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Biofilm formation by *Staphylococcus aureus* clinical isolates correlates with the infection type

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Abstract

Background: Biofilms are involved in many *Staphylococcus aureus* infections. Relation of biofilm forming *S. aureus* strains and the infection types or the clinical outcomes remain unclear.

Methods: We measured biofilm formation, with a microtiter plate assay, of a collection of methicillin-sensitive clinical isolates from 159 invasive *S. aureus* infections, encompassing all cases occurring within a hospital catchment area during two years, and of additional 49 non-invasive skin infection isolates from the same region. These results were related to available clinical and microbiological documentation.

Results: Isolates from medical device infections (intravenous line-associated and prosthetic joint infections), as well as isolates from superficial skin infections, were particularly proficient in forming biofilms. No increased biofilm-forming capacity was seen in isolates from endocarditis, osteomyelitis, or from other infections. There was also a correlation of biofilm formation with the *agr* type of the isolates. Thicker biofilms appeared to be more resistant to antibiotic treatment *in vitro*. No correlation between biofilm formation and clinical outcomes was noted.

Conclusions: *S. aureus* isolates from 'classical' biofilm-related infections, but also from superficial skin infections, are especially proficient in forming biofilms. There is, however, no obvious relation of biofilm-forming capacity of isolates and the clinical outcome of the infection, and more studies on this issue are needed.

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Disclosure of interest

The authors declare no conflicts of interest.

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Keywords

biofilm; *Staphylococcus aureus*; clinical outcome; skin; antibiotic sensitivity; *agr*

Introduction

Many *Staphylococcus aureus* infections involve formation of biofilms, that is, sessile communities of bacteria attached to surfaces and encased in extracellular matrix [1]. Staphylococcal biofilms appear on implantable medical devices (catheters, prosthetic joints, implants, etc.), but also on host tissues in biofilm-like infections of chronic wounds, endocarditis, or osteomyelitis [1]. As biofilms are resistant to host immune system and antibiotics, they contribute to the persistent and hard-to-treat character of staphylococcal diseases [1].

Despite the importance of biofilms, systematic research on the biofilm-forming capacity of *S. aureus* clinical isolates from human infections is limited [2]. Several studies investigated correlations of disease types and biofilm formation [3, 4, 5, 6, 7, 8, 9, 10, 11], but these usually compared isolates from only two different infection types, and frequently involved only methicillin-resistant *S. aureus* (MRSA). Moreover, almost nothing is known about the correlation of the clinical outcomes with the biofilm forming capacity of the infecting isolates.

In this study, we examined *in vitro* biofilm formation by methicillin-sensitive *S. aureus* (MSSA) clinical isolates from a wide range of invasive infections, and demonstrated that it correlates with the infection type, but not with the clinical outcome.

Materials and methods

A previously described collection of 159 *S. aureus* isolates from invasive infections (that is, from blood or other normally sterile body sites) treated in Skaraborg Hospital, Sweden, in 2003–2005, encompassing all cases occurring within the hospital catchment area, was used [12, 13, 14, 15]. Additional 49 isolates from non-invasive (superficial) skin infections were collected in the same region in 2011 [14]. All collected isolates were MSSA.

Biofilm formation by isolates in tryptic soy broth (TSA) supplemented with 0.25 % w/v glucose after 24 h at 37°C in wells of 96-well cell culture plate (Sarstedt, Germany) was determined in triplicates using classic crystal violet staining biofilm assay [16], and expressed as the mean absorbance at 570 nm for each isolate (A_{570}). Before the experiment, plates were coated overnight at 4°C with 20 % v/v human plasma in PBS. In case of invasive isolates, biofilm formation values were correlated with the previously collected and published data on their *agr* type, occurrence of the *tst* gene, and the clinical data of the infected patients [12, 13]. Results were analyzed using the Mann-Whitney U test in SPSS (v.22, IBM Corporation, USA). The study was approved by the Ethical Board of Gothenburg.

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To measure sensitivity of biofilms to antibiotics, the 24 h established biofilms were washed with PBS, and the wells were filled with TSA with 512 mg/ml of rifampicin, and incubated for additional 24 h at 37°C. Afterwards, viability of the biofilms was measured with the XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) reduction assay, and expressed as % viability of the control untreated biofilms [17]. Viability differences were analyzed using the unpaired t test in Prism (v.7, GraphPad, USA).

