Effects of positively charged- and plasma-treated surface on biofilm growth of various subtypes of *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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Abstract
Biofilm study is important because approximately 65% to 80% of all infections are caused by biofilms and resistance against antibiotics is up to ten times more when bacteria are in biofilms. Biofilm models using in vitro model systems offer affordable and fast results albeit several limitations to mimic physiological environment for optimum microbial growth. This study aims to address some of the limitations when growing mixed cultures of ATCC and clinical isolates of Staphylococcus aureus and Pseudomonas aeruginosa – the two main pathogens in osteomyelitis. Variables were tested in this experiment: (i) using conditioned surface for biofilm attachment (plasma and poly-L-Lysine, PLL) and (ii) with different incubation times. Biofilm biomass was quantified by determining the crystal violet absorbance (A_{570}). Results showed that at 16 h incubation, S. aureus biofilms did not grow well on PLL-treated plates. In addition, the biofilm biomass of S. aureus strains was reduced at 24 h incubation in all treated groups, except for the strong biofilm producers in untreated plates.

Similarly, biofilm biomass of P. aeruginosa strains was also higher at 16 h than 24 h incubation. Treating plate surfaces with plasma and PLL did not have any effect on P. aeruginosa strains. The study concludes that PLL and plasma-treated plates have no influence in biofilm growth for both S. aureus and P. aeruginosa at 24 h incubation. However, at 16 h incubation, PLL may have inhibited the biofilm growth of S. aureus. The optimum incubation time to grow biofilms in vitro for S. aureus is between 16 and 24 h while P. aeruginosa is best grown at 16 h incubation. Although other factors may influence the bacterial growth in vitro, this study demonstrated that the use of brain heart infusion for various subtypes of bacteria is favorable for antimicrobial study of polymicrobial biofilms.

Keywords: Biofilms, Microtiter plates, Poly-L-lysine, *Pseudomonas aeruginosa, Staphylococcus aureus*.

Introduction

The initial development of biofilms is influenced by bacteria-to-bacteria interaction to form a community and bacteria-surface adherence or attachment by the weak and reversible van der Waals forces. Bacterial attachment is regulated by cell-wall anchored proteins and extracellular factors that can be termed as Microbial Surface Components Recognizing Adhesive Matrix Molecules or MSCRAMMs that are influenced by cell surface charges and hydrophobicity. MSCRAMMs in bacteria causing osteomyelitis had been studied in *Staphylococcus aureus* but limited work is available for other osteomyelitis bacteria such as *Pseudomonas aeruginosa*.

In fact, the co-existence of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in osteomyelitis has not been studied extensively. Other wound studies indicate that these two species are interacting whilst colonizing within the same tissues.[1,20] Osteomyelitis, a microbial-associated inflammation of the bone, is a major concern because approximately 40% of patients will experience recurrent infections.[12] One of the contributing factors for the chronic disease is the formation of biofilms by osteomyelitis organisms. *S. aureus* infections constitute more than 80% osteomyelitis cases with the most frequently agent isolated on monomicrobial infections whereby its complications include cascades of bone destruction and loss of bone vasculature.[5,9]

On the other hand, *P. aeruginosa* is the second most frequently isolated microorganism which can form polymicrobial infection with other species and can easily cause bone infections in intravenous drug users[3] and diabetic mellitus patients.[17] Studies to investigate these two causes of osteomyelitis must appreciate the nature of biofilm pathogenesis of these two species while considering the biofilm models for these pathogens.

Microtitre plate (MTP) assay is a popular and practical *in vitro* biofilm model.[6,13,16,24,30] This uncomplicated and convenient system offers many advantages as it provides a large amount of tests simultaneously and allows quantification of both living and dead cells.[18,22]

While the MTP assay is user-friendly and low in cost, the principle of the assay which allows direct attachment of the bacteria on untreated MTP surface has some limitations. For examples, a few studies found that many clinical *S. aureus* isolates showed poor attachments to the abiotic surface.[2,15]

In addition, one study also found that bacterial biofilm was inhibited when grown on untreated plastic coverslips.[30]

Plasma proteins have been shown to initiate the primary attachment of *S. aureus*.[4,12] Strong opposite electrostatic charges are known to strengthen bacteria-surface adherence.[8,27,29] Therefore, this study aims to investigate the effects of positive charges and plasma-treated surface on the growth of biofilms of the two osteomyelitis pathogens namely *S. aureus* and *P. aeruginosa*.

Material and Methods

Bacteria: Three strains of *S. aureus* and two strains of *P. aeruginosa* biofilm-producing osteomyelitis bacteria were gifted by Dr. Mohd. Affendi Mohd Shafri (Department of Biomedical Sciences, Faculty of Allied Health Sciences, International Islamic University Malaysia). Bacteria used in this study are listed in table 1.

