



An Easy New Modified Method for Detection of Antibacterial Susceptibility in Biofilm-Growing Bacteria

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Abstract

Background: Biofilms are a major challenge in treating infections. Clinically, biofilms are often associated with chronic infections, so rapid and accurate methods of investigating the antibiotic susceptibility of biofilm bacteria are very important for faster diagnosis and treatment. In this study, a new modified microplate method was proposed to evaluate the antibiotic susceptibility of alive bacteria in the biofilm.

Materials and Methods: The study was conducted on clinical isolates of *Pseudomonas aeruginosa* and standard strain over a period of one year. The susceptibility test was conducted according to a modified version of the Calgary biofilm device method. In the last step, to study the living bacteria in the biofilm, oxidative-fermentative (OF) medium was used to measure the acid production following the use of glucose by the bacteria. The biofilm-growing bacteria was determined by observing the color changing and also measuring optical density (OD) at 427 nm of OF medium.

Results: The method in this study could evaluate the effect of antibiotics on biofilm bacteria based on glucose metabolism. The results indicated that this method can quickly and easily identify alive bacteria in biofilm at a low cost and without the need for any special devices.

Conclusion: Although biofilms are involved in most incurable clinical cases, there is currently no guideline to assist physicians in treating biofilm-related infections. Therefore, it is necessary to establish a specific sensitivity test for biofilm or to approve a new method for routine use in diagnostic laboratories.

Keywords: Biofilms, *Pseudomonas aeruginosa*, Minimum inhibitory concentrations, Clinical laboratory test

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Introduction

Biofilm is a microbial community that is attached to abiotic surfaces and mainly attended to microorganisms producing extracellular polysaccharides (1). Biofilm-related infections such as infections associated with medical devices are caused by biofilm-producing microorganisms (2). One of the strategies of bacterial resistance to antimicrobial drugs, environmental factors, and immune responses is biofilm formation (3). Multidrug-resistant bacteria are commonly caused by infections through static medical devices. The implant surface prepares an optimum situation for microbial attachment and growth which brings many benefits to microorganisms with the increased availability of nutrients, likelihood of survival, maturation, and

potential for symbiotic relations. This results in the failure of treatment, increased spread of resistant pathogens, morbidity, as well as mortality and finally removes the device. A recent UK government report outlined that without significant investment in new therapies, deaths due to infection are predicted to rise to more than 10 million by 2050, a figure greater than cancer (4). Bacteria represent versatile strategies for attacking humans. Recently it has been clear that immune and antibiotic resistance of biofilms are extremely influenced by the nature of biofilms at cellular and species levels. According to the US Centers for Disease Control, biofilms account for two-thirds of all bacterial infections that threaten human health (5). In general, most antimicrobial therapies available against microorganisms are developed

and evaluated in planktonic (free-living) mode. Biofilm pathogenic bacteria can be up to a thousand times more resistant to antimicrobial therapies. The problem of biofilm resistance makes it difficult to treat and eliminate them effectively. Hence, new strategies are needed to prevent, disperse, and treat bacterial biofilms (6). As a consequence of the interaction between planktonic bacteria and biofilms, bacteria can better survive in the host (in harsh conditions). In recent years, the number of patients in the health care sector who are prone to biofilm colonization has increased due to the use of implanted biomaterials. Further, biofilms are involved in chronic bacterial infections not related to the device. Treatment of these infections with conventional antimicrobial agents is not always successful, so it often results in surgical removal of the implant, which carries risks and problems (7). The amount of the minimum biofilm eradication concentration (MBEC) has been suggested as a laboratory assay to evaluate antimicrobial activity against mature biofilm (8). The Clinical Laboratory Standards Institute offers bacterial testing in the form of plankton and has no method of testing antimicrobial susceptibility to biofilm-related organisms. Different protocols have been developed to test antimicrobial sensitivity in biofilm including the MBEC assay (9). Biofilm detection of microorganisms is very important to conquer long-lasting infections, determining the antibiotic and antibacterial activity of the agents against the biofilm and the bacteria inside the biofilm as well. Hence, the present study tried to suggest an easy new method for the detection of antibiofilm activity of agents.

Materials and Methods

Sample Collection and Bacterial Screening

In this study, 151 samples consisted of urine, tracheal aspirates, wound, blood, and secretions obtained from Shahid Mohammadi Hospital, Bandar Abbas, Iran. The isolates of *P. aeruginosa* were identified using standard microbiological procedures (e.g., gram staining, oxidase test, and other biochemical tests). In addition, *P. aeruginosa* PTCC 1430 was used as reference strain of positive biofilm producer. Then, ciprofloxacin-resistant isolates were screened by 5µg ciprofloxacin disk (Floka, USA) using the disk diffusion method according to the Clinical Laboratory Standards Institute (2017) guideline.