Results and discussion

Infection type

Most *S. aureus* isolates can form biofilms *in vitro* [2, 5], yet it remains unclear how this ability correlates with clinical infection. Our study, encompassing isolates from all cases of invasive *S. aureus* infections in the hospital catchment area (all of them MSSA, as MRSA have a very low prevalence in Sweden [18]), found a clear correlation of *in vitro* biofilm formation with certain infection types (Table 1). Nearly all isolates showed some capacity to form biofilms, but isolates from ‘classical’ biofilm-related infections (line-associated infections and infected joint prostheses) formed biofilms significantly better than the other invasive isolates. Analyzed separately, isolates from line-associated infections still formed significantly better biofilms ($p=0.004$), and while the number of isolates from infected joint prostheses was too small to reach a significant difference (n=5, $p=0.3$), their mean biofilm formation was notably high.

Previous studies, restricted to MRSA only, showed better biofilm formation by isolates from urinary catheters compared to other urinary tract infections [3], and by isolates from device-related orthopedic infections compared to non-device orthopedic infections [4]. Other studies, however, did not find correlation of biofilm formation with isolates coming from implant-related or unrelated orthopedic infection [6], from catheter-related bacteraemia or nasal colonization [8], or from bloodstream infections with different infection foci [10]. Unlike these studies, comparing only two or three selected groups of isolates, our study included all invasive *S. aureus* isolates from a single hospital. This unbiased approach confirmed previous assumption that isolates from ‘classical’ biofilm-related infections have increased propensity to form biofilms. It remains to be explained if good biofilm forming strains preferably cause biofilm-related infections, or if their ability to form biofilm increases while bacteria adapt to the new environment during infection. Indeed, longitudinal observations (though limited to only 3 patients) suggested that adaptation of *S. aureus* to biofilm lifestyle might sometimes occur in the course of a chronic infection [19].

We observed no statistically significant differences in biofilm formation for isolates from bacteraemia without focus, invasive soft tissue infections, native joint septic arthritis, endovascular infections, or osteomyelitis (Table 1). A marked trend for increased biofilm formation was observed in isolates from vertebral osteomyelitis, probably not reaching significance due to the small number of cases (n=6, $p=0.1$).

Our finding of no differences for osteomyelitis and endovascular isolates might be surprising, as endovascular infections and osteomyelitis are thought to involve biofilms as part of their pathogenesis [1], and as osteomyelitis isolates were previously noted to form

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more biofilm than sepsis or colonizing isolates [9]. This could be due to several factors. First, the lack of significant differences might be due to the small sample size, especially in the case of vertebral osteomyelitis. Second, biofilm formation is only a part of these diseases' pathology, and it might be overshadowed by other properties, such as the ability to persist intracellularly in osteomyelitis [9] or to clump during endovascular infections [20]. Finally, the conditions used for biofilm formation assay can affect the outcome, and it remains unclear how *in vitro* conditions relate to various conditions *in vivo* [5]. Measurements of biofilm formation on plasma-coated plastic surfaces – as used in this study – is probably representative for biofilm formation on artificial surface of catheters and implants, but not for biofilms forming inside the bone matrix or in the clot on a damaged vascular wall, as it happens in osteomyelitis and endovascular infections.

Previous studies noted good biofilm formation by isolates from skin infections [5, 7, 21]. Also in our study, isolates from superficial, non-invasive skin infections formed significantly better biofilms than the invasive isolates ($p=0.031$; Table 1). This suggests that biofilm formation might be important for *S. aureus* skin colonization and for superficial skin infections, but not for promotion of deeper invasion or systemic dissemination. One could even speculate that biofilm formation on skin surface and invasion into deeper tissues are two distinct behaviors controlled by opposite factors – as was shown for example for staphylokinase, which promotes penetration into the skin, but decreases biofilm formation [14, 15].

S. aureus genotype

While some studies showed correlation of biofilm formation with the *agr* types [4, 6, 22], others did not [10, 23]. In our sample, isolates with the *agr* type I had smaller ($p=0.003$) and isolates from the type III had larger ($p<0.001$) capacity to form biofilms than the isolates from other *agr* types (Table 2). The possibility that different *agr* types regulate biofilm formation in different ways warrants further attention. More likely, however, the observed differences barely represent different distribution of *agr* types amongst local clonal lineages, as *S. aureus* clonal lineages are known to differ in biofilm formation [11, 23, 24, 25, 26].

