RESEARCH ARTICLE

Assessment of biofilm formation by pseudomonas aeruginosa and hydrodynamic evaluation of microtiter plate assay

Abdus Samad,[1] Amir Afzal Khan,[2] Muhammad Sajid,[3] Rabaab Zahra[4]

Abstract

Objective: To assess the biofilm formation in clinical and environmental isolates of Pseudomonas aeruginosa and to evaluate the hydrodynamics in microtiter plate assay and compare it with conventional assays for biofilm formation.

Methods: The cross-sectional study was conducted at the Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan, in 2013-14, while the computational work was done at the National University of Science and Technology, Islamabad. The study comprised environmental and clinical isolates of pseudomonas aeruginosa. Pseudomonas citramide agar was used as a selective media, and further confirmation was done by biochemical tests. Biofilm formation was assessed by Congo red assay, air liquid interfaceassay and microtiter plate assay. Computational Fluid Dynamics (CFD) simulations were also used to improve the microtiter plate assay for biofilm formation assessment. Polymerase chain reaction was used for screening of pelA and pelG genes.

Results: Of the 50 isolates, 25(50%) each were environmental and clinical. The number of biofilm producers observed in Congo red assay, air liquid interface assay and microtiter plate assay were 7(14%), 15(30%) and 30(60%) respectively. Biofilm former gene pelA was observed in 22(44%) isolates while 36(72%) isolates showed the presence of pelG gene.

Conclusions: Microtiter plate assay was found to be a reliable method to detect biofilm forming *pseudomonas aeruginosa* isolates which further provides a base for development of methods to detect biofilms readily and accurately.

Keywords: Biofilm, *P. aeruginosa*,Microtiter plate assay, Computational fluid dynamics. (JPMA 69: 666; 2019)

Introduction

Treatment of biofilm-associated persistent infections is an emerging issue for clinicians as bacterial cells adhere with human epithelial cells or indwelling medical devices such as implants and catheters used in urinary tract and respiratory infections.[1] Biofilms are complex surface-associated communities where bacterial cells are enclosed by self-produced extra cellular polymeric substances (EPS), which mainly consist of exopolysaccharides, proteins and extracellular deoxyribonucleic acid (DNA).[2-4] Pseudomonas (*P.) aeruginosa* is an important human pathogen which causes many infections ranging from wound infections to cystic fibrosis. Biofilm formation contributes to pathogenesis of *P. aeruginosa* both in acute as well as chronic infection in clinical settings.[5] Two dissimilar loci, pel and psl, related to EPS component of matrix in non-mucoid strains have been described. The pel gene cluster was first detected in PA14 and PAK strain[6,7] while psl was studied in *P. aeruginosa* strains PAO1 and ZK2870.[8,9] The pel operon is composed of seven genes, pelA- pelG, which are conserved in *P. aeruginosa* isolates. These genes have a key role in biosynthesis of glucose-rich matrix whose production depends upon the pel locus.

A number of methods are in practice to assess the biofilm formation of a bacterial strain. Most of these are phenotypic methods which include Congo red assay (CRA), Air liquid interface (ALI), tissue culture plate (TCP) method and Microtiter plate assay (MTPA). MTPA is considered the standard screening method for comparing adherence pattern and is the most widely used quantitative method for the detection of biofilm formation.[10,11] Mostly the assay is performed under standard static conditions and little is known about the hydrodynamics and flow pattern in MTPs. A few studies have applied computational fluid dynamics (CFD) simulations to describe flow pattern in MTPs during biofilm production and optimised the suitable conditions to detect the biofilm formation which have proven to be efficient.[12-14] It is suggested that higher flow rates increase the nutrient availability, promote cell growth, and attachment pattern with increased production of exopolymer, while the increase in flow velocity increases

[1,2,4]Department of Microbiology, Quaid-i-Azam University, Islamabad Pakistan, [3]School of Mechanical and Manufacturing Engineering (SMME), National University of Sciences and Technology (NUST), Islamabad, Pakistan.
Correspondence: Rabaab Zahra. Email: rzahra@qau.edu.pk

the shear rate, causing erosion and disassembly of biofilm production because of detachment from the surface.[15]

The current study was planned to phenotypically assess the biofilm formation ability of clinical and environmental isolates of *P. aeruginosa* and apply CFD simulations to evaluate the hydrodynamics in MTPAs, and compare the results with routine phenotypic assays used for testing biofilm formation.

