

# Detecting of biofilm formation in the clinical isolates of *Pseudomonas aeruginosa* and *Escherichia coli*: an evaluation of different screening methods

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## Abstract

Biofilm producer bacteria cause nosocomial, chronic, and recurrent human infections. It is hard to treat biofilm-embedded bacteria because they are more resistant to antimicrobials than planktonic bacteria. The present study aimed to investigate different methods for detecting biofilms in the clinical isolates of *Escherichia coli* and *Pseudomonas aeruginosa*. The study was carried out at the Department of Microbiology, Ardabil Branch, Islamic Azad University, Ardabil, Iran, from January 2019 to June 2019. A total of 320 clinical samples were collected from educational and medical centers in Tehran, Iran which from them 100 isolates of *E. coli* and *P. aeruginosa* were identified by standard microbiological procedures and subjected to biofilm detection methods. Biofilm detection was tested by Congo red agar (CRA), tube method (TM), microtiter plate assay (MTPA). The MTPA was considered to be superior to CRA and tube TM. From the total of 100 clinical isolates, MTPA detected 38 (38%) isolates as biofilm-positive phenotype, of which 30 as strong, and 8 as moderate biofilm-forming isolates. It can be concluded from the current study that the MTPA is a more quantitative and dependable assay for the detection of biofilm-forming microorganisms as compared to other methods, and it can be recommended as a general screening method for the detection of biofilm-producing bacteria in laboratories.

**Keywords:** Biofilm detection, Microtiter plate assay, Congo red agar, Tube method

## 1. Introduction

Microorganisms can live in one of two phenotypes: sessile or plankton. Planktonic are free-floating microorganisms. The sessile phenotype results from the binding of microorganisms to solid surfaces, their irreversible binding, and the development of exopolysaccharides with a gradual

increase in cell secretions' thickness [1]. A biofilm is a collection of microbial cells that are irreversibly enclosed in a polysaccharide matrix and bonded together on biotic surfaces such as host cells or abiotic surfaces such as medical devices [2]. Microbial cells that grow in a biofilm are physiologically different from planktonic cells of the same organism, which, in

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contrast, are single-cell and may float or swim in a liquid environment [3].

Biofilms are considered a serious public health problem due to the increased resistance of the constituent organisms to antimicrobial drugs and the ability to cause infection in patients who have been implanted with medical devices [4, 5]. The biofilm matrix blocks the penetration of antibiotics and prevents them from reaching the cells inside the biofilm [6]. Studies have shown that bacterial biofilms cause 80% of microbial infections that are very difficult to eradicate and treat with antibiotics [7]. One of the most important pathogens that produce strong biofilms is *Escherichia coli*. Previous studies on the survival of biofilm-related pathogens also indicate that biofilms may play an essential role in the persistence and release of stress-sensitive organisms into the environment. The results show that *E. coli* resists food deficiency conditions, and the interaction of this bacterium with *Pseudomonas aeruginosa* can increase *E. coli* growth in biofilm [8].

Biofilm formation contributes to the pathogenesis of *P. aeruginosa* in acute as well as chronic infections in clinical cases [9]. *P. aeruginosa* is currently considered the leading cause of ventilator-associated pneumonia (VAP) in the ICU. Infections caused by this bacterium occur mainly in patients with critical condition and immunodeficiency and increase the morbidity and mortality in these patients as well as increase the incidence of resistance of *P. aeruginosa* to antibiotics [10].

To control chronic and recurrent infections, it is essential to identify microorganisms' biofilms, maturity, and dispersion [11]. There are various laboratory methods to evaluate the production of biofilm in bacteria. Biofilm growth and observation with microscopes such as confocal scanning laser microscope (CLSM), quantitative method of closed systems under static conditions such as tissue culture plate (TCP) method and observation with non-specific color, floating biofilms (Air liquid interface (ALI)), bioluminescent assay, observation of bacteria colonies on the surface of a solid medium such as Congo red agar (CRA) and the simple tube method (TM) are among the biofilm measurement methods [12]. Microtiter plate assay (MTPA) is a screening method for comparing adhesion patterns. It is the most widely used quantitative method for detecting biofilm production. In the CRA method, a positive result is

shown by the black colony and the dry crystal consistency. A pink colony appears in the absence of biofilm [13].

