RELATIONSHIP BETWEEN TYPE III SECRETION TOXINS, BIOFILM FORMATION, AND ANTIBIOTIC RESISTANCE IN CLINICAL *PSEUDOMONAS AERUGINOSA* ISOLATES

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ВЗАИМОСВЯЗЬ МЕЖДУ ТОКСИНАМИ III ТИПА СЕКРЕЦИИ, ОБРАЗОВАНИЕМ БИОФИЛЬМА И АНТИБИОТИЧЕСКОЙ РЕЗИСТЕНТНОСТЬЮ В КЛИНИЧЕСКИХ ИЗОЛЯТАХ *PSEUDOMONAS AERUGINOSA*

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Abstract

Background and objective: *Pseudomonas aeruginosa* is considered as a notorious pathogen due to its multidrug resistance and life threatening infections. We investigated the relationship between type III secretion toxins, biofilm formation, and antibiotic resistance among clinical *P. aeruginosa* isolates.

Methods: A total of 70 genetically distinct clinical *P. aeruginosa* isolates were characterized for antibiotic resistance by disk diffusion assay. Biofilm formation was evaluated by microtiter plate method and presence of four *exo* genes (*exoS*, *exoU*, *exoT*, and *exoY*) was investigated by polymerase chain reaction. A P value <0.05 was regarded statistically significant.

Results: The most effective antibiotics were meropenem and piperacillin. Multidrug resistance was more prevalent in the ciprofloxacin -resistant isolates than in the - susceptible isolates. The most frequently identified *exo* was *exoS* (37.1%). Genotype *exoS*/*exoT* was found in 4 isolates, while genotype *exoU*/*exoT* was not found. Prevalence of *exoS* was generally higher in the susceptible isolates than in the resistant isolates. A significant association was found between the formation of strong biofilm and resistance to antibiotics (P<0.05). Prevalence of *exoY* and *exoU* was higher in the non-strong biofilm producers compared to the strong biofilm producers.

Conclusion: Our study revealed formation of strong biofilm along with antibiotic resistance and the presence of *exo* genes in *P. aeruginosa* isolates. Knowledge of virulence gene profiles and biofilm formation may be useful in deciding appropriate treatment.

Keywords: *Pseudomonas aeruginosa*, biofilm, type III secretion system, drug resistance, virulence, exoenzyme

Резюме.

История вопроса и цель: *Pseudomonas aeruginosa* считается печально известным патогеном из-за своей множественной лекарственной устойчивости и вызываемых им опасных для жизни инфекций. Мы исследовали взаимосвязь между токсинами секреции III типа, образованием биопленок и устойчивостью к антибиотикам среди клинических изолятов *P. aeruginosa*.

Методы: Диско-диффузионный анализ был использован для оценки устойчивости к антибиотикам у 70 генетически отличных клинических изолятов *P. aeruginosa*. Образование биопленок оценивали в микротитрационном планшете, а наличие четырех экзогенов (exoS, exoU, exoT и exoY) исследовали с помощью полимеразной цепной реакции. Значение P<0,05 считалось статистически значимым.

Результаты. Наиболее эффективными антибиотиками были меропенем и пиперациллин. Множественная лекарственная устойчивость была более распространена у изолятов, устойчивых к ципрофлоксацину, чем у чувствительных изолятов. Наиболее часто выявляемым экзоном был exoS (37,1%). Генотип exoS / exoT обнаружен у 4 изолятов, а генотип exoU / exoT не выявлен. Распространенность exoS, как правило, была выше у чувствительных изолятов, чем у устойчивых изолятов. Была обнаружена достоверная связь между образованием прочной биопленки и устойчивостью

к антибиотикам (P <0,05). Распространенность exoY и exoU была выше у продуцентов несильных биопленок по сравнению с продуцентами сильных биопленок.

Заключение. Наше исследование выявило формирование прочной биопленки наряду с устойчивостью к антибиотикам и наличием экзогенов у изолятов *P. aeruginosa*. Знание профиля генов вирулентности и образования биопленок может быть полезно при выборе соответствующего лечения.

