Biofilm formation by Scottish clinical isolates of *Staphylococcus aureus*

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The biofilm-forming capacity of 972 clinical isolates of *Staphylococcus aureus* was tested using a high-throughput polystyrene 96-peg plate format. Isolates of *S. aureus* were collected from patients in hospitals throughout Scotland from 2004 to 2006; 763 of these were methicillin-resistant *S. aureus* (MRSA) and 209 were methicillin-sensitive *S. aureus* (MSSA). The biomass of each biofilm was quantified using a crystal violet staining technique. Isolates were divided into those that formed fully established biofilms, moderately attached biofilms and weakly adherent biofilms by comparison with a known biofilm-forming strain. The majority of MRSA (53.8%) and MSSA (43.5%) isolates formed moderately attached biofilms. Fully established biofilms were formed by 20.5% of MRSA isolates and 28.0% of MSSA isolates, whilst 25.7% of MRSA isolates and 28.5% of MSSA isolates formed negligible biofilms. There was no significant correlation between susceptibility to methicillin and biofilm formation ($P=0.77$). MRSA isolates were divided into clonal types (EMRSA-15, EMRSA-16 and sporadic isolates) based on PFGE genotyping results. EMRSA-15 isolates formed significantly more moderately and fully established biofilms than EMRSA-16 isolates ($P<0.001$). *S. aureus* strains isolated from the skin of patients had a significantly greater capacity to form biofilms than isolates from other body sites, including the blood. Microscopic examination of biofilms by scanning electron microscopy (SEM) revealed that poorly adherent biofilm formers failed to colonize the entire surface of the peg, whilst moderately adherent biofilm formers grew in uniform monolayers but failed to develop a mature three-dimensional structure. SEM analysis of an isolate representative of the group that formed fully established biofilms confirmed that this isolate developed a dense biofilm with a textured, multi-layered, three-dimensional structure.

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INTRODUCTION

Health-care-associated infections are a significant problem in today's hospital environment. In the UK, 4–10% of patients are suffering from a nosocomial infection at any one time, treatment of which costs the National Health Service as much as £1 billion a year (National Audit Office Report, 2000). *Staphylococcus aureus* is one of the most frequently isolated Gram-positive bacteria from hospital infections and approximately 45% of *S. aureus* isolated in the UK are resistant to the antibiotic methicillin (Biedenbach *et al.*, 2004; Boyce *et al.*, 2005). Infections caused by methicillin-resistant strains of *S. aureus* (MRSA)

range from those of the skin and surgical sites, to infections relating to catheters and prosthetic implants, to endocarditis and pneumonia (Diekema *et al.*, 2001). There are two dominant clones of MRSA that cause approximately 93% of infections in Scotland. Epidemic MRSA-15 (EMRSA-15) causes 70% of these infections, epidemic MRSA-16 (EMRSA-16) isolates are responsible for approximately 23% of infections and 7% of MRSA infections are caused by sporadically occurring Scottish clones (Morrison, 2003).

The mainstay of treatment for MRSA infections is the antibiotic vancomycin (Michel & Gutmann, 1997). However, two multidrug-resistant strains of *S. aureus* with intermediate resistance to vancomycin have been isolated from patients in Scotland (Hood *et al.*, 2000). This resistance severely limits therapeutic options and increases the already unacceptably high rates of morbidity and mortality in patients. To compound this problem further, *S. aureus* has the ability to colonize and form biofilms on

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Abbreviations: EMRSA, epidemic MRSA; MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *S. aureus*; SEM, scanning electron microscopy; SMRSARL, Scottish MRSA Reference Laboratory.

implanted biomaterials. These biofilm structures are inherently resistant to antimicrobial challenge and difficult to eradicate from the infected host, as they can display susceptibilities towards antimicrobials of 10–1000 times less than equivalent populations of free-floating planktonic cells (Potera, 1999; Donlan, 2001; Mah & O'Toole, 2001; Gilbert *et al.*, 2002; Parsek & Fuqua, 2004). Biofilms can form on almost any abiotic or biological surface and it is estimated that 65 % of all human bacterial infections involve biofilms (Donlan, 2001; Gilbert *et al.*, 2002). *S. aureus* can readily form biofilms on artificial surfaces, such as stents, prostheses and catheters (Donlan, 2001). In recent years, implanted medical devices have been crucial in the advancement of patient care and the management of serious medical conditions; however, they have also inadvertently predisposed patients to biofilm-related infections by organisms such as *S. aureus* (Gilbert *et al.*, 2002).