The same reasoning as for *agr* types applies also to isolates with or without the *tst* gene. When biofilm formation was compared between isolates harbouring the *tst* gene (n=29; $OD_{570}=2.6\pm0.2$) or not (n=130; $OD_{570}=1.2\pm0.1$), the *tst*-positive isolates formed better biofilms ($p=0.001$). The *tst* might therefore be a marker of genetic lineages with increased biofilm formation.

Biofilm sensitivity to antibiotics

Biofilms are considered resistant to antibiotics [1]. When we measured viability of biofilms formed by three representative strains of the 'strong' and 'weak' biofilms formers (Figure 1a) after exposure to high concentration of rifampicin (frequently used in combination therapy of *S. aureus* biofilms [1]), we indeed observed that rifampicin failed to completely eradicate the biofilms. The decline in viability was, however, significantly more pronounced in weak biofilm formers ($p=0.03$, Figure 1b), suggesting that differences in biofilm formation observed between our clinical isolates might translate to altered tolerance to

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antimicrobial treatment. This prompted us to investigate if biofilm formation in clinical isolates correspond to clinical outcomes.

Clinical outcomes

In some studies, MRSA isolates from persistent bacteraemia formed better biofilms than from resolving bacteraemia [27], and patients infected with strong biofilm producing MRSA had higher re-admission rate, but lower mortality rate [24]. Other large size studies, however, failed to find any correlation between biofilm formation and disease outcome in *S. aureus* bloodstream infections [10, 28]. In our comprehensive MSSA sample, no clear correlation of patient characteristics or disease outcomes and biofilm forming capacity was observed. There were no significant differences in biofilm formation depending on sex, nosocomial or community acquired infection, nor presence or absence of any of the comorbidities (diabetes mellitus, rheumatoid arthritis, atopic dermatitis, psoriasis, renal disease, heart disease). The only difference was that isolates from patients with history of previous invasive *S. aureus* infection showed decreased biofilm formation compared to other isolates (previous infection n=12, OD_{570}=1.2±0.3; no previous infection n=130, OD_{570}=2.0±0.1; p=0.038). Biofilm formation also had no statistical impact on any of the disease outcomes (development of complicated bacteraemia or severe sepsis, 28-day mortality, disease recurrence, presence of residual symptoms).

The lack of correlation of disease outcome with *in vitro* biofilm formation is probably not surprising, considering that differences in biofilm formation observed between the isolates were small, and that the invasive infections differ from each other in their severity and prognosis. Microplate biofilm assays, as used in this study, have a good correlation with *in vivo* mouse catheter biofilm models [29], but differences observed between the groups in our study might be too small to translate to meaningful differences in the clinical outcome. Notably, studies reporting significant correlations between biofilm formation and clinical outcome also reported more pronounced differences in biofilm formation between isolates [24, 27]. Moreover, biofilm-related infections differ greatly in respect to prognosis and ease of treatment. This variation might overshadow the clinical impact of differences in biofilm formation. It was also recently suggested that the importance of biofilm formation for the clinical outcome depends on the genotype of the infecting strain [30], what introduces yet another confounding factor. Therefore, future studies aiming at investigating the impact of biofilm formation on disease outcome should preferably use large number of isolates, stratified based on their clonal lineages and infection types.

Summary

This study observed that good biofilm forming *S. aureus* isolates are predominantly associated with ‘classical’ biofilm-related infections (intravenous line-associated infections and prosthetic joints infections), and with superficial skin infections. Correlations with genotype and history of previous invasive *S. aureus* infection were also observed, but not with the disease outcome. These findings indicate a need for future studies to decipher the clinical impact of the capacity to form biofilms.