Materials and Methods

The cross-sectional study was conducted at the Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan, in 2013-14, while the computational work was done at the National University of Science and Technology, Islamabad. The study comprised environmental and clinical isolates of *Pseudomonas aeruginosa*, and was done after approval was obtained from the Board of Advance Studies and Research of the Quaid-i-Azam University.

The environmental isolates were taken from different environmental sites like rivers, lakes, seepage and drinking water around Islamabad, while the clinical isolates were selected based on their resistance profile and carriage of resistance genes.[16] The strains were identified as *P. aeruginosa* by gram staining, colony morphology on P. cetrimide agar (Oxoid) and confirmed by standard biochemical tests such as catalase, oxidase and indole tests.

The biofilm formation assessment assay were planned using CFD simulations on a 3.3mm radius cylindrical domain of 11.6mm height that was filled half-way with water. A 12-block structured mesh was prepared (Figure-1A) consisting of 405,000 hexahedra type cells with a maximum aspect ratio, non-orthogonality and skewness of 2.07, 31.37 and 0.67 respectively. The volume-of-fluid (VoF) method was implemented in a finite volume discretisation using the open source CFD tool OpenFOAM (Open Source Field Operation and Manipulation).[17] The phase field operator was used to capture the air water two-phase flow by determining the fraction of liquid in each mesh element. An accelerating reference frame was used to capture the revolving motion of the orbital shaker by introducing a source term in the momentum equation to capture the effects of the well motion.[14] The simulations were carried out for 120-150 rpm and resulting hydrodynamic characteristics were studied.

Following previously described method,[18] the test organisms were streaked on CRA which comprised blood-base agar supplemented with glucose and Congo red dye. Congo red stain solution was prepared and autoclaved separately from other medium constituents. The assay was performed in triplicate. Test organisms were inoculated on CRA and incubated for 24-48 hours aerobically at 37°C. Biofilm forming strains produced black colonies, while non-biofilm-forming strains produced pink/white colonies.

ALI assay was performed in glass test tubes containing 3mL Luria broth (LB) medium in static conditions.[19] Incubation time was 10 days at 37°C. The pellicle visualisation was done by staining with crystal violet dye.

For MTPA, overnight culture of test strains were diluted in LB medium and 1µl inoculum was added to each well containing 100µl of M63 medium. Each strain was inoculated in triplicate and incubated at 37°C overnight with shaking at 120 rpm. Next day, the wells were washed three times to remove media components and planktonic cells, and stained with crystal violet. The wells were left at room temperature for 10-15 minutes and were again washed to remove excess dye. The plates were blotted on a stack of filter papers, and left for 3 hours to dry. To quantify and solubilise the biofilm, 125µl of 95% solution of ethanol was added to each well. Solution in each well was thoroughly mixed by using micropipette. Plates were left for 15 minutes at room temperature to ensure solubilisation, and optical density was measured at 590nm using MTP reader. Average OD (Optical Density) of the triplicate wells was calculated while mean and standard deviation (SD) of blank well in triplicate was also obtained. For interpretation of results, the strains were divided into three categories; moderate biofilm producers, strong biofilm producers and weak/no biofilm producers.[20]

For pelAand pelG gene detection by polymerase chain reaction (PCR), DNA was extracted by ethanol precipitation method and amplified by conventional PCR. The primer sequence of pelA gene of forward primer was 5`-GCTACGTGCCGTTCGTCAGCA-3` and reverse primer was 5`-CAGGCCGCCGAGGTAGACGTG-3`. ForpelG gene, forward primer was 5`-TATTGCTGGCGACCCTGTTCGATG-3` and reverse primer was 5`-ATGAAACGCAGCAGGTAGGCACAG-3`. PCR conditions were: initial denaturation at 95°C for 5 minute; 95°C for 1 minute, extension at 72°C for 1 minute and annealing at 52°C for 1 minute for 30 cycles. The amplified products of pelA and pelG were 146 and 190 base pairs respectively. Agarose gel electrophoresis was used for analysis of amplified gene product.

