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Research Article

BIOFILM FORMATION AND ANTIMICROBIAL SUSCEPTIBILITY OF *PSEUDOMONAS AERUGINOSA* IN THE INDOOR AND OUTDOOR ENVIRONMENT

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ABSTRACT
As ubiquitous bacteria, *Pseudomonas aeruginosa* can survive various conditions in the form of biofilm. In this investigation, we evaluated the prevalence and antimicrobial susceptibility of *P. aeruginosa* biofilm-producers and non-producers in the indoor and outdoor environment. Microtiter plate assay (MPA) was used for the quantification of biofilm production, and tube method (TM) as a qualitative method. The antimicrobial activity of 11 antibiotics from 4 different classes was tested against biofilm-producers and non-producers *P. aeruginosa* isolates by disk diffusion method. Out of 98 samples, 31 *P. aeruginosa* isolates were obtained from different surfaces such as metal, ceramic, plastics, rock and concrete and soil. The results showed that 74% of isolates were biofilm-producers and 26% non-producers. Most of the isolates were resistant to ampicillin (90.32%), amikacin (70.97%), and tetracycline (64.52%). In contrast, all isolates were susceptible to ciprofloxacin and tazobactam-piperacillin. Although 21 (67.7%) isolates showed resistance to three or more antibiotics, the 54.84% shared mutual multidrug resistance (MDR) profile, TET, AMP, AMC. The MPA method was more effective and reliable than the tube test for the detection of biofilms. We can conclude from our study that *P. aeruginosa* biofilms are widespread in the non-hospital environment and resistant to several antibiotics form different classes.

***Keywords*:** *Antimicrobial Susceptibility, Biofilm Formation, Pseudomonas aeruginosa*

INTRODUCTION
Bacterial biofilms represent a widespread form of survival strategy in the environment. They are comprised of a multitude of microbial cells immersed in the extracellular polymer-matrix (Donlan, 2002). The ubiquity of the biofilm producing microorganisms (Costerton *et al*., 1987; Notermans *et al.*, 1991), and its genetic diversity (Watnick and Kolter, 2000) implying they are an ancient form of life on Earth (Marić and Vraneš, 2007). More often, the sessile form of bacteria, biofilm is the dominant form of phenotype compared to the free-living, planktonic forms (Costerton *et al*., 1995). They can be found on essentially any environmental surface in which sufficient moisture is present, on different biotic and abiotic surfaces both in the environment and in the healthcare setting.
Employing the biofilms in biotechnology (Rosche, 2009) and bioremediation (Usharani and Lakshmanaperumalsamy, 2016/17) as a positive side cannot exceed the devastating impact on the food industry (Kumar and Anand, 1998) and medicine (Costerton *et al*., 2003**)**. Biofilm infections, such as pneumonia in cystic fibrosis patients, chronic wounds, chronic otitis media and implant- and catheter-associated infections, affect millions of people in the developed world each year and many deaths occur as a consequence (Bjarnsholt, 2013).
One of the main causes of long and strenuous infections is biofilm resistance to different antimicrobial agents (Gilbert *et al*., 1997) and components of the immune system (Donlan and Costerton, 2002). *Pseudomonas aeruginosa* is usually recognized as a human opportunistic pathogen and one of the models

for the investigation of biofilm development. *P. aeruginosa* possesses an incredible capacity for development and acquisition of new resistance mechanisms to antibiotics (Vaisvila *et al*., 2001). The low permeability of the outer membrane provides *P. aeruginosa* with natural resistance to several antimicrobial drugs (Angus *et al*., 1982). Besides that, various efflux pumps (Piddock, 2006) and enzymes inactivate antibiotics (eg. β-lactamases) (Poole, 2011). The increasing number of *P. aeruginosa* MDR isolates led to extensive studies on prevention and antimicrobial susceptibility which are difficult to compare due to various multidrug resistance definitions (Hirsch and Tam, 2010). In addition, while being an opportunistic human pathogen, *P. aeruginosa* also infects other organisms such as plants (Rahme *et al*., 1995; Silo-Suh *et al*., 2002), insects (Jander *et al*., 2000), and nematodes (Mahajan-Miklos *et al*., 1999).