Laboratory study of biofilm production by bacteria can be affected by the choice of a specific culture medium. The conditions that the bacterium encounters during growth can encourage or suppress biofilms production or cause biofilms with unusual structures [14]. This study aimed to investigate the methods (microtiter plate methods, tube method, and Congo red agar) for the detection of biofilm formation in the clinical isolates of *E. coli* and *P. aeruginosa*. The sensitivity and specificity of the methods have been determined through statistical analysis. The MTPA method was considered the gold standard for this study and compared with data from TM and CRA assays. True positives were biofilm producers by MTPA, TM, and CRA assays. False-positive were biofilm producers by TM and CRA assay and not by MTPA method. False-negative were the isolates that were non-biofilm producers by TM and CRA but were producing biofilm by MTPA method. True negatives are those which were non-biofilm producers by all the methods.

## 2. Materials and Methods

### 2.1 Sample collection

Out of 320 clinical samples collected from educational and medical centers in Tehran, Iran from January 2019 to June 2019, 50 isolates of *E. coli* and 50 isolates of *P. aeruginosa* were identified and subjected to biofilm formation detection. These isolates were identified by, colonial morphological analysis on Eosin Methylene Blue (EMB) Agar (for *E. coli* isolates) and Cetrimide Agar (for *P. aeruginosa* isolates) and standard microbiological procedures such as Gram staining, catalase test, cytochrome oxidase reaction, motility, and biochemical tests. These isolates were selected for biofilm formation evaluation. Reference strain of positive biofilm producer *P. aeruginosa* ATCC 9027, *E. coli* ATCC 25922 were used as controls.

### 2.2 Microtiter plate assay (MTPA)

Each of the collected and reference strains was cultured on Tryptic Soy Agar (TSA) (Merck, Germany) medium and incubated overnight at 37°C. Fresh colonies were cultured in 10 ml Tryptic Soy Broth (TSB) (Merck, Germany) medium in a sterile tube and

incubated for 15-18 h at 37°C with a shake at 200 rpm. The optical absorption of each liquid culture was adjusted using a fresh medium with an OD of 0.1 at 620 nm. Bacterial cultures were diluted, and a suspension equivalent to half McFarland was prepared in TSB medium. 200 µl of the suspension prepared from each bacterial strain was transferred into 96 polystyrene plate wells. The plates were incubated at 37 °C for 96 h. The control organism was also incubated, diluted, and added to the culture plate. Negative control wells contained inoculated sterile broth. After this time, each well's contents were removed and washed twice with phosphate buffer solution (PBS) (pH 7). This removed free-floating bacteria. After drying, the wells were fixed with 95% Methanol for 10 minutes and then stained with Crystal violet (1%) for five minutes. The plates were emptied, and an excess stain was washed by sterile distilled water. The wells were filled with 100 µl of Glacial acetic acid solution (33%) and biofilm formation has been evaluated at 570 nm with an ELISA auto reader (Biotek ELx800, USA) [9].

### 2.3 Congo red agar (CRA)

The Congo red indicator (8 g/L) was prepared separately from the other medium constituents as a concentrated aqueous solution and autoclaved (121°C for 10 minutes). Then it was added to the autoclaved Muller Hinton agar medium (Merck, Germany) (10 g/ml) with sucrose (50 g/L) at 55°C. Test organisms were cultured on the CRA medium and incubated aerobically for 24 - 48 h at 37 °C. Black colonies with a dry crystalline consistency indicated biofilm production [1].

### 2.4 Tube method (TM)

A loopful of the bacterial suspension was transferred to a tube containing 10 ml of LB broth (Merck, Germany) medium with glucose (2%). The tubes were incubated for 24 h at 37°C. The contents of the tubes were then removed and washed with PBS (pH 7.3). Then the dried tubes were stained with Crystal violet (1%). Excess dye was washed with sterile distilled water. Tubes were dried in the inverted position. Isolates forming slime or biofilm-based on Crystal violet color's thickness on the tube's bottom and the wall was observed and recorded. The observation of a visible thin layer on the wall and bottom of the tube was considered a moderate biofilm.

The observation of a thick layer visible on the wall and bottom of the tube was considered a strong biofilm. The lack of a visible layer on the wall and bottom of the tube was considered non-biofilm formation and negative. The experiment was performed in three replications [3].

### 2.5 Statistical analysis

The results were entered into Microsoft Excel 2016 and were analyzed by using SPSS version 25 (SPSS Inc., Chicago, IL, USA). Spearman's correlation test was used to define the direction of association. *P*-value <0.05 was considered statistically significant.

