

Biofilm formation in invasive *Staphylococcus aureus* isolates is associated with the clonal lineage

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Declaration

I, Preneshni Rochelle Naicker, hereby declare that the work contained in this assignment is my original work and that I have not previously submitted it, in its entirety or in part, at any university for a degree.

Signature:..... **Date:**.....

Biofilm formation in invasive *Staphylococcus aureus* isolates is associated with the clonal lineage

S. aureus biofilm formation and genetic background

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ABSTRACT

Objectives: The ability to form biofilms contributes significantly to the virulence of *Staphylococcus aureus*. Virulence factors may be associated with certain *S. aureus* lineages. However, the contribution of the genetic background of *S. aureus* to biofilm formation is poorly understood. This study investigated the association between the genetic background and the biofilm forming ability of clinical invasive *S. aureus* isolates. Secondary objectives included investigating any correlation with biofilm formation and methicillin resistance or the source of bacteraemia.

Methods: The study was conducted at a 1300-bed tertiary hospital in Cape Town, South Africa. *S. aureus* isolates obtained from blood cultures between January 2010 and January 2012 were included. Genotypic characterization was performed by PFGE, *spa* typing, SCC*mec* typing and MLST. Thirty genotypically unique strains were assessed for phenotypic biofilm formation with the microtitre plate assay. All isolates were tested in triplicate and an average optical density, measured at a wavelength of 490nm, was determined. The biofilm forming ability of isolates with $A_{490} > 0.17$ was considered 'weak positive' and $A_{490} > 0.34$ 'strong positive'. Isolates with $A_{490} \leq 0.17$ were considered non-adherent. ANOVA with Bonferroni-adjusted post-hoc tests and *t*-tests were used for statistical analysis of the association between biofilm forming ability and strain characteristics.

Results: Fifty seven percent of isolates were capable of forming biofilms. Weak biofilm formation occurred in 40% (n=12) and strong biofilm formation in 17% (n=5) of isolates. Thirteen (43%) of the isolates were classified as non-adherent. All 5 isolates capable of strong biofilm formation belong to one *spa* clonal complex (*spa*-CC 064). Strains from *spa*-CC 064 were capable of higher biofilm formation than other *spa* clonal complexes ($p=0.00002$). These 5 strains belonged to MLST CC5 and CC8. Biofilm formation did not correlate with methicillin resistance and was not related to the source of bacteraemia.

Conclusion: Biofilm formation correlates with the *spa* clonal lineage in our population of invasive *S. aureus* strains. High biofilm formation was associated with *spa*-CC 064. MLST CC5 and CC8 strains are capable of strong biofilm formation.

Keywords: Biofilm; *Staphylococcus aureus*; Genetic background

INTRODUCTION

Staphylococcus aureus has ensured its success as an important pathogen worldwide through its versatility, virulence factors and resistance mechanisms. Invasive *S. aureus* infections have a high mortality rate and infections with methicillin-resistant *S. aureus* (MRSA) have even poorer outcomes (Lowy, 1998; Cosgrove & Fowler, 2008). Biofilm formation is a major virulence factor of *S. aureus*.

A biofilm is ‘an assemblage of surface-associated microbial cells that is encased in an extracellular polymeric substance matrix’ (Donlan, 2002). These unique communities form on various indwelling medical devices. Bacteria embedded in a biofilm are more resistant to antimicrobials through several mechanisms. Overall antimicrobial penetration is poor, growth-dependent agents have decreased efficacy due to the slower metabolic state of the bacteria and exchange of resistance genes is easier due to the close proximity of cells. Formation of persister-cells, a subpopulation of bacteria that survive antimicrobial treatment, is also a contributing factor (Cramton & Gotz, 2004).

Due to advances in the medical field, prosthetic devices are increasingly used in patient management. Biofilms may develop on intravascular catheters, prosthetic heart valves and orthopaedic implants. They become reservoirs for persistent infections and foci for metastatic complications such as endocarditis, deep tissue abscesses, septic arthritis, and osteomyelitis (Costerton *et al.*, 1999; Chu *et al.*, 2005). Definitive management of device-related infection frequently requires removal or replacement of the prosthetic material.

The best-described mechanism of biofilm development in *S. aureus* involves the extracellular molecule polysaccharide intercellular adhesin or poly-*N*-acetylglucosamine (PIA/PNAG) (Mack *et al.*, 1996). PIA/PNAG synthesis is regulated by the intercellular adhesion (*ica*) locus (Cramton *et al.*, 1999). PIA/PNAG and *ica*-independent biofilm formation, particularly in MRSA, has also been described (Fitzpatrick *et al.*, 2005; O’Neill *et al.*, 2008). However, the relationship between biofilm formation and the genetic background of *S. aureus* is poorly understood. Different clonal lineages of *S. aureus* may have different biofilm forming capabilities. In the recent literature, differences in biofilm formation were found to be due to the staphylococcus protein A (*spa*) lineage (Atshan *et al.*, 2012a). Other studies found strong biofilm formation correlated with multilocus sequence typing (MLST) clonal complexes (Croes *et al.*, 2009) or Staphylococcal Chromosome Cassette *mec* (SCC*mec*) typing (Lim *et al.*, 2013).

This study investigated the association between the genetic background and the biofilm forming ability of clinical invasive *S. aureus* isolates. In addition, we investigated any correlation with biofilm formation and methicillin resistance or source of bacteraemia.

METHODS

Setting & Design. This was a prospective, descriptive study conducted at the National Health Laboratory Service (NHLS) Microbiology Laboratory, Tygerberg Hospital, which is a 1300-bed tertiary referral hospital in Cape Town, South Africa.

