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ORIGINAL ARTICLE

Relationship between antibiotic resistance, biofilm formation, genes coding virulence factors and source of origin of *Pseudomonas aeruginosa* clinical strains

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Abstract

Introduction and objective. *Pseudomonas aeruginosa* is an opportunistic pathogen that causes difficult with treating infections, especially in the immunocompromised and patients with some underlying disease. The aim of the study is to assess the antibiotic resistance, biofilm formation, and the presence of genes encoding various virulence factors in clinical isolates of *P. aeruginosa.*

Materials and methods. Seventy-three clinical isolates of *Pseudomonas aeruginosa* were tested. Antimicrobial Susceptibility Testing (AST) and carbapenemases production was performed in accordance with the EUCAST guidelines. The ability to form biofilm was assessed by crystal violet assay. Genes encoding selected virulence factors were detected using standard polymerase chain reaction (PCR).

Results. Among the 73 clinical isolates of *P. aeruginosa,* 41.1% were resistant to imipenem, 61.6% to meropenem, 30.1% to ciprofloxacin and 15.1% to tobramycin. Over 20% of isolates were producers of MBL. Antibiotic resistance profiling revealed that 23.3% of strains were sensitive to all antibiotics, 60.3% were LDR phenotype, and 16.4% were MDR phenotype. The majority of strains (73.6%) were strong-biofilm producers, 17.0% were moderate and 9.4% were weak biofilm producers. PCR analysis showed the presence of *lasB, aprE* and *prpL* genes in most of the tested strains (93.1%, 87.7% and 74.0%, respectively). Among strong biofilm producers, 22.2% were MDR, 63.0% of strains represented LDR phenotype, and 14.8 % were sensitive to all antibiotics. Moderate and weak biofilm producers were LDR and sensitive phenotypes only (respectively, 58.3% and 42.9 – LDR, 41.7 and 51.7% – sensitive).

Conclusions. High frequency of MDR strains and their ability of biofilm formation and virulence factors may be a threat to effective therapy, and can increase morbidity and mortality of infected patients.

Key words

antimicrobial resistance, *Pseudomonas aeruginosa,* biofilm, virulence factors

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen that can cause infections in immunocompromised individuals, most commonly in patients being treated in intensive care, surgery, and burn units. *P. aeruginosa* infections most frequently involve the respiratory tract, placenta, urinary system, skin, and soft tissues, where the greatest risk is associated with the infection of postoperative wounds, burns and pressure ulcers [1, 2]. In the USA, *P. aeruginosa* is among the top six bacterial species responsible for nosocomial infections, and the second most common pathogen causing ventilator-associated pneumonia and catheter-related urinary tract infections [2]. Despite advences in critical-care management, *P. aeruginosa* infections are associated with a mortality rate reaching 20% – 50% [3, 4, 5, 6, 7].

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Infections caused by *P. aeruginosa* pose therapeutic difficulties and frequently assume a chronic course. These are attributable to the fact that *P. aeruginosa* produces several virulence factors both – cell surface components (lipopolysaccharides, fimbriae, flagella, mucus, lectins) and extracellular product (exotoxin A; exoenzymes S, T, U, and Y; alkaline protease, type IV protease, elastase, neuraminidase, phospholipase, and the pigment pyocyanin). Toxins and enzymes inhibit the phagocytosis, damage macrophages, and inactivate the immunoglobulins IgG and IgA [8, 9]. Based on its ability to use different energy sources and adhere to various surfaces, *P. aeruginosa* is capable of surviving in nutrient-poor environments. The attachment of motile bacteria to a surface and their subsequent division lead to the formation of microcolonies. Bacterial microcolonies mature and coalesce to form biofilms [10]. Bacterial biofilm protects against the host's immune system and antimicrobial substances [11].

The production of biofilm and virulence factors is regulated by the *Quorum Sensing* (*QS*) system. *QS* is a process of bacterial

communication that regulates gene expression depending on the density of bacterial cell populations. The phenomenon affects genes that are indispensable for bacterial function and growth, horizontal gene transfer, and virulence. The *QS* mechanism in *P. aeruginosa* plays a key role in the synthesis of virulence factors, including protease, pyocyanin, exotoxin A, alkaline protease, lectins, and rhamnosyltransferase [12, 13, 14]. The process of *QS* contributes to the development of chronic pulmonary infections in patients with cystic fibrosis, the spread of infection leading to sepsis, and the development of tissue pathologies [15].