S. aureus and *P. aeruginosa* were sub-cultured overnight on tryptic soy media (Oxoid™, Hamsphire) at 37°C. Working cultures were prepared by inoculating a single bacterial colony into test tubes containing Brain Heart Infusion (BHI) (Oxoid™, Hamsphire) supplemented with 1% glucose (Sigma-Aldrich, St. Louis) and grown overnight at 37°C.

Treatment for tissue culture plate: Ninety-six-well flat-bottomed tissue culture plates (SPL Life Science, Gyeonggido; http://www.bionovatec.com/wp-content/uploads/2014/02/SPL.pdf) were used in all experiments. These plates were treated with poly-L-lysine (PLL) and plasma. For PLL-treated plates, wells were treated with 10% PLL (Sigma Aldrich, St. Louis) and left for 10 minutes. The solution was decanted and the plates were air dried. For plasma-treated plates, 20% of Bactident Coagulase Rabbit Plasma (Merck, Darmstadt) was pipetted into each well and incubated at 4°C for 24 h. Then, plasma was aspirated out and the wells were air dried.

Biofilm formation and quantification: Each well of the microtitre plates was filled with 100 µl of bacterial suspension and the plates were incubated at 37°C. After incubation, bacterial suspensions were aspirated out and washed three times with normal saline. The plates were shaken vigorously to remove planktonic bacteria. Then, the remaining adhered bacteria were fixed with 99% methanol for 15 minutes. To stain the bacteria, 0.5% crystal violet was added into the well for 20 minutes. Then, the plates were washed, air-dried and the stain bound to the adherent cells was resolubilized using 33% glacial acetic acid for 30 minutes. Quantification of biofilm formed was measured using VERSAmax tunable microplate reader with 570 nm wavelengths. Besides the treated microplates, two more parameters were investigated: various incubation times and different initial inoculum.

Statistical analysis: Statistical analysis was performed using SPSS version 20. The significance value between different treatments (plasma, PLL, control) and between *P. aeruginosa* incubation time (16 and 24 h) was determined by Kruskal-Wallis test. Mann Whitney test was used to determine the significance difference between *S. aureus* incubation time (16 and 24 h).

Results

Several factors need to be considered when growing biofilm cultures. The choice of media, the type of surfaces (for biofilm attachments), and incubation period varied depending on the bacterial species. Polymicrobial biofilms would have different requirements than the monomicrobial biofilms. In this study, we looked at three parameters that may influence monomicrobial biofilms of osteomyelitis pathogens: surface treatment, and incubation time. Due to different sources of both clinical and ATCC bacteria that were tested in this study, we expected to see strain-to-strain variability in our findings.

A simple test to determine the adherence potential of bacterial species was conducted based on the method by Stepanović et al[26] All *S. aureus* strains except for the clinical isolate SA005 are considered strongly adherent biofilm producers. *S. aureus* SA005 is a moderately adherent biofilm producer. Biofilm biomass at 16 h incubation varied significantly between strains and between different surface treatments (Figure 1). This could suggest that during 16 h incubation, PLL may have inhibited the biofilm formation of *S. aureus*. At 24 h incubation, the biofilm biomass in all strains was found to be almost similar for plasma- and PLL-treated surfaces.

Table 1
List of bacterial species and strains

Bacteria	Strain	Source
S. aureus	SA 001	Osteomyelitis patient
	SA 003	Osteomyelitis patient
	SA 005	Osteomyelitis patient
	ATCC 29213	Wound
P. aeruginosa	PAE 004	Osteomyelitis patient
	PAE 009	Osteomyelitis patient
	ATCC 700888	Industrial water system
	ATCC 27853	Blood culture

Moreover, clinical isolates SA001 and SA003 showed significantly higher absorbance readings compared to SA005 and the ATCC strain in untreated surface (control) both at 16 and 24 h incubation. SA005 also can be seen to have the lowest absorbance reading in all groups, both at 16 h and 24 h incubation periods. At 24 h incubation, there were about three times increment of absorbance reading on untreated surface for SA001 and SA003 as compared to the plasma and PLL-treated surface (Figure 1). These findings indicate that biofilm formation was inhibited by the additional treatments to the microtiter surface.

Based on the biofilm strength assay suggested by Stepanović et al[26], all *P. aeruginosa* strains in this study were found to be strong biofilm producers. Similar with *S. aureus*, *P. aeruginosa* biofilm formation was tested on plasma and PLL-treated surface at two different incubation time:16 and 24 h. When grown at 16 h incubation, all strains generally showed high absorbance value (>3.50) with no significant differences between different treated groups. However, the absorbance values dropped to <2.00 after 24 h incubation for all strains, with the lowest absorbance values were seen for PAE 004 followed by ATCC 700888 and PAE 009. ATCC 27853 strain had the highest absorbance values in all treated groups including the control. In fact, most strains seemed to do better in the control, compared to treated plates, which could suggest that after 24 h, biofilm integrity of *P. aeruginosa* was suppressed by the plasma- and PLL-treated surfaces.

