	Non/weak	mrMRSA
SAIV321	0.03	Non/weak	nmMRSA
SAIV333	0.03	Non/weak	nmMRSA
SAIV495	0.03	Non/weak	nmMRSA
SAIV533	0.04	Non/weak	nmMRSA

mrMRSA, multiresistant MRSA; nmMRSA, non-multiresistant MRSA.

*Culture medium without bacterial inoculum was used as negative control.

(SAIV70) was negative for the *icaA* gene. None of the strains had the *bbp* or *bap* genes. The prevalence of biofilm-associated genes among the 15 MRSA clinical strains is demonstrated in Table 5.

Molecular typing

PFGE and MLST techniques were used to evaluate the association of the genetic profile of these strains with their ability to form biofilm. The genetic characteristics of the 15 MRSA clinical strains analysed are summarized in Table 5.

Five pulsotypes (A–E) were detected by PFGE. The SCC*mec*II isolates belonged to pulsotypes A and B. The subtype A has two isolates, A1 and A2, and subtype B only has one. The SCC*mec*III strains were classified as pulsotype C, with five subtypes (C–C4) with one isolate each. The SCC*mec*IV strains belonged to three pulsotypes (A3, D and E); the subtype D was the most frequent in these strains, with three isolates (Table 5, Fig. S1). All strains carrying SCC*mec*III were typed by MLST as belonging to ST239, which is the profile of the BECC. The strains carrying SCC*mec*IV were typed as ST5 (Pediatric/USA 800 clone), except for SAIV333, which was untyped, and the only SCC*mec*II strain tested (SAII207) was typed as ST5 (New York/Japan clone).

DISCUSSION

Recent research has shown that antibiotic resistance, production of enzymes and toxins, ability to form biofilm and evasion of the immune system are key factors that contribute to the global spread of *S. aureus* (Chambers & Deleo, 2009; Schlievert *et al.*, 2010; Strandberg *et al.*, 2010; Foster *et al.*, 2014).

MRSA strains, particularly those of nosocomial origin, have multiple genetic elements, mobile or not, that confer resistance to several classes of antimicrobials. In these strains, these elements are mainly associated with SCC*mec* (Chambers & Deleo, 2009; Hiramatsu *et al.*, 2013).

Most studies that evaluate the formation of biofilm by clinical isolates are limited and controversial; particularly concerning the association between biofilm production and the presence of mobile genetic elements associated with the resistance, such as SCC*mec* elements. In order to address this issue, MRSA clinical strains were recovered from a collection previously characterized for the SCC*mec* type. The MRSA clinical strains were characterized in terms of biofilm formation. Fifteen MRSA isolates harbouring SCC*mec* types II (n=5), III (n=5) or IV (n=5) were selected for this study. All strains characterized as strong biofilm producers carried the SCC*mec* type III, with a mean biofilm biomass of OD_{570} 0.53±0.12, compared with OD_{570} 0.04±0.04 for those characterized as

Table 4. Relationship between antimicrobial pattern, SCC*mec* type and source of 15 MRSA clinical strains used in this study

Antibiotype	Resistance pattern	Number of isolates (%)	Source (number of isolates)	SCC*mec* type (number of isolates)
R1	P, MET, GEN, CIP, ERY, CLI, TET, RIF, STX	5 (33.3)	Bloodstream infection (2)	III (5)
			Surgical site infection (3)	
R2	P, MET, CIP, ERY, CLI	6 (40)	Bloodstream infection (5)	II (5)
			Pneumonia (1)	IV (1)
R3	P, MET, ERY	3 (20)	Bloodstream infection (3)	IV (3)
R4	P, MET	1 (6.7)	Surgical site infection (1)	IV (1)

P, Penicillin; MET, meticillin; GEN, gentamicin; CIP, ciprofloxacin; ERY, erythromycin; CLI, clindamycin; TET, tetracycline; RIF, rifampicin; STX, trimethoprim/sulfamethoxazole.