The sensitivity of bacteria in planktonic and biofilm forms to antimicrobial agents is varied. Biofilm forming microorganisms are far more resistant to antibiotics than bacteria growing as individual cells [16]. The biofilm formed by *P. aeruginosa* is considered to be one of the major causes of therapeutic failure [17].

P. aeruginosa has both natural (intrinsic) and acquired resistance to a range of antibiotic classes. Natural resistance applies to β-lactam antibiotics (benzylpenicillin, isoxazolyl penicillins, aminopenicillin and their combinations with β-lactamase inhibitors, first- and second-generation cephalosporins), tetracyclines, tigecycline, chloramphenicol and trimethoprim [3]. Acquired resistance is associated primarily with the production of enzymes that break down β-lactam antibiotics. A significant role in the antibiotic resistance of *P. aeruginosa* is attributed to metallo-β-lactamases (MBLs) production. MBLs are characterized by an extremely broad substrate spectrum, including all penicillins, cephalosporins, and carbapenems. Crucially, these enzymes exhibit a complete lack of susceptibility to all available clinically used β-lactamase inhibitors. An aspect of paramount importance is the ability of MBLs to inactivate carbapenems effectively, often referred to as 'antibiotics of last resort' in the treatment of infections caused by multi-resistant Gram-negative bacterial strains [18, 19].

OBJECTIVE

The aims of the study were to evaluate antimicrobial resistance, biofilm formation and characterization of the genes encoding selected virulence factors of Pseudomonas aeruginosa clinical strains.

MATERIALS AND METHOD

Bacterial strains. Seventy-three strains of *P. aeruginosa* isolated from patients diagnosed with different clinical infection were evaluated. The strains were isolated in the Microbiology Clinical Laboratory at the University Hospital of Lord's Transfiguration in Poznań, Poland, in the period January – November 2018. The sources of isolation of the tested clinical strains are presented in Table 2.

The identification of the genus level was carried out based on cellular morphology and the Gram staining, macroscopic assessment of bacterial colonies, and conventional biochemical tests. Identification to the level of species was performed by VITEK® Compact automated identification system using GN Cards – test for the identification of *Enterobacteriaceae* and a selected group of glucose nonfermenting Gram-negative organisms.

Antimicrobial susceptibility testing. The testing was performed by the disc diffusion method in accordance with the EUCAST [20] recommendations. Ciprofloxacin 5 μg, ceftazidime 10 μg, piperacillin 30 μg, tobramycin 10 μg, imipenem 10 μg, and meropenem 10 μg were used to test the drug susceptibilityto *Pseudomonas* spp. *P. aeruginosa* ATCC 27853 was used as a reference strain. Carbapenemases production was screened using the Carba NP test and phenotypic double disc synergy tests with EDTA (MBL) and boronic acid (KPC).

The following resistance phenotypeswere distinguished: sensitive (sensitivity to all antibiotics tested), low-level resistant (LDR; resistance to < 3 antimicrobial categories;) and multi-drug resistant (MDR; resistance to ≥ 3 categories).

Quantitative assessment of biofilm formation *in vitro*. To evaluate biofilm formation, the crystal violet assay was used. Each well of a sterile 96-well flat-bottom plastic plate was filled with 200 μL of standardized bacterial suspension (density c.a. 10^6CFU/mL). Negative control wells contained broth only. *Pseudomonas aeruginosa* PAO1 was used as a positive control. The plates were closed and incubated aerobically for 24 h at 37°C. Then, the content of each well was aspirated, and each well washed three times with 250 μL of sterile physiological saline. The plates were shaken in order to remove all non-adherent bacteria. After the washing steps, biofilms were stained for 15 min with a 2% solution of crystal violet (200 μL). Each well was then washed three times, once again with 250 μL of sterile physiological saline After the plates were air-dried, 200 μL of 99% methanol per well was added. The absorbance of each well was measured at 590 nm using an Infinite M200 plate reader (Tecan). The test was repeated three times for each strain.

The interpretation of biofilm production was according to the criteria described by [21]. The mean optical density (OD) of the negative control was considered the cut-off. All strains were classified based on the following criteria: non-adherent (OD ≤ ODc), weakly adherent (ODc < OD ≤ 2 × ODc), moderately adherent (2 × ODc <OD ≤4 × ODc), strongly adherent (OD > 4 × ODc).