Even though the production of biofilms in the hospital setting was extensively investigated (Hassan *et al*., 2011; Lima *et al*., 2017; Saxena *et al*., 2014), biofilms in the non-hospital environment are poorly examined.

The main purpose of this research was to investigate the presence, distribution and antibiotic susceptibility of the *Pseudomonas aeruginosa* biofilms in the non-hospital environment.

MATERIALS AND METHODS

Sample collection

Bacterial isolates were obtained from different indoor and outdoor samples such as bathroom and kitchen sink drains, tub and shower drains, pipes, faucets, kitchen counters, toilets., ceramic tiles, gutters, grooves, pebble drainage, concrete walkways, sponges, compost, soil and dust etc. A total of 98 samples were collected using the wet swab method from different abiotic and biotic surfaces (metal 46%, plastic 16%, rocks and concrete 12%, soil and dust 11%, ceramic 6%, soil 4%, sponge 3%, wood 1%, textile 1%).

Chemicals and bacterial media

Cetrimide agar – *Pseudomonas* isolation selective agar (MerckMilipore, Watford, United Kingdom); Tryptic soy broth (Biomerieux, Prague, Czech Republic), Tryptic soy broth (Biomerieux, Prague, Czech Republic) + 0,25 % glucose (Semikem, Sarajevo, Bosnia and Herzegovina), Mueller Hinton Agar (FlukaAnalytical, Munich, Germany), Nutrient agar (HIMEDIA, Pennsylvania, USA), phosphate buffer – pH 7.4, physiological solution, 95% ethanol.

Antibiotics

For antibiotic susceptibility testing of *Pseudomonas aeruginosa*, we used 11 antibiotcs (Oxoid™, United Kingdom): Ciprofloxacin (CIP) 10 µg, Tetracycline (TET) 30 µg, Streptomycin (STR) 10 µg, Ampicilin (AMP) 10 µg, Meropenem (MEM) 10 µg, Piperacillin-Tazobactam (TZP) 40 µg, Amikacin (AMC) 30 µg, Aztreonam (ATM) 30 µg, Ceftazidime (CAZ) 10 µg, Cefotaxime (CTX) 30 µg, Cefepime (FEP) 30 µg.

Isolation and identification of Pseudomonas aeruginosa

All 98 collected swab samples were inoculated on Cetrimide Agar. The non-biofilm producer strain *Pseudomonas aeruginosa* ATCC 27853 served as a negative control. After incubation at 37 ºC for 24-72 h, positive samples were mixed representatives of genus *Pseudomonas*. In addition, standard tests such as oxidase and catalase tests according to Cowan and Steel′s Manual for the Identification of Medical Bacteria (Barrow and Feltham, 1993) were conducted to identify *Pseudomonas aeruginosa*.

Biofilm detection

After strain purification on Nutrient Agar and TSB, biofilm-forming ability of the *P. aeruginosa* isolates were qualitatively evaluated by tube method using (Christensen *et al*., 1982) polystyrene tubes and 2% crystal violet solution. The microtiter plate assay (MPA) was used for the quantification of the biofilm according to the modified protocol by Stepanović *et al.* (2007). The bacterial suspension was prepared from an overnight culture grown in TSB diluted 1:100 in TSB supplemented with 0.25% glucose. To each well, on polystyrene plates containing 96 flat-bottom wells, 100 µL of suspension was added. For each isolate, three wells were inoculated. TSB media was used as a negative control. After incubation at 37°C

for 24 hours, contents of the wells were decanted and the wells were washed thoroughly with phosphate-buffered saline, left to air dry and biofilms were fixed at 60°C for 60 min. Biofilm formation was evaluated by adding 125 µL of 95% ethanol to each well after staining with 125 µL of 2% crystal violet solution. Optical density was measured at 570 nm using microtiter reader Multiread 400 (Athos).