## 3. Results

Of the 100 *E. coli* and *P. aeruginosa* isolates, 38 (38%) isolates showed a biofilm-positive phenotype under the optimized conditions in MTPA (Figure 1) and isolates were further classified as strong, moderate, weak, and no biofilm (Table 1). CRA assay led to different results, only 26.3% of isolates showed black colonies with dry crystalline morphology after 24-48 hours (Table 2). Though the CRA assay can well detect biofilm-producing strains, weak producers were difficult to discriminate from biofilm negative isolates. No direct correlation between colony morphology on CRA and MTPA results was observed. In TM, strong biofilm producers could be easily detected, whereas it was difficult to differentiate between moderate and weak biofilm-forming isolates which affected its performance in terms of sensitivity and specificity. Statistical analysis of TM and CRA methods for detection of biofilm formation in isolates using MTPA as a gold standard (n=100) showed that TM assay has more sensitivity and specificity than the CRA method (Table 3). Biofilm formation in the isolates by either of the methods was 16% and 60% for *E. coli* and *P. aeruginosa*, respectively.

## Discussion

Biofilm-producing bacteria cause many chronic and recurrent infections that are difficult to treat. Treatment and control of biofilm-associated infections is an essential issue in hospitals and medical care. These bacteria exhibit resistance by different assays such as restricted penetration of antibiotics into biofilms and expression of resistance genes by facilitating the exchange of plasmids responsible for drug resistance due to proximity of cells within

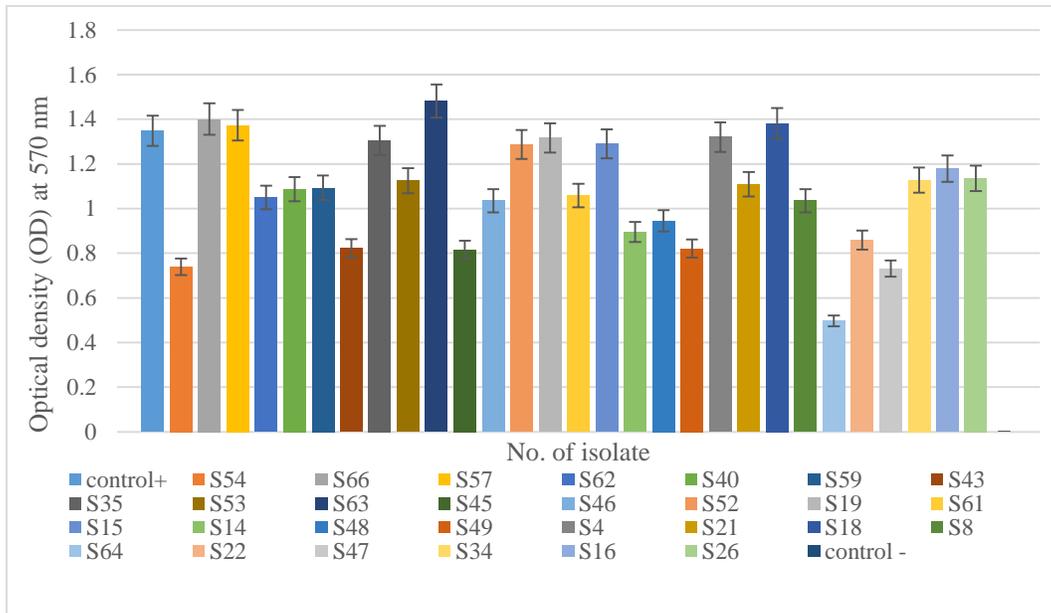


Figure 1. Biofilm formation detection in *Pseudomonas aeruginosa* by microtiter plate assay

Table 1. Interpretation of biofilm formation by average OD value in microtiter plate assay

Biofilm formation	OD isolate (at 570 nm)	ODc	Isolates
Strong	$OD_{isolate} > 0.929$	$OD_{isolate} > 4 \times ODc$	E41, E42, E43, E45, E48, E58, E60, S66, S57, S62, S40, S59, S35, S53, S63, S46, S34, S16, S52, S19, S61, S15, S48, S4, S21, S18, S8, S26, positive controls
Moderate	$0.464 < OD_{isolate} \leq 0.929$	$2 \times ODc < OD_{isolate} \leq 4 \times ODc$	S54, S43, S45, S47, S14, S49, S64, S22
Weak	$0.232 < OD_{isolate} \leq 0.464$	$ODc < OD_{isolate} \leq 2 \times ODc$	-
No biofilm detected	$OD_{isolate} \leq 0.232$	$OD_{isolate} \leq ODc$	-

