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LETTER TO THE EDITOR Open Access

Determination of optimum incubation time for formation of *Pseudomonas aeruginosa* and *Streptococcus pyogenes* biofilms in microtiter plate

Mohammad Abdulraheem Al-kafaween, Abu Bakar Mohd Hilmi*, Norzawani Jaffar, Hamid Ali Nagi Al-Jamal and Mohd Khairi Zahri

Abstract

Pseudomonas aeruginosa and *Streptococcus pyogenes* are the most common pathogens to humans and are able to form a biofilm following ineffective precautionary approach. Biofilm is defined as a surface-attached community of bacterium embedded in an extracellular matrix which leads to tremendous problems in the environment, among humans and animals. This study aims to investigate the ability of *P. aeruginosa* and *S. pyogenes* to form biofilms in 96-well plate before further study in antibiofilm will be done. Initially, the 96-well plate was added with 100 μl of overnight *P. aeruginosa* culture with optical density (OD) 0.1 and *S. pyogenes* culture with OD 0.05. The cultures were incubated for 7 days at 37 °C to justify the formation of biofilm. Subsequently, stained blue biofilm was detached from the plate by using 95% ethanol. Biofilms were finally measured using a micro plate reader at 570 nm and were classified based on the adherence strength formula. *P. aeruginosa* and *S. pyogenes* biofilms strongly adhered to the plates on days three, four, five and six. Interestingly on day three, biofilms showed the highest formation. However, moderate biofilm formation onto the plates by both *P. aeruginosa* and *S. pyogenes* were observed on day two, but non-adherence was observed on days one and seven. Day three is the optimum cultivation period for *P. aeruginosa* and *S. pyogenes* to switch into a strong biofilm in microtiter plate and could be beneficial for antibiofilm experiments.

Keywords: Biofilm, *Pseudomonas aeruginosa*, *Streptococcus pyogenes*

Introduction

A biofilm is an intricate accumulation of microbial colonies. It leads to form a matrix which consists of a highly structured protective layer of polysaccharides (Ford 2014; López et al. 2010). The adherent cell in biofilm is embedded within a slimy extracellular matrix that contained of extracellular polymeric substances (EPS). Microbial cells are the primary components of biofilms that produce EPS which then contributes 50 to 90% of the total organic carbon in biofilms (Zhao et al. 2014; Flemming et al. 2000). EPS is actually composed of polysaccharide and varies in physical and chemical properties, and is neutral or polyanionic in Gram-positive or Gram-negative bacteria. Its size is between 0.2 to 1.0 nm (Kokare et al. 2009; Nyenje et al. 2012, 2013), and its thickness ranges between 10 and 30 nm (Khan et al. 2017). Microbial cells in biofilm are immobilized by EPS (Flemming et al. 2016; Koo and Yamada 2016) which then retains the cells closely and leads the forming of synergistic micro consortia (Flemming and Wingender 2010). The formation and maintenance of structured multi-cellular microbial communities in biofilm crucially depends on the quantity of EPS (Janissen et al. 2015). Usually, a biofilm matrix is composed of carbohydrate, protein, pili, flagella, adhesion fibres and cellulose (Kostakioti et al. 2013; Xiao and Zheng 2016). All these components with the addition of hydrophobic interaction, and action and entanglement of the biopolymers in the culture plate provide sufficient mechanical

* Correspondence: mhilmiab@unisza.edu.my
Faculty of Health Sciences, Universiti Sultan Zainal Abidin, Kuala Nerus, Terengganu, Malaysia

stability to maintain spatial arrangement of the biofilm (Mazza 2016; O'Loughlin et al. 2013). Therefore, our study aims to determine the optimum incubation time for *P. aeruginosa* and *S. pyogenes* to form biofilms in the microtiter plate.

Materials and methods

Bacterial growth

A Gram-positive of *S. pyogenes* (ATCC 19615) and a Gram-negative of *P. aeruginosa* (ATCC 10145) were used for biofilm formation. *P. aeruginosa* and *S. pyogenes* inoculums prepared by picking up two to three morphologically identical colonies from stock culture which were then suspended in 10 mL of sterile Mueller Hinton broth in sterilized universal bottles. The inoculums were incubated at 37 °C for 24 h (Shehu et al. 2016).

