



DOI: 10.1515/rrlm-2017-0027

# Comparison of virulence factors and antibiotic resistance of *Pseudomonas aeruginosa* strains isolated from patients with and without cystic fibrosis

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## Abstract

Its rising incidence, virulence factors and antibiotic resistance rate makes it difficult to treat *Pseudomonas aeruginosa* infections. The aim of this study was to compare virulence factors and antibiotic resistance of *P. aeruginosa* isolates from cystic fibrosis (CF) and other lower respiratory tract infections.

Isolates from patients (n=125) were divided into two groups. The isolates in the first group were from CF patients (n=64). And in the other group isolates were from lower respiratory tract samples, from patients that did not have CF (n=61). The antibiotic susceptibility tests were done by using disc diffusion method. As phenotypic tests; DNase, protease, elastase, hemolysis, and motility test were performed.

The mucoid form of *P. aeruginosa* was detected in 29.7% of CF patients' isolates, whereas in the other group (non-CF) this rate was 9.8% (p=0.011). Motility in the CF patients' isolates was lower (84.4%) than the other group (96.7%). The presence of DNase was significantly low in CF patients' isolates when compared to the other group (p=0.009). When the antibiotic resistance was compared; ceftazidime, imipenem and meropenem and piperacillin resistance was found significantly low in CF patients compared to isolates from the other group (p≤0.05).

Information about virulence factor patterns and antibiotic resistance of *P. aeruginosa* isolates from patients with cystic fibrosis and the patients without cystic fibrosis can prevent the unnecessary usage of antibiotics and lead the way to new approaches in treatment.

**Keywords:** Antibiotic susceptibility, Cystic fibrosis, *Pseudomonas aeruginosa*, virulence

Received: 22<sup>nd</sup> March 2017; Accepted: 20<sup>th</sup> July 2017; Published: 18<sup>th</sup> August 2017

## Introduction

*Pseudomonas aeruginosa* which is an opportunistic Gram-negative bacillus, can easily be isolated from the environmental sources and

even antiseptic solutions due to its hydrophilic nature while it rarely colonizes healthy people (1). *P. aeruginosa* regulates its pathogenicity through a series of virulence factors such as adhesins, pyocyanin, elastase, proteases, hemoly-

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sins, exotoxin and exoenzyme S (2). *P. aeruginosa* exhibit high adaptation ability to enormously different settings owing to its varying potential of virulence factors plus its intrinsic resistance to several antibiotics. Inducible chromosomal AmpC beta - lactamases and efflux pumps systems are among the most frequent mechanisms of intrinsic resistance in *P. aeruginosa* isolates (3,4). The treatment of *P. aeruginosa* infections is challenging since acquired resistance can develop against broad-spectrum penicillins, extended-spectrum cephalosporins, monobactams, aminoglycosides and colistin which are applied as treatment alternatives.

*P. aeruginosa* is the most frequently isolated pathogen from the respiratory tract of patients with cystic fibrosis (CF) (5). Colonization and infection of the respiratory tract with *P. aeruginosa* is persistent in the CF setting. During the chronic colonization/infection process *P. aeruginosa* undergoes some genotypic and phenotypic changes such as mucoid colony formation, loss of motility and hyperexpression of its virulence factor or resistance genes (6). These alterations enable the bacteria to adapt to the stressful environment in CF airways.

It is well-known that *P. aeruginosa* is also a frequent cause of acute nosocomial infections such as bloodstream infections, urinary tract infections, pneumonia or wound infections. Regulation and expression of virulence factors of bacteria are different in acute and chronic infection settings (7). This study was aimed to compare the virulence factors and antibiotic susceptibilities of *P. aeruginosa* strains that isolated from patients with and without CF.

## Materials and methods

### *Pseudomonas aeruginosa* isolates

*P. aeruginosa* isolates obtained from 64 CF patients and 61 non-CF patients with lower respiratory tract infection were included in the

study. The isolates were collected between June 2013 and December 2013 and the study was performed between January 2014 and June 2014. Only one isolate from each patient was included into the study. Oxidase positive, Gram negative non-glucose fermenting bacteria with pigment formation were presumably identified as *P. aeruginosa* and the identification was confirmed with Phoenix Automated Bacterial Identification System (Becton Dickinson, USA). *P. aeruginosa* PAO1, PAO - JP2 and PAO - JP3 were used as the control strains. PAO1 is the wild type strain, PAO-JP2 and PAO-JP3 are the quorum sensing lasI and rhlI mutants and they are negative controls for elastase and protease activity.

