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## Evaluation of Biofilm Formation of *Pseudomonas aeruginosa* Isolated from Al-Shifa Hospital and their Susceptibility to Acetic Acid

Prof. Abdelraouf A. Elmanama,<sup>\*1</sup>  
Renad I. Abu-Dan  
Rewaa N. Eqtifan  
Aisha A. Shomar,  
Mariam R. Rifi

<sup>1</sup> Department of Medical Laboratory Science, Faculty Science, Islamic University of Gaza

Corresponding author:

e-mail address: [elmanama\\_144@yahoo.com](mailto:elmanama_144@yahoo.com)

### Abstract

Biofilm forming ability is a major virulence factor of most of pathogenic bacteria. Over 65% of health care associated infections are caused by biofilm producing organisms. The primary objective of this cross sectional study is to evaluate the biofilm forming ability of *Pseudomonas aeruginosa* isolated from various departments of Al-Shifa hospital and to determine the susceptibility of the isolates for antimicrobial drugs and acetic acid. Swabs moistened with sterile normal saline were used to swab an area of three cm<sup>2</sup>. Samples were cultured and processed by standard microbiological procedures. *P. aeruginosa* isolates were tested for their antimicrobial susceptibility patterns, the minimum inhibitory concentration for acetic acid and the biofilm forming ability were determined using Tube method and Crystal violet assay method. Susceptibility of *P. aeruginosa* isolates for acetic acid was considered as high at Conc. = 0.0097 mg/ml (5%), moderate susceptibility (0.156 to 0.019 mg/ml) (86%) and low susceptibility (>0.312 mg/ml) (9%). The isolates were 100% resistance to Ceftazidime, Piperacillin (97.6%), Ciprofloxacin (28.57%), Norfloxacin (9.5%), Meropenem and Aztreonam (2.38%). All were susceptible to Gentamicin, and Amikacin. The results for biofilm formation showed that 16 sample were strong positive (38.09%), 18 sample moderately positive (42.85%), and eight-samples were negative (19.04%). Biofilm forming ability of the isolates was detected in 80.9% of isolates. In conclusion, both biofilm and antimicrobial resistance were high among the isolates. Acetic acid showed weak/strong/moderate antimicrobial activity against *P. aeruginosa*.

### Keywords:

Biofilm, Acetic acid, Antimicrobials, Bacterial adhesion, *P. aeruginosa*, Gaza- Palestine.

### 1. Introduction:

Bacterial biofilms are structured communities of cells enclosed in self-produced hydrated polymeric matrix adherent to an inert or living surface. Formation of these sessile communities and their inherent resistance to antimicrobials and host immune attack are at the root of many persistent and chronic bacterial infections (J. W. Costerton, Stewart, & Greenberg, 1999), including *Pseudomonas aeruginosa* which remains one of the most important opportunistic cause of nosocomial infections and it

has developed resistance to a wide range of antimicrobial agents in burn centers (Capoor, Sarabahi, Tiwari, & Narayanan, 2010). Infections by *P. aeruginosa* are hard to treat due to its intrinsic resistance to many classes of antimicrobials as well as its ability to acquire resistance. All known mechanisms of antimicrobial resistance can be displayed by this bacterium (intrinsic, acquired, and adaptive) (Gellatly & Hancock, 2013). *P. aeruginosa* has a variety of virulence factors and infections caused by multidrug-resistant *P. aeruginosa* in burn

patients are a major public health problem. In such infections, biofilm production has been measured as an important determinant of pathogenicity (Choy, Stapleton, Willcox, & Zhu, 2008). The simultaneous determination of virulence factors and antimicrobial resistance is an approach for the examination of the microbiological aspects of infections caused by *P. aeruginosa* (Corehtash, Ahmad Khorshidi, Akbari, & Aznaveh, 2015). Over the recent years, several reports confirmed an increasing multidrug resistance among *P. aeruginosa* isolated from burn wound infections from Al-Shifa hospitals. *P. aeruginosa* was the most common pathogen isolated (50%) in the two burn units of Al-Shifa and Naser hospitals (Elmanama, Al Laham et al. 2013). In this work, 42 isolates of *P. aeruginosa* were investigated for their biofilm formation, antimicrobial susceptibility pattern, and tested for their susceptibility to varying concentration of acetic acid.