Detection of genes taking part in *Quorum Sensing* and genes encoding virulence factors. DNA extraction was carried out using the thermal in-house method subjecting the bacteria cell wall to the process of lysis, by warming up (60±2°C) and cooling down (-20±2°C) during six cycles lasting 30 seconds each. Bacterial DNA was extracted using the thermal in-house method, subjecting the bacteria cell wall to the process of lysis, by warming up (60±2°C) and cooling down (-20±2°C) during six cycles lasting 30 seconds each.

Genes encoding various *QS* systems (*lasl, lasR, rhlI, rhlR, ambD, ambE, pqsA, pqsB*), and genes encoding tested virulence factors: elastase (*lasB),* alkaline protease *(aprE),* endopeptidase IV (*prpL*) were detected using single standard polymerase chain reaction (PCR). The specificity of the starters was checked in BLAST Assembled Ref Genomes (https://blast.ncbi.nlm.nih.gov/Blast.cgi program). The following PCR mixture was used: buffer with $MgCl_2$ (1×), dNTP Mix (0.2 mM each), primer pair (10 μM), thermostable Taq polymerase (1U), DNA (100–200 ng) isolated from the tested *P. aeruginosa* strains. As a negative control, the reaction mixture with water was used instead of bacterial

DNA. The positive control was a DNA sample isolated from the strain of *P. aeruginosa* PAO1. Parameters of amplification cycles: denaturation in 94 °C for 3 minutes, hybridization of starters, for 1 minute and extending starters in 72 °C for 1 minute, for 30 cycles (Table 1).

The presence of the amplified products of PCR was evaluated using electrophoretic separation in 1.5% agarose gel against the marker's molecular weight. The electrophoresis was carried out within 40 minutes under constant voltage of 130 V. The resulting bands were visualized by transilluminator in UV light (Syngen).

Table 1. PCR detection of tested genes. Oligonucleotide sequences for identification of genes taking part in Quorum Sensing and genes encoding virulence factors in P. aeruginosa strains

	Sequence (5'-3')	Amplicon size (bp)	Annealing temp. (0C)
***Quorum Sensing* genes**			
lasI	F:GGCTGGGACGTTAGTGTCAT R: CCAGCGTACAGTCGGAAAA	274	59.8
lasR	F: AAGGACAGCCAGGACTACGA R: CCGAGCAGTTGCAGATAACC	490	54.6
rhlI	F: GTTCGACCATCCGCAAAC R: GTCTCGCCCTTGACCTTCT	354	60.9
rhlR	F: CATGGCACCTATCCCAAGG R: GGTCCATTGCAGGATCTCG	396	60.9
pqsA	F: CCTGTTTCCTGCAATACACCTC R: CCATAGCCGAAGAACATCTTG	163	56,5
pqsB	F: GTCTTCGACCTCACCGACT R: GCTGTCCACTTCCAATCCCT	129	56,0
ambE	F: ATGCGGCCTACATGATCTTC R: CGGAGAGCAACTGGAGAATC	201	65,5
ambD	F: GTGTTCACCTCCACCGAGTT R: CGGTGTCTGGCAGTAGAAGTG	263	62,0
Virulence factors genes			
Alkaline protesae (*aprE*)	F: GCAGAACAAGCACCCTACTAC R: AACAGGGGCTTGAACAGGTA	156	60.40
Elastase *(lasB)*	F: CGAGAATGACAAAGTGGAACTG R: CGTAGGTGTACTTGCCGATCTT	218	60.0
Endopeptidase IV (*prpL*)	F: GTCACCTACGACGGGCATAC R: CGCTGAAATCGGAGAAGTAGTC	196	62.2

Statistical analysis. R statistical package [22] was used for statistical analyses. The relationship between the prevalence of the source of isolation, antibiotic resistance phenotype, and biofilm production was determined using Pearson's Chi-square test. A *P* values less than 0.05 was considered significant.