### Antibiotic Susceptibility Tests

The antibiotic susceptibilities of the *P. aeruginosa* strains against amikacin, gentamicin, tobramycin, ceftazidime, cefepime, imipenem, meropenem, ciprofloxacin, levofloxacin, piperacillin and colistin was determined by Kirby - Bauer disk diffusion method according to CLSI criteria (8). *P. aeruginosa* ATCC 27853 was used as the standard control strain for susceptibility testing.

### Phenotypic determination of the virulence factors

Elastase activity: Elastin Congo red (ECR) measurement method was used to investigate elastase activity (9). All clinical strains, *P. aeruginosa* PAO1, PAO - JP2 and PAO - JP3, were incubated at 37 ° C overnight. In order to determine the semi-quantitative elastase production, the centrifugation was done for the prepared suspensions of the clinical *P. aeruginosa* strains and PAO1, PAO - JP2 and PAO - JP3 strains. 1 ml of 30 mM Tris and 10 mg of elastin - Congo red (pH 7.2) were added into tubes containing 0.5 ml of the bacterial supernatants. The tubes were incubated on a shaker at 37 ° C for 24 hours, followed by centrifugation at 3000 x g for 10

minutes. From each tube 200 mL of the supernatant was removed and placed into two wells of a microtiter plate and the absorbance was measured at 495 nm with an optical reader. For each isolate, the average value of the two wells was calculated. In order to determine the cut-off value, two standard deviations were added to the average value from the study with the PAO - JP2 and PAO - JP3 strains which are negative for elastase production. The cut-off value was determined as 0.474 with 95% confidence interval and the isolates with the absorbance value  $> 0.474$  at 495 nm was evaluated as positive for elastase activity.

**Alkaline protease activity:** For the determination of alkaline protease activity, skim milk agar (SMA) was used. 20  $\mu$ l of the bacterial supernatant was placed with sterile Pasteur pipette into the wells opened in SMA. The medium was incubated at 25 ° C for 18 - 24 hours. The presence of a transparent zone around the wells with the bacteria was interpreted as positive for protease activity (10).

**Hemolysis activity:** The strains were inoculated into 5% sheep blood agar and after the incubation at 37 ° C for 24 hours, production of hemolysis was determined.

**Motility test:** The strains were stabbed into the semi-solid motility test medium with an inoculation loop. Cultures were incubated at 37 ° C for 24 - 48 hours. The extension from the stab line and production of turbidity or cloudiness throughout the medium was regarded as a positive motility test. *P. aeruginosa* ATCC 27853 strain was used as the positive control.

**DNase activity:** The bacteria grown in blood agar were inoculated by drawing a line onto DNase agar (Oxoid, UK). Plates were incubated at 37 ° C aerobically for 18 to 24 hours. Lack of clearance around bacterial colonies was considered as DNase negative. The generation of clear zones around inoculation line was considered as DNase positive.

**Mucoid colony morphology:** All clinical samples were inoculated onto Mueller Hinton agar and incubated for 24 hours at 37 ° C for the detection of mucoid colony formation.

Limitation of the study is not to perform virulence gene associated genes.

### **Statistical evaluation**

The difference between groups from categorical variables was studied using Pearson Chi - Square test, Yates adjusted Chi - square and Fisher's exact chi - square test. In the study, p-value lower than 0.05 was considered as statistically significant. All analysis were performed with the help of IBM SPSS (Statistical Package for Social Science) Statistics 21.0 software.

### **Results**

The mean age of the patients with CF was  $13.63 \pm 1.05$  years while it was  $44.57 \pm 3.21$  for non-CF patients. A total of 125 *P.aeruginosa* isolates were included in this study, 64 of them were from sputum samples of patients with CF while 61 were isolated from lower respiratory tract samples of non-CF patients (28 sputum (45.2%), five bronchoalveolar lavage (8.2%), 28 tracheal aspirate samples (45.3 %)).

While presence of elastase and protease was 84.4% and 70.3%, respectively in CF isolates, these rates were 70.5% and 62.3%, respectively, for non-CF isolates ( $p=1.0$ ,  $p=0.448$ ) The rate of motility was higher in non-CF isolates (96.7% ) than the CF isolates (84.4%) and the difference was statistically significant ( $p=0.042$ ). Mucoid colony morphology was detected in a significantly high rate of CF isolates (29.7%) than the non-CF isolates (9.8%), ( $p=0.011$ ). DNase activity was found to be statistically significantly lower for CF isolates than non-CF isolates ( $p=0.009$ ). Regarding the presence of hemolysis, no statistically significant differences were found between the two groups (Table 1).

When the change in virulence factors according to the age of CF patients examined; the presence of DNase was detected in 27.2% of patients older than 18 years and only in 9.5% of patients under 18 ages. Consistent with this result, DNase positivity was found to be 45.1% in adults in the non-CF group while there was no DNase positivity in pediatric group. No significant differences were found in other virulence factors (Table 2).

