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A Method to Quantify Biofilms in Object Glass Using ImageJ

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Abstract

Background: Direct microscopic observation of biofilms is done by observing for the presence of biofilm under a microscope. However, this method is qualitative and not quantitative. Analysis of these biofilm structures using image processing software may provide a method to quantify biofilm production and degradation in glass slides. **Objective:** To quantify the number and area percentage of microbial structures observable on a slide. This study is an experimental in vitro study. **Methods:** Biofilm production was done by submerging slides in petri dishes filled with Brain Heart Infusion with 2% sucrose (w/v) and inoculating it with Staphylococcus aureus. The petri dishes were incubated undisturbed for 48 hours at 37°C (n=3).The slides were then submerged in distilled water (Group 1) or detergent (Group 2) for 5 minutes before staining with 0.1% crystal violet and observed under a light microscope at 1000x. Images from five fields of view were collected. ImageJ was then used to count the number of microcolonies, aggregate cells, and single cells or cell clusters, based on size, and their respective area percentage. Welch's T-Test was performed using JASP version 0.18.3. **Results:** Direct observation of slides shows that microcolonies and cell clusters to be formed in Group 1, and none or little in Group 2, indicating differences in biofilm formation on Group 1 vs Group 2.Based on ImageJ calculation, control slides had an average percentage area for microcolonies, cell agregates, and single cells or clusters of 39.97 ± 9.99%, 8.96 ± 3.19%, and 1.39 ± 0.33%, respectively. Treatment with detergent significantly reduced percentages of microcolonies to 0.33 ± 0.68%, but increased single cell area to 5.27 ± 0.49%. **Conclusion:** ImageJ can be a valuable tool to quantify biofilm production in glass slides based on the number and percentage area of microcolonies, cell aggregates, and single cells or cell clusters.

Keywords - Biofilm, ImageJ, Direct Microscopic Observation, Quantification

Abstrak

Latar Belakang: *Pengamatan mikroskopis langsung terhadap biofilm dilakukan dengan mengamati keberadaan biofilm di bawah mikroskop. Namun, metode ini bersifat kualitatif dan bukan kuantitatif. Analisis struktur biofilm ini menggunakan perangkat lunak pengolahan gambar dapat memberikan metode untuk mengkuantifikasi produksi dan degradasi biofilm pada slide kaca.* ***Tujuan****: Untuk mengkuantifikasi jumlah dan persentase area struktur mikroba yang dapat diamati pada slide. Studi ini merupakan studi eksperimental in vitro.* ***Metode****: Produksi biofilm dilakukan dengan merendam slide dalam cawan petri yang diisi dengan Brain Heart Infusion dengan 2% sukrosa (w/v) dan menginokulasinya dengan Staphylococcus aureus. Cawan petri diinkubasi tanpa gangguan selama 48 jam pada suhu 37°C (n=3). Slide kemudian direndam dalam air suling (Grup 1) atau deterjen (Grup 2) selama 5 menit sebelum diwarnai dengan kristal violet 0,1% dan diamati di bawah mikroskop cahaya pada perbesaran 1000x. Gambar dari lima bidang pandang dikumpulkan. ImageJ kemudian digunakan untuk menghitung jumlah mikrokoloni, agregat sel, dan sel tunggal atau gugus sel, berdasarkan ukuran, dan persentase luas masing-masing. Uji T Welch dilakukan menggunakan JASP versi 0.18.3.* ***Hasil****: Pengamatan langsung pada slide menunjukkan bahwa mikrokoloni dan gugus sel terbentuk pada Kelompok 1, dan tidak ada atau sedikit pada Kelompok 2, menunjukkan perbedaan pembentukan biofilm pada Kelompok 1 vs Kelompok 2. Berdasarkan perhitungan ImageJ, slide kontrol memiliki persentase luas rata-rata untuk mikrokoloni, agregat sel, dan sel tunggal atau gugus masing-masing sebesar 39,97 ± 9,99%, 8,96 ±*

3,19%, dan 1,39 ± 0,33%. Perlakuan dengan deterjen secara signifikan mengurangi persentase mikrokoloni menjadi 0,33 ± 0,68%, tetapi meningkatkan luas sel tunggal menjadi 5,27 ± 0,49%. ***Kesimpulan****: ImageJ dapat menjadi alat yang berharga untuk mengukur produksi biofilm pada slide kaca berdasarkan jumlah dan persentase area mikrokoloni, agregat sel, dan sel tunggal atau gugus sel.*