Biofilm Formation Assay

The ability to produce biofilms in bacteria was assessed by the microtiter colorimetric method. Bacterial colonies were grown for 24 hours in the *tryptic soy* broth (TSB, Merck Darmstadt, Germany at 37 °C). Bacterial suspensions (1:100) were diluted in a new TSB medium and 150 µL of this solution was added to 96-well polystyrene microtiter plates with a sterile flat bottom and incubated for 24 hours at 37°C, then the wells were gently washed

three times with sterile distilled water. The wells were dried in an inverted position at room temperature and finally stained with 125 µL of 0.1% violet crystal solution in water for about 10-15 minutes. Crystal violet powder (Merck, Germany) was discarded. The wells were washed three times to remove excess crystal solution. Finally, violet crystals were released by adding 125 µL of 30% acetic acid (Merck, Germany). The optical density (OD) absorption of each well at 550 nm was measured using an ELISA reader (BioTek ELx800). All assays were repeated three times. As a control, an uninoculated medium was used to determine the background OD. The cut-off OD (OD_c) was defined as three standard deviations above the mean of negatively controlled OD (10). 44

Biofilm forming ability of the strains was classified as follows:

Non biofilm (OD test < OD_c)

Weak biofilm (OD_c < OD < 2 × OD_c)

Moderate biofilm (2 × OD_c < OD < 4 × OD_c)

Strong biofilm (4 × OD_c < OD)

Antimicrobial Susceptibility Test of Biofilm-Growing Bacteria

The susceptibility test was conducted according to a modified version of the Calgary biofilm device method (11). In brief, 20 µL 10⁸ CFU/mL⁻¹ bacterial pellets were resuspended in 180 µL TSB containing 1% glucose and aliquoted into the 96-well culture plate (TPP Techno Plastic Products) and incubated overnight at 37°C without shaking. After washing 3 times with sterile saline solution, plates were incubated at room temperature for 15 minutes. Then, microplates were incubated with each antimicrobial agent at a serial twofold dilution in Mueller–Hinton broth for 24 hours at 37°C (11, 8). Again, plates were washed 3 times with sterile saline solution, and oxidative-fermentative (OF) medium Merck (Germany) was added for 24 hours at 37°C. OF medium and medium antibiotic-free containing bacteria are considered as negative and positive controls, respectively. The MBEC was determined by observing the color-changing (qualitative) and also measuring OD at a wavelength of 427 nm using an ELISA reader (BioTek ELx800) of OF medium (quantitative) in the microplate wells (Figure 1). Finally, all the assays were repeated three times.

Statistical analysis

All data analyses were done with SPSS (version 16, SPSS Inc, USA), and GraphPad Prism 6 software (GraphPad Software, Inc., USA) was used to prepare graphs. A p-value less than 0.05 (two-tailed) were considered statistically significant.

Results

Isolation of Bacteria

Thirty isolates of *P. aeruginosa* resistant to ciprofloxacin

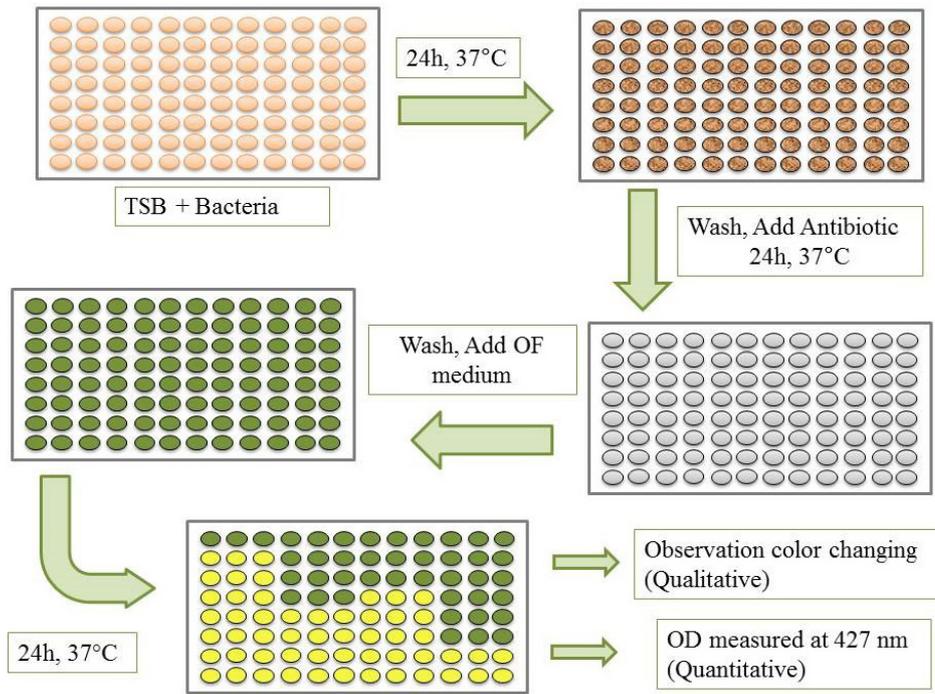


Figure 1. Schematic Chart of Susceptibility Biofilm-growing Bacteria Test. Note. Yellow well: Growth; Green well: No growth.