Results

Of the 50 isolates, 25(50%) each were environmental and clinical. All strains were catalase and oxidase-positive, and

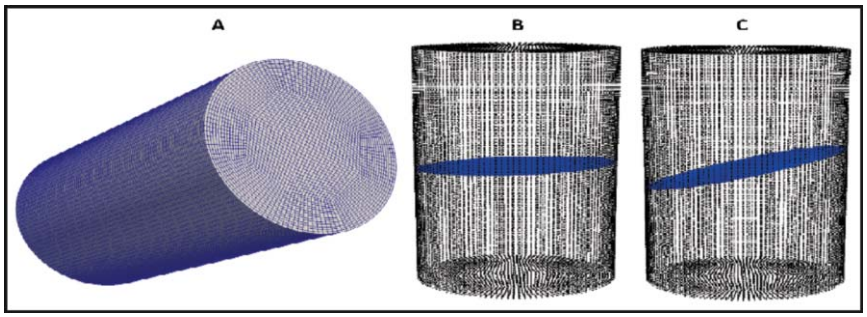

Figure-1: Numerical simulation of hydrodynamics of biofilm formulation (A) Computational domain (B) Air liquid interface at low rpm and (C) Air liquid interface at high rpm.

gave negative results for indole test.

The air-liquid two-phase flow simulations indicated that the rpm affected both the sloshing period and the slope of the air-water interface after stable conditions were achieved. Higher rpm corresponded to greater slopes of the interface, thereby increasing the surface area (Figure-1B-C). Both the unstable sloshing and the stable air-liquid interface contributed to the hydrodynamic mixing of the fluids which led to improved biofilm growth due to improved transport of nutrients in the wells. While sloshing was responsible for direct mixing, the relatively stable phase relied on convective mixing caused by recirculation zones near the air-liquid interface.

On the basis of binding of Congo red dye with EPS, different coloured phenotypes were observed: 7(14%) showed black and 43(86%) showed red, pink and white colours. Among the 25 clinical isolates, 4(16%)showed black and 21(84%) showed red/pink phenotypes, while in the 25 environmental isolates, 3(12%) were black and 22(88%) were of white/pink phenotypes.

In ALI assay, 15(30%) isolates showed strong adherence, 16(32%) exhibited moderate adherence, and 14(28%) isolates showed weak adherence, while 5(10%) showed no adherence. Out of the 25 clinical isolates, 8(32%) showed strong adherence, 12(48%) showed moderate adherence, while 5(20%) showed weak adherence. Among the 25 environmental isolates, 6(24%) showed strong adherence, 9(36%) showed moderate adherence, 7(28%) showed weak adherence and 3(12%) showed no adherence.

CFD simulations suggested optimal shaking conditions for good biofilm formation, so an initial comparison of MTPA under shaking and static conditions was done. *P. aeruginosa* isolates showed moderate or weak biofilm formation in static condition, but strong biofilm formation was observed during shaking conditions (Figure-2). So the assay for all isolates was carried out in shaking conditions. Overall, 42(84%) were positive for biofilm production, while 8(16%) isolates were non-biofilm formers. On the intensities, 30(60%) isolates were strong biofilm producers, 12(24%) were moderate producers, and 8(16%) were weak/non-biofilm formers. Out of the 42 biofilm-producing isolates, 19(45%) were clinical and 23(55%) were environmental. Among the 25 clinical isolates, 13(52%) were strong biofilm producers, 6(24%) were moderate biofilm producers, while 6(24%) showed weak or no biofilm formation. Of the 25