Ключевые слова: *Pseudomonas aeruginosa*, биопленка, система секреции III типа, лекарственная устойчивость, вирулентность, экзофермент.

1. Introduction

Pseudomonas aeruginosa is a ubiquitous gram-negative bacterium capable of causing a wide range of diseases. Prominence of *P. aeruginosa* as a life threatening and a successful opportunistic pathogen is attributed to production of a diverse repertoire of virulence factors and its high resistance to diverse classes of antimicrobial agents [1]. Aminoglycosides, beta-lactams, and fluoroquinolones are three major classes of current anti-pseudomonal agents. Among these, fluoroquinolones are the best available agents for treatment. However, resistance to fluoroquinolones among *P. aeruginosa* has risen dramatically. More seriously, resistance to fluoroquinolones is often associated with cross-resistance to other antibiotics [2].

Furthermore, *P. aeruginosa* is well equipped with numerous pathogenic factors contributing to its virulence. Many of these factors are variable traits and their prevalence may vary from one type of infection to another type (i.e. they are present in some isolates but not in others) [3]. Type III secretion system (T3SS) is an important virulence determinant of *P. aeruginosa* that injects four exotoxins directly into host cells: Exoenzyme S (ExoS), Exoenzyme U (ExoU), Exoenzyme T (ExoT), and Exoenzyme Y (ExoY). The first identified *P. aeruginosa* T3SS toxins, ExoT and ExoS are closely related bifunctional proteins which are able to disrupt the host cell actin cytoskeleton, inhibit phagocytosis, induce host cell rounding, and cause cell death. ExoS and ExoT exhibit activity towards G-proteins of the Rho, Ras, Rac, and Cdc42 families [1]. ExoY is an adenylate cyclase that cleaves the intracellular cAMP in eukaryotic cells and causes cell rounding upon co-cultivation with tissue culture cells. The fourth and the most virulent effector, ExoU, possesses phospholipase activity and disrupts eukaryotic membranes in many cell types. *P. aeruginosa* strains can be divided into two

groups. ExoU and ExoT producing strains are poorly internalized and cause rapid host cell death. While, ExoS and ExoT producing strains are more efficiently internalized and cause slower cell killing. *P. aeruginosa* strains contain either *exoU* or *exoS*, but rarely both [4]. Additionally, some studies reported that *exoS*+ and *exoU*+ strains have different antibiotic resistance patterns [1, 5]; therefore, they may require different therapeutic strategies.

Tendency to form biofilms in *P. aeruginosa* has also been correlated with its ability to cause severe infections. One of the main components of these biofilms is an exopolysaccharide called alginate, which is encoded by *algD* gene. The ability of *P. aeruginosa* to form biofilm further complicates the problem of its high antimicrobial resistance [3].

Knowledge of drug resistance patterns can be helpful in understanding and predicting clinical outcomes of patients and information about virulence gene profiles and biofilm formation may be useful for deciding appropriate antibiotic treatment. Given the importance of T3SS and biofilm in the pathogenesis of *P. aeruginosa* infections, this study was performed to determine relationship between T3SS toxins- encoding genes, antibiotic resistance, and biofilm formation in clinical *P. aeruginosa* isolates

2. Materials and methods

2.1. Bacterial isolates and identification

In this cross-sectional study, 70 *P. aeruginosa* isolates were collected from different specimens of patients admitted to two teaching hospitals in Sanandaj, Iran. Sanandaj is the capital of Kurdistan province in western Iran. Patients are referred to these two hospitals from all over the province. The isolates were

identified as *P. aeruginosa* by the standard tests, including Gram staining, motility, pigment production, oxidase, hemolysis, odor, oxidation and fermentation test, lack of carbohydrate fermentation, and citrate assimilation [6]. The genetic diversity of the isolates was determined using the enterobacterial repetitive intergenic consensus (ERIC)-PCR [7].