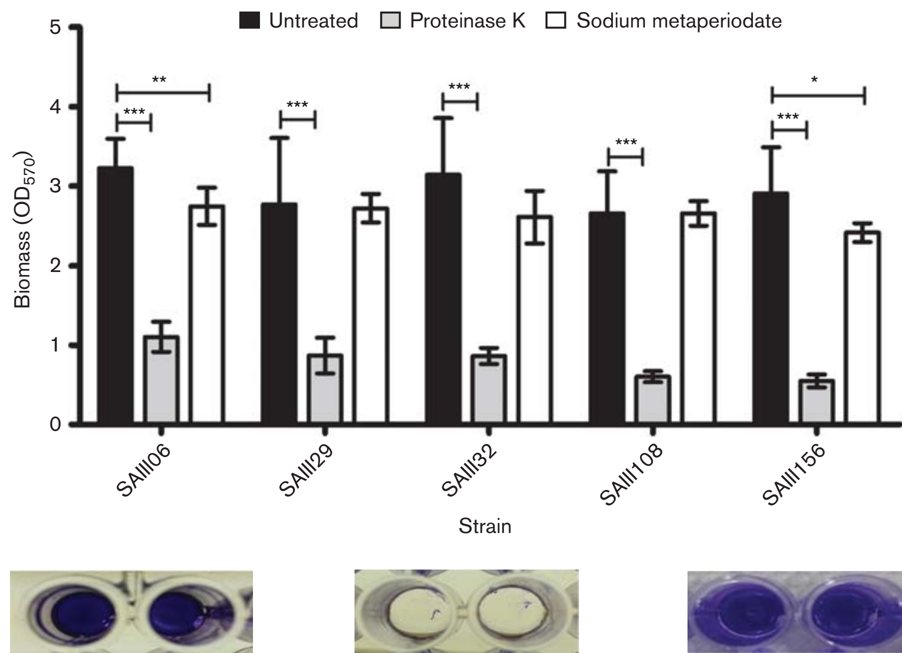

Fig. 3. Effect of proteinase K and sodium metaperiodate treatment on 24 h biofilm structures measured by crystal violet light absorbance at 570 nm (OD_{570}). The culture medium without bacterial inoculum was used as negative control (data not shown). Results represent means plus SD (error bars) of three independent experiments. ***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$ using one-way ANOVA with Bonferroni's post-hoc test or Kruskal–Wallis with Dunn's multiple comparison test.

non-producers/weak producers. These results are consistent with those of Lim *et al.* (2013), who found SCC*mec* type III as a genetic marker for the strong biofilm producers. The ability to form biofilm has been frequently observed in multidrug-resistant strains, and on this point our study differs from the literature (Kwon *et al.*, 2008; Cha *et al.*, 2013), since the MRSA strains harbouring SCC*mec* type II, also characterized as multiresistant, did not present biofilm formation as a striking feature.

The *ica*ADBC operon has often been associated with biofilm formation in species of the genus *Staphylococcus*,

Table 5. Classification of the MRSA clinical strains under study based on genotypic traits related to virulence and biofilm formation

NT, untyped.

PFGE			MLST ST	SCC*mec* type	*agr* group	Biofilm-associated genes							
Type	Subtype	Strain(s)				*icaA*	*icaD*	*fnbB*	IS*256*	*clfA*	*bbp*	*sasC*	*bap*
A	A	SAII68, SAII376	NT	II	II	+	+	−	−	+	−	+	−
	A1	SAII483	NT	II	II	+	+	−	−	+	−	+	−
	A2	SAII397	NT	II	II	+	+	−	−	+	−	+	−
	A3	SAIV333	NT	IV	II	+	+	−	−	+	−	+	−
B	B	SAII207	ST5	II	II	+	+	−	−	+	−	+	−
C	C	SAIII32	ST239	III	I	+	+	+	+	+	−	+	−
	C1	SAIII108	ST239	III	I	+	+	+	+	+	−	+	−
	C2	SAIII156	ST239	III	I	+	+	+	+	+	−	+	−
	C3	SAIII29	ST239	III	I	+	+	+	+	+	−	+	−
	C4	SAIII06	ST239	III	I	+	+	+	+	+	−	+	−
D	D	SAIV495, SAIV533, SAIV321	ST5	IV	II	+	+	−	−	+	−	+	−
E	E	SAIV70	ST5	IV	II	−	+	−	−	+	−	+	−