Bacterial strains. *S. aureus* isolates obtained from pure blood cultures between January 2010 and January 2012 were included. Positive blood cultures were identified using the BACTEC 9240 system (Becton Dickinson, USA). Identification of *S. aureus* was done using Mannitol Salt agar (MSA) and DNase agar plates or Vitek 2 (bioMérieux, Marcy l’Etoile, France). Kirby-Bauer disk diffusion antimicrobial susceptibility testing was performed and Vancomycin Minimum Inhibitory Concentrations (MIC) were determined using ETests (bioMérieux, France) for all MRSA isolates. Clinical and Laboratory Standards Institute (CLSI) 2010 and 2011 interpretative criteria were used. Isolates were stored on Microbeads in cryobroth at -70°C until defrosted and sub-cultured onto blood agar plates for use in this study.

Genotypic characterization. Pulsed-field gel electrophoresis (PFGE) is considered the reference standard for *S. aureus* strain typing and is the most discriminatory typing method (Deurenberg *et al.*, 2007; Oosthuysen *et al.*, 2013). The method described by McDougal *et al.* was followed (McDougal *et al.*, 2003). The clones were classified based on the number of isolates and unique PFGE types as major (> 10 isolates per PFGE type), intermediate (4-9 isolates per PFGE type) or minor (2-3 isolates per PFGE types). In addition, *spa* typing and SCC*mec* typing were also performed (Harmsen *et al.*, 2003; Milheirico *et al.*, 2007). MLST was performed on a representative set of isolates from each major PFGE cluster (Enright *et al.*, 2000).

Isolate selection. From a total collection of 208 non-repeat isolates, blood culture isolates were selected from each major, intermediary and some minor PFGE clusters. Different *spa* types within the same cluster were also selected. For MRSA isolates, isolates from the same PFGE clusters and *spa* type were only selected if they were different on SCC*mec* typing. To include more PFGE clusters, a singleton and different *spa* types, 2 isolates were included even though MLST data was not available (Table 1).

MLST CC	MLST ST	<i>spa</i> -CC	<i>spa</i> types	SCC <i>mec</i>
CC1 (4)	ST1 (4)	cc174(4)	t5471, t174, t8637, t127	-
CC5 (7)	ST5(2) ST6(2) ST461 ST2122(2)	cc002(5) cc064(2)	t002, t045 (2), t071, t570, t701, t2360	I (2)
CC8 (6)	ST8(2) ST 239(2) ST612(2)	cc064(4) cc021(2)	t1443, t1257, t1476, t008, t037(2)	III, IV(2), V, untypeable
CC12	ST12	cc160	t160	-
CC15 (3)	ST2126(3)	cc084(3)	t084, t279, t094	-
CC22 (2)	ST22(2)	cc022(2)	t891, t032	IV

CC30 (2)	ST36, ST1865	cc021(2)	t012, t318	II
CC45 (2)	ST45(2)	cc015/073(2)	t073, t015	-
CC97	ST97	cc267	t359	-
-	-	cc084	t084	-
-	-	singleton	t148	-

Table 1. Genotypic characteristics of strains *Number in parentheses (if >1)

Biofilm assay. Thirty genotypically unique strains were assessed for phenotypic biofilm formation with the microtitre plate assay as described by Christensen *et al* and modified by O'Neill *et al* (Christensen *et al.*, 1985; O'Neill *et al.*, 2007). Briefly, *S. aureus* was grown overnight in Brain Heart Infusion (BHI) broth. The culture was diluted 1:200 in fresh BHI broth. An amount of 200µL was added to a 96-well polystyrene tissue-culture treated microtitre plate (Costar® 3595, Corning Incorporated, NY, USA) and incubated at 37°C for 24 hours. After overnight incubation the wells were manually rinsed three times with Phosphate-Buffered Saline (PBS), using a hand-held pipette. The plates were air-dried at 60°C for one hour to fix the remaining cells. These were then stained with 0.4% Crystal Violet for two minutes and the plate was rinsed three times with PBS once again. The optical density was measured with a MicroELISA plate reader at a wavelength of 490nm (iMark Microplate Absorbance Reader, Bio-Rad, USA). All isolates were tested in triplicate and an average optical density was determined. A cutoff of 0.17 was used as it was three standard deviations above the mean for a clean tissue culture plate stained as above. Isolates with $A_{490} \leq 0.17$ were considered non-adherent. $A_{490} > 0.17$ was considered Weak positive and $A_{490} > 0.34$ Strong positive (twice the cutoff value). *S. epidermidis* ATCC 35984 (American Type Culture Collection, Virginia USA) and Brain Heart Infusion broth were used as positive and negative controls respectively.

Clinical data. Clinical information was obtained by clinical consultation with the attending doctor as part of the routine microbiology service for all positive blood cultures. Informed consent was obtained from patients and clinical data was stored in a database with study numbers used for patient confidentiality. Folder reviews were conducted for select cases where insufficient clinical information was gathered. The source of bacteraemia was determined according to the clinical presentation, radiological features and bacteriological factors i.e. the isolation of *S. aureus* from specimens from the site of infection (Orth *et al.*, 2013). The categories were defined as follows: skin and soft tissue (SSTI) was the clinical presentation of soft tissue inflammation together with bacteraemia. Catheter-related blood stream infection (CRBSI) was determined by the culture of *S. aureus* from the catheter tip ($>10^2$ cfu) and/or clinical evidence of catheter related infection. Pneumonia (PNEUM) was the isolation of *S. aureus* from the respiratory tract with compatible radiological features. Modified Duke's criteria was used to define infective endocarditis (IE). Bone and joint (B&J) was clinical and radiological features of osteomyelitis or septic arthritis. Vascular (VASC) was clinical evidence of endovascular infection and intra-abdominal (IA) was defined as clinical and radiological evidence of deep-seated intra-abdominal source with *S. aureus* cultured from the site (Liao *et al.*, 2008).