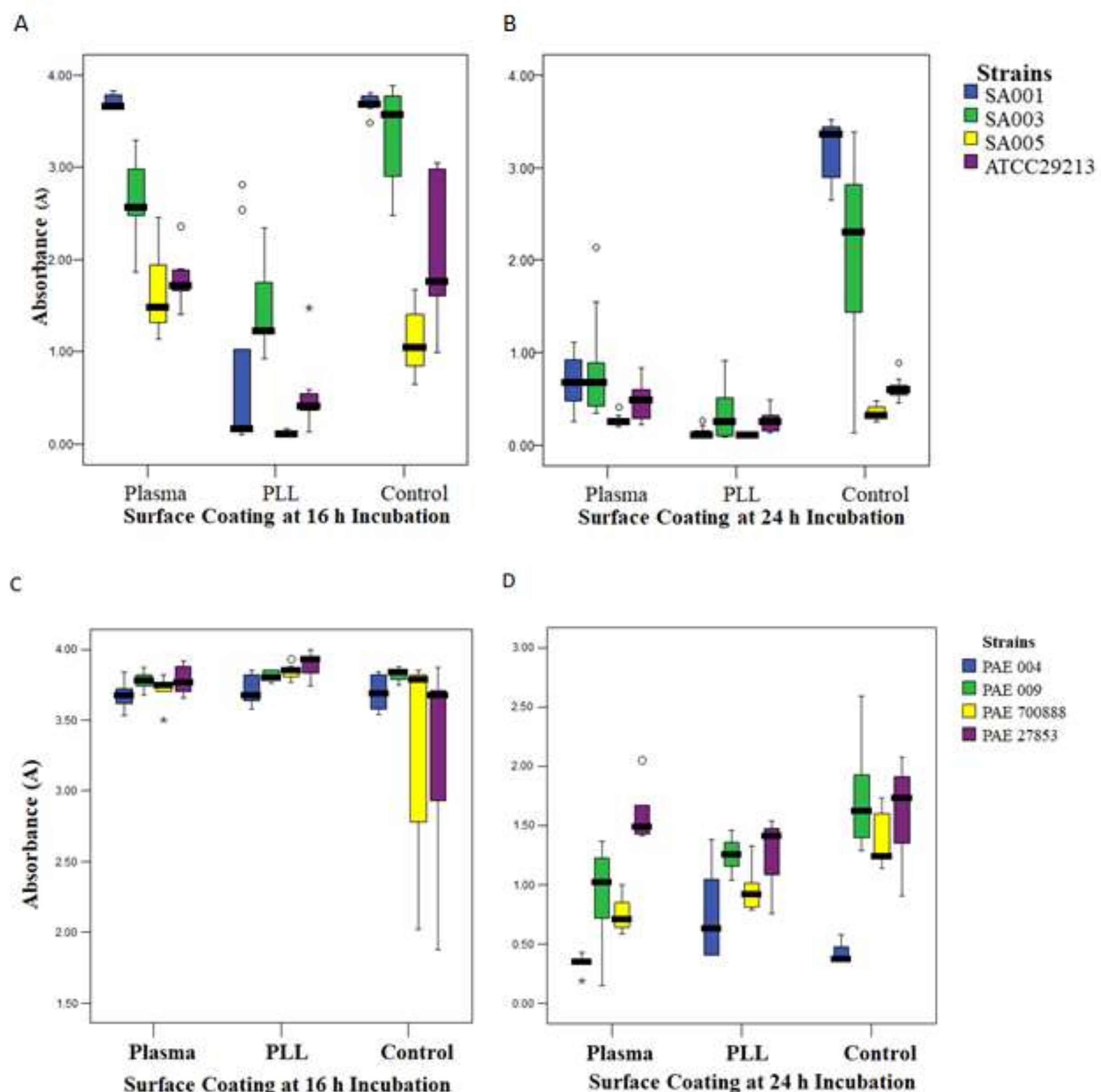

Figure 1: Median absorbance reading (at 570 nm) of *S. aureus* biofilm on plasma, poly-L-lysine (PLL) and control surfaces at 16 and 24 h incubation times

In general, biofilm formation of *S. aureus* for most species was found to be significantly higher during 16 h incubation than 24 h incubation ($p<0.01$) (Figure 1). However, the readings at 24 h were more consistent for all strains in both plasma- and PLL-treated plates as well as in the control plates (except for SA003 and SA005) which could suggest that incubation period between 16 to 24 h would be preferred for *S. aureus*.