The antibiotic susceptibility data revealed that the rates of ceftazidime, imipenem, meropenem and piperacillin resistance were lower in CF isolates than the non-CF isolates ( $p \leq 0.05$ ). Colistin resistance was not determined in any of the *P. aeruginosa* strains tested (Table 3).

## Discussions

*P. aeruginosa* which exhibits a wide range of virulence factors causes a diversity of infections with high mortality and morbidity. These opportunistic pathogens are widespread microorganisms in nature. Although they do not cause disease in healthy individuals, it may lead to life-threatening infections in patients with underlying disease such as cystic fibrosis, malignancies, burns, immunosuppressive and traumatic injuries (11).

Phenotypic analysis of the isolates revealed that mucoid colony formation was 29.7% in CF isolates and this was found to be statistically significantly higher than the non-CF group (9.8%) ( $p = 0.011$ ). This finding supported the view that

**Table 1. The comparison of virulence factors of *Pseudomonas aeruginosa* isolates from patients with and without cystic fibrosis.**

Virulence factor	CF isolates (n = 64)		Non-CF isolates (n = 61)		p-value
	n	%	N	%	
Elastase	54	84.4	43	70.5	1.0
Protease	45	70.3	38	62.3	0.448
Motility	54	84.4	59	96.7	0.042
Hemolysis	47	73.4	51	83.6	0.245
DNase	10	15.6	23	37.7	0.009
Mucoid colony	19	29.7	6	9.8	0.011

**Table 2. The comparison of virulence factors of *Pseudomonas aeruginosa* isolates from pediatric patients and adult patients**

Virulence factor	CF isolates (n = 64)		p-value	Non-CF isolates (n = 61)		p-value
	<18 age (n=42)	>18 age (n=22)		<18 age (n=10)	>18 age (n=51)	
	n (%)	n (%)		n (%)	n (%)	
Elastase	36 (85.7)	18 (81.8)	0.72	8 (80)	35 (68.6)	0.70
Protease	30 (71.4)	15 (68.2)	0.78	6 (60)	32 (62.7)	1.00
Motility	36 (85.7)	18 (81.8)	0.72	9 (90)	50 (98)	0.30
Hemolysis	33 (78.6)	14 (63.6)	0.19	9 (90)	42 (82.3)	0.55
DNase	4 (9.5)	6 (27.2)	0.08	0	23 (45.1)	0.009
Mucoid colony	12 (28.5)	7 (31.8)	0.78	1 (10)	5 (9.8)	0.98

**Table 3. The comparison of antibiotic resistance rate of *Pseudomonas aeruginosa* isolates from patients with and without cystic fibrosis**

Antibiotics	CF isolates (n=64)		Non-CF iso- lates (n=61)		p-value
	(n)	%	(n)	%	
	Piperacillin	10	15.6	24	
Ceftazidime	4	6.3	11	18	0.040
Cefepime	8	12.5	13	21.3	0.180
Imipenem	11	17.2	31	50.8	0.001
Meropenem	10	15.6	27	44.3	0.001
Amikacin	19	29.7	18	29.5	0.980
Gentamicin	17	26.6	22	36.1	0.250
Tobramycin	9	14.1	13	21.3	0.280
Ciprofloxacin	13	20.3	16	26.2	0.430
Levofloxacin	18	28.1	17	27.9	0.970
Colistin	0	0	0	0	-

the emergence of mucoid colonies caused by overproduction of the polysaccharide alginate, which is widely considered to be a marker for the transition to chronic *P. aeruginosa* infection in CF. Thus the microenvironment in CF lung induced the formation of mucoid colonies. Besides mucoid colony formation loss of motility is also a common indicator of adaptation (6). In our study motility feature of isolates from patients with CF was (84.4%) less than that detected in non-CF patients (96.7%) and the difference was statistically significant ( $p=0.042$ ). This indicated that the continuous and selective pressure in the CF environment leads to some phenotypic changes in bacteria to help its adaptation to the host. Non-motile *P. aeruginosa* strains are hardly phagocytosed by macrophages and neutrophils and thus evade from the host immune system leading to chronicity.

The presence of DNase is frequent particularly in the case of severe *P. aeruginosa* infections. In our study DNase positivity for non-CF group was 37.7% whereas it was 15.6% in CF

patients ( $p=0.009$ ). Extracellular DNA in the biofilm matrix could take part in the development of bacterial communities. Since *P. aeruginosa* strains isolated from CF patients have less DNase production, *P. aeruginosa* is unable to disrupt the alginate, which supports biofilm formation. DNase, given as a treatment in CF patients, reduces the mucoid extracellular matrix, making it easier for antibiotics to reach bacterial targets (12).