Statistical analysis. ANOVA with Bonferroni-adjusted post-hoc tests and *t*-tests were used for statistical analysis (Statistica version 10, 2011). The biofilm forming ability was analyzed by methicillin resistance, source of bacteraemia, *spa*, *SCCmec* and MLST type. Statistical significance was defined as $p < 0.05$.

Ethics. This study received ethical approval from the Division of Research Development and Support, Stellenbosch University (N09/01/012).

RESULTS

A. Description of isolates

The 30 isolates selected comprised of 19 PFGE clusters, with 28 *spa* types and 15 MLST sequence types (ST). These were from 9 *spa*-clonal complexes (*spa*-CC) and 9 MLST clonal complexes (MLST CC) (Table 2).

MLST CC	MLST ST	spa-CC	spa type	PFGE cluster	SCCmec (for MRSA)	Clinical*	Average OD	Biofilm
1	1	cc174	t127	Z		SSTI	0.19	WEAK
1	1	cc174	t5471	Z		CRBSI	0.22	WEAK
1	1	cc174	t174	AF		SSTI	0.14	NON
1	1	cc174	t8637	AF		B&J	0.19	WEAK
5	5	cc002	t045	M	I	CRBSI	0.15	NON
5	5	cc002	t570	Y		B&J	0.12	NON
5	6	cc064	t2360	AE		CRBSI	1.06	STRONG
5	6	cc064	t701	AE		CRBSI	0.39	STRONG
5	461	cc002	t045	N	I	CRBSI	0.1	NON
5	2122	cc002	t002	W		SSTI	0.2	WEAK
5	2122	cc002	t071	W		VASC	0.18	WEAK
8	8	cc064	t1476	Q		VASC	0.51	STRONG
8	8	cc064	t008	O	V	SSTI	0.13	NON
8	239	cc021	t037	U	III	SSTI	0.18	WEAK
8	239	cc021	t037	U	untypeable	PNEUM	0.12	NON
8	612	cc064	t1443	S	IV	CRBSI	0.39	STRONG
8	612	cc064	t1257	S	IV	SSTI	0.51	STRONG
12	12	cc160	t160	AB		PNEUM	0.2	WEAK
15	2126	cc084	t084	AA		CRBSI	0.12	NON
15	2126	cc084	t279	AA		IE	0.25	WEAK
15	2126	cc084	t094	AA		SSTI	0.12	NON
22	22	cc022	t891	G		SSTI	0.18	WEAK
22	22	cc022	t032	G	IV	IA	0.26	WEAK
30	36	cc021	t012	B	II	SSTI	0.19	WEAK
30	1865	cc021	t318	A		IE	0.15	NON
45	45	cc015/073	t073	D		IA	0.12	NON
45	45	cc015/073	t015	D		SSTI	0.09	NON

97	97	cc267	t359	P		CRBSI	0.13	NON
		cc084	t084	SINGL [#]		CRBSI	0.3	WEAK
		SINGL [#]	t148	I		PNEUM	0.1	NON

Table 2: Genotypic characterization and biofilm formation

*(SSTI = skin and soft tissue; CRBSI = catheter-related blood-stream infections; PNEUM = pneumonia; B&J = bone and joint;

IA = intra-abdominal; VASC = vascular; IE = infective endocarditis)

[#]Singleton

There were 9 MRSA (30%) and 21 methicillin-sensitive *S. aureus* (MSSA) (70%). The source of infection was determined for all the *S. aureus* bacteraemias (Figure 1). Ten originated from skin and soft tissue infections (SSTI), 9 from catheter-related blood-stream infections (CRBSI), 3 pneumonias (PNEUM), and 2 each from infective endocarditis (IE), bone and joint (B&J), vascular (VASC) and intra-abdominal (IA).

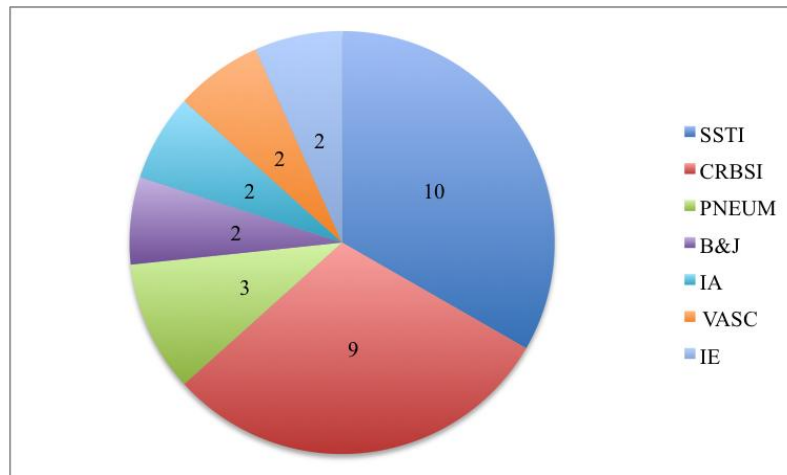


Figure 1. Source of bacteraemia

(SSTI = skin and soft tissue; CRBSI = catheter-related blood-stream infections; PNEUM = pneumonia; B&J = bone and joint; IA = intra-abdominal; VASC = vascular; IE = infective endocarditis)

Biofilm formation occurred in 57% of isolates (Figure 2). Weak biofilm formation occurred in 40% (n=12) and strong biofilm formation in 17% (n=5) of isolates. Thirteen isolates were classified as non-adherent. The reproducibility of the assay was excellent.