Optical density cut-off value (ODc) = average OD of negative control + 4x standard deviation (SD) of negative control = 0.232  
 Standard deviation (SD) = 0.03214  
 E: *E. coli*; S: *P. aeruginosa*

Table 2. Biofilm formation (38 isolates) by each of the methods

Biofilm formation	MTPA No. (%)	TM No. (%)	CRA No. (%)
Strong	30 (78.95)	13 (34.21)	10 (26.32)
Moderate	8 (21.05)	13 (34.21)	14 (36.84)
Weak/none	0	12 (31.58)	14 (36.84)

MTPA: microtiter plate assay, TM: Tube method, CRA: Congo red agar

biofilms. There are a lot of different assays for detecting biofilm [15, 16].

In this study, we evaluated 100 isolates by three screening methods for their ability in biofilm

Table 3. Statistical analysis of TM and CRA methods for detection of biofilm formation in isolates using MTPA as a standard (n=100)

Detection methods	Sensitivity	Specificity
TM	70%	83%
CRA	50%	71%

MTPA: microtiter plate assay, TM: Tube method, CRA: Congo red

formation. In the MTPA, the number of isolates showing biofilm formation was 38 (38%) (30 strong and 8 moderate) and no weak biofilm producers were detected. A previous study showed from the 50 clinical

isolates, 32 were biofilm producers (3 strong and 29 moderate) and 18 were weak/non-biofilm [17]. In another study, of the 50 isolates, 25 each were environmental and clinical. The number of biofilm producers reported in CRA and MTPA was 7 (14%) and 30 (60%), respectively which is consistent with the results of the current study [12]. In the current study with the CRA assay, 29 (76.32%) were found to be biofilm-producing bacteria and 9 (23.68%) as non-biofilm producers. The CRA method showed little correlation with the other methods. Siddhiqui *et al.* reported that the CRA method is not appropriate for biofilm detection. Out of 112 clinical isolates, CRA detected only 14.25% as biofilm producers as compared to TM which detected 29.46% as biofilm-forming bacteria [1]. The tube method also detected 38% isolates as biofilm producers. This method correlated with MTPA for identifying biofilm producers, but it was difficult to differentiate between high, moderate, and weak producers due to the variability in the results detected by different observations. Several studies recommend the analysis of biofilm formation via TM by different observers [18, 19]. TM and CRA are considered to be less sensitive than the MTPA, as some clinical isolates that were non-adherent, weak, or moderate biofilm producers in the TM and CRA assays showed strong biofilm patterns in the MTPA. So MTPA was selected as a gold standard for statistical analysis. In the current study sensitivity and specificity of TM were 70% and 83%, respectively. For the CRA assay, sensitivity and specificity were lower at 50% and 71%, respectively. So following the previous studies, TM and CRA cannot be recommended as general screening tests to identify biofilm-producing isolates since they can yield false positive or false negative results [12, 16, 20]. No significant relationship can be reported from the correlation of biofilm formation with organisms isolated because other factors such as the isolation source or the type of infection, antibiotic resistance, and genetic, physiological, and biochemical differences of the isolates should be considered to investigate the relationship between the genus of the isolates and the techniques used to identify biofilm formation. Conclusively, MTPA is a more reliable quantitative assay for detecting biofilm formation among clinical isolates. The tube method may also be considered in a fast screening of biofilm producers. Hence, measures for the early detection of biofilm

producers in hospital environments and patients should be considered. This will decrease the rate of mortality of infections that are seemingly untreatable due to the presence of biofilms. Limitations encountered in this study were sampling, analytical and equipment limitation to investigate other screening and confirmatory assays of biofilm formation and not considering the effect of gene factor in biofilm formation.

Prevention and control against potential biofilm forming clinical pathogenic isolates is an essential step towards the management of chronic and recurrent infections. There are different biofilm detection methods like MTPA, TM, and CRA. A suitable method that is cost-effective easy to do and requires less technical expertise is the need of the hour. Results of the current study showed that MTPA is a suitable and reliable assay that is more efficient than the other detecting biofilm formation methods. Considering the ease of doing the test, rapidity, and cost-effectiveness, the CRA method and TM can be considered for biofilm detection but not as general and reliable detection methods.

### **Author Contributions**

All authors contributed equally to this manuscript and approved the final version of the manuscripts.

### **Conflict of Interests**

The authors declare that they have no conflicts of interest.

### **Ethical declarations**

This study received ethics approval by Ardabil University of Medical Sciences (Approval code: IR.ARUMS.REC.1398.022).

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