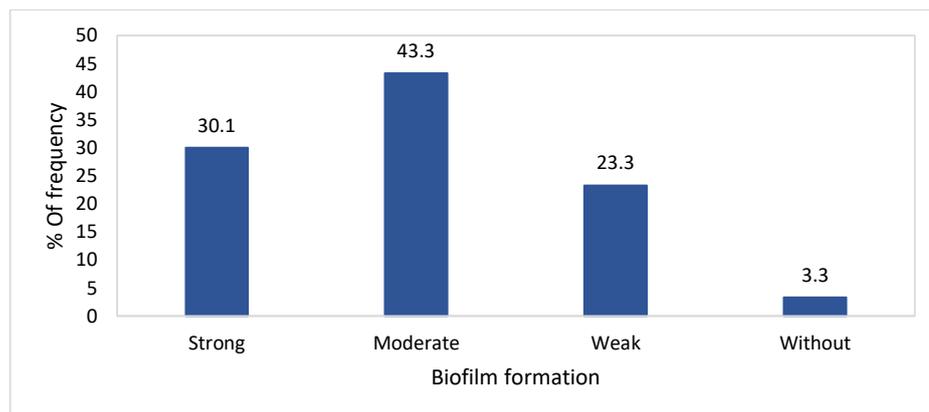


Figure 2. Frequency of *Pseudomonas aeruginosa* Isolates Based on Biofilm Production.

were recovered from samples. The percentage of bacterial load from each sample was as follows: urine (43.3%), tracheal aspirates (20%), wound (16.7%), blood (10%), and secretions (10%).

Biofilm Formation

As illustrated in Figure 2, 29 isolates (96.7%) were biofilm formers. Among these, 7 isolates (23.3%) proved to be weak biofilm (+), 13 (43.3%) were moderate isolates (++), and 9 isolates (30.1%) were strong biofilm (+++).

Susceptibility Testing of Biofilm-Growing Bacteria

The OF test was applied to indicate the oxidation or fermentation of glucose by gram-negative rods. The concentration of acid produced during the process of glucose metabolism turned the bromothymol blue

Table 1. Frequency and Percentage of Isolates Based on Qualitative MBEC in Various Concentrations of Ciprofloxacin

Concentration of Ciprofloxacin (µg/mL)	No. (%)
≥ 5120	4 (13.8)
2560	9 (31.1)
1280	5 (17.2)
640	2 (6.9)
320	3 (10.3)
160	6 (20.7)
80	-
≤ 40	-

Note. MBEC: Minimum biofilm eradication concentration.

indicator from green to yellow in OF medium. Therefore, if the bacteria are alive and they use the glucose in the medium, they will change the color from green to yellow.

Therefore, yellow wells were considered positive.

Table 1 presents the MBEC qualitative results, where the highest frequency for ciprofloxacin was 2560 µg/mL, and MBEC50 of ciprofloxacin was calculated at 1280 µg/mL.

According to Figure 3, the cut-off for bacterial growth was obtained by calculating the mean ± standard deviation of the negative control at a wavelength of 427 nm, which is the maximum adsorption of bromothymol blue reagent in an acidic state (yellow). Therefore, absorptions higher than the cut-off value were considered positive wells. Table 2 represents the data related to two samples. For example, in isolate No. 71 with strong biofilm, the adsorption rate in the well with a concentration of 640 µg/mL was equal to 0.957 nm, which was higher than the cut-off value. Therefore, a slightly isolated MBEC of 71 was recorded for the antibiotic ciprofloxacin 1280 µg/mL. A small MBEC was also recorded for isolate No. 75 with an average biofilm of 640 µg/mL.