As the recalcitrance of biofilm-mediated infections has an adverse effect on patient health, the main objective of this study was to investigate the capacity of Scottish clinical isolates of *S. aureus* to form biofilms. These isolates were representative of those that cause the majority of infections in the health-care environment in Scotland, and the ability to form biofilms was compared for the different clonal groups.

METHODS

Staphylococcal isolates. Nine hundred and seventy-two clinical isolates of *S. aureus* were collected over an 18-month period (2004–2006) from hospitals throughout Scotland and submitted to the Scottish MRSA Reference Laboratory (SMRSARL; Stobhill Hospital, Glasgow, UK). These consisted of 763 MRSA isolates and 209 methicillin-sensitive *S. aureus* (MSSA) isolates. Isolates were genotyped at the SMRSARL using PFGE for MRSA isolates (HARMONY method) or a PCR ribotyping technique for MSSA isolates (Kostman *et al.*, 1995). MRSA isolates were made up of 538 EMRSA-15 isolates (70.5 %), 161 EMRSA-16 isolates (21.1 %) and 64 sporadic Scottish isolates (8.4 %). The isolates were collected from the blood, nose, wounds, skin, mouth/throat, urine, sputum, eye, in-dwelling instruments and genitourinary tract of Scottish patients (Fig. 1). *Staphylococcus epidermidis* RP62A (ATCC 35984), a well-characterized biofilm-forming strain, was used as one of two positive controls in biofilm assays. A clinical EMRSA-15 isolate (isolate 1) that formed fully established biofilms was used as a *S. aureus* positive control. Isolates from patients were cultured on blood agar and stored in Microbank storage vials at -70°C . Isolates were freshly subcultured on brain heart infusion (BHI) agar (Oxoid) prior to each assay.

Biofilm model. One colony from each staphylococcal isolate was used to inoculate 5 ml BHI broth (Oxoid). The culture was incubated for 18 h at 37°C with aeration at 200 r.p.m. Following incubation, the number of cells in each culture was adjusted to 1×10^6 c.f.u. ml^{-1} and 200 μl each culture was transferred to eight wells of a 96-well microtitre plate (Nunc). Eight replicates were used for each isolate in each biofilm assay. The biofilm-forming *S. epidermidis* strain RP62A and *S. aureus* clinical isolate 1, known to form fully established biofilms, were added to each plate as positive controls. BHI broth was incorporated as a negative control. An Immuno Maxisorp 96-peg plate (Nunc) was then positioned in the wells of the microtitre plate, allowing the pegs to be submerged within the bacterial culture. The inoculated peg plate was transferred to a 96-well microtitre plate containing fresh BHI broth and

Fig. 1. Isolation sites of the *S. aureus* clinical isolates included in this study.

incubated for 48 h at 37°C on a rocking platform, at 20 oscillations min^{-1} , to allow mature biofilms to establish. Each biofilm assay was repeated on a further two occasions.

Semi-quantification of biofilm biomass. Biofilm biomass was quantified using a modification of a methodology described by Mowat *et al.* (2007). Following incubation, the peg plate was removed from the microtitre plate, rinsed twice in PBS to remove loosely attached planktonic cells and dried for 30 min at 37°C . Each replicate peg was stained with filtered 0.5 % (w/v) crystal violet for 5 min. Excess crystal violet was removed by gently washing the peg plate twice with distilled water. Replicate pegs were detached from the plate using needle-tipped pliers and added to 1 ml 70 % ethanol to leach the crystal violet from the stained biofilms. The A_{570} was measured using a microtitre plate reader (LUMIstar; BMG). As the polystyrene pegs were suspended in the wells of the microtitre plate, any biomass that remained bound to the surface following the washing steps could be viewed as a genuine biofilm. The wells of the microtitre plate were not sampled for the presence of biofilm biomass as this may not have been a biofilm but rather a deposit of planktonic cells.

Analysis of biofilm formation. The capacity of each strain to form a biofilm was compared with that of the confluent biofilm-forming *S. aureus* control by analysing the absorbance of the crystal violet stain obtained for each biofilm. This allowed each isolate to be assigned a percentage value depending on the proportion of biofilm biomass it was able to establish after 48 h in comparison with the control (taken as 100 %). Eight replicate pegs were included for each isolate in each biofilm assay and the assay was carried out three times. Isolates were also divided into three groups depending on whether they formed fully established biofilms with $>75\%$ of the biomass of the positive control, moderately adherent biofilms with 25–75 % biomass or weak biofilms with $<25\%$ of the biomass of the positive control.