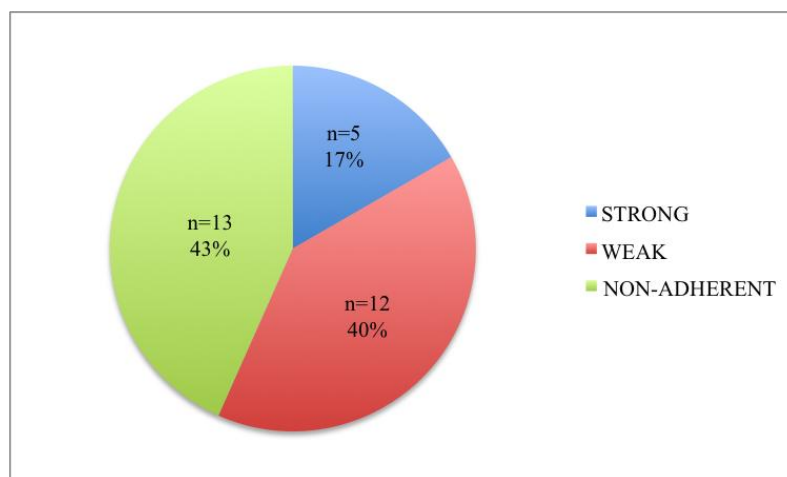


Figure 2. Biofilm categories

B. Analysis by genetic background.

i) *spa* typing

The *spa* types t701, t1275, t1443, t1476 and t2360 were strong biofilm producers. There were too many individual *spa* types for further analysis.

All 5 isolates capable of strong biofilm formation belong to one *spa* clonal complex (*spa*-CC 064). Strains from *spa*-CC 064 were capable of higher biofilm formation than all other *spa* clonal complexes ($p=0.00002$) (Figure 3).

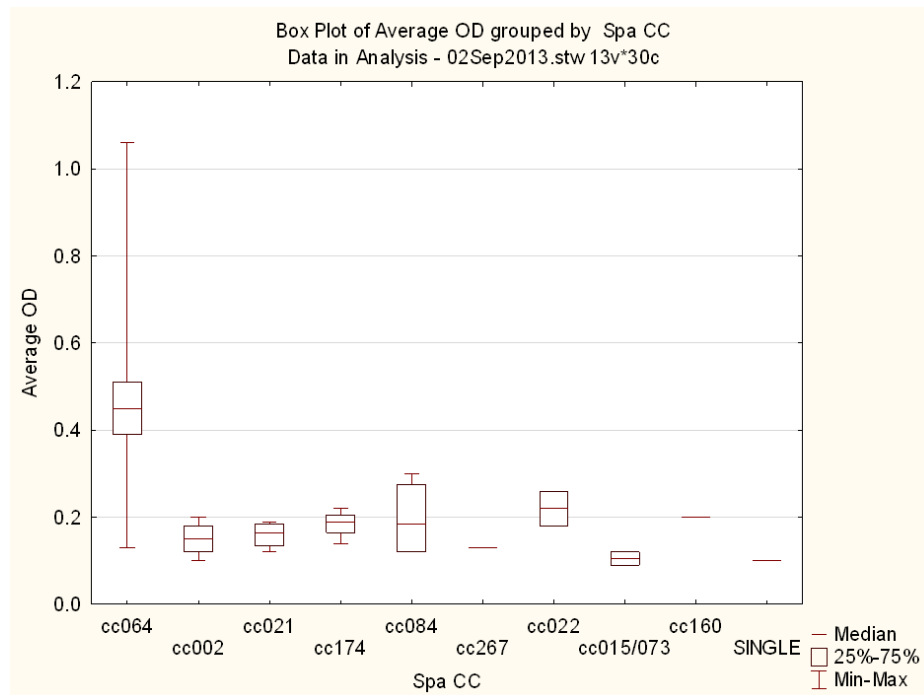


Figure 3. Optical density and *spa*-CC

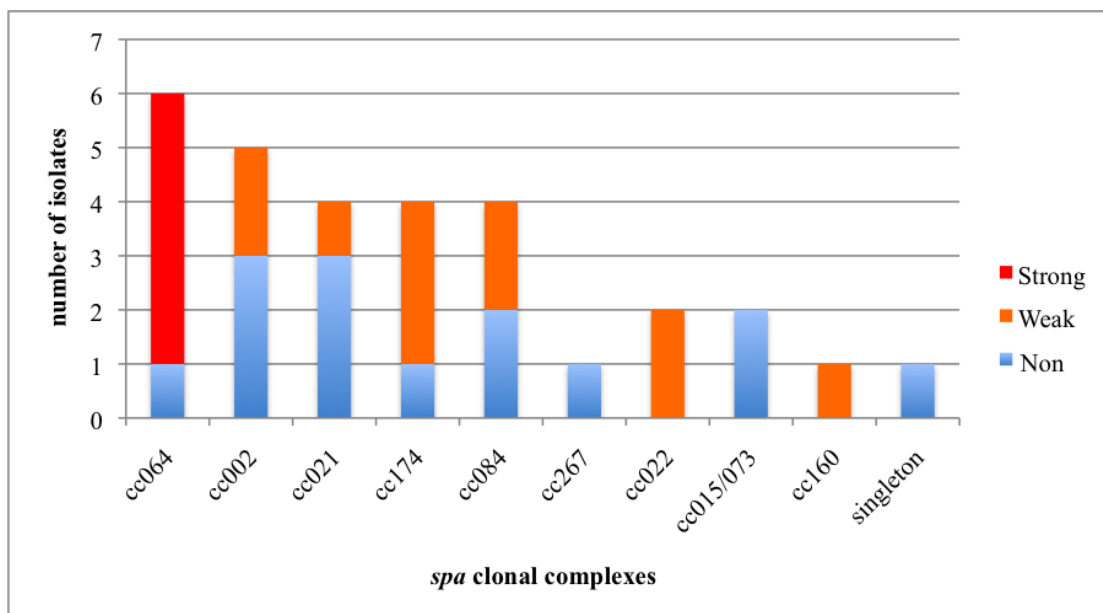


Figure 4. Biofilm categories and *spa*-CC

ii) MLST

MLST ST6, ST8 and ST612 produced strong biofilms. Generally the category of biofilm production correlated with the MLST sequence types.