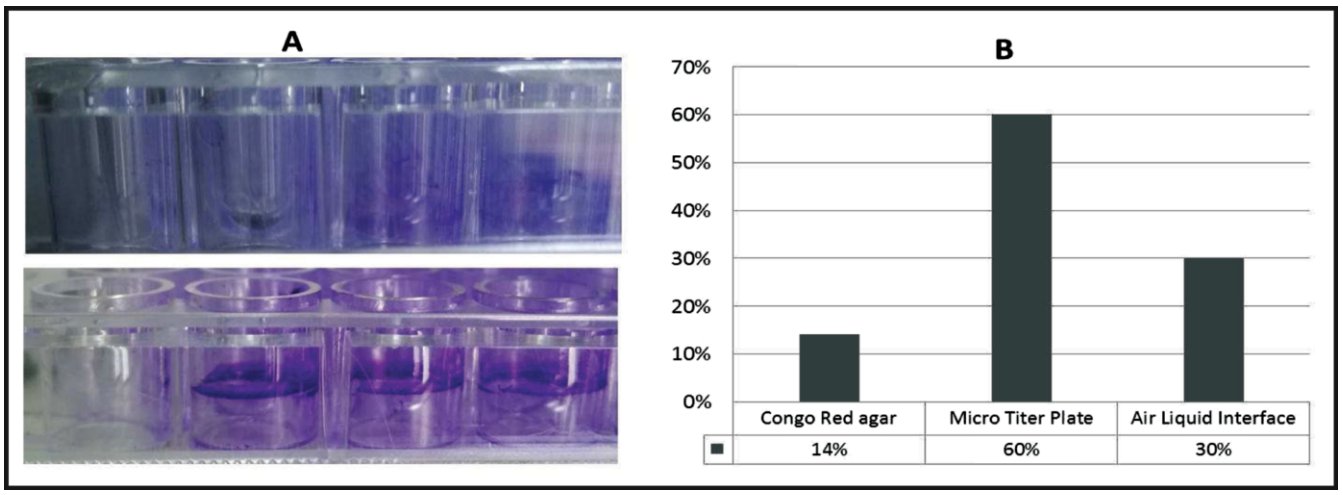

Figure-2: (A) Optimization of MTPA using CFD simulations. Top panel shows the assay done in static conditions and bottom panel shows the assay done in shaking conditions. (B) Comparison of Biofilm formation assay in Congo red agar. Microtiter plate assay and Air liquid interface assay.

