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Research Paper

Biofilm formation and antibiotic resistance: An ongoing challenge?

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ABSTRACT

Biofilms are formulations of a particular group of microorganisms, sophisticated collections of polysaccharides, DNA and proteins. It is an entity found on many living and non-living surfaces. With biofilms productions, bacteria can gain a defensive shield against disinfectants, antibiotics and host defence. The resistant potency to antibiotics acquired when a bacteria form a biofilm, complicate the treatment and even becomes ineffective after being the right choice of medicine to the same strain of bacteria in its planktonic format. Biofilms appears to be a significant virulence factor to strengthen the colonization of bacteria in medical devices and living tissues, and weaken the process to have an effective treatment. The aim of this study is to investigate the effect of biofilm formation and antibiotic susceptibility using statistical analysis via One-way ANOVA and Tukey post-test to highlight significant difference between a collection of bacterial strains.

Key words: Biofilms formation, bacteria, and antibiotic susceptibility.

INTRODUCTION

A biofilm is defined as microbial cells assemblage associated with a specific surface in an irrecoverable way (cannot be detached by tender rinsing) and found to be enclosed within an extracellular polymeric substance (EPS), a matrix that mainly composed of polysaccharides and function as a protective layer (Donlan, 2002). Biofilms formation may exist in various surfaces such as human tissues, indwelling medical equipment, and more frequently associated with domestic environment (Donlan and Costerton, 2002). An estimation was released from UK based study stating that 9 million people are suffering from intestinal diseases annually, much of which has a home-origin due to humans' excreta (Curtis et al., 2003). Furthermore, Hoiby et al. (2010) showed that biofilm has numerous advantages for bacteria, such as the ability to provide resistance to antibiotics, dynamic surrounding environment, and disinfectants. Quorum sensing (QS) in a biofilm triggers gene expression by up and down regulations thereby enhancing adaptation such as developing an ability to survive harsh environment (e.g., nutrient deficient) and phenotypic variations (Koch *et al.,* 2001).

According to Laxminarayan et al. (2013), antimicrobial susceptibility testing (AST) is an essential test to guide therapeutic decisions, and there are two mostly adopted methodologies worldwide: Clinical Laboratory Standards Institute (CLSI), and European Committee on Antimicrobial Susceptibility Testing (EUCAST). Apart from that, AST goals are to test potential drug resistance and to ensure susceptibility to particular drugs for certain infections. The most common testing approaches include a rapid automated instrument or broth microdilution. In addition, manual methods provide resilience and saving of costs such as gradient diffusion and disk diffusion. All procedures are able to qualitatively assess drugs in a scale of categories as 'susceptible, intermediate, or resistant' and some other methodologies can provide quantitative yields (e.g., Minimum Inhibitory Concentration). Over and above, biofilm has significant concern for public health due to their association in inducing infections and firmly, patients in indwelling medical devices besides their resistance to antibiotics that contribute a more significant burden challenging health care worker worldwide (Donlan, 2001).

The aim of this report is to draw attention to a public

health problem, because a greater understanding of biofilms can contribute to effective biofilm-control strategies, resulting in enhancements of patient management that go in parallel with AST. Moreover, aims will be fulfilled via analytical assessment of biofilm-forming ability in relation to particular microorganisms and AST results, as well as applying statistical analysis using one-way ANOVA test with Tukey post-testfor P-value determination.

METHODOLOGY

96-well plate assay

Microtiter plate assay was used to evaluate biofilm formation which occurs when bacteria adhere to an abiotic surface. Bacteria were inoculated into the 96-well microtiter plates and subsequently incubated. After incubation, all microtiter were rinsed to remove planktonic bacteria. The remaining bacteria after rinsing process were stained with crystal violet to visualization the biofilm formation. Visualisation was followed with quantification using spectrophotometry.

Antibiotic Susceptibility Testing

A culture broth was prepared for each organism under study to inoculate bacteria into Muller Hinton agar. After inoculation, filter paper disks were distributed in all MH media. Disk diffusion test was calibrated to EUCAST clinical breakpoint.

