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Quantifying *Staphylococcus aureus* biofilm formation on stainless steel and titanium alloy used in orthopedic fracture fixation

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Running Title: Quantification of biofilms on implant surfaces

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ABSTRACT

In order to assess and compare the antibacterial property of implants surfaces, a standard method is needed to quantify bacterial load. This study evaluated the effectiveness of three quantifying methods, namely, (I) crystal violet staining analysis, (II) ultrasound detachment with viable cell counts, and (III) confocal laser scanning microscopy for characterizing *S. aureus* Seattle 1945 (ATCC 25923) biofilm on metallic coupons. The accuracy of the results, time for completion, and ease of use of methods were compared. The crystal violet method is relatively faster and more straightforward for analyzing biofilm formation. However, the accuracy of the confocal laser scanning microscopy method is found to be

considerably higher than that of the other methods. Confocal laser scanning microscopy method is considered to be more time-consuming for data collection and analysis and costlier. The ultrasound detachment followed by viable cell count of recovered cells is recommended for biofilm quantification analysis on orthopedic materials when there is a large number of samples (more than ten samples). This info could provide guidelines that would facilitate the selection of suitable method for quantifying biofilm formation on orthopedic implants based on investigators' consideration on method accessibility, assay cost, assay time, and complexity of method.

Keywords: Biofilm, *Staphylococcus aureus*, orthopedic implants, fracture fixation, infection

INTRODUCTION

Biofilms consist of a group of microorganisms attached to a surface within an extracellular polymeric substance matrix. When the film structure is established, the microorganisms inside it are more resistant to various antimicrobial agents¹. Post-surgery biofilm formation on orthopedic implants is a major problem in the treatment of the patients². A biofilm delays the healing process and increases the probability that revision surgeries may be needed to remove the contaminated implant². The attachment of the biofilm has been shown to be a complex process that is influenced by different environmental factors and surface characteristics³. Surface roughness, and associated changes in effective surface area, is one of the known factors that affect microorganism's colonization. It appears that colonization increases with escalations in surface roughness⁴. In addition to roughness, surface hydrophobicity can also play a role in the initial attachment. Some studies showed that microorganisms attach faster to hydrophobic, nonpolar surfaces such as Teflon than hydrophilic surfaces like glass or metals^{3,4}.

New implant materials or surface modification methods have been developed to reduce biofilm formation⁵⁻⁷. Prior to *in vivo* application of these materials or surface modifications, the anti-biofilm properties need to be evaluated *in vitro*. Currently, there is a range of methods that have been used to quantify biofilms on solid surfaces *in vitro* and these can be classified into three groups: (I) microscopic

methods, (II) bacterial culture-based procedures, and (III) biochemical investigations⁸. However, researchers need to consider the limitations and accuracy of each method prior to their application of these within a study. While there have been some comparison reviews that sought to rank these techniques, there is a need to compare the benefits to employing these techniques for larger scale, specifically for orthopedic modified surfaces.

In this study, three different quantification methods were compared for characterization of biofilm formed on the surfaces of stainless steel and titanium disks, two of the most commonly used alloys in fracture fixation implants⁹. Based on the experimental needs of accessibility and the initial estimates of cost and required time for each method, crystal violet (CV) staining, confocal laser scanning microscopy (CLSM) and sonication with viable cell count (VCC) were selected for evaluation.

Crystal violet staining, a low cost, simple and fast method for analysis of biofilm, was first described by Christensen *et al.* 1985¹⁰. CV is a basic protein dye that binds to negatively charged surface molecules and polysaccharides in the extracellular matrix¹¹. The method is basically based on staining the biofilm formed on a surface with CV, then rinsed a few times to remove non-attached bacterial cells, washed with 95% v/v ethanol to release the bound CV and then the optical density (OD) is recorded with a spectrometer. The concentration of CV recovered is considered to be proportional to the amount of biofilm on metal surfaces¹². It should be noted that CV stains both live cells, dead cells and their matrix and therefore, it can not be used to evaluate a functional biofilm¹³.