## 2. Material and Methods

### Bacterial culture media

Eight types of media were used for carrying out this research; MacConkey agar (Mac), Muller Hinton agar (MH), Blood agar (BA), Nutrient agar (NA), Triple sugar iron agar (TSIA), Simmon's citrate agar, Brain Heart Infusion Broth (BHIB), Brain Heart Infusion Broth with 2% glucose, and Nutrient broth (NB) medium (HiMedia, India). All media were prepared according to manufacturer's recommendations.

### Antimicrobials

In table (1) is a list of antimicrobials used in the susceptibility testing.

**Table 1: List of antimicrobials and their potency used in antimicrobial susceptibility testing.**

Antimicrobial	Code	Potency
Amikacin	AK	30 µg
Aztreonam	ATM	30 µg
Ceftazidime	CAZ	30 µg
Ciprofloxacin	CIP	5 µg
Gentamicin	GM	10 µg
Meropenem	MRP	10 µg

Norflloxacin	NOR	10 µg
Piperacillin	PRL	100 µg

## Methods

### Isolation of *P. aeruginosa* from various departments of Al-Shifa hospital

Forty-two isolates of *P. aeruginosa* were obtained from the microbiology departments and from the environment of Al-Shifa hospital. A cotton swab was moistened with sterile normal saline and used to swab an area of three cm<sup>2</sup> of various environmental surfaces. The swab is then placed in 1 ml sterile normal saline tube to maintain the viability of microorganisms. All samples were transported from collection area in an icebox within two hours to the Medical Microbiology Laboratory, IUG for processing.

A 0.1 ml of the suspension was aseptically pipetted and transferred in to pre-labeled MacConkey agar. The inoculated plates were incubated at 37±0.5 °C for 24 hours, after which their cultural characteristics were observed and recorded. Isolates from MacConkey agar were sub cultured to obtain pure isolates. The isolates were then being identified by colony morphology and characteristic growth, gram stain, TSIA and pattern of biochemical profile (catalase, oxidase, motility, and citrate) in accordance with the standard methods.

### Antimicrobial susceptibility by agar diffusion method

Each isolate was grown in BHIB and suspensions were standardized by matching with 0.5 McFarland turbidity standards to give a resultant concentration of about 1.5×10<sup>8</sup> cfu/mL. The antimicrobial susceptibility testing was determined using the modified Kirby-Bauer diffusion technique, by swabbing the Mueller-Hinton agar (MHA) plates with the resultant Brain Heart Infusion Broth suspension of each strain, antimicrobials alone and their combinations taking care not to allow spillage of the solutions on to the surface of the agar.

The plates were allowed to stand for 30 min before being incubated at 37 °C for 24 h. The diameter of inhibition zone produced around each antimicrobial disk was measured and interpreted using the Clinical Laboratory Standardization Institute (CLSI) zone diameter interpretative standards (Wayne, 2008).

### Determination of antimicrobial activity of acetic acid Minimal Inhibitory Concentration assay (MIC)

Acetic acid MICs were determined in triplicate using the micro broth dilution method in Mueller-Hinton broth according to CLSI (Tin, Sakharkar, Lim, & Sakharkar, 2009). The concentrations were prepared by serial dilution. Each well of a 96-micro titer plate was inoculated with 100 µl of each of the bacterial strains. Blank Mueller-Hinton broth was used as negative control. The micro titer plates were incubated aerobically at 37 °C for 24 h. A 25 µl of 0.1% Triphenyl tetrazolium chloride (TTC) was added to each well, incubated for 15 minutes. Plates were read and MIC was determined by recording the concentration of the well that preceded the red color appearance.

### In Vitro determination biofilm formation of *P. aeruginosa*

#### Tube method

A 0.1 mL of bacterial culture (obtained by adjusting turbidity to 0.5 McFarland standards) was transferred to glass test tubes containing 10 mL BHIB with 2% glucose tubes, which were incubated at 37 °C for 72 hours. The medium was then removed and the tubes were washed with distilled water, air-dried and biofilm formation were assayed by crystal violet (Pour et al., 2011). All tests were carried out in triplicates.

