Cent. Eur. J. Med. • 9(2) • 2014 • 187-192
DOI: 10.2478/s11536-013-0241-y

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Central European Journal of **Medicine**

Biofilm formation and serum susceptibility in Pseudomonas aeruginosa

Research Article

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Received 14 January 2013; Accepted 22 May 2013

Abstract: *Pseudomonas aeruginosa* (*P. aeruginosa*) is one of the most important opportunistic pathogens. The pathogenicity of *P. aeruginosa* has been associated with multiple bacterial virulence factors. The aim of this study was to evaluate the association between *P. aeruginosa* strains obtained from various clinical samples and resistance to antibiotics and pathogenicity factors, such as resistance to serum bactericidal activity and biofilm formation. This study included 121 *P. aeruginosa* strains isolated from clinical samples; 65 of the isolated *P. aeruginosa* strains were carbapenem-resistant, and 56 were carbapenem-sensitive. Carbapenem-resistant *P. aeruginosa* strains were more often resistant to the majority of tested antibiotics, compared to carbapenem-sensitive strains. We did not find any statistically significant difference between resistance to carbapenems and serum resistance and ability of tested P. aeruginosa strains to produce biofilms. Carbapenem-resistant *P. aeruginosa* strains were recovered from the urinary tract significantly more often (75.0%) than carbapenem-sensitive *P. aeruginosa* strains (25.0%). Carbapenem-sensitive *P. aeruginosa* strains were recovered significantly more often from the respiratory tract than carbapenem-resistant strains, 60.0% and 40.0%, respectively. All the *P. aeruginosa* strains recovered from blood were serum-resistant. *P. aeruginosa* strains recovered from the respiratory tract and wounds were significantly frequently serum sensitive, 95.6% and 56.6%, respectively. We did not find any differences in biofilm production among the *P. aeruginosa* strains recovered from different sources.

Keywords: *P. aeruginosa* • *Antibiotic susceptibility* • *Biofilm* • *Carbapenem-resistance* • *Serum-bactericidal activity*

1. Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is one of the most important opportunistic pathogens causing a variety of severe acute and chronic infections in hospitalized, immunocompromised hosts [1]. These gram-negative, non-fermenting bacteria continue to be a major cause of nosocomial infections, predominantly pneumonia and infections of the urinary tract, skin and soft tissue. Furthermore, they are the most prevalent pathogens isolated from patients with chronic lung infections, including cystic fibrosis, with high rates of associated morbidity and mortality [2-4].

The predisposition of *P. aeruginosa* to development of resistance to antibiotics and expression of multiple virulence factors contributes to the frequent ineffectiveness of current therapies. The pathogenicity of *P. aeruginosa* has been associated with multiple bacterial virulence factors, including biofilm formation and the expression of adhesions, endotoxin and hydrolytic exotoxins, which cause tissue destruction. The resistance to serum bactericidal effect is one of the major virulence factors of *P. aeruginosa* [5,6]. The host innate immune system includes serum components, such as antibodies and proteins of the complement system that mediate the bactericidal effect of serum. This phenomenon is seen with a higher frequency of serum resistance among *P. aeruginosa* strains isolated from blood, wounds, urine [7,8] than among strains isolated from the sputum of asymptomatic patients with cystic fibrosis [9-11]. Serum resistance might be an important microbial phenotype, which could conceivably differentiate between invasive and non-invasive strains and isolates [12].

Therapy is complicated by the organism's potent ability for adaptation, mutation, and gene acquisition [13]. This diversity of *P. aeruginosa* infections is due

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to the development of various adaptive mechanisms such as the nutritional and metabolic pathways, besides the regulation of gene expression.

P. aeruginosa can form bacterial biofilm that protects the organism from defenses and antimicrobial therapy [14]. *P. aeruginosa* biofilm is difficult to eradicate, and it causes bacterial persistence, leading to infection chronicity and morbidity [15].

In addition, its ability to form biofilm provides greater protection against host immune defense systems and susceptibility to various antimicrobial agents [16,17]. P. aeruginosa is a multidrug resistant (MDR) organism and is considered a phenomenon of bacterial resistance. This is demonstrated by different types of antibiotic resistance. It is also commonly believed that in MDR *P. aeruginosa* isolates, reduced virulence may result due to decreased biofilm. However, recent data suggest otherwise, and MDR *P. aeruginosa* may remain fully pathogenic [18].

The aim of this study was to evaluate the association between the resistance of *P. aeruginosa* strains obtained from various clinical samples and antibiotics and pathogenicity factors such as resistance to serum bactericidal activity and biofilm formation.