CLSM is a technique for capturing high-resolution optical images at different depths. As it can provide depth sectioning of thick specimens, it is a suitable method for analyzing thickness, biomass and structure through the three-dimensional (3D) images or cross-sectional views of the biofilm. It has been used extensively to increase the understanding of biofilm characteristics¹⁴. Development of new analytical methods and software increased the quantitative output of these images¹⁴. An automatic threshold can be used in MATLAB to separate the bacteria and surface sequences^{15,16}.

Ultrasound detachment combined with viable cell count is another method used to analyze biofilm formed on surfaces. It is more complicated and time-consuming than the CV method, but less costly and

faster than laser confocal microscopy^{17,18}. The method is based on recovering the live bacteria from the surface of biofilm by detaching them through a sonication technique followed by a plate counting method⁸. The low-moderate sonication (20-40 kHz) of the bacterial suspension in the water bath for a short duration (2-10 min), has been shown to have the optimum effect on bacterial detachment. Longer exposure time or higher ultrasound power leads to disruption of bacterial cell wall^{19,20}.

In this study, three different quantification methods were compared for the characterization of biofilm formed on the surfaces of stainless steel and titanium disks. A fluorescent strain of *Staphylococcus aureus* (*S. aureus*) expressing intracellular green fluorescent protein (GFP) was used to facilitate the observation of colonization and biofilm formation on metal coupons via CV staining, laser confocal microscopy and viable cell count methods. Protocols for cultivation and quantification of biofilms on stainless steel and titanium alloys surfaces were developed and validated.

MATERIALS AND METHODS

Titanium and stainless steel cylindrical coupons

Stainless steel and titanium are the most common alloys used for fracture fixation implants⁹. In this study, biofilms were grown on titanium-6Al-4V and 316 stainless steel cylindrical disks (Lisnabrin Engineering, Ireland) and will be referred to in this manuscript as Ti and SS coupons respectively. Each coupon (1 cm diameter, 1 mm thickness) were laser cut from 1 mm thick sheets of either grade 5 Ti-6Al-4V or 316 SS and no additional steps were taken to polish their respective surfaces. Surface roughness (Ra) of Ti ($n = 30$) and SS ($n = 30$) was measured in six randomized locations on each sample. The average surface roughness of Ti coupons was 0.56 μm (SD = 0.10 μm) and SS coupons was 0.20 μm (SD = 0.01 μm).

Bacterial strain

For quantifying the growth of the biofilm on the SS and Ti coupons, the *S. aureus* Seattle 1945 strain (ATCC 25923) was used. It is a clinical isolate often used as a standard laboratory testing control²¹⁻²³. This bacterium was transformed with plasmid pGFPuvr, which encodes green fluorescent proteins. Thus, the presence, growth and colonization of bacteria can be evaluated using fluorescent microscopy^{24,25}.

Bacterial growth on the coupons

S. aureus stock cultures were streaked onto the tryptic soy agar (TSA) +1% glucose plates and incubated overnight at 37 °C to obtain isolated colonies. A single colony was then transferred onto tryptic soy broth (TSB) media and grown overnight in a shaker incubator at 37 °C and 125 rpm to the concentration around 1×10^9 CFU/mL. The overnight cultures were then used to inoculate the experimental coupons.

Coupon cleaning procedure

Prior to use, coupons were cleaned and sterilized by soaking in a 70% v/v ethanol solution for 10 min, rinsed in 1x PBS (0.01 M) solution, then soaked in ethanol solution and finally rinsed with PBS and air dried in sterile petri dishes.

Coating the underside surface of the coupons with VaLaP

To be able to quantify the amount of biofilm formed on just the upper side of the SS and Ti coupons, the underside of individual coupons was coated a mixture of vaseline, lanolin and paraffin wax in equal rations (VaLaP). This is a biologically inert material that has been shown to prevent bacterial attachment²⁶. Covering one side of the surface is important when quantifying biofilm using the CV and ultrasound with VCC methods, while it is not necessary when using the CLSM method as only the upper surface is imaged.

