To Biofilm or Not to Biofilm?

Valentina Živković[1], Tomislav Kurevija[1], Ivana Haršanji Drenjančević[1,2], Maja Bogdan[1,3], Maja Tomić Paradžik[1,4] Jasminka Talapko[1], Domagoj Drenjančević[1,2]

[1] Faculty of Medicine, Josip Juraj Strossmayer University of Osijek, Osijek, Croatia

[2] University Hospital Center Osijek, Osijek, Croatia

[3] Institute of Public Health Osijek-Baranja County, Osijek, Croatia

[4] Institute of Public Health Brod-Posavina County, Slavonski Brod, Croatia

Corresponding author: Domagoj Drenjančević, MD, PhD - domagoj@mefos.hr

Abstract

Aim: The goal of this research is to examine the biofilm forming ability of Staphylococcus aureus and Pseudomonas aeruginosa clinical isolates in different in vitro conditions using Meuller-Hinton and Luria-Bertani broths.

Material and methods: 30 strains of Pseudomonas aeruginosa and 30 strains of Staphylococcus aureus obtained from clinical specimens were used. After preparing the suspensions of bacteria inoculated on broths, they were set on microtiter plates and the biofilm production was measured using the spectrophotometric reader on 550 nm. Strains were classified into four categories: non-producing, weak producers, moderate and strong producers, based on the comparison of optical density of samples and negative control.

Results: Both tested species successfully formed a biofilm in both broths ($p<0.01$). P. aeruginosa strains had a higher percentage of strong producers in both in vitro conditions, in comparison with S. aureus strains (3.3% vs 50%). Nevertheless, there is no statistically significant difference in biofilm formation between the strains, regardless the used broths, and there is no statistically significant difference between the biofilm forming ability of both species observed separately regarding in vitro conditions either.

Conclusion: Both species have an ability to produce biofilm, which likely contributes to the pathogenicity and virulence of these bacteria and also leads to a better understanding of their in vivo characteristics to cause infections related to biofilm.

(Zivkovic V, Kurevija T, Harsanji Drenjancevic I, Bogdan M, Tomic Paradzik M, Talapko J, Drenjančević, D. To Biofilm or not to Biofilm? SEEMEDJ 2018;2(1);12-19)

Received: September 29, 2017; revised version accepted: April 3, 2018; published: November 27, 2018

KEYWORDS: biofilm, P. aeruginosa, S. aureus

Introduction

Biofilm formation is one of the additional bacteria virulence factors which is still an interesting subject for numerous researches. Biofilm infections are becoming a major health problem in chronic infections and implants. Biofilm is a multicellular structure that protects bacteria from adverse environmental factors, making them highly resistant to different antibiotics. It also stores nutrients, which serve the bacteria to survive, protects them from phagocytosis, and secures survival in the host organism. Resistance to disinfectants is a very important characteristic of biofilm because it prevents removing bacteria from the surface, enabling such microorganisms to permanently colonize the human organism with pathological consequences. Biofilm should be considered as a mobile functional community with the features of a complete microorganism because, among other things, they have homeostasis, circulatory system, genetic material exchange and metabolic activity, which ensure their further development (1). In addition, biofilm-protected bacteria are capable to disperse individual bacterial cells and decomposing parts of biofilm into the surrounding tissues and circulation system. But most importantly, on the surfaces of medical devices or in the human body, biofilm is made by microorganisms with the ability to produce an extracellular polymeric substance. These polymeric substances have an ability to incorporate a large amount of water into their structure and become highly hydrated (2). These solid-liquid barriers between the surface and the aqueous environment allow the community of biofilms optimal conditions for the growth and survival of microorganisms. Also, biofilm is formed exclusively by the cells that produce polysaccharides in sufficient quantity (3). Several environmental and genetic signals control each step of biofilm development and dispersal. Accumulation of signal molecules in the environment allows each bacterial cell to estimate cell density or the total number of bacteria at that time – the quorum detection or quorum sensing phenomenon. Colonization of medical devices is proportionally increased by surface irregularity and microorganisms bond more rapidly to hydrophobic surfaces such as plastic, rather than hydrophilic ones. (2,4). The appearance of biofilm on implants and various surgical implantable devices causes chronic infections, rejection of implants, ineffectiveness of the embedded device, organ damage, and sometimes even lethal outcome for the patient.

The aim of this research is to examine the biofilm forming ability of Staphylococcus aureus and Pseudomonas aeruginosa clinical isolates in different in vitro conditions using Mueller-Hinton and Luria-Bertani broths.