Antimicrobial testing susceptibility

The antimicrobial activity of 11 antibiotics from 4 different classes were tested against biofilm-producers and non-producers *Pseudomonas aeruginosa* isolates by disk diffusion method respecting the National Committee for Clinical Laboratory Standards (2015) guidelines. Inoculum density was adjusted according to the 0.5 McFarland scale (~1.5 x10^8 CFU/mL).

The samples were classified according to Stepanović *et al*.(2007). The mean value of the optical density for each isolate (ODi) was compared to the optical density of the negative control (ODc). The isolates were classified into the following categories:: non-producing if ODi ≤ ODc; weakly producing if ODc < ODi ≤ 2 x ODc; moderately producing if 2 x ODc < ODi ≤ 4 x ODc; or strongly producing if 4x ODc < Odi.

RESULTS AND DISCUSSION

Out of 98 samples, 31 were verified *Pseudomonas aeruginosa* isolates. The isolates were found on different surfaces such as metal 29%, soil and dust 22.6%, ceramic 13%, plastic 13%, rocks and concrete 13%, soil 9.7%.

After quantitative method of microtiter plate assay (MPA), isolates were classified according to Stepanović *et al*.(2007). Out of 31 isolates, 23 (74%) isolates were biofilm-producers and 8 (26%) isolates were non-producers. Of the isolated biofilm producers, most isolates belonged to the category of moderately-producing, while the least isolates were in the category of strongly-producing strains.

We compared classification results of qualitative and quantitative technique (Table 1).

Table 1: Classification results of 31 *Pseudomonas aeruginosa* isolates using microtiter plate assay and tube method

	Biofilm formation	MPA		TM	
		No	%	No	%
No of isolates	Non-producer	8	25.8	5	16.1
	Weakly-producer	8	25.8	9	29
	Moderately-producer	11	35.5	10	32.2
	Strong producer	4	12.9	7	22.6

In this study, the majority of *Pseudomonas aeruginosa* biofilm-producers were discovered on metal surfaces (30.43%) and soil and dust (21.74%), while the smaller number was detected in soil, plastics and ceramic surfaces (13.04%). Biofilm-producers on rock and concrete surfaces (8.70%) were scarce.

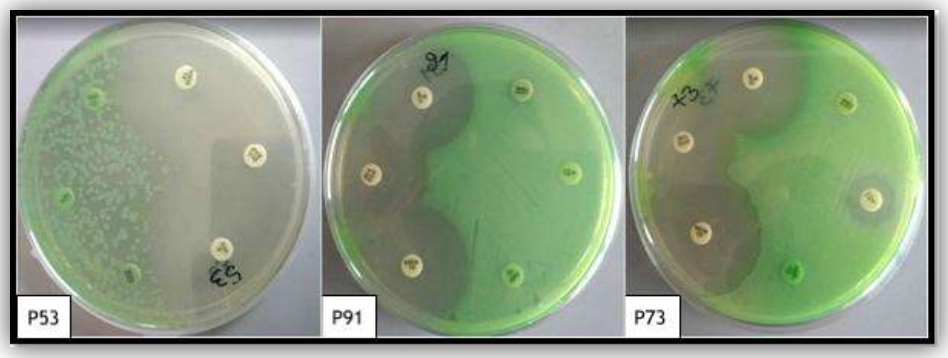


Figure 1: P53–Non-producer colonies overgrowed AMP, STR and TET antibiotic; P91–Non-producer bacterial colonies overgrowed TET, STR, and AMP; P73–Strongly biofilm producer bacterial colonies overgrowed TET and AMP and partialy STR.

Antibiogram

All tested antibiotics expresed certain antibacterial effect (Table 2). The large number of isolates showed resistance to ampicilin (90.32%), amikacin (70.97%), and tetracycline (64.52%) (Figure 1). All isolates were susceptible to ciprofloxacine and tazobactame-piperacilin.