2. Materials and methods

2.1 Bacterial strains and susceptibility testing

A total 121 strains of *P. aeruginosa* were included in this study. All these strains were isolated from clinical samples of patients treated in the Hospital of Lithuanian University of Health Sciences during the period 1 January 2011 to 31 June 2012. Sixty-five (53.7%) strains showing resistance to meropenem and/or imipenem by routine disk diffusion method and 56 (46.3%) strains sensitive to meropenem and imipenem were included in this study. Only one strain per patient was included.

The susceptibility testing to meropenem, imipenem, piperacillin, ceftazidime, ciprofloxacxin, gentamicin, and amikacin was performed by the E-test method according to the recommendations of the manufacturer (Liofilchelm, Italy). Detected minimal inhibitory concentrations were evaluated according to The European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints [19]. Minimum inhibitory concentration (MIC) values, which were detected between the sensitive and resistant breakpoint, were interpreted as sensitive. The susceptibility testing to piperacillin/tazobactam, cefepime, cefoperazone/sulbactam, tobramycin, aztreonam was performed by disk diffusion method (BBL, USA), and zones were interpreted according to EUCAST recommendations.

All *P. aeruginosa* strains according to interpreted MIC results were divided into carbapenem-sensitive and carbapenem-resistant groups. The strains resistant to meropenem and imipenem were attributed to the carbapenem-resistant group.

2.2 Serum bactericidal assay

The ability of *P. aeruginosa* strains to resist serum bactericidal effect was tested as described earlier in the literature [20,21].

Bacterial ability to stay viable under human serum effect was evaluated after 1 hour, 2 hours and 3 hours and graduated to 6 levels. *P. aeruginosa* strains assigned to 1-4 levels were interpreted as serum sensitive and at 5-6 levels as serum resistant. Every strain of P. aeruginosa strain was tested 3 times. A strain was considered sensitive or resistant if the detected level was the same in all experiments.

2.3 Biofilm formation

The tube method, described by Christensen, was used to detect biofilm formation [22]. One to two overnight growth colonies of tested microorganisms were inoculated with 10 ml Trypticase Soy Broth (EMAPOL, Poland). The tubes were incubated at 37°C for 24 h. After incubation, tubes were washed with phosphate buffer saline and dried, then stained with crystal violet (0.1%) for 20 min. The excess stain was washed with deionized water. Tubes were dried in an inverted position at room temperature. Biofilm formation was considered positive when a visible film lined the bottom of the tube. The strains were grouped as non-biofilm producers (no visible film line), moderate-biofilm producers (medium intense film line), and high-biofilm producers (intense film line).

2.4 Statistical analysis

Proportions were compared with nonparametric statistical criterion chi-square or Fisher's exact test. Differences between groups were considered significant if P was <0.05. Statistical package IBM SPSS Statistics Version 20 was used for the data analysis.

3. Results

In our study P.aeruginosa strains (n=121) were divided into two groups. The first group consisted of 65 carbapenem-resistant strains (resistant to imipenem and meropenem), and the second group consisted of 56 *P. aeruginosa* carbapenem-sensitive strains.

Carbapenem-resistant *P. aeruginosa* strains were more often resistant to the majority of tested antibiot-

ics, except cefepime and aztreonam, compared to carbapenem-sensitive strains, and the difference between groups was statistically significant (Table 1).

Table 1. Resistance of Carbapenem-Resistant and Carbapenem-Sensitive *Pseudomonas aeruginosa* strains to Various Antibiotics.

Antimicrobial Agent	Carbapenem-Resistant Strains, n (%)	Carbapenem-Sensitive Strains, n (%)	χ^2	*P*
Piperacillin	46/65 (70.8)	16/56 (28.6)	21,44	<0.001
Piperacillin/ tazobactam	16/38 (42.1)	7/43 (16.3)	6,62	0.01
Ceftazidime	35/65 (53.8)	14/56 (25.0)	10,39	0.001
Cefepime	11/38 (28.9)	7/42 (16.7)	1,73	0.189
Cefoperazone/ sulbactam	19/38 (50.0)	11/43 (25.6)	5,16	0.023
Ciprofloxacin	57/65 (87.7)	12/56 (21.4)	53,90	<0.001
Gentamicin	51/65 (78.5)	10/56 (17.9)	44,20	<0.001
Amikacin	25/65 (38.5)	3/56 (5.4)	18,54	<0.001
Tobromycin	21/37 (56.8)	4/41 (9.8)	19,73	<0.001
Aztreonam	9/59 (15.3)	4/51 (7.8)	1,44	0.23

All 121 *P. aeruginosa* strains were tested for human serum bactericidal effect; 85 (70.2%) of *P. aeruginosa* strains were found to be sensitive to serum and 36 (29.8%) were resistant.