Strains from MLST CC 1, 5, 8, 12, 15, 22 and 30 were capable of biofilm formation. Although there was no difference found with biofilm formation and MLST CC ($p=0.89$), MLST CC5 and CC8 isolates had a higher biofilm forming capacity. The 5 strong biofilm producers (all from *spa*-CC064) fell into MLST CC5 and CC8 (Figure 5 & 6).

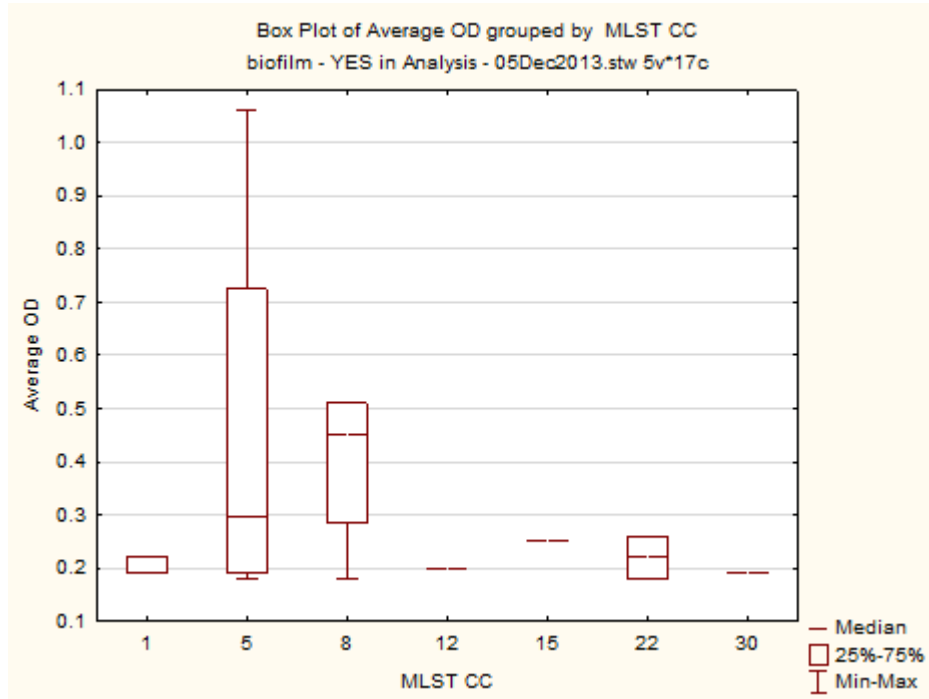


Figure 5. Average OD and MLST CC

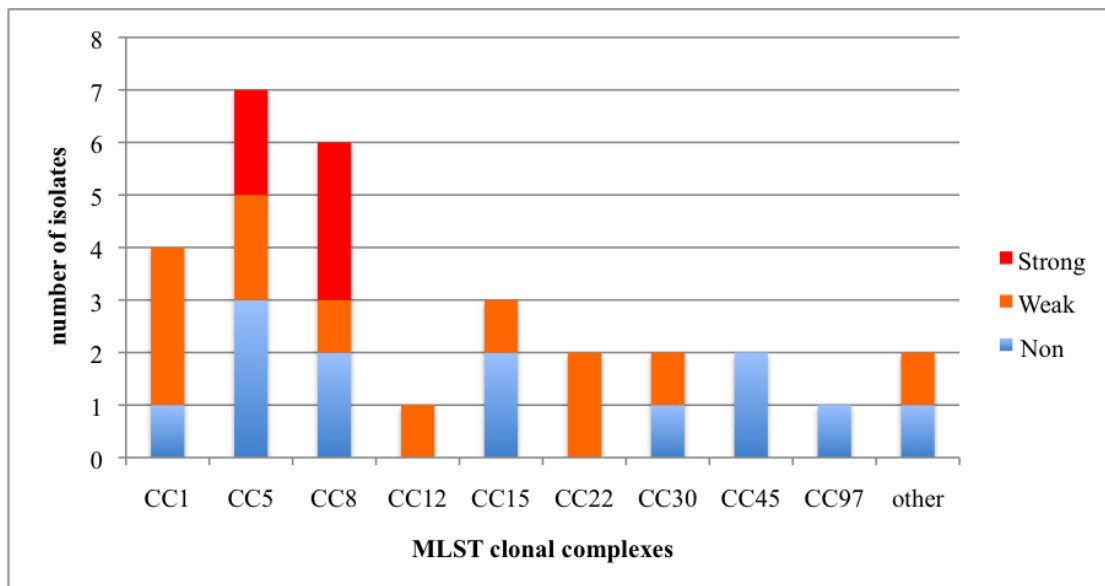


Figure 6. Biofilm categories by MLST CC

iii) SCC*mec* typing

There were only 9 MRSA isolates for SCC*mec* typing and further analysis could not be performed.

iv) PFGE

As PFGE strain typing was the basis of selection of isolates, biofilm formation could not be analysed by PFGE clusters due to the large variation.