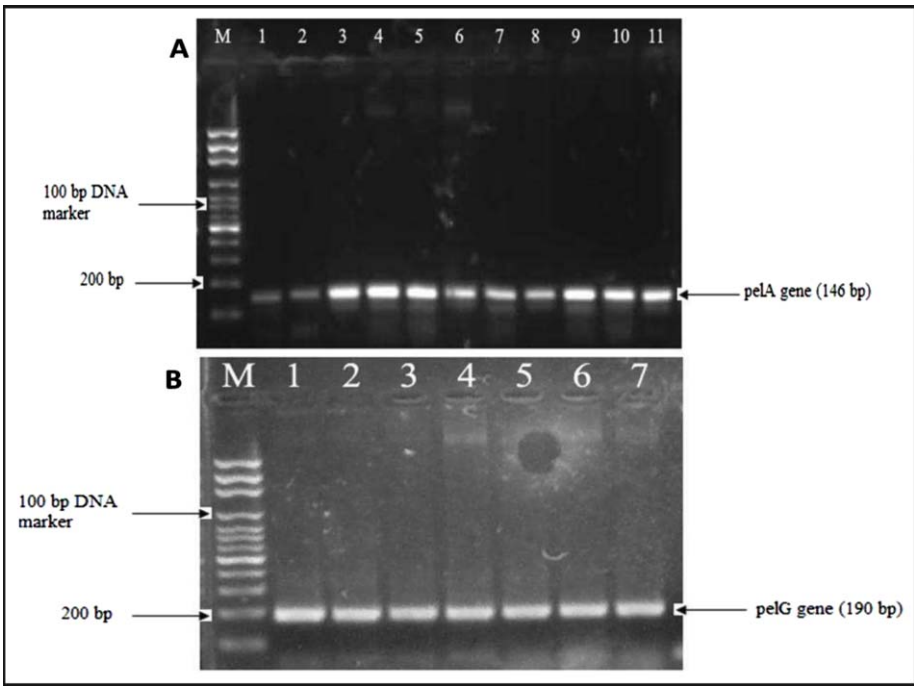

Figure-3: Representative gel image showing pelA gene amplification (A) and 190 bp PCR product of pelG gene amplification (B). M indicates 100 bp DNA marker.

environmental isolates, 2(8%) showed weak or no biofilm formation, 6(24%) showed moderate biofilm formation, while 17(68%) were strong biofilm producers.

By comparing all three phenotypic methods, MTPA was found to be more reliable as 30(60%) isolates showed strong biofilm formation whereas in CRA and ALI, 7(14%) and 15(30%) showed strong biofilm formation.

The strong biofilm-forming isolates by MTPA were grown on M63 media supplemented with 1% lactose, 1% glucose and 1% maltose. Eleven (38%) isolates showed moderate biofilm formation, while 19(62%) isolates produced weak biofilm. The isolates showed more biofilm formation on media supplemented with glucose compared to lactose and maltose.

Out of 50 isolates, 22(44%) were identified to be positive for pelA gene. Of them, 12(54.5%) were clinical, and 10(45.5%) were environmental. Besides, 28(56%) isolates were negative, and, of them, 13(46.4%) were clinical and 15(53.6%) were environmental. For pelG gene, 35(70%) isolates were positive and 15 (30%) were negative. Among the 25 clinical isolates, 16(64%) were positive and 9(36%) were negative for pelG gene. Of the 25 environmental isolates, 19(76%) were positive and 6(24%) were negative for pelG gene. Isolates positive for both pelA and pelG genes were 29(58%).

Discussion

Biofilm-producing bacteria have a key role in pathogenesis because of decreased access of antibiotics and difficulty to remove from the surfaces. Several phenotypic tests are used to evaluate the biofilm formation of bacteria but the studies related to biofilm formation in *P. aeruginosa* and their efficient methods are limited. The current study started off evaluating the potential of different methods to accurately and reliably assess the biofilm formation in clinical and environmental isolates. Using CRA, 7(14%) isolates were detected as strong biofilm producers which is similar to a study which showed 14% isolates to be strong producers, while 34(68%) were found to be moderate.[21] Another report found 43.5% *Staphylococcus (S.) epidermidis* isolates to be positive using CRA.[22] Using MTPA, 42(84%) isolates were observed to produce different intensities of biofilm in the current study. One study in India reported that out of 152 isolates, 53.9% were biofilm producers[10] while another study reported 49% to produce biofilm using MTPA.[23] The high number of biofilm producers in our study with MTPA could be due to the difference in assay conditions where using CFD simulations MTPA was optimised to be carried out under shaking conditions compared to static conditions in other studies. Hydrodynamics have great impact on biofilm production in terms of influence on cell removal and attachment to surfaces with transfer of oxygen and nutrients. During shaking, air-liquid interface area increases and oxygen transfer rate is relatively high which promotes microbial growth.[24] One study showed that in static culture *P.aeruginosa* strain FRD1 developed a non-mucoid motile phenotype due to enhanced fliC gene production, while in shaking conditions this enhanced gene production was reversed to non-motile variant, suggesting the role of oxygen availability.[25] We speculate that good biofilm formation under shaking conditions suggests the relation of strong biofilm formation with the availability of oxygen. The current study found that 45(90%) isolates were capable of producing different patterns of

pellicle using ALI method. It was difficult to differentiate between the moderate and weak adherence in the assay, but comparing that 30% isolates giving strong adherence in ALI with 14% in CRA suggests that the biofilm formation carried out in liquid media give more reliable results. One study reported that MTPA was better for biofilm detection than CRA[26] and another reported MTPA as a reliable and quantitative method for the measurement of biofilm formation process.[23] Another study reported that there was no correlation between CRA and other methods of biofilm detection.[21]