Microorganisms' strains

Organisms were readily available for routine lab work and obtained purely from Quality Control media to ensure better results. Organisms were *Pseudomonas aeruginosaMPA01*, *S. marcescens310258*, *E. coli310217*, *E. coli704476/2* and *Coliform B706498* (a mixture of *Klebsiella*, *Enterobacter and E. coli*)

Statistical analysis

P-value was determined to be a value of significance when it yielded <0.05. One-way ANOVA and Tukey post-test were utilized to state a clinical significance difference.

RESULTS

Formation of biofilm was demonstrated through the 96 well plate assay. The figures illustrate the mean absorbance at 550 nm for five different microorganisms, *P. aeruginosa MPA01*, *S. marcescens310258*, *E. coli310217*, *E. coli704476/2* and *Coliform B706498* (a mixture of *Klebsiella*, *Enterobacter* and *E. coli*).

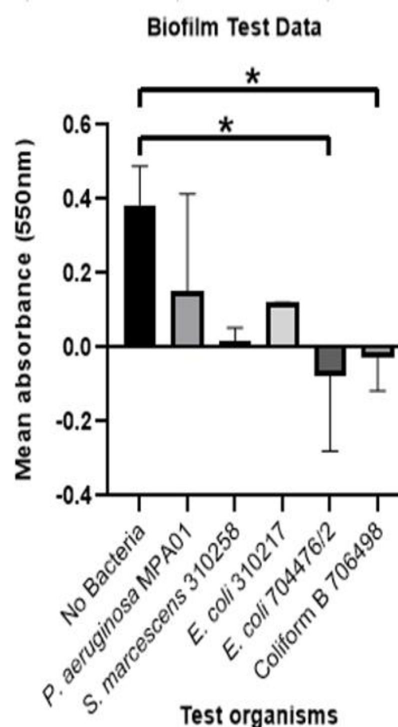

Figure 1: The mean absorption values at 550 nm for P. aeruginosa MPA01, *S. marcescens 310258*, *E. coli 310217*, *E. coli 704476/2* and *Coliform B 706498* test organisms. The values were obtained from the 96 wel plate assay. The bar chart shows mean, +/- SD and P value using one-way ANOVA test with a Tukey post-test with a comparisons (***P value ≤0.05**).

From the one-way ANOVA (Figure 1), it was clear that no-well bacteria and *E. coli* 704476/2 had significant difference (*P*=0.02). Moreover, no-bacteria well and *coliform B 706498* had significant difference (*P*=0.04). However, no statistically significant differences (*P*>0.05) were shown between other well microplates which indicating that no relationship was obtained.

Figure 2 shows that there were highly significant differences (*P*<.0001) when comparing *P. aeruginosa MPA01* with no-bacteria well, *S. marcescens 310258*, *E. coli 310217*, *E. coli 704476/2* and *Coliform B 706498*. Furthermore, the comparison of no-bacteria well with *S. marcescens 310258* indicated that there was significant difference (*P*=0.03).

EUCAST disk diffusion test was used for antibiotic susceptibility testing for four organisms *S. marcescens 310258*, *E. coli 310217*, *E. coli 704476/2*, and *coliform B 706498* followed by control organism, against all four