C. Analysis by methicillin resistance

There were no significant differences observed in biofilm formation of MRSA isolates compared with MSSA isolates ($p=0.9$) (Figure 7 & 8).

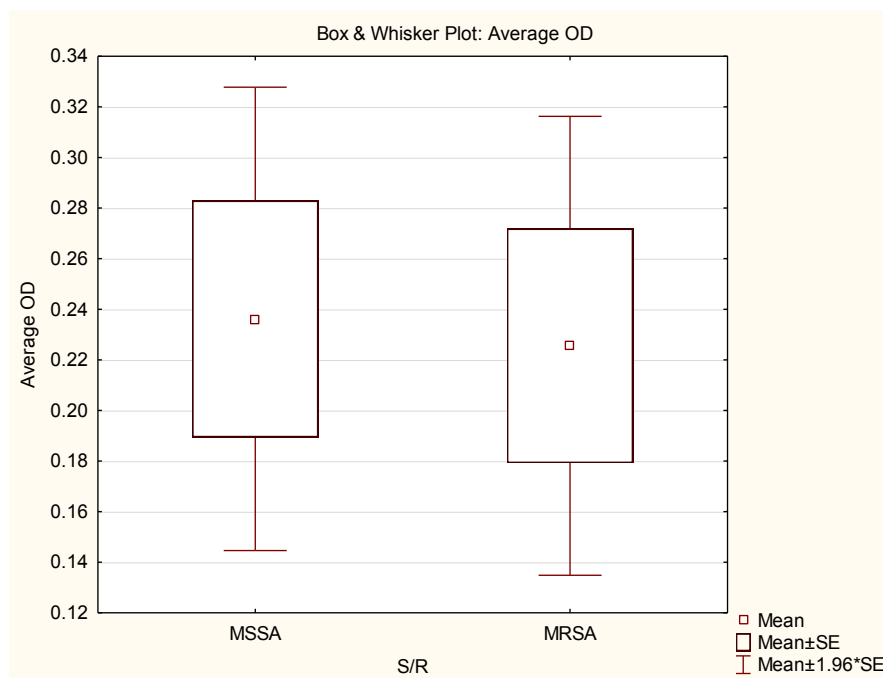


Figure 7. Optical density for MRSA and MSSA isolates

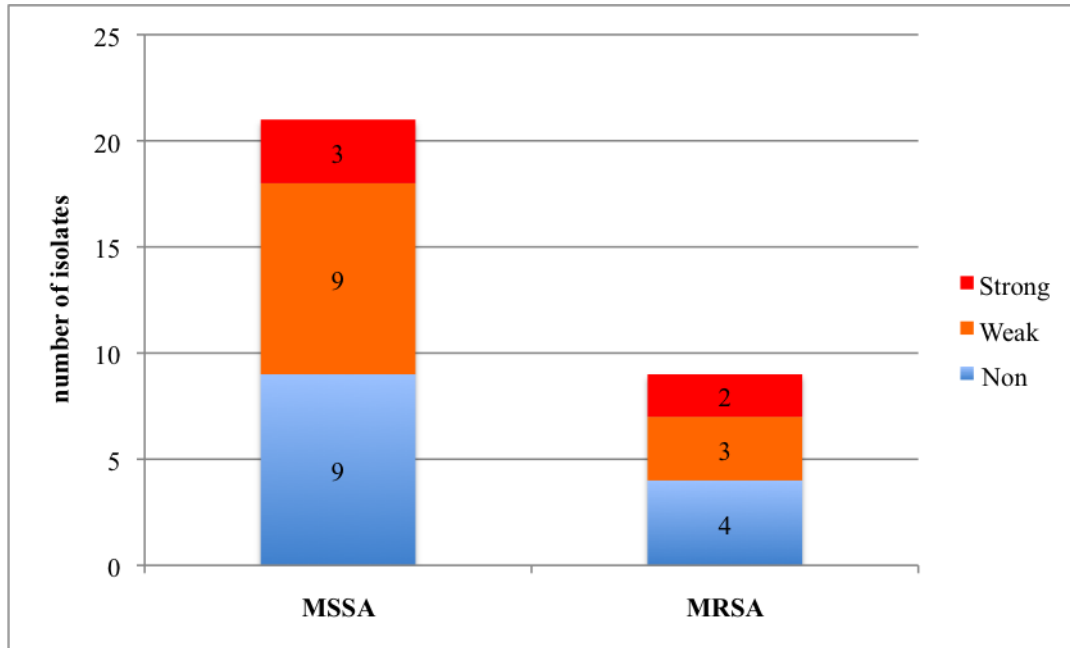


Figure 8. Biofilm categories by Methicillin resistance

D. Analysis by source of bacteraemia

There were no differences in biofilm formation relating to the source of bacteraemia ($p=0.64$). However, as clinically expected, it was observed that VASC and CRBSI infections had higher median optical densities. The sources of bacteraemia for strong biofilm producers were CRBSI, SSTI and VASC (Figure 9 & 10).