Material and methods

Sample preparation

This study included 60 bacterial strains, 30 Pseudomonas aeruginosa and 30 Staphylococcus aureus strains, obtained from different clinical specimens from 2007 to 2015 and isolated in microbiological laboratories at University Hospital Center Osijek, Croatia and in the General Hospital Slavonski Brod, Croatia. All bacterial strains are part of the collection of microbial strains kept at the Department of Microbiology and Parasitology, Faculty of Medicine, University of Osijek. Microorganisms were identified according to standard microbiological methods and biochemical tests to the species level (5). After the bacteria had been grown on the blood agar plate during 18-24 hours incubation, two to three individual colonies of bacterial cultures were taken and inoculated into vials with 3 ml of Mueller-Hinton (MH) (Becton Dickinson and Co., Cockeysville MD, USA) and Luria-Bertani (LB) (Difco R Luria-Bertani broth, Becton Dickinson, USA) broth. The suspensions were incubated in the thermostat at 37°C for another 18-24 hours. After incubation, the tubes were well mixed (vortexed) and 20 μl from each suspension was transferred into new tubes with 2 ml MH and LB broth, which yielded suspensions of approximately 5x105 CFU/ml concentrations. After the preparation, suspensions were planted on flat bottom

polyester microtiter plates (Copan, Brescia, Italy). Wells with 100 μl of uninoculated MH and LB were used as the negative control and the remaining wells had 50 μl MH or LB broths which were planted with 50 μl of the prepared suspensions. The biofilm-producing strain Acinetobacter baumannii ATCC 19606 was used as a positive control. The microtiter plate was incubated in the thermostat for 18-24 hours at 37°C. After the incubation, the broth was shaken out and wells were washed three times with distilled water. At the end of the experiment, coloring with 0.1% crystal violet and solubilization with 95% ethanol was done (6). All measurements were done in triplicate.

Quantification of biofilm

The final step was a spectrophotometric measurement of biofilm production on an enzyme immunoassays plate reader (BioRad 93200 PR3100 TSC Microplate Reader) at 550 nm. The optical density (OD) values were measured in every well of the plate and they represent biofilm production. The final results were reported as the optical density cut-off value (ODc), which was calculated as average OD for each sample made in triplicate increased by three standard deviations of negative controls. The results were classified into the following categories: non-producers, weak, moderate and strong biofilm producers (6,7) according to the criteria presented in Table 1.

Table 1. The criteria for evaluating biofilm production

Criterion	Category
$OD < ODc$	Non-producers
$ODc < OD < 2 \times ODc$	Weak producers
$2 \times ODc < OD < 4 \times ODc$	Moderate producers
$4 \times ODc < OD$	Strong producers

OD = average optical density value of biofilm production in a single well; ODc = limit value of biofilm production (at least some biofilm produced)

Statistical Analysis

The results were processed using the statistical software package SPSS 19.0 (IBM Corp., Armonk, NY, USA), and the data processing was carried out by checking normality distribution and calculation of descriptive data, including the frequencies, percentages, median and interquartile ranges. Wilcoxon test of equivalent pairs, $\chi 2$ test with Fisher's exact test and Cramer's V (ϕ) coefficient were utilized for the statistical significance testing of the differences between two or more independent groups.

Results

The biofilm production ability data for both bacterial species regarding the in vitro nutrient condition (incubation in Mueller-Hinton and Luria-Bertani broths) are shown in Table 2. Data are presented as the average of triplicate measurement of optical density and includes medians and interquartile ranges for each variable used.

Table 2. The amount of biofilm formed, presented as the average optical density for *S. aureus* and *P. aeruginosa* using Luria-Bertani and Mueller-Hinton broths, in comparison to control

Staphylococcus aureus		C	Q
Luria-Bertani broth	control	0.059	0.014
	OD (AR)	0.085	0.031
Mueller-Hinton broth	control	0.076	0.011
	OD (AR)	0.097	0.043
Pseudomonas aeruginosa			
Luria-Bertani broth	control	0.032	0.017
	OD (AR)	0.318	0.481
Mueller-Hinton broth	control	0.070	0.07
	OD (AR)	0.330	0.602

Legend: C = median; Q = interquartile range; OD (AR) = average optical density

By comparing the data for both bacteria and cultivation media (Table 2), it can be seen that interquartile dispersal is greater for P. aeruginosa than for S. aureus strains.