especially *Staphylococcus epidermidis* (O'Gara, 2007). This operon enables the production of the PIA, which mediates intercellular adherence and the accumulation of multilayer biofilms. Almost all strains were positive for *icaA* and *icaD* genes. Additionally, an assessment of the haemagglutination ability was used for indirect inference of the level of PIA expression. No strains tested were able to cause haemagglutination. To confirm these results, the strong biofilm producers were treated with proteinase K and sodium metaperiodate to determine the proteinaceous composition of the extracellular matrix. Our findings suggest that, under the experimental conditions tested, other genes may play a more significant role in biofilm formation than the *icaADBC* operon in these strains.

Considering this, we evaluated the presence of genes encoding fibronectin-binding protein B (FnBPB), biofilm-associated protein (Bap), clumping factor A (ClfA), bone sialoprotein-binding protein (Bbp) and *S. aureus* surface protein (SasC), which have been described as alternative mediators of the accumulation phase of biofilm formation or as responsible for cell adhesion to the host matrix (Cucarella *et al.*, 2001; Tristan *et al.*, 2003; O'Neill *et al.*, 2008; Schroeder *et al.*, 2009; Vergara-Irigaray *et al.*, 2009; McCourt *et al.*, 2014). The fibronectin-binding proteins, especially FnBPB, can mediate the attachment to tissue or synthetic surfaces of medical devices coated with plasma proteins, such as fibronectin (Vergara-Irigaray *et al.*, 2009; Speziale *et al.*, 2014). Once attached, staphylococcal biofilms grow by proliferation and production of a proteinaceous extracellular matrix. Our results emphasize the importance of this *ica*-independent biofilm formation pathway in the MRSA clinical strains tested, since only strong biofilm producer MRSA strains were positive for the *fnbB* gene. Concerning Bap, our findings seem to be consistent with other researchers, which have not reported this gene in any *S. aureus* isolate of human origin (Cucarella *et al.*, 2001, 2004; Vautor *et al.*, 2008). The *clfA* and *sasC* genes were widely distributed among strains without association with biofilm production, while *bbp* was absent in all tested strains.

Another aspect to be regarded when assessing the biofilm formation is the initial adhesion of the micro-organism to the surface through Van der Waals forces, hydrophobic and electrostatic interactions. These non-specific forces allow direct cell adhesion to the surface, leading to the formation of an ideal environment for the formation of the biofilm (Katsikogianni & Missirlis, 2004).

All MRSA clinical strains studied were characterized as hydrophilic ($\Delta G iwi > 0$, $\gamma^- \leq 25.5$ mJ m^{-2}) and adhered to an unmodified polystyrene surface, independently of the degree of hydrophilicity. This fact is corroborated by previous studies (Cerca *et al.*, 2005; Sousa *et al.*, 2009) and suggests that other cell surface factors, such as bacterial adhesins, can contribute to the initial adhesion process. However, when the evaluation was carried out according to the SCC*mec* type, strains harbouring SCC*mec* types III and IV were more weakly hydrophilic and adhered better than SCC*mec*II strains. The analysis of the strains according to the SCC*mec* type increased the number of replicates, which may evidence the difference in adhesion ability between the groups. Indeed, a micro-organism may adhere to a substratum via the hydrophobic effect when apolar areas on a bacterial surface interact with hydrophobic sites on the substratum (Doyle, 2000). Polystyrene has a hydrophobic nature, therefore it is understandable that the least hydrophilic group presents higher adhesion rates.

All MRSA surfaces predominantly showed electron donation, with higher values of electron donor parameter γ^- compared with the low values of the electron acceptor parameter γ^+. This polar character reinforces the theory that biological surfaces have predominantly an electron donor feature as a result of the presence of oxygen in the atmosphere and residual water of hydration (van der Mei *et al.*, 1998). The analysis by SCC*mec* type showed that the higher the γ^+ component values, the higher the numbers of adhered cells. The surfaces of polystyrene and polymers used in the manufacture of medical devices, such as acrylic and silicone, have electron donor features (Sousa *et al.*, 2009). Increased interactions between electron-donor groups of the substrata and electron-acceptor groups of cells may explain this result.