#### Crystal violet biofilm assay:

Three wells of sterile 96-microtiter U-bottomed plate (name of manufacturer of these plates?) were filled with 200 µl of bacterial suspension (dilution 1:100 with 2% glucose BHIB). Negative control contained broth only. After 24 hrs

incubation at 37°C, wells were washed three times with 250 µl of DW. After 15 min, plates were stained for 5 min with 0.2 ml of 2% crystal violet per well. Excess stain was removed and rinsed off by placing the plates under running tap water. The plates were air-dried. The adherent cells were re-solubilized with 160 µl of 3% (V/V) glacial acetic acid per well (Christensen et al., 1985). The optical density (OD) of each was measured at 570 nm. Results interpreted according to the followings criteria; OD <0.005 (-), OD 0.500<1.500 (+), OD >1.500 (++) (Alcaráz, Satorres, Lucero, & Centorbi, 2003).

### 3. Result

#### Isolation of *P. aeruginosa* from various departments of Al-Shifa Hospital

*P. aeruginosa* forms yellow green or bluish pigment on nutrient agar media. The organism is Gram negative, capable of growing on MacConkey agar and with not fermentation on TSIA. It was motile and can grow at 37 °C. API 20E kit (*bioMérieux, France*) was used to confirm the identity of *P. aeruginosa*.

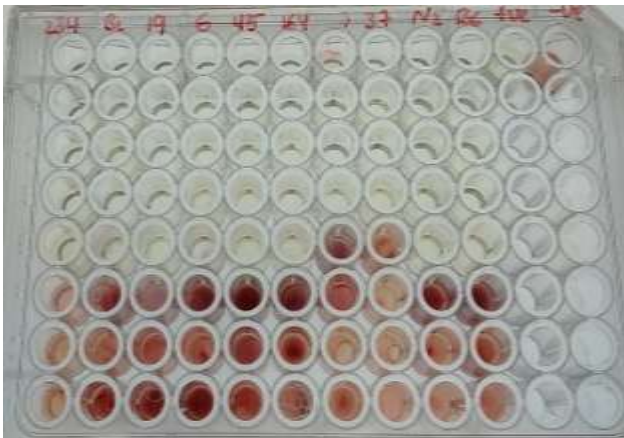
#### Determination of antimicrobial activity of acetic acid Minimal Inhibitory Concentration assay (MIC)

The activity was divided into three categories; (5%) of *P. aeruginosa* isolates showed high susceptibility for acetic acid at concentration of 0.0097 mg/ml, (86%) showed moderate susceptibility (conc. 0.156 to 0.019 mg/ml) and (9%) showed low susceptibility (conc. >0.312 mg/ml) as shown in figure (1).

**Figure (1)** Antimicrobial activity of acetic acid against *Pseudomonas aeruginosa* expressed as PMIC activity. A-J are the test samples.

#### Antimicrobial susceptibility assay

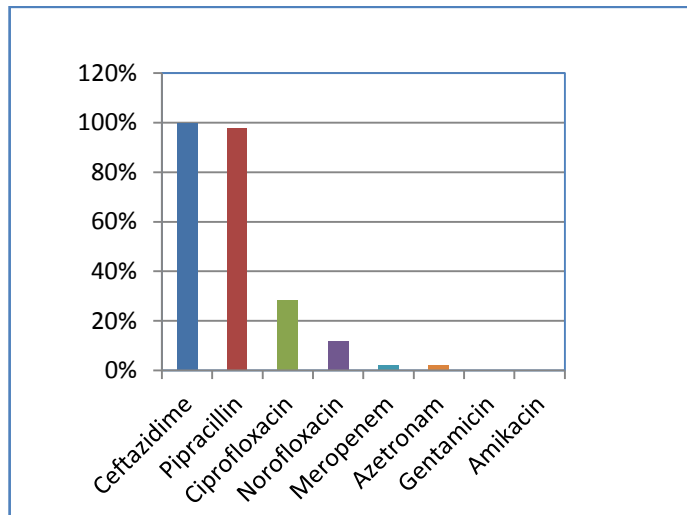
All of the 42 *P. aeruginosa* isolates showed 100% resistance to ceftazidime, similarly 97.6% resistance to piperacillin, 28.57% resistance to ciprofloxacin, 9.5% resistance to norfloxacin, 2.38% resistance to meropenem and aztreonam. All of the 42 *P.*



**Table 2:** Antimicrobial resistance among clinical and environmental isolates of *P. aeruginosa*

Antimicrobial	% of Resistance				P Value
	Clinical isolates (N=31)		Environmental isolates (N=11)		
	No	%	No	%	
Amikacin	0	0.00	0	0.00	NC
Aztreonam	1	03.2	0	0.00	0.363
Ceftazidime	31	100	11	100	NC
Ciprofloxacin	10	32.2	2	18.2	0.788
Gentamicin	0	0.00	0	0.00	NC
Meropenem	0	0.00	1	09.1	2.887
Norfloxacin	2	06.4	2	18.2	1.296
Piperacillin	30	96.8	11	100	0.363

*aeruginosa* isolates screened showed 100% susceptibility to gentamicin, and amikacin (figure 2).