Cultivation and quantification of biofilm

Cultivation of biofilm growth were conducted using the following protocol. Briefly, 24-well flat-bottom non-tissue-culture treated microtiter plates were used to minimize binding of bacteria onto the wells

and to facilitate the cultivation of biofilm on metal coupon surfaces. Coupons were dipped gently into melted VaLaP until the bottom side was covered. Each coupon was then placed into a separate well of the 24-well plate. 600 μ L of TSB+1% glucose was added to each well to cover the entire surface of the coupon. The concentration of the overnight *S. aureus* was determined spectrophotomically and 10^3 bacterial cells were added to each well containing coupon and the plate was incubated at 37 °C for 24 h. Washing of metal coupons after growth of biofilms was carried out by dipping the coupons in wells containing 500 μ L of deionized (D.I.) water. This process was repeated three times to wash away planktonic bacteria. The biofilms on metal coupons were then observed using an epi-fluorescence microscope or a laser confocal microscope. For quantification of biofilm, the CV staining method or viable cell count experiment was used. A positive control, i.e., a well containing TSB and bacterial inoculum, and a negative control, i.e., a well containing TSB without bacteria were included for each individual experiment^{14,16}.

Quantification of biofilm via CV staining

For quantification of biofilm growth on the coupons using CV method, they were processed as follows: After the last wash to remove unbound planktonic cells, the coupons were placed into empty wells and allowed to air-dry at 37 °C for 30 min. Metal coupons were placed into wells containing 600 μ L of 0.1% (w/v) aqueous CV solution and stained for 5 min at room temperature (22°C). The metal coupons were then washed three times in wells containing 600 μ L of D.I. to remove excess stains and air-dried at 37 °C for 30 min. 600 μ L of 100% ethanol was added to the wells containing coupons to dissolve the bound CV and the plate was placed on a shaker (50 rpm) at room temperature for 10 min to detach the adherent biofilm growing on the coupons. 100 μ L of each solution was transferred into a 96-well microtiter plate and the biofilm quantified using a Synergy H1 Hybrid Microplate Reader reading at 590 nm¹².

Quantification of biofilm using confocal laser scanning microscopy

The fluorescence property of *S. aureus* Seattle 1945^{GFPuvr} strain was verified using a Zeiss laser confocal microscope. CLSM is a technique for obtaining high-resolution optical images with depth selectivity (Z-stacks). The main feature of confocal microscopy is its ability to acquire in-focus images from selected depths. Washing metal coupons after the growth of biofilms is completed by dipping the coupons in wells containing 600 μ L of D.I. water. This process is repeated three times to wash away planktonic bacteria. The biofilms on metal coupons were then observed using a laser confocal microscope. Images were acquired point-by-point and reconstructed with the computer, allowing 3D reconstructions of topologically complex objects. 3D images enable us to exam biofilm structure on different coupons. Interpreting the images through measuring some features of bacterial biofilm using COMSTAT gave us the ability to determine how the biofilm formed on different surfaces^{15,16}.

Biofilm thickness and biomass analysis

To analyze the average thickness and biomass of biofilm formed on coupon surfaces, multiple laser confocal image stacks were analyzed using the COMSTAT program. The image acquisition and data analysis were standardized and optimized, e.g., size of inoculum, incubation time, and the number of stacks. There are some features that are calculated by COMSTAT including Bio-volume, which is defined as the number of biomass pixels in all images of a stack multiplied by the voxel size $[(\text{pixel size})_x \times (\text{pixel size})_y \times (\text{pixel size})_z]$ and divided by the substratum area of the image stack. The resulting value is biomass volume divided by substratum area ($\mu\text{m}^3/\mu\text{m}^2$). Bio-volume represents the overall volume of the biofilm and provides an estimate of the biomass in the biofilm. Area occupied by bacteria in each layer was fraction of the area occupied by biomass in each image of a stack and the substratum coverage is the area coverage in the first image of the stack. Substratum coverage reflects how efficient the colonization of bacteria is on the substratum. Thickness distribution and mean thickness: This function locates the highest point (μm) above each (x, y) pixel in the bottom layer containing biomass. Hence, the thickness is defined as the maximum thickness over a given location, ignoring pores and voids inside the biofilm. Mean biofilm