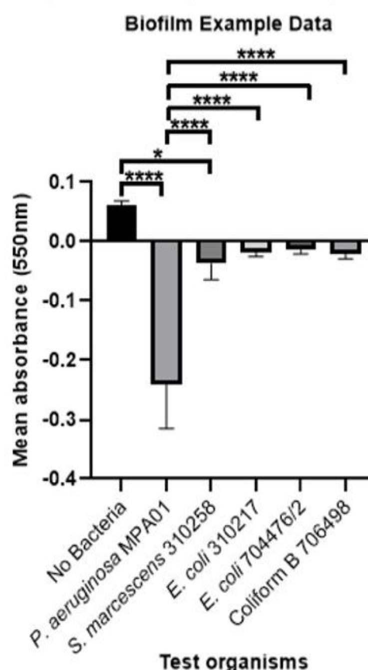

Figure 2: The mean absorption values at 550 nm for P. aeruginosa MPA01, *S. marcescens 310258*, *E. coli 310217*, *E. coli 704476/2* and *Coliform B 706498* test organisms. The values were obtained from the 96 wel plate assay. The bar chart shows mean, +/- SD and P value using one-way ANOVA test with a Tukey post-test with a comparisons (***P value ≤ 0.05***), (*****P value ≤ 0.0001***).

Table 1: EUCAST zone diameters of cephalosporins for each test organisms reported susceptible (S) or resistance (R) responses with the expected zonediameter ofthe control organism.

Test organisms	**EUCAST zone diameter breakpoints (mm) of Cephalosporin antibiotics**							
	CFX		**CXM**		**CTX**		**CPM**	
	Test 1	**Test 2**	**Test 1**	**Test 2**	**Test 1**	**Test 2**	**Test 1**	**Test 2**
S. marcescens 310258	2 (R)	0 (R)	15 (R)	18 (R)	22 (S)	26 (S)	30 (S)	33 (S)
E. coli 310217	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)
E. coli 704476/2	15 (S)	16 (S)	22 (S)	23 (S)	28 (S)	29 (S)	34 (S)	31 (S)
Coliform B	14 (S)	15 (S)	18 (R)	17 (R)	23 (S)	23 (S)	35 (S)	38 (S)
E. coli 10418 (Control)	18 (S)	18 (S)	22 (S)	26 (S)	26 (S)	29 (S)	33 (S)	37 (S)

generations of cephalosporin antibiotics Cefalexin (CFX), Cefuroxime (CXM), Cefotaxime (CTX), and Cefepime (CPM) as clearly shown in Table 1.

Table 1 shows that control organism and *E. coli 704476/2* were susceptible to all fourantibiotic discs, however, *S. marcescens* and *coliform B* were resistant to CXM and susceptible to CTX and CPM antibiotics. Interestingly, *E. coli 310217* was observed to be resistant to all cephalosporins.

DISCUSSION

Public health and clinical microbiologists recognised biofilms to be ubiquitous in nature, and many infectious diseases appear to be associated with biofilm formation. Namely, central nervous system shunt infection, contact lens associated keratitis, chronic sinusitis, chronic otitis, cystic fibrosis, chronic wound infection, recurrent urinary

tract infection, chronic osteomyelitis, and Crohn's disease (Del-Pozo, 2018; Claret et al., 2007). Based on the National Institute of Health, approximately 80% of human bacterial infections are due to biofilm-associated microorganisms (Romling and Balsalobre, 2012). Although biofilm has an increased prevalence among bacterial infections, it is still might be underestimated. It was shown that a distinctive spectrum of medical devices has a significant potential to harbour biofilms, leading to a considerable rate of device-related infections that complicate people at high risk of disease as immunocompromised patients (Costerton et al., 1999; Donlan, 2001; Lynch and Robertson, 2008).

Biofilm formation ability is exhibited as shown in the experiment, a p-value of a significance *(P*≤.05) which triggered the rejection of the null hypothesis by default that states no statistical significance difference between two variables. One-way ANOVA clearly showed statistically significant difference in two test organisms (refer to Figure 1), as compared with six values of significance (refer to Figure 2), with a diverse degree of significance. Apart from that, variability in biofilms formation may occur due to many factors. More specifically, incubation conditions (e.g., temperature), washing procedure, an organism's ability to adhere to a surface, and defect on the growth of an organism might be easily mistaken for a deficiency in the attachment capability, such as the factors that could interfere with results' interpretations (Merritt et al., 2005). In addition, growth time of a biofilm and staining pattern may emerge to be distinct from one organism to another, and that's why it comes essential to empirically determine the proper condition to grow a biofilm. Such as in the case reported by Merritt et al. (2005), when *P. aeruginosa* developed in both aerobically and anaerobically (carbon source), positive crystal violet staining pattern is exhibited, while when only grown aerobically, it forms a ring of staining (air medium interface). This report is supported by Sawyer and Hermanowicz (1998) and Hunt et al. (2004) who reported that crystal violet stain in 96 well plate assay may face a gradual loss of biomass attaching to the assay surface due to a progressive lack of nutrients, which stimulates microorganism to detach. Another condition that can emerge variations in biofilm formation, is the availability of nutrients. For example, *S. marcescens* biofilm forming ability is nutrient-dependent as suggested by Rice et al. (2005).