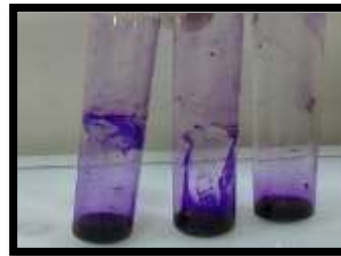
**Figure (2)** Antimicrobial susceptibility of *P. aeruginosa* isolates.

There were no significant differences in the susceptibility patterns among clinical and environmental isolates as shown in table 2

### Assessment of biofilm formation.

#### Tube method:

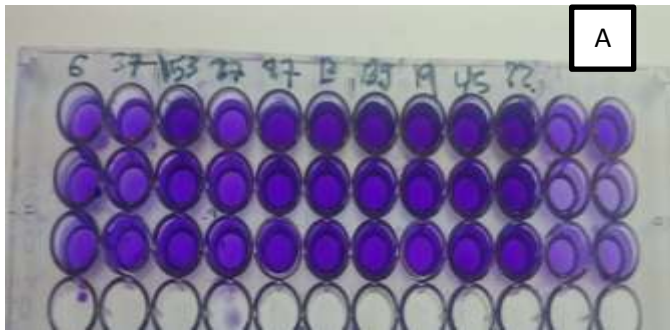
In tube method for biofilm evaluation, the results were comparable with plate method as illustrated in figure (3).



**Figure (3)** Biofilm on tube method for *P. aeruginosa*.

#### Crystal violet assay method

Most isolates were found to be biofilm-forming and showed thick blue ring at the liquid-air interface. Figure (4) is showing the quantitative measurements of adherent biofilm stained with crystal violet dye using the 96 micro titer plates, which is read by an ELISA reader.



**Figure (4)** Biofilm activities assessment against *Pseudomonas aeruginosa*, crystal violet staining method, (A) Negative control.

The results of the tube and crystal violet assay methods were partially correlated. For the micro titer plate method, the isolates were classified as highly biofilm producers (strongly adherent), moderate biofilm producers (moderate adherent) and non-biofilm producers (weak/non-adherent). Biofilm formation was strongly positive in sixteen (38.09%) isolates of *P. aeruginosa*. While the remaining isolates were either moderate biofilm producers (18, 42.8%) or weak/non-biofilm producers (8, 19.04%) *P. aeruginosa*.

In addition, there was no significance difference in the ability of *P. aeruginosa* to form biofilm regardless of the source as shown in table 3.

**Table 3:** Biofilm formation among clinical and environmental isolates of *P. aeruginosa*

Biofilm formation	Clinical isolate (N=31)		Environmental isolate (N= 11)		P value
	No	%	No	%	
Negative	5	16.1	3	27.3	0.688
Intermediate	14	45.16	4	36.36	0.688
Strong	12	38.7	4	36.36	0.688

#### 4. Discussion

This study illustrates biofilm production by both clinical and environmental isolates of *P. aeruginosa*. The ability to produce biofilm is an important virulence factor by facilitating establishment of resistant infections by hampering elimination of infection by the immune system and antimicrobials

(Pye, 2013). One reason for choosing *P. aeruginosa* is that in recent years, nosocomial infections caused by these bacteria have been recognized as an acute problem in hospitals due to its intrinsic resistance to many antimicrobial classes and its capacity to acquire practical resistance to all effective antimicrobials (Fazeli et al., 2012). Moreover, the risk of infection with *P. aeruginosa* in burns is of great concern.