The isolates were stored in Trypticase soy broth (TSB, Que -lab, Canada) containing 15% glycerol at -70°C until further tests. This study was approved by Research Ethic Committee (REC) at Kurdistan University of Medical Sciences [IR.MUK.REC.1396.328].

2.2. Antibiotic susceptibility test

Antimicrobial susceptibility of the isolates was determined by the disk diffusion method according to the 2019 Clinical and Laboratory Standards Institute (CLSI) guidelines [8]. The following antibiotic disks (all from Rosco, Denmark) from three antipseudomonal categories were tested: aminglycoside [amikacin (30 µg) and gentamicin (10 µg)], beta-lactam [cefepime (30 µg), piperacillin (100 µg), aztreonam (30 µg), ceftazidime (30 µg), meropenem (10 µg), and imipenem (10 µg)], and fluoroquinolone [ciprofloxacin (5µg)].

Briefly, Mueller-Hinton agar (Que-lab, Canada) plates were inoculated with the bacterial suspensions equal to the turbidity standard of 0.5 McFarland (1.5×10^8 colony forming unit/mL). Antibiotic disks were placed onto the inoculated plates with the appropriate distance and the plates were then incubated at 35°C for 16-18 h. The inhibition zones around the disks were measured and interpreted according to the 2019 CLSI criteria [8].

2.3. DNA extraction and detection of genes

Genomic DNA was extracted using the boiling method. Overnight cultures of the isolates in TSB were centrifuged and Tris-EDTA (TE) buffer was added to the pellets. The suspensions were boiled at 100 °C for 10 min and centrifuged. The supernatants were then collected and after qualitative evaluation on agarose gel (SinaClon, Iran) and quantitative evaluation by measuring the absorbance at 260 nm and by calculating the ratio of 260/280 to determine purity, were used as the DNA templates for subsequent experiments. The ratio of 260/280 within the range of 1.6-2 indicated the purity of DNA [9].

The *algD* and T3SS toxins- encoding genes *exoY, exoS, exoT,* and *exoU* were amplified by polymerase chain reaction (PCR) method using the specific primers shown in Table 1. PCR reaction was performed in a total volume of 25 µL as follows: 1X reaction buffer, 0.2 mM of each dNTP, 0.4 µM of each primer, 1.5 mM $MgCl_2$, 0.5 U Taq DNA polymerase (SinaClon, Iran), and 3 µL template DNA. The DNA was amplified in a thermal cycler (Eppendorf, Germany) using the following conditions: initial denaturation step (94°C for 5 min), followed by 30 cycles of denaturation (1 min at 94°C), annealing (1 min at different temperatures (Table 1)), and extension (1 min at 72°C), with a final extension at 72°C for 5 min.

The PCR products were analyzed by electrophoresis on a 1.5% agarose gel in 0.5X Tris-Borate EDTA (TBE) buffer, stained with Safe Stain (SinaClon) and visualized by UV transilluminator. A 100 bp Plus DNA ladder (SinaClon) was used as a size marker.

[Table 1 is here]

2.4. Biofilm formation assay

Biofilm formation was performed according to the method of O'Toole [10]. Overnight cultures of the isolates were diluted to the turbidity equal to a

McFarland 0.5 standard in TSB medium and 100 μL of the each dilution were loaded into the wells of a flat-bottom 96-well microtiter plate (Jet Biofil, China). After 24 h incubation at 37°C for biofilm formation, the supernatants were removed and the wells were washed twice with distilled water. After discarding the planktonic cells, the wells were stained with an aqueous solution of crystal violet (0.1%, w/v) for 15 min at room temperature and washed twice with distilled water. The microtiter plates were then dried for a few hours. The bound dye was solubilized in 125 μL of 30% (v/v) acetic acid and the plates were kept for 15 min at room temperature to extract bound dye. The optical density (OD) of each well was measured by using a microplate reader (Anthos Labtec, Netherlands) at 550 nm. *S. aureus* ATCC 25923 (biofilm forming) and *Staphylococcus epidermidis* ATCC 12228 (not biofilm-forming) were used as controls. Sterile TSB was used as the negative control. For biofilm formation assay, 4 wells per strain were used and each test was repeated three times.