The same findings can be seen for *P. aeruginosa* biofilm formation - biofilm biomass at 24 h incubation was significantly less than the 16 h incubation periods for all strains ($p<0.01$). In fact, biofilm formed by *P. aeruginosa* at 24 h incubation was found to be easily detached from the surface. Based on the diversity of the interquartile range, data at 16 h incubation is preferred.

Discussion

Biofilm formation begins with the interaction of the bacterial cells to the surface of biotic and abiotic surface for attachment. In general, all strains of *S. aureus* attached poorly to the PLL-treated surface as compared to the plasma and control surfaces. A study suggested that PLL is toxic for bacteria and can cause inhibition of bacterial cell growth.[7] Gottenbos[10] also suggested that the positively charged surfaces could result in antimicrobial activity due to the absence of bacterial growth. It was postulated that PLL can penetrate the cytoplasmic membrane as well as the outer cell membrane.

Another possible reason is that PLL may form a repulsive force on the abiotic surface and this may have inhibited the biofilm formation. On the other hand, plasma-treated surface was a preferred surface structure for *S. aureus* biofilm formation ($p<0.05$). Previous studies showed that a thicker biofilm was formed when using human plasma treated surface compared to unconditioned surface[30] and that plasma enhanced the adhesion of staphylococci by increasing the expressions of MSCRAMMs, namely fibrinogen and fibronectin.[4]

We observed that most of *P. aeruginosa* strains formed approximately similar biofilm biomass on plasma, PLL-treated and the control surface. The exopolysaccharides, the components of the outer layer that are responsible for the biofilm formation in *P. aeruginosa* are known to have a neutral charge.[11,21,28] This might explain why the addition of PLL on the surface had no effect on the *P. aeruginosa* strains. Additionally, the greater absorbance value in any treated surface might be due to the ability of this species forming biofilm layer not only at the bottom of the well, but also at the wall itself.

This can be due to the morphology and special characteristics of *P. aeruginosa* which have extracellular appendages such as flagella and type IV pili that enhance cell-to-surface attachment.[23] These appendages allow the bacteria to interact with abiotic surface and twitching motility by the type IV pili. This element allows and subsequently stabilizes the cells attachment to abiotic surface that were initiated by flagella. Moreover, type IV pili-mediated twitching motility also contributes to the cell migration across the surface and recruiting cells for aggregation.[19] Hence, these features might have promoted the formation of *P. aeruginosa* biofilm as can be seen *in vitro*.

Other factors that may affect biofilm growth period include availability of the nutrients and development of biofilms itself. Different bacterial species may possess different optimum incubation time to grow biofilm. Previous studies recommended more than 24 h period of incubation for *S. aureus* and *P. aeruginosa* (72 h) in tryptic soy media.[26] In this study, *S. aureus* biofilms were found to grow better at 16 h than 24 h incubation using BHI media, but based on the variability of the interquartile range, an incubation period between 16 and 24 h would be recommended.

P. aeruginosa was also found to grow best at 16 h incubation. We can now recommend the use of BHI supplemented with glucose for faster biofilm formation. Earlier study also reported a faster growth of biofilm for *Staphylococcus epidermidis,* another pathogen of osteomyelitis, when using BHI as a growth medium.[25]

Other than limited nutrients, the reduction in biofilm absorbance of *P. aeruginosa* at 24 h incubation, might be explained due to the detaching process known as sloughing.[14] When incubated at 16 h, biofilm seemed to remain attached at the surface bottom during biofilm quantification. However, as the incubation time increases, the thickness of biofilms also increases and biofilms were seen to detach from the surface and this was possibly a mechanism to prevent further nutrient deprivation.

Conclusion

The study reiterates the diversification of subtypes of bacteria that produce different outcomes in a controlled *in vitro* experiment. This diversification is hypothesized to be a survival strategy for the different physiological conditions, such as immune responses from the host and the environmental factors. Environmental conditions and host defenses cause differing stresses on the bacteria, and to survive in vastly different environments, *S. aureus* and *P. aeruginosa* must be able to adapt to its surroundings. It is noted that within the same species, subtypes of bacteria react differently and it was difficult to generalize a trend based on the origin of the strains.

The study is also limited to only two incubation time readings. Our study also did not look at the synergistic effect when both *S. aureus* and *P. aeruginosa* are grown together in one culture system.

However, we concluded that all bacterial subtypes from both species can grow well in BHI media, hence a polymicrobial

study is probable. Future work can be done to investigate this aspect to optimize the biofilm model for osteomyelitis pathogens.

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