Table 3. Distribution of biofilm production in *S. aureus* and *P. aeruginosa* strains using Luria-Bertani and Mueller-Hinton broth ($p<0.01$, Wilcoxon's Equivalent Pair Test)

Staphylococcus aureus	**non-producers**	**weak producers**	**moderate producers**	**strong producers**
	f (%)	f (%)	f (%)	f (%)
LB	17 (56.7)	13 (43.3)	0 (0)	0 (0)
MH	19 (63.4)	9 (30)	1 (3.3)	1 (3.3)
Pseudomonas aeruginosa	**non-producers**	**weak producers**	**moderate producers**	**strong producers**
	f (%)	f (%)	f (%)	f (%)
LB	0 (0)	8 (26.7)	4 (13.3)	18 (60)
MH	4 (13.3)	6 (20)	5 (16.7)	15 (50)

Legend: LB = Luria-Bertani broth; MH = Mueller-Hinton broth; f = frequency

It has been found that both bacterial species, S. aureus and P. aeruginosa successfully (to a statistically significant degree) created biofilm in both cultivation media ($p<0.01$, Wilcoxon's Equivalent Pair Test).

Table 4. The contingency table for biofilm production of *P. aeruginosa* and *S. aureus* strains in Luria-Bertani and Mueller-Hinton broth.

Pseudomonas aeruginosa		**Luria-Bertani broth**			
		weak producers	moderate producers	strong producers	**Total**
Staphylococcus aureus	non-producers	**2** **6.7%**	**2** **6.7%**	**13** **43.3%**	**17** **56.7%**
	weak producers	**6** **20.0%**	**2** **6.7%**	**5** **16.7%**	**13** **43.3%**
	total	**8** **26.7%**	**4** **13.3%**	**18** **60.0%**	**30** **100.0%**

Pseudomonas aeruginosa		**Mueller-Hinton broth**				
		non-producers	weak producers	moderate producers	strong producers	**Total**
Staphylococcus aureus	non-producers	**3** **10%**	**3** **10.0%**	**3** **10.0%**	**10** **33.3%**	**19** **63.3%**
	weak producers	**0** **0%**	**2** **6.7%**	**2** **6.7%**	**5** **16.7%**	**9** **30.0%**
	moderate producers	**0** **0%**	**0** **0%**	**0** **0%**	**0** **0%**	**0** **0%**
	strong producers	**0** **0%**	**1** **3.3%**	**0** **0%**	**0** **0%**	**1** **3.3%**
	total	**4** **13.3%**	**6** **20.0%**	**5** **16.7%**	**15** **50.0%**	**30** **100.0%**

The correlation between the tested bacterial species according to their biofilm production ability is shown in Table 4. There is no statistically significant difference in biofilm formation between S. aureus and P. aeruginosa strains in Luria-Bertani (Fischer's exact test, p=0.075) or in Mueller-Hinton broth (Fischer's exact test, p=0.359).

The ability to produce biofilm depending on different cultivation conditions is shown in Figures 1 and 2. Staphylococcus aureus strains had very modest biofilm production in both broths: 43.3% of the strains seem to be weak producers and the remaining are non-producers in Luria-Bertani broth. There is even a smaller number of biofilm weak producers (30%) in Mueller-Hinton broth, and almost all remaining ones are biofilm non-producers, with the exception of one moderate (3.3%) and one strong (3.3%) producer. P. aeruginosa strains belong to strong biofilm producers in both in vitro conditions. In Luria-Bertani broth, all tested strains were shown as biofilm producers. Weak producers accounted for 26.7% of the strains, moderate ones accounted for 13.3%, and 60.0% were strong producers. There was 20.0% of weak producers, 16.7% of moderate producers and 50.0% of strong producers in Mueller-Hinton broth, and 13.3% of the strains were biofilm non-producers.

There was no statistically significant difference between cultivation conditions and the ability to form biofilm either in S. aureus or in P. aeruginosa strains.

Figure 1. Biofilm production ability of S. aureus in Luria-Bertani and Mueller-Hinton broths (Fischer's exact test, p=0.664).