RESULTS

Antimicrobial susceptibility. The drug susceptibility profile is shown in Figure 1A. The current study demonstrated strains resistant to beta-lactam antibiotics, especially to carbapenems: imipenem (41.1%) and meropenem (61.6%). Among the tested strains, 30.1% were resistant to ciprofloxacin and 15.1% to tobramycin. 23.3% of the strains were producers of Metallo-β-lactamases. None of the strains produced Klebsiella pneumoniae carbapenemase (KPC). Sensitivity to all antibiotics was found in 23.3% of strains, 60.3% of strains showed a phenotype LDR, and 16.4% were MDR phenotype

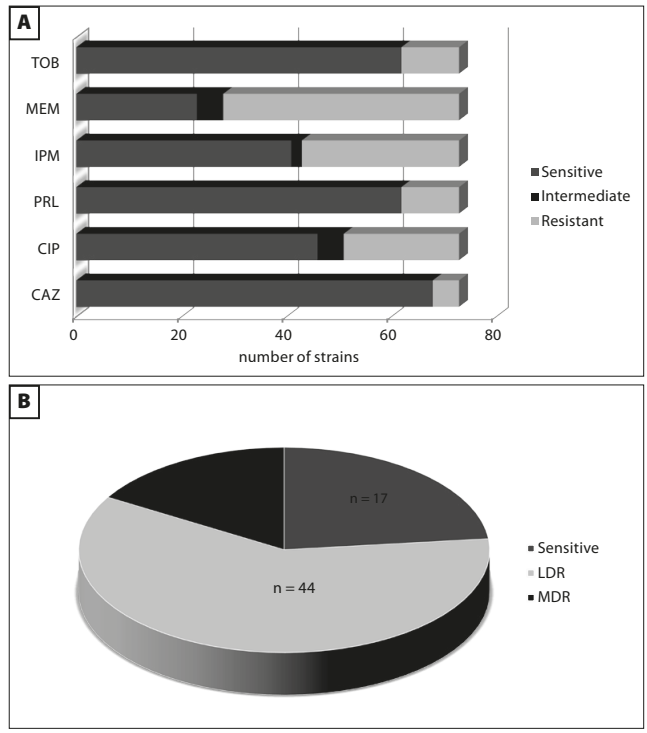

Figure 1. Antibiotics susceptibility.
A – Antimicrobial susceptibility profile: CAZ – ceftazidime; CIP – ciprofloxacin; PRL – piperacillin; IPM – imipenem; MEM – meropenem; TOB – tobramycin.
B – Antimicrobial resistance phenotypes: Sensitive (sensitivity to all antibiotics tested) – LDR (low-level resistant; resistance to < 3 antimicrobial categories) and MDR (multidrug resistant; resistance to ≥ 3 categories).

(Figure 1B). All MDR strains were carbapenem (meropenem and imipenem) resistant.

The ability of biofilm formation. The ability to biofilm formation of 73 strains of *P. aeruginosa* was tested using the spectrophotometric method with crystal violet. Based on the absorbance value, following the biofilm formation criteria, all bacteria strains were qualified into appropriate groups in terms of the biofilm-forming rate. The majority (73.6%) of *P. aeruginosa* strains were found to be strong biofilm producers. Only 9.6% of strains showed a weak biofilm-forming ability (Tab. 2).

Prevalence of genes encoding virulence factor and Quorum Sensing. Qualitative analysis showed that the *lasB* gene was present in 93.1%, *aprE* in 87.7%, and *prpL* in 74.0% of the tested strains (Tab. 2). Analyzing the results, it was noted that 67.1% of the strains studied had all the genes responsible for the production of virulence factors. Only 2 strains of *P. aeruginosa* did not have any of the identified genes. These strains belonged to strong biofilm producers. Four strains did not have 2 of the analyzed genes simultaneously. Three of these strains had the ability to form a strong biofilm, and one to a weak degree (Tab. 2). Qualitative identification of genes involved in the *QS* system showed that 42 strains had all the genes participating in the *QS* (Tab. 2).

Correlation of results. As a correlation of the obtained results, the examined strains were analyzed for the source of isolation and their ability to create biofilm. Out of 53 strains of *P. aeruginosa* isolated from the lower respiratory tract, 73.6% exhibited a strong biofilm-forming ability, while 17.0%

Table 2. Qualitative identification of genes taking part in e *Quorum Sensing* (QS) and genes encoding virulence factors, biofilm formation rate, and phenotype resistance in *Pseudomonas aeruginosa* strains isolated from different clinical infection