In the present study, 22(44%) isolates were identified to be positive for pelA gene, while 28(56%) were negative. For pelG gene, 35(70%) isolates were positive, while 15(30%) were negative. PCR results of pel genes well correlated with biofilm formation by MTPA.

Studies related to pel genes and biofilm formation in pseudomonas from Pakistan are occasional, but we can infer some association from biofilm-responsible genes in other microorganisms such as *S. aureus* and its icaA and icaD genes. Our results correlated with the study in which 42.2% *S. aureus* were positive for icaA and icaDgenes.[27] In another study, 45% *S. epidermidis* isolated from catheters were found positive for icagenes. The pel operon codes for glucose-rich EPS during biofilm formation process. It was reported that carbon source, used in culturing media, greatly influences the swarming motility and quorum sensing. We also found that the addition of glucose compared to other sugars affected the biofilm formation positively.

Conclusions

MTPA under shaking was found to be a quantitative and efficient method to detect biofilm formation in *P. aeruginosa* compared to MTPA in static conditions or CRA and ALI methods. This can be suggested as a routine screening method for the detection of biofilm-producing bacteria in clinical laboratories.

Disclaimer: None.

Conflicts of Interest: None.

Source of Funding: None.

References

1. Driscoll JA, Brody SL, Kollef MH. The epidemiology, pathogenesis and treatment of Pseudomonas aeruginosa infections. Drugs 2007;67:351-68.
2. Moskowitz SM, Foster JM, Emerson J, Burns JL. Clinically feasible biofilm susceptibility assay for isolates of Pseudomonas aeruginosa from patients with cystic fibrosis. J clin microbiol 2004;42:1915-22.
3. Ryder C, Byrd M, Wozniak DJ. Role of polysaccharides in Pseudomonas aeruginosa biofilm development. Curr opin microbiol 2007;10:644-8. doi: 10.1016/j.mib.2007.09.010
4. Steinberger RE, Holden PA. Extracellular DNA in single- and multiple-species unsaturated biofilms. Appl Environ microbiol 2005;71:5404-10.
5. Schaber JA, Triffo WJ, Suh SJ, Oliver JW, Hastert MC, Griswold JA, et al. Pseudomonas aeruginosa forms biofilms in acute infection independent of cell-to-cell signaling. Infection immunity 2007;75:3715-21.
6. Friedman L, Kolter R. Two genetic loci produce distinct carbohydrate-rich structural components of the Pseudomonas aeruginosa biofilm matrix. J Bacteriology 2004;186:4457-65.
7. Vasseur P, Vallet-Gely I, Soscia C, Genin S, Filloux A. The pel genes of the Pseudomonas aeruginosa PAK strain are involved at early and late stages of biofilm formation. Microbiology 2005;151(Pt 3):985-97.
8. Friedman L, Kolter R. Genes involved in matrix formation in Pseudomonas aeruginosa PA14 biofilms. Mol microbiol 2004;51:675-90.
9. Jackson KD, Starkey M, Kremer S, Parsek MR, Wozniak DJ. Identification of psl, a locus encoding a potential exopolysaccharide that is essential for Pseudomonas aeruginosa PAO1 biofilm formation. J bacteriology 2004;186:4466-75.
10. Mathur T, Singhal S, Khan S, Upadhyay DJ, Fatma T, Rattan A. Detection of biofilm formation among the clinical isolates of Staphylococci: an evaluation of three different screening methods. Indian J Med Microbiol 2006;24:25-9.