thickness provides a measure of the spatial size of the biofilm and is the most common variable used in biofilm literature²⁷.

Quantification of biofilm using viable cell count

A viable count test was performed to quantify biofilm formation on the coupons since this method yields more consistent and sensitive results than the CV method. We followed the Bjerkan method with modification; briefly, after cultivation of biofilm on the coupon surface, each disk was placed into a 15 mL sterile conical tube containing 5 mL of PBS and gently vortexed for 15 seconds to rinse off planktonic cells. After addition of 5 mL of PBS, each tube was then subjected to sonication for 5 min at full wave (40 KHz) in a DK Sonic water bath ultra-sonic cleaner. The power was enough to detach the bacterial cells from the surface, but not to damage the cells. This wash 1 solution (W1) was serially diluted 10^{-1} , 10^{-2} , 10^{-3} , and 10^{-4} folds. 100 μ L from each undiluted and diluted sample was plated on TSA and incubated at 37 °C for 48 h. Each disk was washed a second time in 5 mL PBS in ultrasound water bath for another 5 min yielding the second wash solution (W2). The washing process was repeated for the third time and generated a third wash solution (W3). Viable counts of W2 and W3 was conducted the same way described for W1. Numbers of colonies were counted and the total cell number recovered from each coupon was calculated¹⁹.

Validation of the methods using glass cover slips

To validate the biofilm quantifying methods, biofilms grown on round glass coverslips (Prod No. 260368, Ted Pella INC) with a diameter of 10 mm and thickness of 0.16-0.19 mm were analyzed using the same quantifying methods described for the metal coupons. The results were obtained to determine if the methods utilized were reproducible and consistent. Glass coverslips have been used to quantify biofilm formation as it provides a proper surface for confocal microscopy imaging, and it has been shown to be a robust and reproducible method for biofilm evaluation²⁸. A typical Z-stacks of CLSM of the biofilm formed on the glass coverslip is shown in **Figure 1**.

Figure 1 A typical Z-stacks of CLSM of the biofilm formed on the glass coverslip. Intensity of green color is proportional to the biofilm presence in the randomly selected location on the glass coverslip

Statistical analysis

IBM SPSS statistic software (v24) was used for statistical analysis. To analyze the significance of data obtained from individual biofilm quantifying methods, a one-way analysis of variance (ANOVA) test was run with a Tukey post-hoc analysis. The one-way ANOVA test was used to determine whether there were significant differences between the means of data.

RESULTS

Biofilm quantification results

The *S. aureus* biofilm formation on individual metal disk surfaces over time was quantified via CV staining, laser confocal microscopy, and viable cell count methods and the results presented as follows.

Analysis of biofilm formation using crystal violet method

S. aureus Seattle 1945 formed biofilms on the surface of the metal coupons. CV stained biofilm was dissolved into ethanol and then the CV concentration was determined with a microplate reader to estimate the amount of biofilm formed on each surface. The experiment was performed with three replications for each of the Ti and SS coupons. Based on the collected data, no significant difference was observed among the two different coupons. Ti coupons exhibited better biofilm suppression than SS (Figure 2 a).