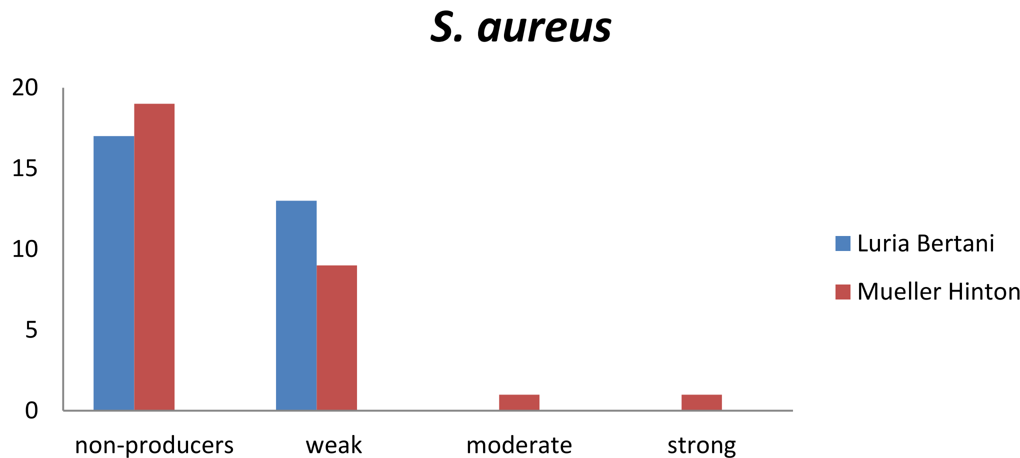
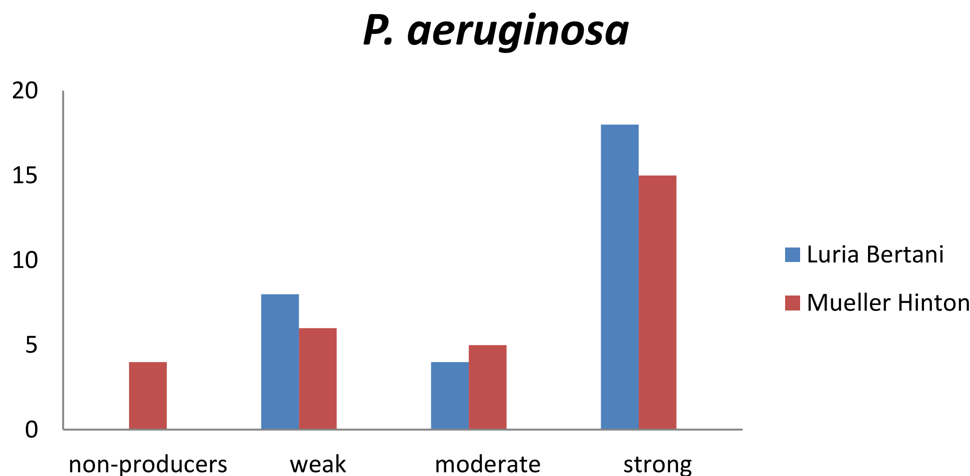


Figure 2. Biofilm production ability of P. aeruginosa in Luria-Bertani and Mueller-Hinton broths (Fischer's exact test, p=0.476).



Discussion

The main observation which arises from this study is that both bacterial species, gram-positive S. aureus and gram-negative P. aeruginosa, successfully and to a statistically significant degree form biofilm in both tested broths (Table 4). Another important observation is that interquartile dispersal is greater in P. aeruginosa compared to S. aureus strains. P. aeruginosa strains have a higher incidence of extreme values and thus a greater range of results. However, no statistical difference was observed with regard to the medium in which biofilm production was measured. Both species showed that biofilm production is more pronounced in Luria-Bertani medium by comparing the percentage, but no statistical significance has been established in statistical tests. Although Luria-Bertani medium is a medium in which higher production is expected, other authors have also pointed out the possibility that biofilm production may be unexpectedly expressed depending on the conditions of bacterial growth. Biofilm formation can be strongly affected both by growth media and by temperature (8,9). Another study (10) has also shown that both of these bacterial species are biofilm producers, independently of the clinical specimen isolation origin (sputum, urine, urine catheter, etc.). In this study, which involved the application of Congo agar and Tube method,

influence of the different in vitro conditions on biofilm forming ability of these two bacterial species was visible. (10) Both of the bacterial species have been shown to be strong producers of biofilm, with more than 80% of strong producers found (10). In our study, P. aeruginosa strains were strong producers in 55% cases, equally in both broths, whereas S. aureus strains had only one strong producer (3.3%). By comparing the results of this small series of experiments, it is reasonable to assume that the biofilm forming ability is greatly influenced by cultivation conditions, that it is nutrient dependent and also has a significant role in antimicrobial susceptibility of biofilms. (11,12,13)

Also, it is very important to emphasize that the role of biofilm in the genesis of infections associated with medical devices is indisputable. Microorganisms isolated from the samples of patients with these infections often exhibit the apparent ability to generate biofilms, as has been shown in many studies. (14,15) Additionally, it is known that multiple bacterial species can cooperate and form complex networks with many defending mechanisms and built-in sophisticated protection against the human immune system and antimicrobials as well. (16) Such polymicrobial biofilms are nowadays recognized as a significant factor in the pathogenesis of multiple infections in humans.