Biofilm density was classified according to the scheme of Stepanovic *et al.* [11]. The cut-off value (ODc) for each microtiter plate was defined as three standard deviations (SD) above the mean OD of the negative control: ODc=average OD of negative controls+ (3×SD of negative controls). Isolates were then classified into the following categories, based on the average OD of the strain:

OD≤ODc=no biofilm producer; ODc≤OD≤2ODc=weak biofilm producer; 2ODc≤OD≤4ODc=moderate biofilm producer; and 4ODc≤OD=strong biofilm producer.

2.5. Statistical analysis

SPSS software version 16 (SPSS Inc., USA) was used for statistical analysis. Pearson chi-square test and Fisher's exact test (where appropriate) were used to

determine the relationships. A P value <0.05 was regarded statistically significant. Multidrug-resistant (MDR) was defined as non-susceptible isolates to at least one agent in three or more different antimicrobial categories [12]. The isolates were classified as strong biofilm producers or non-strong (moderate and weak) biofilm producers for statistical purposes [13].

3. Results

In this study, 70 genetically distinct strains of *P. aeruginosa* were isolated from different clinical specimens. The mean age of the patients was 56.3 years old. The youngest patient was a 27 year old male and the oldest was a 91 year old male. The age distribution of patients was as follows: 27-46 years (n= 22, 31.4%), 47-66 years (n= 30, 42.8%), and 67-86 years (n= 16, 22.8%).

Of the 70 isolates, 47 (67.1%) were isolated from males and 23 (32.9%) were from females. The majority of the strains were isolated from urine (n=54, 77.1%) followed by tracheal secretions (n=11, 15.7%), and blood (n=5, 7.1%).

3.1. Determination of antibiotic susceptibility

The most effective antibiotics were meropenem (n=62, 88.6% sensitivity) and piperacillin (n=58, 82.8% sensitivity). They were excluded from statistical analysis of difference among groups. The sensitivity to other antibiotics was as follows: amikacin 55 (78.6%), imipenem 54 (77.1%), ceftazidime 53 (75.7%), cefepime 49 (70%), and aztreonam 41 (58.6%). The least effective antibiotics were ciprofloxacin (n=29, 41.4% sensitivity) and gentamicin (n=27, 38.6% sensitivity).

A total of 27 resistance patterns were detected in the 70 isolates, while 21 isolates (30%) were susceptible to all tested antibiotics. The patterns ranged from resistance to one antibiotic to all the 9 antibiotics. Of the 70 isolates, 7 isolates (10%) were resistant to one agent, 31 (44.3%) to 2 to 6 agents, and 11 (15.7%)

isolates showed resistance to 7 to 9 agents. The most frequently detected pattern was resistance to ciprofloxacin and gentamicin combination (10/70, 14.3%) followed by resistance to gentamicin (6/70, 8.6%). Of the 70 isolates, 29 were MDR (41.4%). Co-resistance to three antipseudomonal categories (fluoroquinolone, beta -lactam, and aminoglycoside) was found in 25 of the 70 isolates (35.7%) (Table 2).

Furthermore, pattern of resistance to other antibiotics was determined in the ciprofloxacin - susceptible and -resistant isolates (Table 2). In the 29 ciprofloxacin -susceptible isolates, 8 isolates (27.6%) were resistant to 1 to 3 agents and no isolate was resistant to 5 to 9 agents. However, in the 41 ciprofloxacin -resistant isolates, 18 isolates (43.9%) were resistant to 1 to 3 agents, and 23 isolates (56.1%) showed resistance to 5 to 9 agents. Multidrug resistance was found in 27 (65.9%) of the 41 ciprofloxacin -resistant isolates, while only 2 (6.9%) of the 29 ciprofloxacin -susceptible isolates were MDR.