Kata kunci *- Biofilm, ImageJ, Pengamatan Mikroskopis Langsung, Kuantifikasi*

I. INTRODUCTION

Microbial biofilms are a current problem particularly in medical device-related infections, particularly from invasive medical devices such as catheters and endotracheal tubes[1,2]. Biofilms are a constant source of bacterial nosocomial infection and major cause of septicemia or sepsis which increases the health burden in hospitals[3]. Furthermore, it is known that biofilms can also withstand antibiotic therapy, whether by the dissemination of antibacterial genes from persistent bacteria in a biofilm community, by reducing antibiotic exposure inside the biofilm due to the production of extracellular polysaccharide substances in the biofilm, or by the antibiotic unable to work on metabolically dormant cells located in the base of the biofilm[4]. Thus, efforts to screen for agents that can reduce biofilm, either by inhibiting its production or eradicating mature biofilm, are currently being undertaken worldwide. This effort requires methods to quantify and observe microbial biofilm *in vitro*.

There are different states of the microbial cells in a biofilm produced on an object glass, which also coincides with the current theory or observation of biofilm production. Biofilm production is initiated when a bacterium attaches to a surface and begins to replicate. This would form several cells as well as provide a platform for other cell recruitment during the initial phase of extracellular polymeric substance production[5]. After more cells are attached, a structural community of bacteria is established, which, as well as being comparatively large in size, also provides nutrition and means to communicate with other cells in this community[2,6]. When viewed using a confocal laser scanning microscope (CLSM), it has been discovered that microbial biofilms on an apatitic surface would have various configurations, consisting of microbial in single cells (around 1 – 5 μm), a cluster of cells (5 – 20 μm), an aggregate of cells (20 – 300 μm), or a large microcolony (>300 μm)[7].

Several methods to produce and quantify biofilm in a laboratory setting has been explored in other papers[8]; however, no golden standard for biofilm production and quantification has been established. In one method, an object glass is used as an abiotic surface for bacterial attachment and biofilm production and stained with crystal violet (usually 0.1%)[9,10]. Crystal violet is reported to be able to stain cell walls as well as extracellular polymeric substances (EPS) which compose the biofilm matrix[11], and therefore enable the visualization of biofilm-related microscopic structures.

Image files acquired from microscopic observation can be analyzed digitally by using image processing software. One of these, often used in various cell-based research projects, is ImageJ, a free java-based image processing software available for most operating systems. ImageJ can be used to calculate area, measure distances and angles, and create density histograms[12]. ImageJ has been used in many research studies to identify and calculate abnormal cell numbers, cell size, and malformation[13], and bacterial cell counts on a plate[14]. However, the use of ImageJ in biofilm related research is limited.

This study aims to provide a novel method to quantify biofilms from images obtained using direct microscopic observation of biofilm produced in object glass. Firstly, we produced biofilm in object glass in two groups. In one group, the object glass was treated with detergent prior to staining, and the other was treated with water. We then use ImageJ to quantify biofilm based on the area size and the number of large structures (microcolonies and cell aggregates) and small structures (single cells) and compare the biofilm between those two groups.

II. RESEARCH METHODS

A clinical *Staphylococcus aureus* strain was used in this study, which was known to be able to produce biofilm. Bacterial was grown in a Nutrient Broth (Himedia) for 24 hours and plated in nutrient agar (Himedia) every month. For this study, several colonies were taken and mixed with normal saline and standardized using a 0.5 McFarland Standard, or equivalent to 0.5 x 108 CFU/ml. However, contamination by an unknown basil bacteria occurred during the production of the biofilm, which occurred despite the production of biofilm on different slides on different days. The data collected by this unknown basil bacteria was used in this study.