The resistance of *P. aeruginosa* serum-sensitive and serum-resistant strains to various tested antimicrobial agents is shown in Table 2. We did not find any statistically significant difference between resistance to carbapenems and serum resistance of tested *P. aeruginosa* strains.

Table 2. Resistance of Serum Sensitive and Serum-Resistant *Pseudomonas aeruginosa* strains to Various Antibiotics.

Antimicrobial agent	*Pseudomonas aeruginosa* strains		χ^2	*P*
	Serum sensitive (grades 1-4) N=85 n (%)	Serum resistant (grades 5-6) N=36 n (%)		
Carbapenems	48 (56.5)	17 (47.2)	0.87	0.351
Piperacillin	41 (48.2)	21 (58.3)	1.03	0.310
Ceftazidime	32 (37.6)	17 (47.2)	0.96	0.327
Ciprofloxacin	50 (58.8)	19 (52.8)	0.38	0.539
Aminoglycosides	45 (52.9)	17 (47.2)	0.33	0.565

Resistance to serum of *P. aeruginosa* strains had no statistically significant correlation with resistance to tested antibiotics.

All 121 *P. aeruginosa* strains were tested for formation of biofilm; 52 (43.0%), 30 (24.8%), and 39 (32.2%) of the strains were found to be no, moderate and high biofilm producers, respectively. The resistance of non-biofilm producers and moderate and high biofilm producers to various antimicrobial agents is shown in Table 3. We did not find any statistically significant differences between carbapenem-resistant and carbapenem-sensitive *P. aeruginosa* groups and their variant ability to produce biofilm.

Table 3. Resistance of *Pseudomonas aeruginosa* Strains with Different Biofilms Production Level to Various Antibiotics.

Antimicrobial agent	*Pseudomonas aeruginosa* strains			*P*
	non-biofilm producer N=52 n (%)	moderate biofilm producer N=30 n (%)	high biofilm producer N=39 n (%)	
Carbapenems	33 (63.5)	15 (50.0)	17 (43.6)	0.152
Piperacillin	31 (59.6)	15 (50.0)	16 (41.0)	0.212
Ceftazidime	28 (53.8)	18 (60.0)	16 (41.0)	0.260
Ciprofloxacin	32 (61.5)	18 (60.0)	19 (48.7)	0.441
Aminoglycosides	19 (36.5)	12 (40.0)	18 (46.2)	0.651

Biofilm formation of *P. aeruginosa* strains also had no statistically significant correlation with resistance to any tested antibiotics.

In our study, 53 out of 121 (43.8%) *P. aeruginosa* strains were recovered from infections of wounds, 45/121 (37.2%) from the respiratory tract, 20/121 (16.5%) from the urinary tract, and 3/121 (2.5%) from blood.

The carbapenem-resistant *P. aeruginosa* strains were recovered from the urinary tract significantly more often than carbapenem-sensitive *P. aeruginosa* strains, 75.0% and 25.0%, respectively, p=0.037. Carbapenem-sensitive *P. aeruginosa* strains were recovered from the respiratory tract significantly more frequently than carbapenem-resistant strains, 60.0% and 40.0%, respectively (Table 4).

Table 4. Proportion of Pseudomonas aeruginosa Strains Recovered From Various Sources in Relation to Carbapenem Resistance.

Source	Carbapenem-Resistant Strains, n (%)	Carbapenem-Sensitive Strains, n (%)	χ^2	*P*
Urinary tract (N=20)	15 (75.0)	5 (25.0)	4.37	0.037
Wounds (N=53)	31 (58.5)	22 (41.5)	0.86	0.353
Blood (N=3)	1 (33.3)	2 (66.7)	–#	0.596
Respiratory tract (N=45)	18 (40.0)	27 (60.0)	5.42	0.020

–# Fisher exact test was employed for small sample size.

The *P. aeruginosa* strains recovered from different sources had different serum resistance (Table 5). All the strains recovered from blood were serum resistant. *P. aeruginosa* strains recovered from respiratory tract and wounds were significantly frequently serum sensitive, 95.6% (n=45) and 56.6%, (n=53), respectively.

Table 5. Serum-Resistance of *Pseudomonas aeruginosa* Strains Recovered From Various Sources.

Source	% of tested *Pseudomonas aeruginosa* strains (n)		χ^2	*P*
	Serum sensitive (grades 1-4)	Serum resistant (grades 5-6)		
Urinary tract (N=20)	60.0 (12)	40.0 (8)	1.20	0.273
Wounds (N=53)	56.6 (30)	43.4 (23)	8.40	0.004
Blood (N=3)	0	100.0 (3)	–#	0.025
Respiratory tract (N=45)	95.6 (43)	4.4 (2)	21.96	<0.001

–# Fisher exact test was employed for small sample size.