[Table 2 is here]

3.2. Prevalence of genes

The genes encoding Exo toxins were found in 33 of the 70 isolates (47.1%), while 35 isolates (50%) carried no virulence genes. Of the 70 isolates, 26 (37.1%) carried the *exoS* gene, 9 (12.8%) the *exoY*, 6 (8.6%) the *exoU*, and 4 isolates (5.7%) carried the *exoT* gene. The *algD* was found in 5 isolates (7.1%). The simultaneous presence of two genes was found in 11 isolates and only 2 isolates carried three genes simultaneously (*exoS*, *exoY*, and *exoT*). Both strains were isolated from urine and showed susceptibility to all tested antibiotics. No isolate carried the simultaneous presence of four or five genes. Genotype *exoS*/*exoT* was found in 4

175 isolates, while genotype *exoU*/*exoT* was not found. None of the *exoU* + isolates
176 harbored the *exoS* (Table 3).

177 **[Table 3 is here]**

178

179 Because there are studies that reported *exoS*+ and *exoU*+ strains have different
180 antibiotic resistance patterns [1, 5]; we determined the prevalence of *exoS* in the
181 antibiotic- susceptible and -resistant isolates. With the exception of gentamicin, the
182 prevalence of *exoS* was higher in the susceptible isolates than in the resistant
183 isolates, although it was not significant. The *exoS* was found more frequently in the
184 isolates susceptible to ciprofloxacin followed by cefepime, imipenem, and
185 amikacin (Figure 1). Multidrug resistance was found in 10 of the 26 *exoS*+ isolates
186 (38.5%).

187 **[Figure 1 is here]**

188 **3.3. Biofilm assay**

189 Of the 70 isolates studied, all formed biofilm; of which 35 isolates (50%) formed
190 strong biofilm, 28 isolates (40%) moderate biofilm, and only 7 isolates (10%)
191 formed weak biofilm.

192 The resistant isolates formed strong biofilm more frequently compared to the
193 susceptible isolates. The formation of strong biofilm was more frequently found in
194 the isolates resistant to cefepime followed by ceftazidime, and aztreonam.
195 Significant associations were seen between formation of strong biofilm and
196 resistance to cefepime (P=0.019), aztreonam (P=0.008), and ciprofloxacin
197 (P=0.008) (Figure 2).

198 **[Figure 2 is here]**

In addition, the prevalence of *exo* genes was determined in the 35 strong and the 35 non-strong (moderate+ weak) biofilm producers. The *exoS* and *exoT* were equally distributed between the two groups (37.1% and 5.7%, respectively); however, the prevalence of *exoY* and *exoU* was higher in the non-strong biofilm producers compared to the strong biofilm producers (20% vs. 5.7% for the *exoY*, and 11.4% vs. 5.7% for the *exoU*, respectively). All of the five *algD* - positive isolates formed strong biofilm.

4. Discussion

P. aeruginosa is considered as a notorious pathogen due to its multidrug resistance and life threatening infections [1]. Our strains were mostly isolated from inpatients and the majority of patients (42.8%) were between 47-66 years old, which can be explained by the fact that *P. aeruginosa* infections mostly occur in people in the hospital and /or with the weakened immune systems [14].

Fluoroquinolones, aminoglycosides, and beta lactams are three main antimicrobial classes with reliable antipseudomonal activity. Among these, fluoroquinolones are the best available treatment option [2]. A relatively high percentage of our isolates (58.6%) showed resistance to ciprofloxacin which is in agreement with previous studies in Pakistan [15] and Egypt [16]. The widespread use of fluoroquinolones both in human and veterinary medicine may be responsible for the high resistances to this class. In addition, resistance to fluoroquinolones was significantly associated with cross resistance to other agents [2]. In our study, MDR isolates were more frequently detected in the ciprofloxacin -resistant isolates.