Biofilm assay

Initially, 2 ml of inoculums was removed aseptically from the universal bottle and poured into a micro cuvette (Eppendorf, Germany). The optical density (OD) 0.1 for *P. aeruginosa* and OD 0.05 for *S. pyogenes* were adjusted by using sterile Mueller Hinton broth (MHB) at 600 nm with a spectrophotometer (Genesys 20, Thermo Scientific). Subsequently, *P. aeruginosa* and *S. pyogenes* biofilms were prepared by transferring 100 μl of adjusted inoculums into sterile 96-well plates (Fisher Scientific, UK). As a negative control, broth without bacteria was prepared. The incubation was done at 37 °C for 7 days. The media were then removed by slightly tapping the plate. The plate was washed three times with sterile distilled water to remove free-floating planktonic bacteria and was then drained off by inverting to allow it to air dry. The biofilms were stained with 100 μl 0.1% (*w*/*v*) crystal violet for 10 min. To remove the crystal violet, the plate was washed three times with phosphate-buffered saline. To detach the biofilms, 100 μl of 95% ethanol was added into each well. The solubilized biofilm formations were finally measured by the micro plate reader (Tecan Infinite 200 PRO, Austria Gmbh) at the wavelength of 570 nm (Jaffar et al. 2016). The experiments were performed in triplicate. The following formulas were used to classify the biofilm formation. Non-adherent [NA = OD ≤ ODC)], weak adherent [WA = ODC < OD ≤ (2 × ODC)], moderate adherent [MA = (2 × ODC) < OD ≤ (4 × ODC)] and strong adherent [SA = (4 × ODC) < OD)] (Nyenje et al. 2013).

Statistical analysis

Quantitative analysis of biofilm formation was statistically tested by using SPSS (version 20.0: IBM). Mean difference was determined by using an independent *t* test. Statistical significance was set at $p < 0.05$. Data are represented as mean values ± standard deviation.

Results and discussion

The qualitative assay showed the formation of *P. aeruginosa* and *S. pyogenes* biofilms in the microtiter plate (Fig. 1). Biofilm was stained blue in colour.

After 7 days of incubation in MHB OD 0.1 and MHB OD 0.05, a variety of biofilm phenotypes were demonstrated. Figure 2 shows biofilm formation by *P. aeruginosa.* Strong biofilm formation were determined on days three, four, five and six, (1.0 ± 0.10), (0.78 ± 0.04), (0.64 ± 0.02) and (0.37 ± 0.03), respectively. Biofilm moderately adhered on day two (0.12 ± 0.01). However, on days one and seven the biofilms were non-adherent, (0.06 ± 0.00) and (0.09 ± 0.01), respectively.

The biofilm formation by *S. pyogenes* has shown similar characteristics with *P. aeruginosa* biofilm (Fig. 3). The strongest adherent *S. pyogenes* biofilms were on days three, four, five and six, (0.96 ± 0.05), (0.70 ± 0.02), (0.58 ± 0.02) and (0.33 ± 0.01), respectively. Biofilm moderately

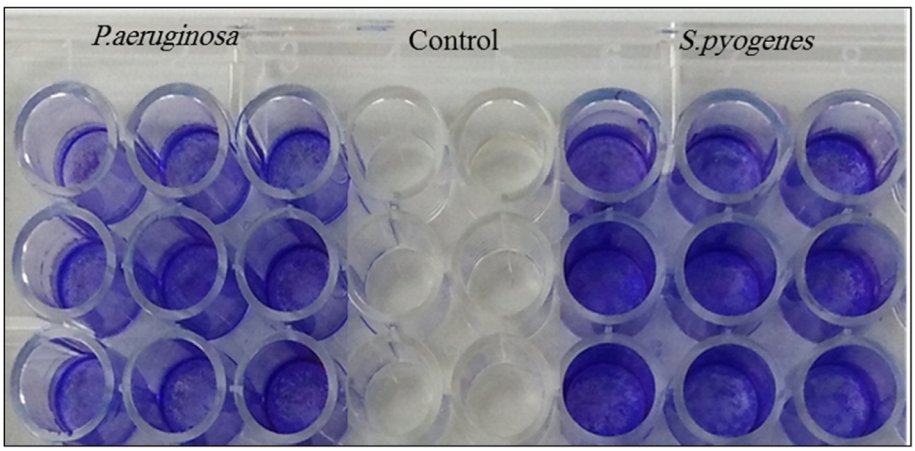

Fig. 1 *P. aeruginosa* and *S. pyogenes* biofilms. Biofilm formation qualitative assay in microtiter plate determined by using crystal violet. Biofilm was stained blue.