Specimens		Biofilm	QS genes								Virulence genes			Resistance phentype
			lasI	*lasR*	*rhlI*	*rhlR*	*ambD*	*ambE*	*pqsA*	*pqsB*	*lasB*	*aprE*	*prpL*	
1	resp. tract	S	+	-	+	-	-	+	+	+	+	+	+	LDR
2	resp. tract*	S	+	+	+	+	-	+	+	-	+	+	+	LDR
3	wound	S	+	+	+	+	+	+	+	+	+	+	+	Sensitive
4	resp. tract*	S	+	-	+	-	+	+	+	+	+	+	-	MDR
5	wound	W	+	+	+	+	+	-	+	+	+	-	+	Sensitive
6	wound	M	+	+	+	+	+	+	+	+	+	+	+	LDR
7	resp. tract	S	+	+	+	+	+	+	+	+	+	-	+	Sensitive
8	blood	M	+	+	+	+	+	+	+	+	+	+	+	Sensitive
9	resp. tract*	M	+	+	+	+	+	+	+	+	+	+	-	Sensitive
10	resp. tract	M	+	+	+	+	+	+	+	+	+	+	+	LDR
11	resp. tract*	S	+	+	+	+	+	-	+	+	+	+	-	Sensitive
12	resp. tract*	M	+	+	+	+	+	+	+	+	+	+	+	LDR
14	resp. tract*	S	-	-	-	-	-	+	+	-	+	+	-	Sensitive
15	resp. tract*	S	+	+	+	+	-	+	+	+	+	+	+	MDR
16	blood	S	+	+	+	+	+	+	+	+	+	+	+	Sensitive
17	urine	S	+	+	+	+	+	+	+	+	+	+	+	MDR
18	resp. tract*	M	+	+	+	+	+	+	+	+	+	+	+	Sensitive
19	resp. tract*	W	+	+	+	+	+	+	+	+	+	+	+	Sensitive
20	resp. tract*	S	+	+	+	-	+	+	+	-	+	+	+	MDR
21	resp. tract*	S	+	+	+	+	+	+	+	+	+	+	+	MDR
23	resp. tract	W	+	+	+	+	+	-	+	-	+	+	+	Sensitive
24	resp. tract*	M	+	+	+	+	+	+	+	+	+	+	+	Sensitive
25	resp. tract	W	+	+	+	+	+	+	+	+	+	-	-	LDR
28	resp. tract*	S	+	+	+	+	+	-	+	+	+	+	+	MDR
29	resp. tract*	S	+	+	+	+	+	+	+	+	+	+	-	LDR
30	wound	S	+	+	+	+	+	+	+	+	+	+	+	Sensitive
31	resp. tract*	W	+	+	+	+	+	+	+	+	+	+	-	Sensitive
32	resp. tract*	M	+	+	+	+	+	+	+	+	+	+	+	LDR
33	urine	S	-	-	-	-	+	+	+	+	+	+	+	MDR
34	urine	S	-	+	-	+	+	+	+	+	+	+	+	LDR
35	wound	M	+	-	-	+	+	+	+	+	+	+	+	Sensitive
36	resp. tract	S	+	-	+	+	+	+	+	+	+	+	+	LDR
37	blood	S	+	-	+	+	-	+	+	+	+	+	+	Sensitive
38	urine	S	+	+	+	+	+	+	+	+	+	+	-	MDR
39	resp. tract*	S	+	+	-	+	+	+	+	+	+	+	+	MDR
40	urine	S	+	+	+	+	+	+	+	+	+	+	-	LDR
41	resp. tract*	S	+	+	+	+	+	+	+	+	+	+	+	LDR
42	wound	S	+	+	+	+	+	+	+	+	+	+	-	LDR
43	resp. tract*	S	+	+	+	+	+	+	+	+	+	+	+	MDR
44	urine	S	+	+	+	+	+	+	+	+	+	+	+	LDR
45	wound	S	+	+	+	+	+	+	+	+	+	+	+	LDR
46	wound	S	+	+	-	+	+	+	+	+	+	+	+	LDR
48	resp. tract*	W	+	+	-	+	+	+	+	+	+	+	+	LDR
49	wound	S	+	+	-	+	+	-	+	+	+	+	-	LDR
50	resp. tract	S	+	+	+	+	+	+	+	+	+	+	+	LDR
51	resp. tract*	S	+	+	+	+	+	+	+	+	+	+	+	LDR
52	resp. tract	S	+	+	+	+	+	+	+	+	+	+	+	LDR
53	resp. tract*	S	+	+	+	+	-	+	+	+	+	+	+	LDR
54	resp. tract*	S	+	+	+	+	+	+	+	+	+	+	+	LDR