P. aeruginosa is well equipped with numerous pathogenic factors contributing to its virulence. T3SS in *P. aeruginosa* is an important virulence factor that transports

four proteins: ExoU, ExoS, ExoY and ExoT [1]. In our study, *exoS* showed the highest prevalence (37.1%). In southern Iran [17] 35.8% and in Bulgaria [18] 37.6% of the isolates carried *exoS*, which were similar to our report. However, studies in central Iran and Poland reported the prevalence rates of 77.7% [19] and 88.4% [20] for *exoS*, respectively, which were higher than that in our study.

While in our work the prevalence of *exoT* was 5.7%, which was similar to a study in northwestern Iran (5%) [21], it was markedly lower than those reported by others. For example, in central Iran [22] and in Poland [20], the prevalence rates of 20.4% and 94.4% were reported, respectively. Moreover, we observed a low prevalence of the *exoY* (12.8%); while, in the northwest of Iran, 55% [21] and in India, 91.3% [23] of *P. aeruginosa* isolates carried the *exoY*. The prevalence of the fourth gene, *exoU* in our study was 8.6%. However, in a study from Iran a rate of 42.8% [19] and in Egypt a prevalence of 33% [16] were reported for *exoU*. The lower prevalence of *exo* genes in our study may be due to differences in the source of isolates or geographical regions. Many of the *P. aeruginosa* virulence factors are variable traits and they are found in some isolates, but not in others [3]. For example, Choy *et al.*, reported that the *exoU* is commonly found in *P. aeruginosa* strains isolated from keratitis [24], whereas it occurs at low prevalence in the non-ocular isolates [5] or the prevalence of *exoS* was significantly higher in isolates from blood than those obtained from respiratory infections [18]. In addition, it is possible that our strains were isolated from chronic infections. The expression of the T3SS has been found to be downregulated in isolates from chronic phase of infection, which is consistent with the notion that persistence of bacteria in the host requires the down-regulation of many virulence factors [4].

In our study, a disagreement was seen in the distribution of *exoS* and *exoU* genes, in consistent with other studies [1, 4, 5]. The *exoU* gene is located within a genomic island and its acquisition may cause loss of the *exoS* [25]. The T3SS system and its effectors were probably acquired by horizontal DNA transmission and antibiotic rich environments could promote the evolution of more virulent strains [4]. The prevalence of *exoS* in our study was generally higher in the susceptible isolates compared to the resistant isolates. The higher prevalence of this gene may contribute to the pathogenesis of antibiotic susceptible isolates. The *exoS*+ strains may be protected from the action of antibiotics due to their ability to invade mammalian cells and their residence inside cells [4]. Due to the small number of *exoU*+ isolates, we couldn't determine association between the presence of *exoU* and drug resistance; however, the presence of *exoU* has been correlated to antibiotic resistance phenotypes in *P. aeruginosa* [5, 26]. The *exoU*+ strains may have a survival advantage by having the potential to be more resistant.

Biofilm production has been considered as an important determinant of pathogenicity in *P. aeruginosa* infections by facilitating the emergence of antimicrobial drug resistance and by generating chronic infections. The alginate is the most important component of *P. aeruginosa* biofilms [3]. A low prevalence of *algD* (7.1%) was found in our isolates. The prevalence rates of 0 to 98% were reported for *algD* in different studies [16, 27, 28]. The low prevalence of *algD* in our study might be attributed to the high number of strains isolated from UTI. There are reports that contribution of alginate in the urinary tract is thought to be minimal [29, 30]. In addition, production of some other exopolysaccharides like Psl and Pel may contribute to the formation of biofilm in *P. aeruginosa* [3]. The prevalence of strong biofilm producers in our study was higher in the resistant isolates than in the susceptible isolates, especially for cefepime, aztreonam, and

ciprofloxacin. The ability to form strong biofilm along with resistance to antibiotics may cause high rates of failure in therapy of *P. aeruginosa* infections. Furthermore, we found that *exoY* and *exoU* were more prevalent in the non-strong biofilm producers. These results may indicate the importance of *exoY* and *exoU* in the pathogenesis of non-strong biofilm producers of *P. aeruginosa*.