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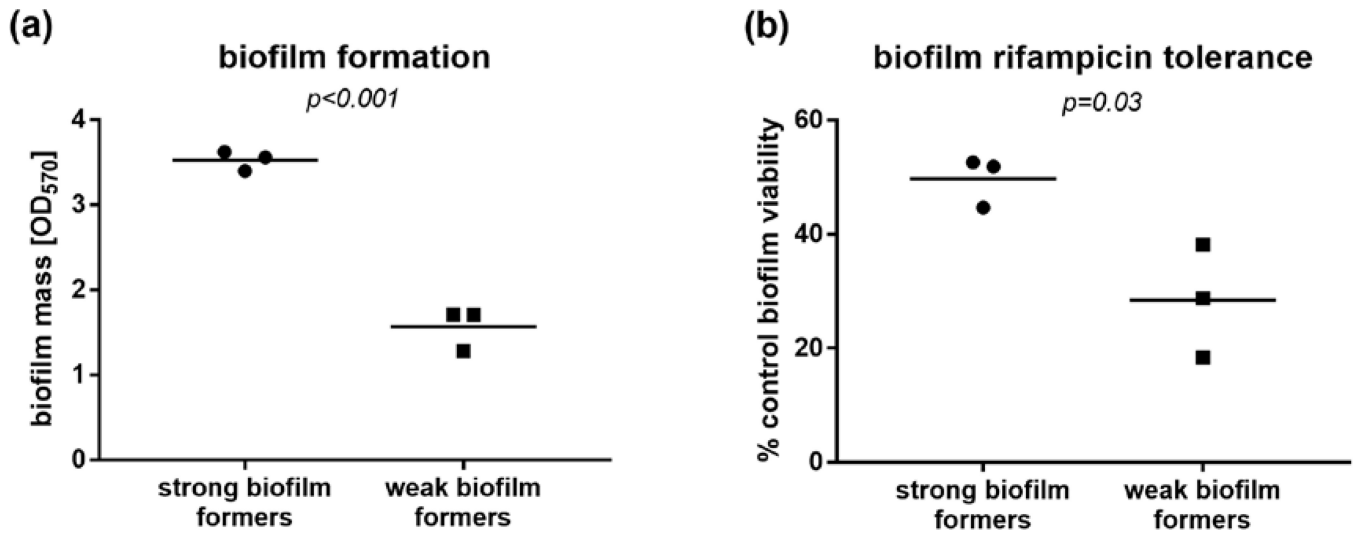

Figure 1.

Biofilm formation in the in 96-well plate crystal violet assay (a) and viability after 24 h exposure to rifampicin, measured with XTT assay (b) of representative ‘strong’ and ‘weak’ biofilm-forming *S. aureus* strains from the invasive isolate collection. *P* values calculated with the unpaired t test.

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Table 1.

Correlation of infection type and biofilm formation in collection of *S. aureus* clinical isolates. Biofilm formation was measured as A_{570}. Mann-Whitney U test was used for statistical comparisons. $p>0.05$ was considered non-significant (n.s.).

infection type	n	Biofilm formation [mean ± SEM]	*p* compared to other invasive isolates
all invasive	159	1.9 ± 0.1	-
bacteraemia without focus	31	1.7±0.2	n.s.
invasive skin and soft tissue	37	1.9 ± 0.2	n.s.
biofilm – related:	27	2.5 ± 0.2	0.002
- line-associated	22	2.6 ± 0.2	0.004
- prosthetic joint	2	2.4 ± 0.5	n.s.
native joint arthritis	17	2.0±0.3	n.s.
endovascular	11	1.7±0.4	n.s.
osteomyelitis (all):	13	1.9±0.3	n.s.
- vertebral osteomyelitis	6	2.3 ± 0.6	n.s.
respiratory tract infection	7	1.2 ±0.4	n.s.
urinary tract infection	8	1.7±0.5	n.s.
intraabdominal	7	1.1±0.2	n.s.
other type	1	2.1	n.s.
non-invasive skin infections	49	2.3 ± 0.2	0.031

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Table 2.

Correlation of *agr* type and biofilm formation in collection of *S. aureus* clinical isolates from invasive infections. Biofilm formation was measured as A_{570}. Mann-Whitney U test was used for statistical comparisons. $p>0.05$ was considered non-significant (n.s.).

***agr* type**	**n**	**Biofilm formation [mean ± SEM]**	***p* compared to other isolates**
all typed	159	1.9 ± 0.1	-
agr I	58	1.5 ± 0.1	0.003
agr II	39	1.6 ± 0.2	n.s.
agr III	53	2.6 ± 0.2	<0.001
agr IV	9	1.6 ±0.4	n.s.