The relationship between *P. aeruginosa* virulence factors and the body parts bacteria isolated was examined in various studies which tried to clarify the pathogenesis of *P. aeruginosa* infections. In their study, Ciragil et al. studied elastase, protease and alginate properties of *P. aeruginosa* isolates isolated from different parts of the body. They found that alkaline protease was detected in 52% of CF and 65% of non-CF patients (13). In our study protease positivity was 64% for the CF isolates and 80% for the non-CF isolates. This supports the view that protease activity is seen more in acute *P. aeruginosa* infections and it decreases when the infection becomes chronic.

The importance of elastase production in the pathogenesis of lung infections due to *P. aeruginosa* was demonstrated clearly and shown that the 73%-95% of *P. aeruginosa* isolates from respiratory tract samples produce this enzyme (14-15). In isolates from chronic CF patients, the production of elastase is reduced (16). In our study there was no significant difference between CF and non-CF group ( $p \geq 0.05$ ). This may be, because of the younger ages in the CF group. A study by Amitani et al. revealed that elastase produced by *P. aeruginosa* leads to delay in mucociliary clearance in patients with chronic bronchial infection (17). Also in our study, elastase positivity was found as 84% for the *P. aeruginosa* isolates from the patients with CF.

Faraji et al. compared the prevalence of three virulent genes in *P. aeruginosa* be-



tween CF patients' isolates and the burn wound isolates. They found that the number of *toxA*, *lasB* and *exoS* were significantly higher in the bacteria from the patients with CF (18). In our study we performed phenotypic tests and found high rates of elastase, protease and mucoid formation in CF patients. It is clear that virulence factors play an important role in CF infections by *P. aeruginosa*.

The CF *P. aeruginosa* isolates in this study population revealed lower beta-lactam resistance than their non-CF counterparts. In our study resistance rates of ceftazidime, imipenem, meropenem and piperacillin antibiotics were lower in CF isolates than the non-CF isolates ( $p \leq 0.05$ ). Intrinsic and acquired antibiotic resistance also aid to the virulence and adaptation of *P. aeruginosa* in the infection setting (19). This might be explained by the higher mucoidity thus biofilm production of these more susceptible isolates. It has been previously shown that mucoid *P. aeruginosa* isolates, that characterize the chronic lung infection in CF patients, are more susceptible to antibiotics since they produce less beta-lactamase than the non-mucoid paired isolates. The authors proposed that these mucoid isolates are exposed to a relatively lower antibiotic pressure and thus produce less amount of beta-lactamase (20).

In our study we did not detect any colistin resistance while ceftazidime resistance was 6.3%. Consistent with our findings in a study that aims to determine antibiotic resistance of CF in *P. aeruginosa* isolates, ceftazidime resistance was detected as 14% while there is no resistance for colistin (21). While previous study was detected resistance for imipenem, meropenem piperacillin and tobramycin as 20%, 21.4%, 23.2 and 23.5% respectively; we detected resistance for these antibiotics as 17.2%, 15.6%, 15.6% and 14.1% respectively. In their study Manno et al. detected amikacin resistance as 30.2%, similar to our study showing 29.7% resistance (21).

Both studies also found high resistance for amikacin. In a multicentre study in Australia, they also found that resistance for aminoglycoside as 43% in the pediatric patient group and 53% in adult patient group (22).

Bosso et al. compared the antibiotic resistance of *P. aeruginosa* isolates obtained from patients with CF with other groups and found that resistance rate for amikacin, gentamicin, tobramycin and levofloxacin was lower in patients with CF (23). Similar results were also obtained in another Turkish multi-center study comparing the antibiotic resistance of *P. aeruginosa* isolates from sputum samples of patients with CF and lower respiratory tract samples of patients without CF (24). In contrary to this Rao et al. showed that isolates from blood samples of CF patients were more resistant to antibiotics than those of non-CF patients (25). It may be because of several classes of antibiotics used in the management of CF airways infection. These data also illustrate the complexity of antibiotic susceptibility of *P. aeruginosa* within CF.

The results of this study supported the view that progressive evolution of *P. aeruginosa* strains in CF patients may lead to the development of a less virulent phenotype in terms of expression of specific virulence determinants. However, mucoid colony conversion seems to be the major determinant in the chronic colonization/infection process. While *P. aeruginosa* isolates from CF patients lose their ability to provoke acute infections, they gain the ability to persist in the form of biofilm nature. These findings emphasize the need for further studies on the virulence factor and antibiotic resistance patterns of CF isolates in order to establish improved treatment strategies in CF lung infections.

### Conflict of interest

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

This study follows the principles of the Declaration of Helsinki.

## Abbreviations

CF: Cystic fibrosis  
 ECR: Elastin Congo red  
 DNase: Deoxyribonuclease  
 SMA: Skim milk agar

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