Table 2. Qualitative identification of genes taking part in e *Quorum Sensing* (QS) and genes encoding virulence factors, biofilm formation rate, and phenotype resistance in *Pseudomonas aeruginosa* strains isolated from different clinical infection (continuation)

Specimens		Biofilm	QS genes						Virulence genes				Resistance phentype
			lasI	*lasR*	*rhlI*	*rhlR*	*ambD*	*ambE*	*pqsA*	*pqsB*	*lasB*	*aprE*	*prpL*	
55	resp. tract*	S	-	-	+	-	-	-	+	+	-	+	+	LDR
56	resp. tract*	S	+	+	+	+	+	+	+	+	+	+	-	LDR
57	resp. tract*	M	+	+	+	+	+	-	+	+	+	+	+	LDR
58	resp. tract*	S	-	-	-	-	-	+	+	+	-	-	-	MDR
59	resp. tract*	S	+	+	+	+	+	+	+	+	+	+	+	LDR
60	resp. tract*	S	+	+	+	+	+	+	+	+	+	+	+	LDR
61	resp. tract*	S	+	+	+	+	+	+	+	+	+	-	-	LDR
63	resp. tract*	S	+	+	+	+	+	+	+	+	-	-	-	LDR
64	resp. tract*	S	+	+	+	+	+	-	+	+	-	-	+	LDR
65	resp. tract*	S	+	+	+	+	+	+	+	+	+	+	+	LDR
66	resp. tract*	S	+	+	+	+	+	-	+	+	+	+	+	LDR
67	wound	W	+	+	+	+	-	-	+	+	+	+	-	LDR
68	resp. tract	S	+	+	+	+	+	-	+	+	+	+	+	LDR
69	resp. tract*	M	+	+	+	+	+	-	+	+	+	+	+	LDR
70	resp. tract*	S	+	+	+	+	+	-	+	+	+	+	+	LDR
72	wound	S	-	-	-	-	-	-	+	+	-	-	+	LDR
76	resp. tract*	S	+	+	+	+	+	+	+	+	+	+	-	LDR
77	resp. tract*	S	+	+	+	+	+	+	+	+	+	+	+	LDR
78	resp. tract*	S	+	+	+	+	+	+	+	+	+	-	-	LDR
79	resp. tract*	S	+	+	+	+	+	+	+	+	+	+	+	LDR
80	resp. tract*	S	+	+	+	+	+	+	+	+	+	+	-	MDR
81	resp. tract*	M	+	+	+	+	+	+	+	+	+	+	+	LDR
82	resp. tract*	S	+	+	+	+	+	+	+	+	+	+	+	LDR
83	resp. tract*	S	+	+	-	+	+	+	+	+	+	+	+	Sensitive

* patients with cystic fibrosis.
S – strong; M – moderate; W – weak; CAZ – ceftazidime; CIP – ciprofloxacin; PRL – piperacillin; IPM – imipenem; MEM – meropenem; TOB – tobramicin; MBL – metallo-β-lactamases; KPC – Klebsiella pneumoniae carbapenemase: Sensitive (sensitivity to all antibiotics tested); MDR – multidrug-resistant (non-susceptible to ≥ 3 categories); LDR – low-level resistant (< 3 antimicrobial categories).

were moderate and 9.4% weak biofilm producers. Similarly, bacterial strains isolated from wounds were dominated by strong biofilm producers (63.6%), followed by strains with a moderate (18.2%) and weak (18.2%) biofilm-forming capacity. All the strains of *P. aeruginosa* isolated from urine (6 in total) exhibited a strong biofilm-forming ability. Two strains isolated from blood showed a strong biofilm-forming ability, and one exhibited weak biofilm forming potential. The observed differences were not found to be statistically significant (p=0.6331) (Tab. 3).

Analysis of the prevalence of different resistance phenotypes among the strains with varying biofilm-forming ability revealed that all strains with the resistance phenotype MDR belonged to the group of strong biofilm producers. They accounted for 22.2% of strains with strong biofilm-forming capacity. Furthermore, strains with the resistance phenotype LDR represented 63.0%, and strains showing sensitivity to all antibiotics – 14.8%. Regarding the group of moderate biofilm producers, 58.3% of strains represented the resistance phenotype LDR, while 41.7% were sensitive to all antibiotics studied. Among the weak biofilm producers, 42.9% of strains had the LDR phenotype, while 57.1% of strains were susceptible to all antibiotics. The results were found to be statistically significant (p=0.02151) (Tab. 3). No statistical relationship was confirmed for the remaining features (Tab. 3).