Biofilm production was done according to the direct microscopy observation method as described in previous literature with several modifications[9], conducted with three biological replicates on different days. A sterilized glass object was inserted into the middle of a sterilized petri dish under aseptic conditions. Each petri dish was then filled with 10 ml of Brain Heart Infusion Broth (HiMedia) supplemented with 2% sucrose (w/v). From the standardized bacterial stocks, 100 µl was transferred into the petri dish and was incubated undisturbed for 48 hours at 37°C. After incubation, washing was performed on each slide by submerging it in distilled water or detergent, respectively, for 5 minutes before staining. The object glass was transferred into a 0.1% Crystal Violet (CV) solution, left for 1 minute, and then washed by submerging in distilled water. The object glasses were then left to dry overnight before microscopic observation.

Observation of the object glass was done on a trinocular microscope at 1000x magnification using emersion oil. From each object glass, five different fields of view were obtained and saved as a .TIFF image. Quantification was performed using ImageJ to determine the number of single cells, cell clusters, cell aggregates, and microcolonies, as well as the percentage area size.

ImageJ was used to analyze the images according to the manual[12]. Briefly, each picture was transformed into an 8-bit image. The unit of measurement was changed into µm, based on the scale on each image (each image had a 200 µm scale line) (using the 'set scale' function). The threshold was then adjusted to remove background, resulting in a black and white image. The function 'analyze particle' was used to calculate the number of structures detected, by inserting the area sizes for each structure, as described below. The circularity was left at default at 0.00 – 1.00. The option to 'include holes' was selected, and each result was summarized.

Prior to calculation, a sample microscopic image was used to calibrate the area size measurement used to identify microcolonies, aggregate cells, and single cells. The definition of each structure was obtained from previous literature; however, it should be noted that not all those structures were perfect circles. As far as we are aware, ImageJ calculates and identifies objects using area size, not diameter. Thus, the area determined was tweaked from preselected images so as to select only the intended structure, which fulfills the size requirement based on literature. The obtained area sizes for each structure were as follows: microcolonies (>15.000 μm^2), aggregate cells (200 – 14.999 μm^2), and cluster cells or single cells (1 – 199 μm^2). The scale for each image was 1.13 pixels/µm.

Statistical analysis was conducted using JASP version 0.18.3[15] using Welch's t-test, with a p-value considered to be < 0.05.

III. RESULTS

A. MICROSCOPIC OBSERVATION SHOWED BACTERIAL MICROCOLONY AND CELL AGGREGATE FORMATION ON SLIDE

Similar to previous literature[9,10], our results similarly show that microcolonies and cell clusters or aggregates were formed on the surface of the slides, as shown in Fig. 1.

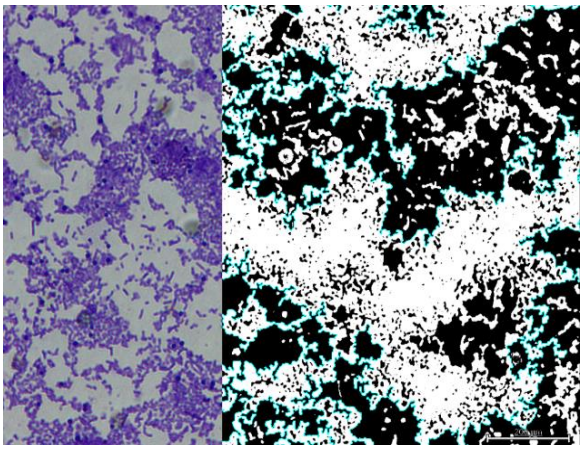

FIGURE 1. PRE-PROCESSING (LEFT) AND POST-PROCESSING (RIGHT) OF IMAGES USING IMAGE-J TO QUANTIFY BIOFILM PRODUCTION, WITH A BLUE OVERLAY INDICATING A MICROCOLONY STRUCTURE.

Aside from microcolonies, smaller structures such as single cells and cell clusters were also visible. The definitions of a cell cluster and aggregate were a collection of cells with a diameter of 1 - 5 μm and 5 - 50 μm, respectively[7]. However, using ImageJ, identification of structure was done using area size instead of diameter, and therefore, the selection based on structural diameter of these structures was difficult to obtain. Hence, in this paper, both cell clusters and single cells were grouped into one.