Statistical analysis of biofilm formation. Statistical analysis of biofilm formation was performed using SPSS software. For the multi-group comparisons (comparison of biofilm formation in MRSA subtypes, and comparison of biofilm formation and isolation site), Kruskal–Wallis and χ^2 tests were used to determine whether any of the groups exhibited a statistically significant different percentage of biofilm formation. If the Kruskal–Wallis test demonstrated at least

one of the groups to be statistically different, a *post hoc* analysis using the Mann-Whitney U-test and Bonferroni correction was used to adjust the significance (*P*) value for the number of comparisons.

Evaluation of biofilms on polystyrene pegs by scanning electron microscopy (SEM). Three MRSA isolates were selected, based on crystal violet staining, as representative strains that formed weak, moderate and fully established biofilms on the polystyrene pegs. Following biofilm formation, the pegs were rinsed twice with PBS and removed from the plate using needle-tipped pliers. Each peg was fixed with 2% (w/v) glutaraldehyde/2% (w/v) paraformaldehyde/0.15 M sodium cacodylate/0.15% (w/v) alcian blue for 3 h at room temperature. Pegs were then rinsed three times with 0.15 M sodium cacodylate buffer, immersed in 1% (v/v) osmium tetroxide in 0.15 M sodium cacodylate and incubated for 1 h at room temperature. Pegs were rinsed three times with distilled water. Specimens were then dehydrated with a series of ethanol solutions from 30% ethanol in distilled water in 10% increments to 100% absolute ethanol. Biofilms were then treated twice with hexamethyldisilazane for 5 min and dried overnight in a desiccator. Finally, pegs were coated with a thin layer of gold using a Polaron SC515 SEM sputter coating system and viewed with a JEOL 6400 scanning electron microscope.

RESULTS AND DISCUSSION

Biofilm-mediated infections in the hospital environment have a significant negative impact on patient health and place an enormous burden on the resources of the health services (Potera, 1999; Donlan & Costerton, 2002). The ability of nosocomial pathogens, such as *S. aureus*, to form biofilms is of significant clinical interest, as biofilm formation influences the efficacy of antimicrobial therapy and the subsequent outcome of an infection (Gilbert *et al.*, 2002; Gotz, 2002; Parsek & Fuqua, 2004). *S. aureus* is a common cause of biofilm-mediated life-threatening infections associated with intravenous catheters, artificial heart valves, stents and prosthetic joints (Davey & O'Toole, 2000). The aim of this study was to determine the biofilm-forming capacity of 972 clinical isolates of *S. aureus* taken from patients in hospitals throughout Scotland.

Evaluation of the biofilm model

Biofilm formation by clinical isolates was evaluated using a high-throughput peg plate platform and semi-quantified by crystal violet staining. This screening platform has recently been used to study biofilm-mediated tolerance to antibiotics in diverse pathogenic organisms such as *Candida albicans* and *Staphylococcus lugdunensis* (Ramage *et al.*, 2001; Frank *et al.*, 2007). Our study has shown that this methodology is also an effective tool for the study of biofilm formation in *S. aureus*. The 96-peg plate has the capacity to examine a large number of isolates simultaneously and allows the formation of highly reproducible biofilms. Biofilm biomass can be detected easily by crystal violet staining and biofilm structure can be examined in detail by microscopy. The peg plate platform used in this study is similar to other well-published systems (Harrison *et al.*, 2005) and in our opinion is superior to a microtitre plate format for the development of bacterial biofilms. The

polystyrene pegs are suspended in the bacterial culture and therefore any cells that adhere to the peg surface and develop a multi-layered structure can be taken as a biofilm. In the case of a microtitre plate, gravitational force automatically deposits cells on the bottom of the wells, leading to a structure that may not be a genuine biofilm.

The *S. aureus* isolates included in this study were collected from individuals in hospitals throughout Scotland between 2004 and 2006. The SMRSARL receives in excess of 1000 MRSA isolates and 1000 MSSA isolates from the blood-stream of patients per annum, and this figure does not include the large number of isolates that are received from wounds, nasal swabs, and surgical and other infected sites (Health Protection Scotland, 2007). The 972 isolates were divided into MSSA (209 strains) and MRSA (763 strains) isolates and each then subdivided into three groups: those that produced fully established biofilms (>75% biofilm biomass), those that formed moderately adherent biofilms (25–75% biofilm biomass) and those that formed negligible biofilms (<25% biofilm biomass) in comparison with the biofilm-forming control strain.