DISCUSSION

Antibiotics dedicated to fighting infections caused by *P. aeruginosa* lose their therapeutic efficacy because of the growth of multi-drug resistance in *P. aeruginosa* strains [2, 23, 24, 25]. The presented study found a high degree of resistance to carbapenem antibiotics. 41.0% of the studied strains were resistant to imipenem, and 61.6% to meropenem. Other authors have also reported high levels of resistance to carbapenems.

According to studies conducted by the International Nosocomial Infection Control Consortium (INICC) in a total of 36 countries (Latin America, Asia, Africa, and Europe), 47.2% of *P. aeruginosa* strains isolated from infections were imipenem resistant [24]. Studies conducted on strains isolated from patients hospitalized with pneumonia in the United States and Europe showed that 23.7% and 34.2% of the evaluated strains were resistant to meropenem, respectively. In Brazil, the Surveillance and Control of Pathogens of Epidemiological Importance found that 35.8%, 36.8%, 36.6% of *P. aeruginosa* strains were resistant to meropenem, imipenem, and ceftazidime, respectively [26]. Studies conducted in Poland on *P. aeruginosa* isolates obtained from patients with cystic fibrosis showed 72.2% of mucoid strains, and 56.2% of non-mucoid strains were meropenem resistant [27].

Table 3. Numbers of *Pseudomonas aeruginosa* strains according to analyzed features. Pearson's Chi-square test was performed to determine the dependence between variables

A) Biofilm formation and drug resistance phenotype			
Biofilm/Resistance phenotype	MDR	LDR	Sensitive
S	12	34	8
M	0	7	5
W	0	3	4
p-value		0.02151	
B) Source of samples and biofilm formation			
Source/Biofim	S	M	W
blood	2	1	0
resp. tract	6	1	2
resp. tract*	33	8	3
urine	6	0	0
wound	7	2	2
p-value		0.6331	
C) No. of virulence genes and drug resistance phenotype			
No. of virulence genes/Resistance phenotype	MDR	LDR	Sensitive
0	1	1	0
1	0	5	0
2	3	8	6
3	8	30	11
p-value		0.3447	
D) No. of QS genes and drug resistance phenotype			
No. of QS genes/Resistance phenotype	MDR	LDR	Sensitive
0	0	0	0
1	0	0	0
2	0	1	0
3	1	1	0
4	1	0	0
5	0	5	0
6	2	4	3
7	3	10	3
8	5	27	10
p-value		0.5821	
E) No. of virulence genes and biofilm formation			
No. of virulence genes/Biofilm	S	M	W
0	2	0	0
1	4	0	1
2	13	1	3
3	35	11	3
p-value	0.4047		
F) No. of QS genes and biofilm formation			
No. of QS genes/Biofilm	S	M	W
0	0	0	0
1	0	0	0
2	2	0	0
3	2	0	0
4	1	0	0
5	1	0	0
6	6	1	2
7	12	2	2
8	30	9	3
p-value		0.956	

* patients with cystic fibrosis.
S – strong, M – moderate, W – weak, Sensitive (sensitivity to all antibiotics tested), MDR – multidrug-resistant (non-susceptible to ≥ 3 categories), LDR – low-level resistant (< 3 antimicrobial categories)

The prevalence of carbapenem resistance among Gram-negative bacilli is increasing worldwide. The acquisition of MBL-encoding genes is considered to be the most important mechanism underlying carbapenem resistance [28]. Among the strains evaluated in the present study, 23.3% had the capacity to produce these enzymes. Brazilian researchers showed that 73.53% of *P. aeruginosa* strains isolated from a variety of infections were MBL-positive [29]. On account of the coexistence of MBL-encoding genes with other resistance genes, MBL-producing strains usually have a complex MDR phenotype. The occurrence of MBL-producing *P. aeruginos*a strains in a hospital setting has been reported and confirmed worldwide by several authors [25, 30, 31].

Both multiple resistance to antibiotics and biofilm formation may lead to difficulties in the treatment of infections where *P. aeruginosa* is the etiological factor. The present study showed that all *P. aeruginosa* strains isolated from clinical specimens were capable of biofilm formation under *in vitro* conditions. However, they differed in the degree of biofilm formation. Most strains showed a strong biofilm-forming capacity (74.0%), while significantly fewer strains exhibited moderate (16.4%) and weak (9.6%) biofilm-forming potential. Similar findings were reported by other authors [32, 33, 34]. Studies conducted in 2018 by J. Lima *et al.* [33] to assess the biofilm-forming ability showed that 77.5% of clinical strains of *P. aeruginosa* were capable of producing biofilm. Da Silva Carvalho *et al.* [34] found that 86.5% of *P. aeruginosa* strains studied were classified as strong or moderate biofilm producers.