TABLE 1. SUMMARY OF BACTERIAL STRUCTURES CALCULATED USING IMAGE-J (THREE SLIDES FROM DIFFERENT DAYS WITH FIVE FIELDS OF VIEW PER SLIDE)

No.	Parameters	Group 1[a]	Group 2[a]
1.	Average Single and Cluster cells		
	○ Number (cell units)	415.06 ± 139.85	1,754.93 ± 689.52*
	○ Percentage Area Size (%)	1.34 ± 0.33	5.27 ± 0.49*
2.	Average Aggregate		
	○ Number (cell units)	120.47 ± 32.31	193.34 73.87
	○ Percentage Area Size (%)	8.96 ± 3.19	7.22 ± 2.39
3.	Average Microcolony		
	○ Number (cell units)	4.60 ± 2.41	0.20 0.4*
	○ Percentage Area Size (%)	39.97 ± 9.99	0.33 ± 0.68*

[a]Group 1 was slides treated with water and Group 2 was slides treated with detergent. (*) notation signifies a significant difference between Group 1 and Group 2.

B. QUANTIFICATION OF BACTERIAL STRUCTURES USING IMAGEJ SHOWED WATER-TREATED SLIDES HAVING A HIGH PERCENTAGE OF MICROCOLONY AND CELL AGGREGATE STRUCTURES

As shown in Fig. 1, ImageJ was able to detect both microcolonies as well as smaller structures. The results of this calculation are shown in Tab. 1. In our case, slides washed with water (Group 1) showed <10 microcolonies per field of view at every slide, as the close proximity of the cells caused it to form one continuous structure. The microcolony had an average microcolony percentage area size of around 39.97 ± 9.99% of the image, which represented the size of the microcolony. The number of clusters or aggregates, on the other hand, was around 120.47 ± 32.31 structures per field of view for every slide, with a total area size of around 4.60 ± 2.41%, and the single cells were 415.06 ± 139.85 with a total area of 1.34 ± 0.33%. Direct microscopic observation shows that detergent treatment was able to reduce the number of microcolonies observed in each slide, as shown in Fig. 2. Quantification using ImageJ shows that slides treated with detergent showed a significant reduction in

the microcolony number and area percentage, and an increase in single cell number and area percentage (Fig. 3). However, no significant difference was observed in aggregate cell number or area percentage.

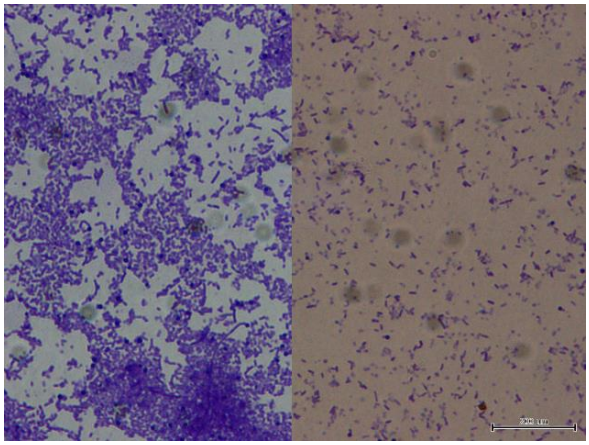

FIGURE 2. A SIDE-BY-SIDE COMPARISON OF TYPICAL MICROSCOPIC IMAGES OF DIFFERENT SLIDES TREATED WITH WATER (LEFT) AND DETERGENT (RIGHT). LITTLE OR NO MICROCOLONIES WERE OBSERVED IN MOST SLIDES TREATED WITH DETERGENT.

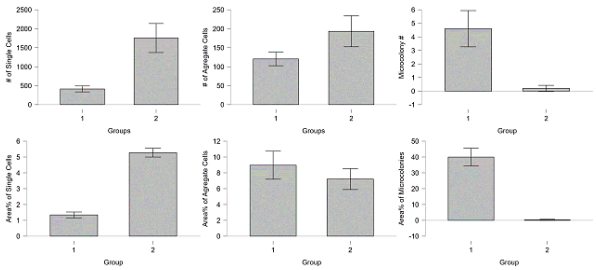

FIGURE 3. BAR-PLOTS COMPARING THE NUMBER AND AREA SIZE PERCENTAGE OF SINGLE CELLS, CELL AGGREGATES, AND MICROCOLONIES USING IMAGE-J ON GROUP 1 (TREATED WITH WATER) AND GROUP 2 (TREATED WITH DETERGENT). BARS DENOTE A CONFIDENCE OF INTERVAL 95%.