We did not find any differences in biofilm production among the *P. aeruginosa* strains recovered from different sources (Table 6).

Table 6. Biofilm Production of *Pseudomonas aeruginosa* Strains Recovered From Various Sources.

Source	% of tested *Pseudomonas aeruginosa* strains (n)			
	non-biofilm producer	moderate biofilm producer	high biofilm producer	*P*
Urinary tract (N=20)	45.0 (9)	25.0 (5)	30.0 (6)	0.970
Wounds (N=53)	41.5 (22)	22.6 (12)	35.8 (19)	0.739
Blood (N=3)	66.7 (2)	33.3 (1)	0	0.476
Respiratory tract (N=45)	42.2 (19)	26.7 (12)	31.1 (14)	0.933

4. Discussion

Many studies are focused on clinical significance of antibiotic - resistance bacteria. Virulent organisms are able to produce clinical symptoms of infection in human and animal hosts and should therefore be exposed more frequently to antimicrobial drugs, and the risk of resistance is expected to be higher [23]. In the study by Drahovska et al., enterococci isolated from human infections and from the traditional Slovak sheep cheese, bryndza, were compared, and a higher level of resistance was found in clinical than in food strains; differences were found in the distribution of virulence-associated cylA gene, as well [24]. The relationship between antibiotic resistance and virulence factors in urinary enterococcus isolates were found in the study by Baylan et al.: hyaluronidase asa1 gene positive *Enterococcus faecalis* (*E. faecalis*) isolates were more resistant to ciprofloxacin, norfloxacin and levofloxacin; esp gene positive *E. faecalis* isolates were more resistant to doxycycline; and hyl gene positive *E. faecalis* isolates were more resistant to nitrofurantoin than these gene negative isolates [25].

Bacterial strains that have acquired resistance to one antibiotic can develop resistance to other classes of antibiotics. Our results showed that *P. aeruginosa* clinical isolates resistant to carbapenems were more resistant to piperacillin, piperacillin/tazobactam, ceftazidime, cefoperazone/sulbactam, ciprofloxacin and aminoglycosides. The data of Lagatolla et al. [26], similar to those in other studies [27,28], demonstrate that most of the blaVIM positive isolates of *P. aeruginosa* exhibited a multidrug-resistant phenotype, including imipenem, meropenem, ceftazidime, piperacillin, aztreonam, amikacin, gentamicin, tobramycin and ciprofloxacin, except polymixin B. Antibiotic resistance alone cannot explain the virulence of bacteria. A study by Doina et al., concluded that strategies could be developed to target virulence factors of pathogens instead of whole bacteria, such as the development of drugs that target the plasmids containing resistance genes or drugs that target the adhesion of virulent bacteria to tissue [29]. It is very important to detect associations between bacterial pathogenic factors and antibiotic resistance. Many researchers have reported that bacterial biofilm is associated with resistance to a wide range of antimicrobial agents [30]. However, we found one study by Hostacka et al. that did not confirm these findings; it showed the same percentage of production of biofilm in the strains sensitive to ciplofloxacin and aminyglycosides compared with the resistant one [31]. Serum sensitivity/resistance might be an important microbial phenotype, which could conceivably differentiate between invasive and non-invasive strains [12]. Approximately 1/3 of our tested single clinical isolates were resistant to serum bactericidal effect and were high biofilm producers. In our study relationship was not found with resistance to serum, biofilm formations and resistance to carbapenems and other classes of tested antibiotics. A single isolates being sensitive in vitro to antibiotics may run into resistance in case of the bacteria manage to become a productive member of a biofilm producing community. This could explain why no association was found between in vitro sensitivity to antibiotics and biofilm formation. Unfortunately, we did not investigate genes that are responsible for resistance to carbapenems. Further studies are needed to assess the importance of other pathogenicity factors of *P. aeruginosa*. However, Hostacka et al., showed that the resistance to antibiotics has not always been associated with changes in the production of the pathogenicity factors such as motility, biofilm N-acylhomoserine lactone signal molecules production and response to oxidative stress.

5. Conclusion

In our study, the source of *P. aeruginosa* infection was related to carbapenem-resistance. Carbapenem-sensitive strains were isolated most frequently from the respiratory tract, and carbapenem-resistant strains were isolated from the urinary tract. We observed an association between the source of recovery of strains and their resistance to serum bactericidal effect. All *P. aeruginosa* strains isolated from blood were serum-resistant. No correlation was observed between biofilm formation

Acknowledgments

This research was funded by a grant (No. MIP 047/2012) from the Research Council of Lithuania.

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