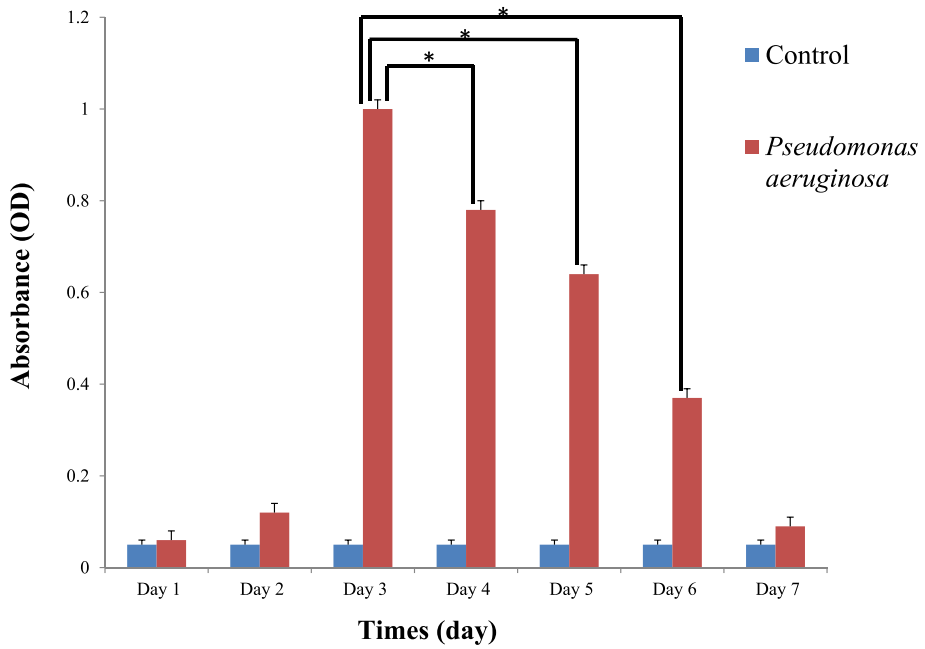

Fig. 2 Biofilm formation by *P. aeruginosa*. Quantitative biofilm analysis for 7 days in microtiter plate. Asterisk* indicates the mean differences were significant ($p \leq 0.05$)

adhered on day two (0.13 ± 0.01). Meanwhile, on days one and seven, the biofilms were non-adherent, (0.05 ± 0.00) and (0.08 ± 0.01), respectively.

P. aeruginosa biofilms on day three were significantly higher compared to days four, five and six, $p < 0.014$, $p < 0.016$ and $p < 0.017$, respectively. Similarly *S. pyogenes* biofilms on day three were significantly higher compared to days four, five and six, $p < 0.018$, $p < 0.014$ and $p < 0.017$, respectively. Thus, we suggest on day three, a large number of bacteria in the plate were switched to biofilm. An optimum number of bacteria adherence occurred in microtiter plate resulting strong biofilm formation on that day (Jama et al. 2017; Rossi et al. 2016). In addition to day three, we observed that the biofilm