IV. DISCUSSION

A. MICROSCOPIC OBSERVATION SHOWED BACTERIAL MICROCOLONY AND CELL AGGREGATE FORMATION ON SLIDE

Studies have stated that Crystal Violet (CV) can stain bacterial cell walls as well as extracellular polysaccharides (EPS) produced in biofilm formation[16]. However, any EPS staining was not observable in our findings. Bacterial cells were observed to be in close proximity to each other and forming a large structure. One limitation of this study, compared to previous studies in this subject, is the inability to observe three dimensional structures; thus, it is unknown whether the structure form is also constructed vertically as it would in a typical microcolony structure in biofilm production. However, as the diameter of the structure formed was well beyond 300 μm, we conclude that the large structures observed were one or several microcolonies.

In this paper, both cell clusters and single cells were grouped into one. This is done considering that aside from the difference in diameter, these two structures are not known to have a different role in the formation of biofilm, they can both be considered as transitional states between single cells, which are the singular or small cell structures that would initiate biofilm production, and the microcolony structure, which is often considered to be the main key structure in bacterial biofilms. On the other hand, it can be hypothesized that an increase in the number of these smaller structures, especially with the absence of large microcolonial structures, would indicate a breakdown of the current biofilm structure.

B. QUANTIFICATION OF BACTERIAL STRUCTURES USING IMAGEJ SHOWED WATER-TREATED SLIDES HAVING A HIGH PERCENTAGE OF MICROCOLONY AND CELL AGGREGATE STRUCTURES

Disinfectants are often used in laboratories for the sterilization of laboratory equipment. In the last 5 years, there has been an increasing interest in comparing the efficacy of disinfectants in removing biofilm[17]. Based on our results, submerging in disinfectant was able to reduce the microcolony area by up to 99%, however, the number and percentage area of single cells increased after treatment. This may indicate that the detergent was able to breakdown

microcolonies and cause the destabilization of preformed biofilms. As the protocol used in this study attempts to reduce any mechanical cleansing of the slides (the slides are submerged and not passed through running water or detergent), we speculate that the cells in this protocol would remain on the surface of the slides instead of being displaced. In normal cleaning, this detergent would be effective in removing and sterilizing equipment from biofilms and bacterial cells.

Biofilm production is influenced by many different factors - different bacteria would have different biofilm production capabilities, the type of media used in biofilm production (as well as the addition of different sugars), and incubation time play a role in the success of biofilm production[18]. This is usually crucial in the search for antibiofilm compounds, i.e., compounds that can prevent the production of biofilm by the bacteria. Some researchers screen for antibiofilm compounds against biofilm that are not yet established, whereas a compound of interest is added during the early stages of bacterial life (some times at the same time as the bacterial inoculation)[19,20]. A more established biofilm would be harder to remove, as it would have defenses against chemical compounds through the formation of an extrapolymeric substance (EPS) layer that prevents any chemical compounds from interacting with the bacterial layer. Furthermore, bacteria located deeper in the biofilm structure would be metabolically inactive, preventing the metabolic action of certain antibacterial agents.

In this study, we show that ImageJ can be a valuable tool in determining biofilm production and size, as well as be used to measure antibiofilm bioactivity of certain compounds, and should be used in conjunction with direct microscopic observation for biofilm production. Increased biofilm production would be expected if a bacterial agent with high biofilm production was used as well as with optimized growth media and incubation time. As with the 96 well plate method, the number of washes used would influence final biofilm production, where increased washing would physically remove biofilm. Finally, as this method submerges the slides, a large volume of test compounds is required if this method is to be used as antibiofilm screening, therefore, this method might not be suitable for small sample volumes.

V. CONCLUSION

ImageJ can be used to quantify biofilm production by calculating the number of and area size of microcolonies (area size of >15.000 μm^2), cell aggregates and single cells. In biofilm, detergent application was found to reduce the number of large structures and increase the number of smaller structures, showing a possible mechanism of biofilm breakdown. This method can be included in the repertoire of antibiofilm activity screening, and possibly be used to simulate biofilm production and destruction in clinically important mixed cultures found in various medical devices.

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