Figure 2 Quantification results of biofilm formed on Ti and SS unmodified coupons **a)** Crystal violet OD₅₉₀ results of three SS and Ti unmodified coupons. There was no statistical difference between the biofilm growth on the SS and Ti coupons (n=3); **b)** Average biofilm thickness of SS and Ti unmodified coupons. There was no statistical difference between the biofilm growth on the SS and Ti coupons (n=3); **c)** Average biomass on SS and Ti unmodified coupons. There was no statistical difference between the biofilm growth on the SS and Ti coupons (n=3); and **d)** Average cell recovered from SS and Ti unmodified coupons. There was no statistical difference between the biofilm growth on the SS and Ti coupons (n=3). All statistical analysis was based on t-test two-tailed means comparison analysis

Analysis of biofilm formation using confocal laser scanning microscopy method

After bacterial growth on two different surface coupons for 24 hrs, the coupons were washed three times with D.I. water to eliminate unattached bacteria from the surfaces. The coupons were then placed into glass bottom chambers upside down while using glass coverslips as spacers to elevate the coupons thus preventing direct contact of biofilms to the bottom of the chamber to avoid changing the biofilm structure. Images were taken using CLSM with a 63x objective and oil immersion. For each coupon, three image stacks were taken in three discrete randomly picked locations. **Figure 3 (a, b)** shows a 3D structure of biofilm on the metal coupon surface, **and Figure 3 (c, d)** shows the wide field images of biofilm on the metal coupon surface. To analyze the average thickness and biomass of biofilm formed on coupon surfaces, multiple laser confocal image stacks were taken and analyzed using COMSTAT program. From the collected data (**Figure 2 b, c**), Ti and SS material surfaces showed different amounts of biofilm formed on them. However, no significant difference was observed between the material surfaces.

Figure 3 3D structures of biofilm on **a)** SS and **b)** Ti unmodified coupons. Intensity of green color is proportional to the biofilm present in the randomly selected location of each coupon. Wide-field fluorescence images of biofilm formed on **c)** SS and **d)** Ti unmodified coupons diameter equal to 1 cm

Analysis of biofilm formation using viable cell count method

Evaluation of biofilm on Ti and SS surfaces using viable cell count method indicated that the number of cells recovered from two surface types were not significantly different from each other. Viable

counts of biofilm recovered from SS and Ti coupons for three complete sets are shown in **Figure 2 (d)**. For both experiments, ANOVA analysis with SPSS software of the collected data did not show significant difference between the SS and Ti coupons ($p = 0.05$).

Comparison of biofilm characterization among methods

To validate the biofilm quantifying methods, round shape glass coverslips were used with three different quantifying methods, i.e., CV staining, CLSM, and viable cell count as previously described for the metal coupons. These experiments were conducted to determine whether the used methods are reproducible and consistent in terms of analyzing the amount of biofilm formed on the coupons.

To validate the consistency and reproducibility of the methods, biofilm was grown on the glass coverslips and analyzed using the same three quantification methods used to evaluate biofilm on metal coupons. The results showed that the biofilm formed successfully on the glass coverslips (**Figure 4**). Quantification of biofilm formed on the glass coverslips demonstrated the consistency and reproducibility of the methods used for enumeration of biofilms. All the data fall within the range of quantified biofilm of the metal coupons. In the CV test, the OD results of three glass coverslips were in the range of (0.40 - 0.52) with a standard deviation of 0.06. The viable cell count for total cells recovered from the glass coverslips result fell within ($7.59\text{E}+05$ - $1.21\text{E}+06$ cells) with a standard deviation of 231902.7. CLSM data showed the biofilm thickness range of (3.28 - 4.90 μm) with a standard deviation of 0.82 μm . Biomass evaluation of biofilm formed on the glass coverslips were within (1.37 - $2.23 \mu\text{m}^3/\mu\text{m}^2$) with a standard deviation of $0.45 \mu\text{m}^3/\mu\text{m}^2$. All in all, the obtained data for each different method are close (based on small standard deviation, which indicates that the data points are close to the mean, and 90% of the obtained data fall within the standard deviation) and this demonstrates the consistency and reproducibility of the methods.