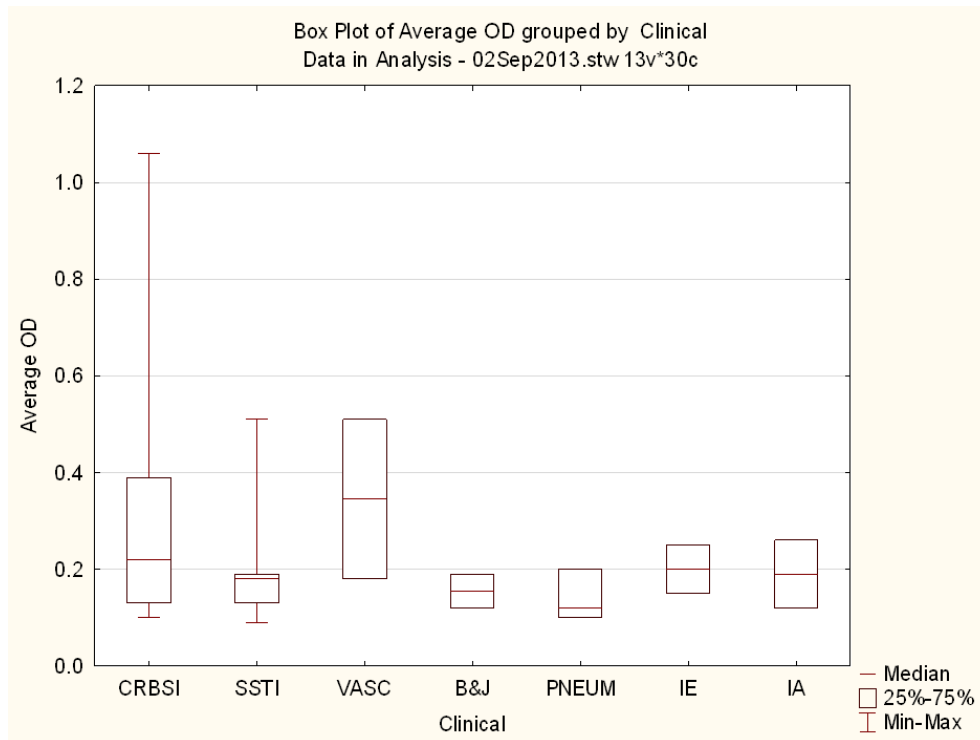


Figure 9. Optical density and source of bacteraemia

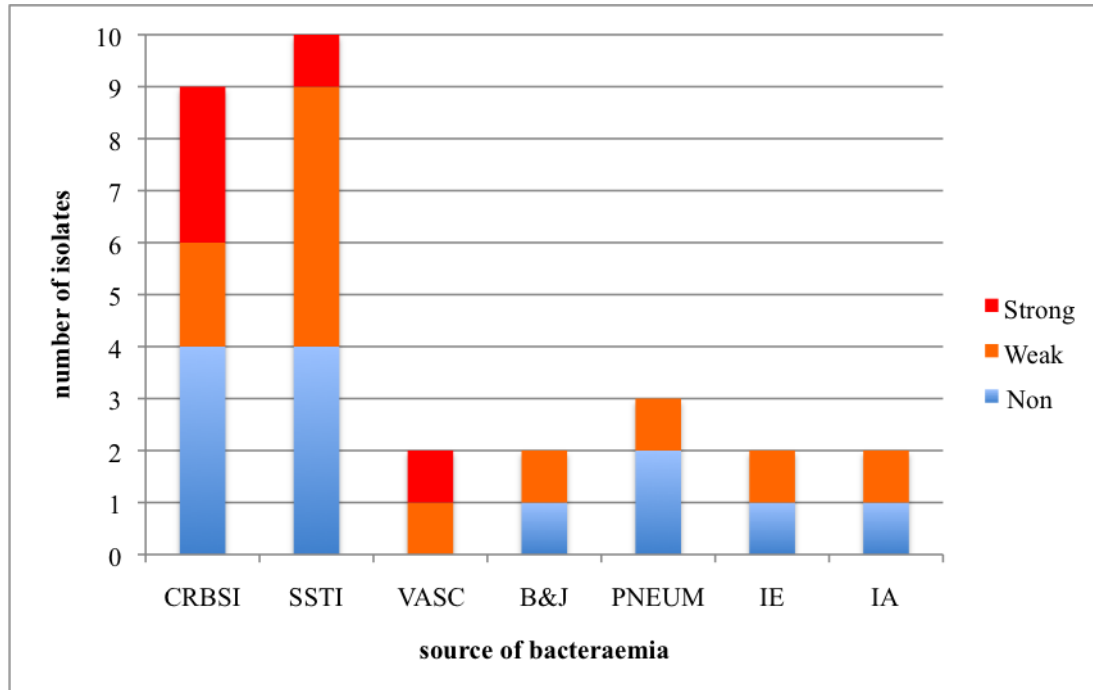


Figure 10. Biofilm categories and source of bacteraemia

DISCUSSION

Biofilm-associated infections caused by *S. aureus* are a significant cause of morbidity and mortality (Archer *et al.*, 2011). In this study strong biofilm formation was associated with strains from *spa*-CC 064 and MLST CC 5 and 8. Although MLST CC8 has now been incorporated into MLST CC5, it is considered separately in this analysis (Oosthuysen *et al.*, 2013). As MLST CC8 is now incorporated into MLST CC5, MLST CC5 may be associated with high biofilm formation. This suggests that certain clones are more prone to biofilm formation and that *spa*-typing and MLST may be a predictor of biofilm formation.

S. aureus demonstrates predominantly clonal evolution. Virulence factors may be associated with specific *S. aureus* lineages. Certain virulence factors such as collagen adhesion (CNA) and toxic shock syndrome toxin 1 (TSST1) are known to be associated with certain clonal lineages (Deurenberg *et al.*, 2009). Therefore some clones are considered more virulent than others (Melles *et al.*, 2004). Biofilm formation is also a major virulence factor and several other observations have supported the link between the clonal lineage and biofilm formation.