AST results showed a different pattern of susceptibility and resistance even for the same species as *E. coli* (e.g., 704476/2, and 310217 test organisms, and 10418 as a control). Control organism and *E. coli704476/2* were all susceptible to cephalosporins. Moreover, *S. marcescens* and *coliform B* were resistant to cefuroxime and susceptible to third and fourth generation cephalosporins. It was reported by Verbist (1976) that cefuroxime, cefazolin, and cefamandole were the most effective cephalosporins against 344 isolates of *Enterobacteriaceae* except *S. marcescens*. Additionally, it was observed that cefoxitin was the only effective antibiotics against *S. marcescens* and by far most suitable for cephalothin resistant *E. coli, Proteus mirabilis* and *Klebsiella*. AST of test *E. coli310217* revealed a resistant pattern to all four generations of cephalosporins that suggests the presence of ESBL or AmpC beta-lactamases. It was demonstrated that resistance to Cefotaxime and Cefepimecan work as a surrogate marker to identify ESBL or AmpC beta-lactamase-producing *E. coli* (Rossolini et al., 2008). ESBL and AmpC beta-lactamase strains showed increasingly noted alarming rates worldwide (Coque et al., 2008; Rodriguez-Villalobos et al., 2011). According to Pitout (2010), ESBL and AmpCbeta-lactamase bacteria can cause life-threatening infections, limited therapy options, high costand deleterious effect on clinical outcomes. Similarly, Park et al. (2009) and Tumbarello et al. (2007) reported the negative effects of ESBL and AmpCbeta-lactamase bacteria. However, in a typical or conventional resistance to antimicrobial agents, biofilms acquire the unique type of resistance against innate host defence, making it difficult to be eradicated (Anderl et al., 2000).

Another consideration needs to be filled to reach a better understanding. Indeed, a comprehensive recognition of a pathogenic organism's growth and interaction in a biofilm can lead to the development of an infectious disease. A study by Donlan (2002) showed a list of the characteristics of biofilms which play an essential role in the infectious disease process. For instance, in biofilms resistance to immune system, some biofilm-associated microorganism may induce endotoxins production and less antimicrobial susceptibility of biofilm cells. As well as within a biofilm, cells can exchange resistant plasmids and bloodstream infection, urinary tract infection or emboli may occur when biofilm or its cells get detached. As a prospectus for future studies, researches should focus on genes expressed by biofilm-producing microorganisms, control measures for indwelling medical devices (e.g., coated with antimicrobial agents) to prevent colonisation spread of these organisms in healthcare settings, emphasise on the resistance exhibited by biofilms and more advanced treatments. Continual work between CLSI and EUCAST to be always in agreement and avoid missing detection of cases for countries selecting one approach over the other. However, Kassim et al., (2016) showed comparable AST breakpoints between CLSI and EUCAST. And since EUCAST is freely available, it provides an easy access for laboratories with low income and poor settings.

In conclusion, the experiment was conducted to investigate biofilm formation and AST of test organisms. Biofilms emerge to pose a critical situation for microbiologists and public health due to an elevated resistance rate to antimicrobials. It is found to complicate patients on indwelling devices. Thus, more appreciation of the biofilms and its part in infectious diseases can improve

the entire clinical process in terms of decision making.

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