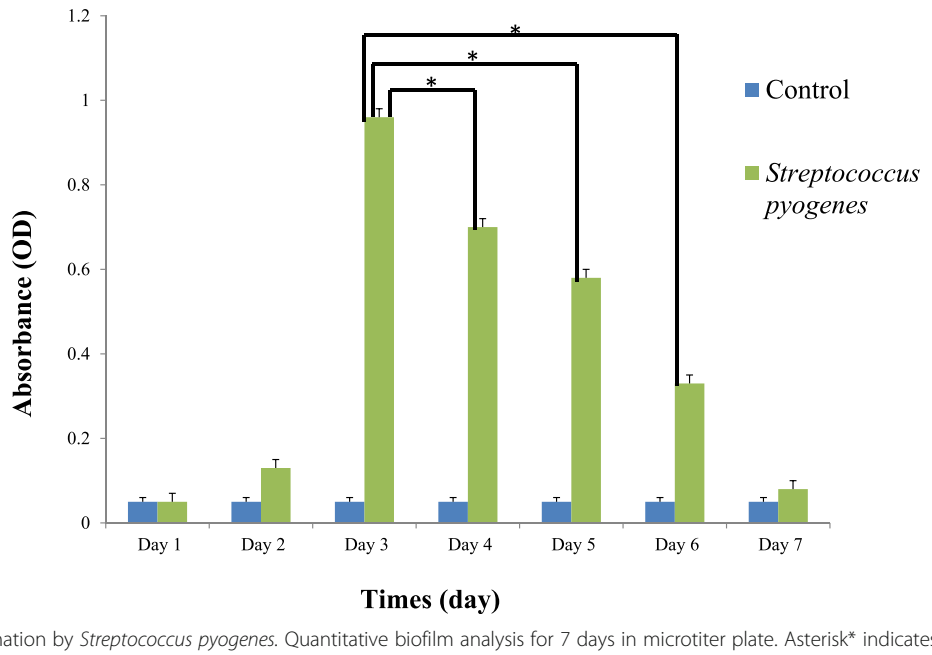

Fig. 3 Biofilm formation by *Streptococcus pyogenes*. Quantitative biofilm analysis for 7 days in microtiter plate. Asterisk* indicates the mean differences were significant ($p \leq 0.05$)

formation by *P. aeruginosa* and *S. pyogenes* were strong on days four, five and six. *P. aeruginosa* and *S. pyogenes* were not able to produce biofilm on days two and seven. Many factors such as integration of diverse signals from the environment might play a role in biofilm formation, concurrent with other events such as phenotypic and genetic switching during biofilm production and also EPS production (Ismael 2013; Bakar et al. 2018). Commonly, biofilm formation is enhanced by cell motility particularly when it is mediated by flagella. Under certain environmental conditions, flagella is necessary for biofilm formation by *P. aeruginosa* and *S. pyogenes* (Priya and Brundha 2013). However, the rapid decrease of biofilm-forming capacity that we observed on day seven could be attributed to the loss of exopolymers from the biofilm and in particular of exopolysaccharides, which may suggest that an active process of detachment was occurring, probably mediated by enzymatic degradation (Allison et al. 1998). Previous studies showed that *P. aeruginosa* produced a great biofilm on day three, while *Escherichia coli* was produced biofilm on day six (Culotti and Packman 2014). Another study found that day three was the preferable day in producing strong biofilm by *Proteus mirabilis* (Emineke et al. 2017). A study in 2001 showed that an ideal cultivation period for producing biofilms by *Candida albicans* was at 72 h and *Saccharomyces cerevisiae* was at 60 h (Chandra et al. 2001). The current study shows that many types of pathogenic *Leptospira* biofilms were classified either as non-adherent or weak adherent after the first and second days of incubation and the strongest biofilm production was found from the third to seventh days (Pui et al. 2017). Another study showed that day five was the optimum day for *Leptospires* to produce greater biofilm formation (Apun et al. 2018). Previous studies also reported that *Listeria monocyte* and *Listeria* sp. produced strong biofilms by which they gradually increased after 2 to 7 days of incubation (Adetunji and Isola 2011; Mueller et al. 2007). A study by Jaffar et al. (2016) demonstrated that *Actinomycetemcomitans* and *Porphyromonas gingivalis* produced biofilm from days two to seven, with differing attachment ability. The present study demonstrates that strong biofilms formed on day three. The ability to adhere to a solid surface and the consecutive formation of an organized bacterial biofilm community are crucial for the formation of *P. aeruginosa* and *S. pyogenes* biofilms. This is because the formation of biofilm depends on the ability of bacteria to attach on the surface for 96-well plates (Merritt et al. 2006). It is well known that the switching from a planktonic to a biofilm mode of growth is an intricate process, which occurs in response to environmental changes. As the first step of biofilm formation is bacterial adhesion to surface, we can hypothesise that the strains showed a high ability to create hydrophobic interactions with the microtiter plate surface (Woo et al. 2012). Moreover, physical and chemical plate properties are the main factors that regulate the initial adhesion process (Lemos et al. 2014) .

Conclusion

This study demonstrated that *P. aeruginosa* and *S. pyogenes* successfully formed biofilms on the 96-well plate by using MHB after 3 days of cultivation. The cultivation period had significantly affected biofilm formation for both bacteria. Importantly, the study has proven that a duration less than 3 days or more than 3 days was not the optimum condition to form biofilm for both *P. aeruginosa* and *S. pyogenes* in microtiter plate.

Abbreviations

ATCC: American type culture collection; EPS: Extracellular polymeric substances; IBM: International Business Machines Corporation; MA: Moderate adherent; MHB: Mueller Hinton broth; NA: Non-adherent; OD: Optical density; *p*: Probability value; SA: Strong adherent; SPSS: Statistical package for the social sciences; *w/v*: Weight per volume; WA: Weak adherent

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MAA collected all the data and prepared the manuscript. ABMH prepared a part of the manuscript. ABMH, HANA, MKZ, and NJ designed the whole experiment. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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