One peg each from MRSA isolates that were characterized as weakly adherent, moderately adherent and fully established biofilm formers was examined by SEM, following 48 h incubation. The weak biofilm former failed to colonize the majority of the surface of the polystyrene peg. Small clusters of cells were observed, but these did not aggregate to form a monolayer or a more mature biofilm structure (Fig. 2a). The representative moderately adherent isolate grew in a uniform monolayer, but did not form a mature multi-layered biofilm (Fig. 2b). SEM analysis of the isolate that formed >75% of the biofilm biomass of the control confirmed that this isolate developed a dense, mature biofilm on the polystyrene peg, with a multi-layered three-dimensional structure (Fig. 2c). The SEM images supported the results achieved by crude crystal violet staining of biofilm biomass and highlight the value of the 96-peg plate format for high-throughput analysis of biofilm formation in clinical isolates of *S. aureus*.

Comparison of biofilm formation in MRSA and MSSA isolates

The majority of the 763 MRSA isolates examined (53.8%) formed moderately adherent biofilms. Negligible biofilms were formed by 25.7% of the MRSA isolates, whilst 20.5% of isolates produced fully established biofilms. Several other groups have also examined the ability of clinical isolates of MRSA to form biofilms; however, these studies have usually been limited to a small number of isolates. O'Neill *et al.* (2007) studied biofilm formation in 114 clinical isolates of MRSA and found that only 9% had the ability to form fully established biofilms. Our study, which examined a much larger number of isolates collected consecutively from patients throughout Scotland, showed that more than twice as many isolates had the capacity to form biofilms on our polystyrene peg plate platform. These

Fig. 2. SEM images of clinical isolates of MRSA that form weak (a), moderately adherent (b) and fully established (c) biofilms on polystyrene pegs following 48 h incubation at 37 °C.

differences may relate to variation in isolation site or the geographical differences in the most commonly isolated genotypes of MRSA included in each study.

A large proportion of the 209 MSSA isolates examined (43.5 %) also formed moderately adherent biofilms when compared with the control strain. Non-quantifiable biofilms were produced by 28.5 % of MSSA isolates, whilst 28.0 % of isolates formed fully established biofilms. When these results were compared using the statistical tests outlined in Methods, there was no significant difference in biofilm formation between *S. aureus* isolates that were susceptible and resistant to meticillin ($P=0.77$). These results suggest that there is no correlation between meticillin susceptibility and the ability to form biofilms, as both types of isolate had the capacity to form substantial biofilm structures on the polystyrene pegs.

In Scotland, approximately 250 isolates of MRSA and MSSA are collected from the bloodstream of patients with bacteraemia every quarter (Health Protection Scotland, 2007). The ability of a large proportion of MRSA and MSSA isolates to form moderately adherent and fully established biofilms may explain why these organisms can facilitate infection of the host and survive in the hospital environment. Although *S. aureus* isolates with sensitivity and resistance to meticillin have similar prevalence in the Scottish health-care environment, the drug-resistant phenotype of MRSA isolates complicates treatment. Studies have shown that, although there is no significant difference between rates of disseminated infection by MRSA and MSSA in hospitals, the mortality rate is significantly higher in infections caused by MRSA due to the added interference of antimicrobial resistance (Melzer *et al.*, 2003).

Analysis of biofilm formation by clonal types of MRSA

The 763 MRSA isolates were further divided into clonal types (specifically EMRSA-15, EMRSA-16 and sporadic

clones) based on PFGE genotyping results, and biofilm formation within these clonal types was analysed. In Scotland, EMRSA-15 is responsible for approximately 70 % of MRSA infections, EMRSA-16 isolates cause 23 % of infections and 7 % of MRSA infections are associated with sporadic clones (Morrison, 2003). The distribution of isolates included in this study was representative of the range of strains within the health-care environment in order to provide clinically relevant epidemiological data on biofilm formation in the Scottish *S. aureus* population.