P. aeruginosa has 4 *QS* systems *(las, rhl, pqs,* and *iqs)*, which are interconnected and organized in a hierarchical manner [35]. Because the systems are interlinked, the absence of any one component does not affect the *QS* expression ability of cells [9]. Some *QS*-deficient clinical strains were found to be capable of causing infection in humans [14]. The design of this study involved the identification of *QS* genes as well as virulence-determining genes, the expression of which is regulated by the *QS* system.

The present study shows that 57.5% of the strains had all the studied genes linked to the *QS* system. Karatuna and Yagci [36] observed similar results. Their studies of *P. aeruginosa* strains isolated from respiratory tract infections showed that 68.7% of isolates had all 4 genes, while 18.7% of strains did not have any of the studied genes. In their analysis of *P. aeruginosa* strains isolated from different clinical specimens, Kadhim and Ali [37] found that 81.6% of the isolates had at least one of the studied genes. The results obtained by Perez-Iberreche [38] for strains isolated from respiratory infections also show that 90.1% of the strains had all the genes studied. Only one isolate derived from cystic fibrosis patients, and one from non-cystic fibrosis patients did not have any of the analyzed genes involved in the *QS* [39]. Different findings were reported by Sabharwal *et al.* [9] for strains isolated from urinary tract infections. Of the strains studied, only 33.3% of the isolates had all the genes under analysis.

A qualitative analysis of genes conditioning the production of virulence factors in *P. aeruginosa* cells showed that the gene for elastase was present in the majority of strains studied (93.1%). Comparable results were obtained by Senturk S. et al. [14] investigating strains isolated from urinary tract infections. The gene responsible for the synthesis of elastase was identified in 86.59% of all *P. aeruginosa* strains [12]. A slightly lower percentage (75%) of isolates possessing the

gene encoding elastase synthesis was reported by Sabharwal et al. [9] in their analysis of strains originating from urinary tract infections.

The gene determining the production of alkaline protease was present in 87.7% of *P. aeruginosa* isolates evaluated in the current study. Similar results were reported by Mittal R. *et al.* [39], who identified the alkaline protease gene in 50% of strains isolated from urinary tract infections. The strains showed the capacity to colonize kidney tissues [40]. In contrast, a study conducted by Sabharwal et al. [9] on *P. aeruginosa* strains isolated from urinary tract infections, showed the presence of alkaline protease in just 16.6% of the strains. The authors hypothesized that alkaline protease might not play an important role in the pathogenesis of urinary tract infections. The lowest proportion of bacterial strains analyzed in the study (74.0%) had the gene encoding the production of endopeptidase IV. Similar results were obtained by researchers determining the presence of the gene in strains isolated from lower leg ulcers. The percentage of isolates having the gene was found to be 61.5%. Kadhim and Ali [37] recorded slightly different results. They showed that 46.6% of *P. aeruginosa* strains isolated from various clinical infections had the gene responsible for endopeptidase IV production.

CONCLUSIONS

Based on the correlation of study findings, it was observed that the biofilm-forming ability was significantly higher among strains with the resistance phenotype MDR. It was also found that among the MBL-producing strains, there was only one weak biofilm producer, while the remaining strains exhibited a strong biofilm-forming ability. Similar findings have been reported by other authors [40, 16] who observed a correlation between the MDR phenotype and the ability of *P. aeruginosa* strains to form a biofilm.

Resistance to antimicrobial agents and the ability to grow as a biofilm are the main problems in the treatment of infections triggered by *P. aeruginosa*. The high degree of this resistance, and growth in the biofilm form, as well as the presence of various virulence factors, are the reasons for difficulties in managing infections caused by *P. aeruginosa*. The ability of *P. aeruginosa* to grow as a biofilm is believed to explain the weak relationship between antibiotic sensitivity under *in vitro* conditions and clinical response. A better understanding of the genes and mechanisms involved in biofilm formation by *P. aeruginosa* strains, as well as gaining insights into its structure, can assist in the development of new therapies to eliminate biofilm formation.

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