Conclusion

The obtained results are in agreement with previous medical and microbiological knowledge of biofilm formation, which plays a pivotal role in numerous infections such as periodontitis, chronic prostatitis, bacterial vaginosis, chronic otitis media, osteomyelitis, chronic pulmonary infections in cystic fibrosis patients, and chronic wound infection, considering that the investigated bacterial species, S. aureus and P. aeruginosa, are the most common etiological pathogens of these infections.

Funding
We greatly appreciate the financial support of University of Osijek, Faculty of Medicine (research grant VIF2016-MEFOS-27).

Transparency declaration
Competing interests: None to declare.

References

1. Milanov D, Ašanin R, Vidić B, Krnjajić D, Petrović J. Biofilm – organizacija života bakterija u prirodnim ekosistemima. Arhiv veterinarske medicine 2008;1:5-15.
2. Rodney MD. Biofilms: Microbial Life on Surfaces. Journal list. 2002;8:881–90.
3. Irie Y, Roberts AEL, Kragh KN, Gordon VD, Hutchison J, Allen RJ et al. The *Pseudomonas aeruginosa* PSL Polysaccharide Is a Social but Noncheatable Trait in Biofilms. MBio 2017;8:00374-17.
4. Characklis WG, McFeters GA, Marshall KC. Physiological ecology in biofilm systems. New York: John Wiley & Sons; 1990. p. 341–94.
5. Jorgensen JH, Pfaller MA, Carroll KC, Funke G, Landry ML, Richter SS, Warnock DW. Manual of Clinical Microbiology. Washington, DC, USA: ASM Press, 2015.
6. Milanov D, Bugarski D, Petrović J, Rackov O. Primena testa na mikrotitracionim pločama i mikroskopskih tehnika u ispitivanju sposobnosti nekih bakterijskih vrsta izolovanih od životinja da formiraju biofilm. Arhiv veterinarske medicine 2010;3:23-37.
7. Ghellai L, Hassaine H, Klouche N, Khadir A, Aissaoui N, Nas F et al. Detection of biofilm formation of a collection of fifty strains of *Staphylococcus aureus* isolated in Algeria at the University Hospital of Tlemcen. J Bacteriol Res 2014;6:1-6.
8. Labrie J, Pelletier-Jacques G, Deslandes V, Ramjeet M, Auger E, Nash JH, et al. Effects of growth conditions on biofilm formation by *Actinobacillus pleuropneumonia*. Vet Res 2010;41:3.
9. Nucleo E, Steffanoni L, Fugazza G, Migliavacca R, Giacobone E, Navarra A et al. Growth in glucose-based medium and exposure to subinhibitory concentrations of imipenem induce biofilm formation in a multidrug-resistant clinical isolate of

Acinetobacter baumannii. BMC Microbiol 2009;9:270.
10. Rewatkar AR, Wadher BJ. *Staphylococcus aureus* and *Pseudomonas aeruginosa*- Biofilm formation methods. IOSR-JPBS 2013;8:36-40.
11. Rochex A, Lebeault JM. Effects of nutrients on biofilm formation and detachment of a *Pseudomonas putida* strain isolated from a paper machine. Water Res 2007;41:2885-92.
12. Henry-Stanley MJ, Hess DJ, Wells CL. Aminoglycoside inhibition of *Staphylococcus aureus* biofilm formation is nutrient dependent. J Med Microbiol 2014;63:861-9.
13. Bogdan M, Drenjancevic D, Harsanji Drenjancevic I, Bedenic B, Zujic Atalic V, Talapko J et al. In vitro effect of subminimal inhibitory concentrations of antibiotics on the biofilm formation ability of *Acinetobacter baumannii* clinical isolates. J Chemother 2018;30:16-24.
14. Pour NK, Dusane DH, Dhakephalkar PK, Zamin FR, Zinjarde SS, Chopade BA. Biofilm formation by *Acinetobacter baumannii* strains isolated from urinary tract infection and urinary catheters. FEMS Immunol Med Microbiol 2011;62:328-38.
15. Rodríguez-Baño J, Martí S, Soto S, Fernández-Cuenca F, Cisneros JM, Pachón J, et al. Biofilm formation in *Acinetobacter baumannii*: associated features and clinical implications. Clin Microbiol Infect. 2008;14:276–8.
16. Hotterbeekx A, Kumar-Singh S, Goossens H, Malhotra-Kumar S. In vivo and In vitro Interactions between *Pseudomonas aeruginosa* and *Staphylococcus spp*. Front Cell Infect Microbiol 2017;7:106. doi: 10.3389/fcimb.2017.00106. eCollection 2017.