Atshan *et al.* found that strains from the same *spa* type had similar adherence properties on the microtitre plate assay. In another study by the same group, MRSA strains from the same MLST, *spa* and SCC*mec* type had similar biofilm forming abilities, however there was more variation by *spa* type. In contrast to our findings, they found *spa* type t037 (ST239-CC8-IIIa) strains form strong biofilms (Atshan *et al.*, 2012a,b). Croes *et al.* suggested that MLST CC8 was a predisposing factor for a strain to produce strong biofilms (Croes *et al.*, 2009). Furthermore, the Brazilian clone (also MLST CC8) was shown to have increased adherence (Amaral *et al.*, 2005). The

EMRSA-15 clone in Scotland was found to form stronger biofilms than EMRSA-16 isolates (Smith *et al.*, 2008).

In two South African academic hospitals it was shown that MRSA bacteraemia had a higher mortality rate than MSSA (Perovic *et al.*, 2006). The prevalence of MRSA in bacteraemia at Tygerberg hospital has been reported to be 30% (Orth *et al.*, 2013), which is reflected in the percentage of MRSA in the collection of strains that we tested. Certain pandemic MRSA clones are spread worldwide. Recent publications describe the ST612-MRSA-IV clone (MLST CC8) as the most widespread MRSA clone in Cape Town, and South Africa (Jansen van Rensburg *et al.*, 2011; Orth *et al.*, 2013; Oosthuysen *et al.*, 2013; Moodley *et al.*, 2010). In this study ST 612 formed strong biofilms, which may contribute to its dominance in nosocomial infections in South Africa.

Some studies found SCCmec typing a predictor of biofilm formation. SCCmec IV isolates were capable of higher biofilm formation (Kwon *et al.*, 2008). In contrast to the findings by Lim *et al.* where SCCmec III was found to be a genetic risk factor for strong biofilm formation (Lim *et al.*, 2013), we found SCCmec IV isolates produced the strongest biofilms (only three isolates tested). In our study the one SCCmec III isolate did form a weak biofilm. The differences found could also be attributed to the biofilm assay methodology. A larger collection of MRSA isolates should be tested to make any meaningful conclusions.

Kwon *et al.* demonstrated that MRSA clinical isolates had a greater likelihood of developing biofilms. Approximately 37.9% of the 66 MRSA formed biofilms compared to 14.3% of the 35 MSSA tested ($p < 0.05$) (Kwon *et al.*, 2008). Other studies showed no difference in biofilm formation between MRSA and MSSA isolates (Smith *et al.*, 2008; Indrawattana *et al.*, 2013). In our study there was also no difference in biofilm formation between MRSA and MSSA isolates, although the mean optical density for MSSA was higher. Atshan *et al.* also found MSSA clones had slightly higher biofilm formation than MRSA. Croes *et al.* found that MRSA or MSSA with an MRSA associated CC, were more capable of strong biofilm formation in the presence of 0.1% glucose. Our study did not supplement glucose which may have affected the MRSA biofilm capabilities. Our selection criteria was based on the genotypic classification which is also a limiting factor as some clones may be associated with methicillin-resistance (Deurenberg *et al.*, 2007).

Several studies have investigated the source of isolates as a predictor of biofilm formation. Isolates from blood cultures showed a higher frequency of biofilm formation compared to isolates from other sites (Kwon *et al.*, 2008). In the study by Smith *et al.*, the isolates derived from skin had a greater ability to form fully established biofilms (Smith *et al.*, 2008). In our study, the source of bacteraemia as established by clinical data for *S. aureus* blood-stream infections was not a predictor of biofilm formation. However, we only tested blood culture isolates, these being the most clinically important specimens, and did not collect isolates from the source of infection.

The variations in biofilm capacity may be due to differences in surface proteins or gene expression in different *S. aureus* clonal lineages. Microarray analysis showed variation between different lineages is, in particular, due to surface adhesion genes

and their regulators. However there were no specific genes to differentiate carriage versus invasive isolates (Lindsay *et al.*, 2006). Kuhn *et al.* also found no difference with adhesion genes in epidemic and sporadic MRSA clones (Kuhn *et al.*, 2006). Gene expression studies and whole genome sequencing may assist to elucidate the reason for these differences (Costa *et al.*, 2013).

The strength of this study was that biofilm formation was tested on a collection of genotypically well characterized strains, all of them isolated from blood cultures. They were selected to be genetically diverse. PFGE, the gold standard for *S. aureus* strain typing, was the basis of the selection to ensure that we did not test the same strain as outbreaks of MRSA may occur in hospital settings. Strains within the same PFGE cluster were chosen if they were different *spa* or *SCCmec* types. The limitations were the small numbers included. By testing more isolates we could have also assessed if certain *spa*-types are more prone to biofilm formation. Another limiting factor was not using supplementary glucose in the biofilm assay. This may influence the MRSA biofilm formation (Croes *et al.*, 2009; O'Neill *et al.*, 2007). Although we did analyse our collection by methicillin resistance and source of bacteraemia, these were secondary objectives. The limitation of selecting isolates based on their genetic background is that conclusions cannot be made about methicillin resistance and source of bacteraemia. Further studies should be conducted with different selection criteria to specifically address these questions. Further investigations for the presence of adhesion genes, gene expression and accessory gene regulator (*agr*) groups are required.

Biofilm formation correlates with genetic background in our population of invasive *S. aureus* strains. The propensity to form biofilms may be linked to the epidemic potential of certain successful clones of *S. aureus*. The *spa* lineage may serve as a genetic predictor of biofilm formation. MLST can also be used to determine clones with higher biofilm formation. Further studies are required to identify these clones with high biofilm formation and investigate the prevalence and expression of adhesion genes.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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ADDENDUM

Declaration of contribution to laboratory work

The biofilm assay was performed by Preneshni Naicker.

Clinical data collection and folder reviews were conducted by Preneshni Naicker.

PFGE, MLST, *spa* typing and SCC*mec* typing was performed by Karayem Karayem.