Scientists from divergent fields are investigating alternative antimicrobial agents (plants, and others) for their antimicrobial usefulness. One study in Gaza strip investigated several potential plants against *P. aeruginosa* as antibiofilm agents (Elmanama and Al-Reefi 2017). Acetic acid has been commonly used in medicine for more than 6000 years for the disinfection of wounds (Ryssel et al., 2009). Effective alternative or adjunct non-antimicrobial antimicrobial agents could improve treatment as well as reduce the use of antimicrobials and contribute to a reduction in antimicrobial selective pressure and the further development of antimicrobial resistance (Carson, Ash, & Chakera, 2017). In this study, Acetic acid was used at different concentrations to investigate its effect on *P. aeruginosa* isolated from burns, wounds or soft tissue wounds of 31 patients and 11 environmental swab samples. The results of this study indicated the susceptibility of *P. aeruginosa* to acetic acid as follows; 9.52% of the isolates were classified as low activity and showed MIC of 0.312, 85.71% of the isolates were classified as moderate and showed MIC of 0.039 and 0.078, and 4.76% of the isolates were classified as high and showed MIC of 0.0097. This is a somewhat incompatible with other studies conducted in Germany by Ryssel et al. (2009), who reported that acetic acid in a concentration of 3% has excellent bactericidal effect on *P. aeruginosa*. In addition, by Fearn, Ahmed, & Hasan (1977), 1% of acetic acid is effective against *P. aeruginosa* in 88 % of cases. In addition, other study conducted by Sloss, Cumberland, & Milner (1993), reported that all strains of *P. aeruginosa* exhibited a minimum inhibitory concentration of 2%. According to our results, 85.7% of *P. aeruginosa* isolates were moderately susceptible of acetic acid. Therefore, this similarity in susceptibility of the tested isolates may indicate that they are of a common source.

One major objective of this study was to determine the antimicrobial susceptibility patterns for *P. aeruginosa*. Emerging resistant strains of *P. aeruginosa* are repeatedly linked to non-prudent use of antimicrobials leading to ineffective empirical therapy and in turn, appearance of even more resistant strains of this bacterium. In this study, a notable observation was that Gentamicin and Amikacin demonstrated excellent antibacterial activity against all 42 *P. aeruginosa* isolates. The result obtained by Rio et al., (2002), reported 50% of the isolated strains to be resistant to Gentamicin, 25% to Ciprofloxacin, 9% to Amikacin, and 2% to Ceftazidime. Their result is compatible with our result for Ciprofloxacin resistance but incompatible with the others. Another study conducted in Egypt reported 78% from 147 isolates were sensitive to Gentamicin and 88% of the isolates were sensitive to Norfloxacin and 82% were sensitive to Piperacillin by using MIC by agar dilution method (Hashem, Hanora, Abdalla, & Saad, 2016). These variations are expected and may be due differences in usage of antimicrobials, infection control practices and differences in sites of tested isolates.

Most of the *Pseudomonas* isolates examined in this study produced biofilm; Only (19.0%) showed negative biofilm forming abilities. Factors affecting biofilm formation by these isolates were not evaluated in this study.

The result for biofilm from our isolates was 16-sample strong positive (38.09%), 18-sample moderate positive (42.85%), and eight-sample negative (19.04%). This may indicate that biofilm forming potential is one survival strategy for these microbes in hospital stress environment and may become a source of hospital-acquired infections. The result obtained by Gurung et al., (2013), is in agreement with our study, biofilm was detected in 16/49 (33%) of *P. aeruginosa*. The result obtained by Perez, Costa, Freitas, & Barth (2011), indicated the absence of strong-biofilm producing *P. aeruginosa*. The results are incompatible with our study. According to our study, 80% of *P. aeruginosa* were biofilm producer (strong + Moderate). Mechanisms responsible for antimicrobial resistance in organisms producing biofilms includes delayed penetration of the antimicrobial agents through the biofilm matrix, altered growth rate of biofilm

organisms (slower growth), and other physiological changes due to the biofilm mode of growth. Thus, the ability to form biofilm could be an effective strategy to enhance the survival and persistence under stressed conditions like host invasion or following antimicrobial treatment (Gurung, et al., 2013).

## 5. Conclusions and Recommendations

Biofilm forming ability of the isolates was detected in 80% of isolates. High resistance to common antimicrobials was also observed. Acetic acid showed moderate antimicrobial activity against all *P. aeruginosa* isolates. Further research is required to evaluate the genotypic characteristics of biofilm forming microbes in order to determine the major genes responsible for biofilm formation. Acetic acid may be used in the treatment because of its effectiveness *in vitro* at low concentrations. It is also necessary to reduce the use of antibiotics in a regulated and subjective way to reduce the resistance of bacteria and prevent or slow down the emergence of new strains.

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