The complexity of the dissemination and persistence of MRSA in the hospital environment also involves the acquisition of resistance mobile genetic elements, which could give a selective advantage to the micro-organism over other nosocomial pathogens. As mentioned earlier, several genes have been studied, including the insertion sequence IS*256*, which seems to have an important role in increasing the resistance, mainly to aminoglycosides, and in the biofilm formation ability of healthcare-acquired MRSA strains (Lyon *et al.*, 1987). In our study, it was observed that all gentamicin-resistant strains carried the IS*256* and were also resistant to ciprofloxacin, erythromycin, clindamycin, tetracycline, rifampicin and trimethoprim/sulfamethoxazole (Fig. S2). Beyond this resistance profile, all these strains were strong biofilm producers. The association between biofilm production and the IS*256* is clearly demonstrated in studies such as those of Kwon *et al.* (2008) and Lim *et al.* (2013), who showed a high frequency of this gene in biofilm-producing strains.

Other studies have associated mutations caused by the presence of the IS*256* and the occurrence of a nonfunctional *agr* quorum-sensing system (McEvoy *et al.*, 2013). As a result, *agr*-dysfunctional strains have a tendency to form biofilms *in vitro*, which has been attributed to, among other factors, the decreased production of proteases responsible for digestion of biofilm extracellular matrix, overexpression of protein A and the increased accumulation of fibronectin-binding proteins (Toledo-Arana *et al.*, 2005; Merino *et al.*, 2009; Vergara-Irigaray *et al.*, 2009; Ferreira *et al.*, 2013). The literature differs as regards the association

between the type of *agr* and biofilm production (Cafiso *et al.*, 2007; Lim *et al.*, 2013). However, more important than the *agr* type seems to be its functionality. In this study, all strong biofilm producers belonged to the *agr* group I; however, at the present status of our experimental work, the additional IS*256* insertion within the *agr* operon remains open for investigation.

Epidemiologically, biofilm-forming MRSA infections are associated with nosocomial infections. Therefore, it is essential to investigate the spread of MRSA clones in the hospital environment. The analysis in this study showed five major pulsotypes according to the PFGE, with a large genomic diversity, as shown by the number of subtypes in each pulsotype with the SCC*mec*III strains, strong biofilm producers, classified as PFGE clone C. According to MLST results, the strains carrying SCC*mec*IV were typed as ST5 (Pediatric/USA 800 clone) and the only SCC*mec*II strain tested (SAII207) was typed as ST5 (New York/Japan clone). All strains carrying SCC*mec*III were typed as belonging to ST239, which is the profile of the BECC. Although several MRSA clones circulate in Brazilian hospitals (Rodríguez-Noriega & Seas, 2010), the BECC remains the most important Brazilian clonal complex.

Of concern is the fact that during the evolutionary process driven mainly by environmental selective pressure, the most resistant strains become even more virulent, for example, through the production of biofilm. The BECC has been shown to be widely spread throughout Brazil, South America and Europe, and its dissemination can be explained, in part, by its greater ability for adhesion and biofilm production besides the multiresistance profile (Amaral *et al.*, 2005; Rodríguez-Noriega & Seas, 2010; Costa *et al.*, 2013). However, it should be further investigated whether strong biofilm formation is a characteristic of a subpopulation that is prevalent in Brazil or whether it is a general characteristic of strains harbouring this set of genes.

Collectively, our results corroborate previous findings and contribute additional evidence suggesting that SCC*mec* type III, *agr*I, *fnbB* and IS*256* are genetic determinants associated with strong biofilm-formation in clinical MRSA strains by an *ica*-independent biofilm pathway. This study contributes to the understanding of the biofilm phenotype as an aggravating factor potentially involved in the severity and persistence of infections caused by *S. aureus* belonging to the BECC (SCC*mec*III, ST239).

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