In this study, EMRSA-15 isolates showed a propensity to form moderately adherent and fully established biofilms (334/538 EMRSA-15 isolates). The majority of EMRSA-16 isolates formed negligible or moderately adherent biofilms (154/161 EMRSA-16 isolates). Sporadic Scottish isolates were

Fig. 3. Comparison of the biofilm-forming capacity of 538 EMRSA-15, 161 EMRSA-16 and 64 sporadic Scottish subtypes of MRSA. Error bars represent the standard deviation among the results for different isolates. The line in each box plot represents the mean biofilm-forming capacity (%) of the MRSA subtypes. ○, Outlying values; *, $P < 0.001$.

present in roughly equal numbers in the negligible, moderately attached and fully established biofilm groups (Fig. 3). Statistical analysis using the Kruskal–Wallis and Mann–Whitney U-tests confirmed that EMRSA-15 isolates had a significantly greater ability to form biofilms than EMRSA-16 isolates ($P < 0.001$). When EMRSA-15 isolates were further divided into subtypes, no one variant was associated with an increased ability to form biofilms. Isolates that cause infections sporadically in Scottish patients also had a significantly enhanced capacity to form established biofilms compared with EMRSA-16 isolates ($P < 0.001$); however, a larger number of sporadic isolates would have to be examined to determine whether this difference is true for the sporadic isolate population as a whole.

EMRSA-15 and EMRSA-16 are the dominant epidemic strains of MRSA isolated from patients in Scottish hospitals. The emergence of these clones has coincided with a substantial increase in the incidence of MRSA infections, from 1–2 % in the early 1990s to approximately 45 % of all *S. aureus* infections in UK hospitals today (Johnson *et al.*, 2001). Of these two dominant clones, EMRSA-15 isolates are responsible for the majority of infections (70 %). These strains appear to have a greater ability than other clonal types to survive and spread in hospitals, although the reasons behind this increased virulence are as yet unknown. In this study, EMRSA-15 had a greater tendency to form fully established biofilms. In comparison, EMRSA-16 isolates tended to be weak biofilm formers with few isolates (4.5 %) demonstrating the capacity to form thick adherent layers. This would suggest that the ability to form superior biofilm structures may be an important virulence factor in EMRSA-15 isolates and may explain why this clone has developed into such a highly successful nosocomial pathogen.

Comparison of biofilm formation and isolation site of *S. aureus*

When the biofilm-forming capacity of strains was compared with the site from which they were collected from the patient, an obvious trend emerged (Fig. 4). *S. aureus* isolates that originated on the skin had a significantly greater ability to form fully established biofilms than isolates taken from the blood or other body sites ($P = 0.0002$). This suggests that, within this habitat, the ability to form a fully established biofilm is an important virulence factor that helps the organism to survive. *S. aureus* has been shown by confocal laser-scanning microscopy to form a biofilm-like glycocalyx and to congregate in microcolonies in samples taken from serious clinical skin infections, such as impetigo (Akiyama *et al.*, 2003). This biofilm mode of growth provides cells on the skin with increased protection against antimicrobial agents routinely prescribed to treat such conditions. When skin is penetrated by surgery or injury, bacteria such as *S. aureus* can colonize the wound site, establish an infection and cause systemic disease. This pathogen may utilize the biofilm mode of growth to inhabit the precarious environment of the

Fig. 4. Comparison of biofilm-forming capacity and isolation site of *S. aureus*. The error bars represent the standard deviation among the results for the different isolates. Each box plot represents the spread of biofilm-forming capacity (%) in isolates collected from the ten isolation sites. The line in each box represents the mean biofilm formation in isolates from each of these sites. ○, Outlying values; *, $P < 0.001$.

human skin as a commensal organism, placing the host at risk of more serious disease if the skin defences are breached. This highlights the importance of the biofilm mode of growth for the survival of *S. aureus* in the human host.

This investigation has provided vital information on the capacity of clinical isolates of *S. aureus* to form biofilms, the relationship between meticillin resistance and biofilm formation, and the ability of different genotypes of MRSA to utilize the biofilm mode of growth. Understanding the ability of isolates to form biofilms is the first step towards understanding the mechanisms employed in biofilm formation in *S. aureus*. The *ica* operon, which encodes a polysaccharide intercellular adhesin, is currently the best-understood mediator of biofilm development (Cramton *et al.*, 1999); however, *ica*-independent biofilm development, biofilm-associated protein (Bap) and the *S. aureus* surface protein (SasG) have all been implicated in biofilm development and regulation (Cucarella *et al.*, 2001; Corrigan *et al.*, 2007; O'Neill *et al.*, 2007). Further investigation of the genetic mechanisms of biofilm formation in *S. aureus* will ultimately help in the management of biofilm-mediated infections and in the reduction of morbidity and mortality in patients suffering from *S. aureus* infections.

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