Conclusion

In conclusion, findings of the present study showed a relatively different distribution of *exo* genes in clinical *P. aeruginosa* isolates from western Iran. The formation of strong biofilm along with antibiotic resistance and presence of *exo* genes may lead to severe diseases. Further in-depth studies are needed to determine whether gene linkage on mobile genetic elements underlies the relationships observed in our study. Knowledge of virulence gene profiles and biofilm formation may be useful for deciding appropriate treatment.

FIGURES

Figure 1. Prevalence of *exoS* virulence gene in antibiotic -resistant and -susceptible isolates of 70 clinical *Pseudomonas aeruginosa*. *: Ciprofloxacin Cp, Aztreonam AT; Imipenem IP, Ceftazidime TZ; Cefepime PM, Gentamicin Gm, Amikacin Ak.

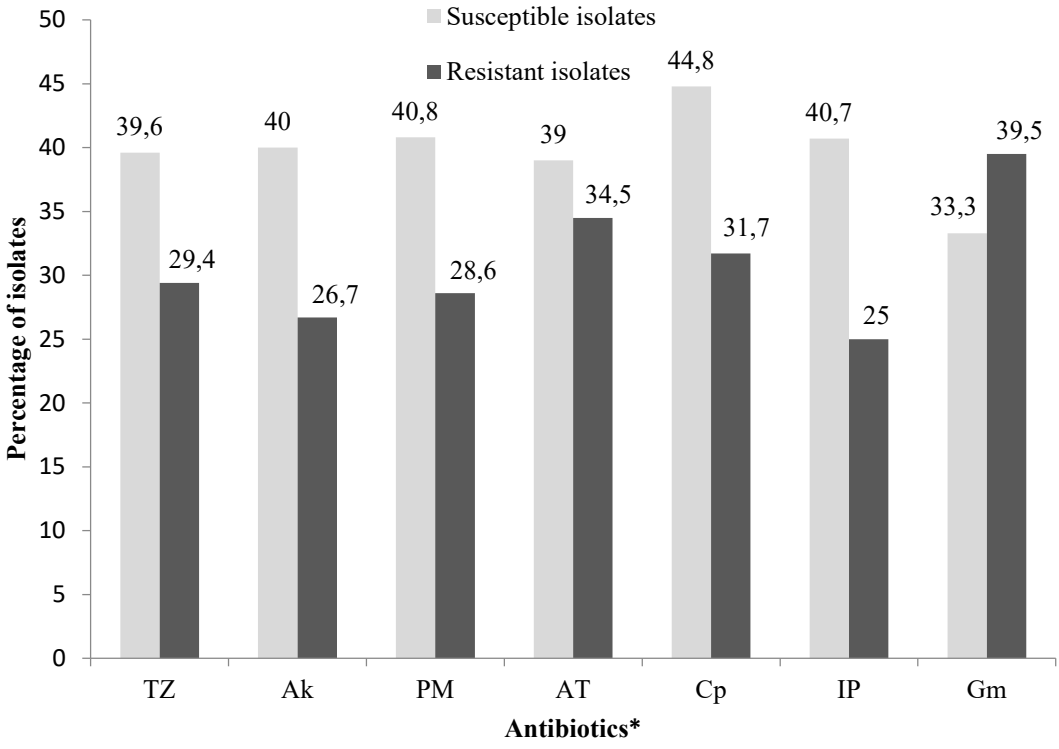
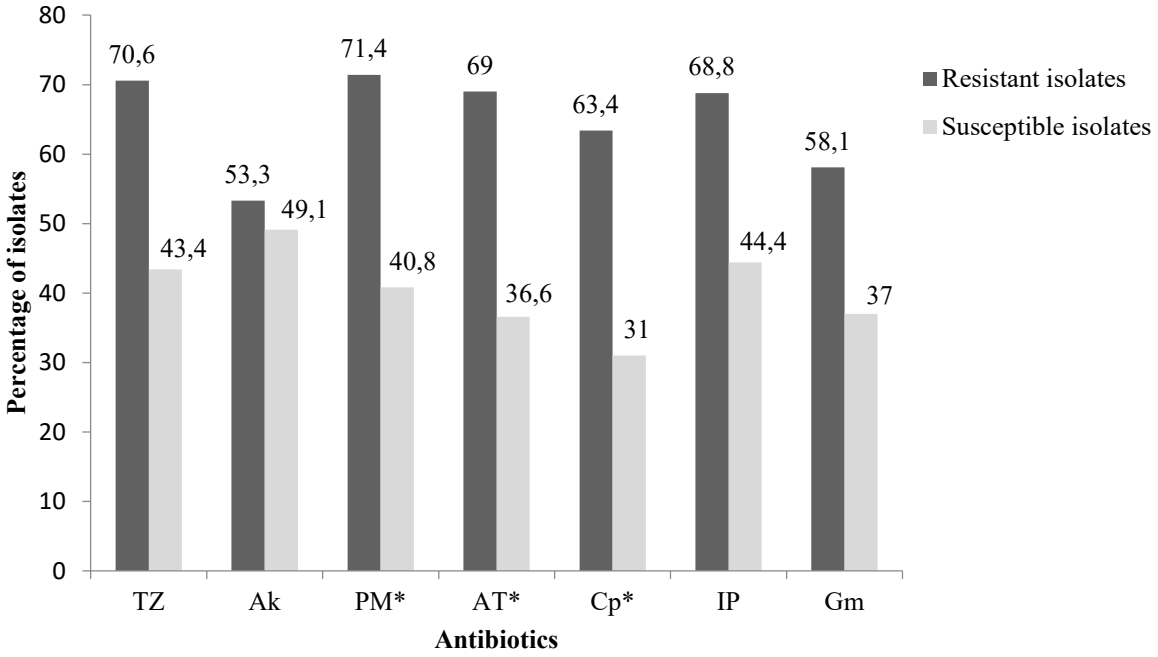