Table 2: List of antibiotics and number of resistant, intermediate and susceptible isolates

Class of Antibiotic	Antibiotics	Resistant			Intermediate			Susceptible		
		Total	BP	NP	Total	BP	NP	Total	BP	NP
	AMC	22	16	6	-	-	-	9	7	2
Aminoglycoside	STR	3	2	1	-	-	-	28	22	6
	ATM	18	13	5	8	5	3	5	5	-
Beta-lactam	AMP	28	21	7	-	-	-	3	2	1
Beta-lactamase inhibitor	TZP	-	-	-	-	-	-	31	23	8
Carbapenem	MEM	4	3	1	1	1	-	26	19	7
Cephalosporine	CAZ	1	1	-	-	-	-	30	22	8
Cephalosporine	FEP	-	-	-	13	7	6	18	16	2
Cephalosporine	CTX	5	3	2	19	13	6	7	7	-
Fluoroqinolone	CIP	-	-	-	-	-	-	31	23	8
Tetracycline	TET	19	12	7	7	7	-	6	5	1

The presence and distribution of the MDR Pseudomonas aeruginosa isolates

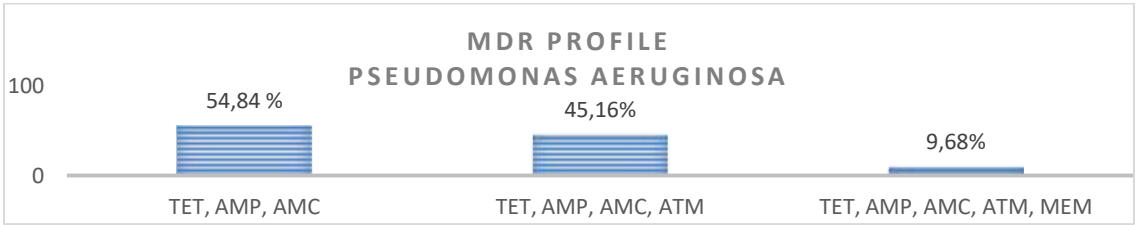
Out of 31 isolates, 21 (67.7%) isolates *Pseudomonas aeruginosa* showed resistance to three or more antibiotics from different classes. From the overall number of multidrug resistance isolates, 15 (71.4%) were biofilm-producers and 6 (28.6%) non-producers. Most isolates expressed resistance to 4 different antibiotics (9 or 42.9%), while only 2 (9.5%) isolates were resistant to 6 antibiotics (Table 3). The highest number of MDR isolates was detected on a metal surfaces (38.09%), and the lowest was on plastics and ceramic (9.52%).

Table 3: The ratio of MDR in different categories of biofilm formation isolates

			Resistance profile			
			3 AB	4 AB	5 AB	6 AB
Biofilm formation	**Total**	**MDR**				
Non- producers	8	6	1	2	2	1
Weakly producers	8	6	1	4	1	-
Moderately producers	11	6	1	3	2	-
Strongly producers	4	2	1	-	-	1

Determination of the most common MDR profiles

As shown by the Figure 2, we defined three mutual multidrug-resistant profiles among the isolates based on the antibiogram results.



TET-Tetracycline; AMP-Ampiciline; AMC-Amikacin; ATM-Aztreonam; MEM-Meropenem

Figure 2: Percentage of isolates that share the identical MDR profile

In this research, we evaluated *Pseudomonas aeruginosa* biofilm-formation in the indoor and outdoor environment on various abiotic and biotic surfaces and their antimicrobial susceptibility. *P. aeruginosa*, as a ubiquitous bacteria, can survive on the most different surfaces, and nutrient deficiency conditions (Hardalo and Edberg, 1997). With respect to the previous investigations, samples are taken from the soils, pipes, drainages, waste disposals and other wet places. From the 98 samples, 31 isolates were (31,63%) identified as *P. aeruginosa*. Similarly, Gad *et al*. (2007), collected 28,5% *Pseudomonas* isolates from the clinical settings and environment, among them 19,5% *P. aeruginosa* strains.