11. Gad GF, El-Feky MA, El-Rehewy MS, Hassan MA, Abolella H, El-Baky RM. Detection of icaA, icaD genes and biofilm production by Staphylococcus aureus and Staphylococcus epidermidis isolated from urinary tract catheterized patients. J Infect Dev Ctries 2009;3:342-51.
12. Gomes LC, Moreira JM, Simoes M, Melo LF, Mergulhao FJ. Biofilm localization in the vertical wall of shaking 96-well plates. Scientifica 2014;2014:231083.
13. Gomes LC, Silva LN, Simoes M, Melo LF, Mergulhao FJ. Escherichia coli adhesion, biofilm development and antibiotic susceptibility on biomedical materials. J Biomed Mater Res A 2015;103:1414-23.
14. Moreira JMR, Gomes LC, Araujo JDP, Miranda JM, Simoes M, Melo LF, et al. The effect of glucose concentration and shaking conditions on Escherichia coli biofilm formation in microtiter plates. Chem Eng Sci 2013;94:192-9. doi: 10.1016/j.ces.2013.02.045
15. Pereira MO, Vieira MJ. Effects of the interactions between glutaraldehyde and the polymeric matrix on the efficacy of the biocide against Pseudomonas fluorescens biofilms. Biofouling 2001;17:93-101.
16. Nahid F, Khan AA, Rehman S, Zahra R. Prevalence of metallo-beta-lactamase NDM-1-producing multi-drug resistant bacteria at two Pakistani hospitals and implications for public health. J Infect Public Health 2013;6:487-93.
17. Jasak H. OpenFOAM: Open source CFD in research and industry. Int J Nav Arch Ocean 2009;1:89-94.
18. Freeman J FF, Keane CT. New method for detecting slime production by coagulase negative staphylococci. J Clin Pathol 1989;42:872-4.
19. Merritt JH, Kadouri DE, O'Toole GA. Growing and analyzing static biofilms. Curr Protoc Microbiol 2005;Chapter 1:Unit 1B. doi: 10.1002/9780471729259.mc01b01s00
20. Stepanovic S, Vukovic D, Hola V, Di Bonaventura G, Djukic S, Cirkovic I, et al. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. APMIS : acta pathologica, microbiologica, et immunologica Scandinavica

2007;115:891-9. doi: 10.1111/j.1600-0463.2007.apm_630.x

21. Baqai R, Aziz M, Rasool G. Urinary tract infections in diabetic patients and biofilm formation of uropathogens. Infect Dis J Pak 2008;17:21-4.
22. Růžička F, Hola V, Votava M, Tejkalova R, Horvát R, Heroldová M, et al. Biofilm detection and the clinical significance ofStaphylococcus epidermidis isolates. Folia microbiologica 2004;49:596.
23. Hassan A, Usman J, Kaleem F, Omair M, Khalid A, Iqbal M. Evaluation of different detection methods of biofilm formation in the clinical isolates. Brazilian J Infectious Dis 2011;15:305-11.
24. Duetz WA. Microtiter plates as mini-bioreactors: miniaturization of fermentation methods. Trends microbiol 2007;15:469-75.
25. Wyckoff TJ, Thomas B, Hassett DJ, Wozniak DJ. Static growth of mucoid Pseudomonas aeruginosa selects for non-mucoid variants that have acquired flagellum-dependent motilitya. Microbiology 2002;148:3423-30.
26. Knobloch JK, Horstkotte MA, Rohde H, Mack D. Evaluation of different detection methods of biofilm formation in Staphylococcus aureus. Med Microbiol Immunol 2002;191:101-6.
27. Satorres SE, Alcaraz LE. Prevalence of icaA and icaD genes in Staphylococcus aureus and Staphylococcus epidermidis strains isolated from patients and hospital staff. Cent Eur J Public Health 2007;15:87-90.