Discussion

To screen the efficacy of antibiotics against *Paeruginosa* biofilms, we optimized a microtiter-based protocol. This

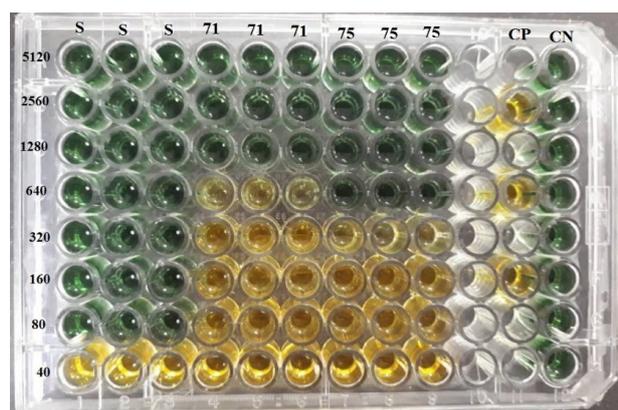


Figure 3. Qualitative MBEC Test Based on Oxidative-Fermentative Method. Note. MBEC: Minimum biofilm eradication concentration.

methodology was selected because it requires few steps and is user-friendly. This method is based on cellular metabolism as well as the production of acid from sugar. The method does not require special materials and equipment, and all materials are available in any laboratory, so it is more economical. It also takes less time to perform the test compared to other methods. It can be examined qualitatively (observationally), and the results can be quantitatively confirmed. Another method used in this study is the modified calgary apparatus. In this method, to check for live bacteria, each well should be cultured on an agar medium at the end of the assay. The advantage of this method is that the number of colonies can be measured in each concentration. In our method, the number of steps and days of testing was less. In addition, as illustrated in Table 3, like the method mentioned, it did not require special devices such as ultrasonic water baths (1). Comparing the results of biofilm-growing bacteria by qualitative and quantitative methods using OF medium, no significant difference was found.

The microbial community (biofilm) can produce extracellular polysaccharides after binding to non-biological surfaces. Microbial cells that grow in biofilms are physiologically different from planktonic cells of the same organism (1). The researchers have challenges with biofilm complexity and developmental mechanisms (12). Due to the lack of reliable and accurate methods for testing new antibiotics, their development is hampered. One of the methods of evaluation and determination of MBEC for different antibiotics is the resazurin method which has both advantages and disadvantages. Using this method as a screen test requires ptimizing resazurin for each bacterial strain. The test results must also be interpreted carefully because this method is less valuable. This method cannot detect the number of cells less than 10⁶ CFU per biofilm, so a supplementary method is required (13). Based on current instructions or antibiotic treatments, Plankton

Table 2. OD Observation of Isolates Based on Quantitative MBEC in Various Concentrations of Antibiotics

Isolates	Concentration (µg/mL)								PC	Average NC	SD Control Negative	Cut- off
	5120	2560	1280	640	320	160	80	40				
PTCC 1430	0.396	0.371	0.348	0.347	0.339	0.367	0.302	0.855	2.085			
PS 71	0.338	0.406	0.313	0.957	1.129	1.193	1.144	1.522	1.692	0.279	0.139	0.418
PS 75	0.343	0.335	0.329	0.333	1.249	1.071	1.204	1.390	1.992			

Note. MBEC: Minimum biofilm eradication concentration. OD: Optical density; PC: Positive control; NC: Negative control; SD: Standard deviation; OF: Oxidative fermentative; Horizontal wells: Isolates with three repetitions; Vertical wells: Different concentrations of antibiotics (µg/mL).

Table 3. Comparison of the New Method With the Previous and Basic Methods

Methods	New Method	Basic Method
Number of steps	6	7
Accuracy	Visual inspection is possible. Liveing bacteria can be easily evaluated by color chenging.	Probability of contamination. Error reading turbidity (may read dead bacteria and biofilms)
Time of test	3 days	4 days
Simplicity	OF medium and simple microplate	Calgary plates and ultrasonic water bath

Note. OF: Oxidative fermentative.

bacteria are often unable to successfully deliver a pathway for the treatment of biofilm infections. The biofilm bacteria are inherently more tolerant to antibiotics than planktonic cells. Therefore, the determination of specific biofilm antibiotics is an essential step for predicting treatment success (14). In the study on different methods of antibiofilm, it is necessary to examine the bacteria present in the biofilm. Both the matrix and the bacteria in it are essential for the formation and maintenance of a mature biofilm. When a potential treatment only destroys the matrix, the remaining bacteria can create a new biofilm by producing new extracellular matrix components (15). For the results to be generalizable, other methods must be evaluated, but due to the time limitation of the study, it was decided to carry out them in future studies.

Conclusion

In this study, OF medium microtiter plate test has been developed and optimized to detect antimicrobial effect of drugs on planktonic cell in biofilms. As such, OF medium, which was a new, simple, and reproducible method, was used to study biofilm growing bacteria. Moreover, this procedure can be used to investigate the antimicrobial activity of a wide range of biocides.

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Authors' Contribution

FF, NB, SS, and SA: Conceptualization, the original draft writing, investigation, and formal analysis; FF and NB: Conceptualization, supervision, and project administration; FF and SS: Conceptualization, and project administration; FF, NB and SS: Investigation; FF, NB and SS: Writing including reviewing, editing, and investigation.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

Disclaimer

The content of this research is only the views and achievements of the authors.

Ethical Statement

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