When discussing the microtiter plate assay (MPA), we were following Stepanović *et al*., 2007 protocol**.** After staining the biofilm with 2% Crystal Hucker violet, as an indicator of biofilm formation, we recognised the violet ring in point of contact between air and liquid. Using the protocol according to Stepanović *et al*. (2007), it appears that 95% ethanol provides better results in the resolubilization of biofilm and its measurement indirectly.

Tube method (TM) used for the qualitative detection of biofilms produced comparable results to the quantitative method, but it was difficult to discriminate between weak, moderate and strong biofilm producers because of different observers**.** This method should not be considered as a primary method in biofilm detection (Christensen *et al*., 1982; Mathur *et al*., 2006). Although we used TM, we conducted this method as confirmation to MPA.

Considering the harsh environment conditions and limited nutrient sources on different surfaces (metal, plastics, ceramic, and rock surfaces) (Olsen, 2015), it was expected to have more biofilm-producer (74%) than the non-producers (26%) isolates knowing the biofilm represents one way of survival strategy (Leid, 2009). Although the samples were taken from the non-hospital conditions, the ratio between non-producers-producers was similar to Lima *et al*. (2017), findings in clinical settings (75% biofilm producers and 25% non-producers).

Most of the isolates (90.32%) exhibited resistance to the antibiotic ampicillin. These results are similar to the Saxena *et al*. (2014), findings where 95% of isolates were resistant ampicillin. The high degree of resistance to ampicillin can be explained with the natural resistance of *P. aeruginosa* to the beta-lactam antibiotics due to the inducible AmpC beta-lactamases (Livermore, 1995). Furthermore, a lot of isolates were resistant to amikacin (70.97%) and tetracycline (61.29%). Aminoglycoside-modifying enzymes (AME) are inhibiting the effects of aminoglycosides (Vaziri *et al*., 2011), in our case amikacin. Because of the MexAB/Mex/XY efflux system, *P. aeruginosa* is essentially resistant to the tetracycline (Morita *et al*., 2001.; Dean *et al*., 2003).

Cefotaxime produced the highest number of intermediates (61.29%). Saeed and Awad, (2009) also reported a significant percentage (45%) of *Pseudomonas aeruginosa* intermediates to cefotaxime. Even though cefotaxime exhibits certain activity against *P. aeruginosa*, it should not be prescribed for antipseudomonal therapy (Carmine, 1983).

In contrast, all isolates were susceptible to ciprofloxacin and piperacillin-tazobactam. Ciprofloxacin is still successfully in use for the treatment of *Pseudomonas aeruginosa* infections (Manno *et al*., 2005). Piperacillin-tazobactam is often employed for *P. aeruginosa* therapy infection in critical patients (Lodise *et al*., 2007), and that explains its effective antimicrobial activity in this research.

In this study, we determined the number of MDR *Pseudomonas aeruginosa* isolates resistant to three or more antibiotics from different classes. Based on the antibiogram results, 17 (54.84%) isolates shared mutual multidrug-resistant profile: TET-AMP-AMC. Considering the tetracycline, ampicillin and amikacin are in different classes of antibiotics, the MDR criteria are fulfilled.

Conclusion

In this study, we examined the presence of *Pseudomonas aeruginosa* biofilms in the indoor and outdoor environment and their antimicrobial susceptibility. The significant number of isolates were recovered from soil and metal surfaces, indicating they are capable of living on biotic and abiotic surfaces. Obtained results have shown that the biofilm production was not connected with antibiotic susceptibility profile for the studied *P. aeruginosa* isolates. Most isolates exhibited resistance to tetracycline, ampicillin and

amikacin, while all were susceptible to ciprofloxacin and piperacillin-tazobactam. Considering the fact more than half of the isolates in our investigation showed resistance to three or more antibiotics from different classes, we can conclude that *P. aeruginosa* biofilms in the non-hospital environment are widespread and show resistance profiles similar to isolates from clinical settings.

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