Figure 4 Quantification results of biofilm formed on glass coverslip **a)** Crystal violet OD₅₉₀ results of three glass coverslips; **b)** Average biofilm thickness on glass coverslips; **c)** Average biomass on glass coverslips; and **d)** Average cells recovered from glass coverslips. * means there is significant difference between two mean groups based on t-test two-tailed means comparison analysis.

DISCUSSION

Crystal violet is shown to be a suitable staining method for biofilm quantification. Although it can quantify the amount of biofilm on the surface, it stains both live and dead bacterial cells so, cannot determine the functional (live cells) biofilm^{12,13}. In comparison with two other biofilm quantifying methods, crystal violet is a quick and simple method best for initial analysis of the modified surfaces. It is sensitive to pipetting and evaporation of the liquids and thus, keeping the experimental conditions consistent for all

samples is challenging and not feasible for a single researcher when evaluation of higher number of samples is needed. For these reasons, using viable cell count method in a larger research study is a more feasible and reliable method for quantification of biofilms. CLSM was the other method used to evaluate biofilm formation. This method enabled us to exam the biofilm structures and biofilm characteristics such as biofilm distribution, thickness and biomass. Although it has been shown that CLSM method provides a detailed evaluation of biofilm, and it can differentiate live and dead cells⁸, this method may not be appropriate for large-scale studies due to the relatively longer time required for each sample analysis and the higher cost associated (both equipment and beam time) when compared to the other methods. Minimum area of at least 100,000 μm^2 should also be imaged to have representative data of the biofilm formed on one surface¹⁵. Each stack area is about 10,000 μm^2 and it takes 30 min to 1 h to collect an entire Z-stacks for one area. With at least 10 different Z-stacks of each sample type to be imaged and many different sample types to be evaluated, the required time to complete such task is considerable. In terms of cost comparison, crystal violet and viable cell count method are relatively low-cost methods when compared to CLSM. Viable cell count method can determine the number of live cells in the biofilm and also can be handled better when a large number of samples are being compared at the same time. So, based on the purpose of the work, each of the three different studied methods can be picked to analyze biofilm on orthopedic surfaces. For initial analysis of limited number of samples CV can be used. If time and cost is not a major concern, CLSM is suggested which also can be a proper method for evaluation of the effectiveness of treatments on bacterial viability. When there are a large number of samples to be processed and time and cost are important factors, ultrasound detachment followed by viable cell count is recommended.

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organisms or toxins, the investigator adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories. The U.S. Army Medical Research Acquisition Activity, 820 Chandler Street, Fort Detrick, MD 21702-5014 is the awarding and administering acquisition office.

Conflict of interest statement

No conflict of interest declared.

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412

Figures

Figure 1

A typical Z-stacks of CLSM of the biofilm formed on the glass coverslip. Intensity of green color is proportional to the biofilm presence in the randomly selected location on the glass coverslip

Figure 2

Quantification results of biofilm formed on Ti and SS unmodified coupons a) Crystal violet OD590 results of three SS and Ti unmodified coupons. There was no statistical difference between the biofilm growth on the SS and Ti coupons (n=3); b) Average biofilm thickness of SS and Ti unmodified coupons. There was no statistical difference between the biofilm growth on the SS and Ti coupons (n=3); c) Average biomass on SS and Ti unmodified coupons. There was no statistical difference between the biofilm growth on the SS and Ti coupons (n=3); and d) Average cell recovered from SS and Ti unmodified coupons. There was no statistical difference between the biofilm growth on the SS and Ti coupons (n=3). All statistical analysis was based on t-test two-tailed means comparison analysis

Figure 3

3D structures of biofilm on a) SS and b) Ti unmodified coupons. Intensity of green color is proportional to the biofilm present in the randomly selected location of each coupon. Wide-field fluorescence images of biofilm formed on c) SS and d) Ti unmodified coupons diameter equal to 1 cm

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