Figure 2. Prevalence of strong biofilm producers in antibiotic-resistant and susceptible isolates of 70 clinical *Pseudomonas aeruginosa*. Ciprofloxacin Cp, Aztreonam AT, Imipenem IP, Ceftazidime TZ , Cefepime PM, Gentamicin Gm, Amikacin Ak. *: *P* value less than 0.05.



TABLES

Table 1. Primer sequences, annealing temperatures and *expected* amplicon *size*

Target gene	**Primer sequence (5′-3′)**	**Annealing temperature (°C)**	**Size of fragments (bp)**	**Reference**
exoS	CTTGAAGGGACTCGACAAGG/ TTCAGGTCCGCGTAGTGAAT	58	504	[37]
exoU	CCGTTGTGGTGCCGTTGAAG/ CCAGATGTTCACCGACTCGC	61	134	[38]
exoT	CAATCATCTCAGCAGAACCC/ TGTCGTAGAGGATCTCCTG	55	1159	[39]
exoY	TATCGACGGTCATCGTCAGGT/ TTGATGCACTCGACCAGCAAG	61	1035	[39]
alg D	ATGCGAATCAGCATCTTTGGT/ CTACCAGCAGATGCCCTCGGC	57	1310	[40]

Table 2. Antimicrobial cross resistance pattern of ciprofloxacin-resistant versus ciprofloxacin -susceptible isolates of clinical *Pseudomonas aeruginosa*.

Agents	**Resistance pattern***	**In Cp-susceptible isolate (n=29)**	**In Cp-resistant isolate (n=41)**	**In total (N=70)**
One	Cp	0	1	1
	Gm	6	0	6
two	AT, Cp	0	2	2
	Cp, Gm	0	10	10
Three	Ak, Cp, Gm	0	1	1
Three, MDR**	PM, AT, Cp	0	1	1
	AT, IP, Gm	1	0	1
	PM, Cp, Gm	0	1	1
	Cp, Gm, Pi	0	1	1
	AT, IP, Pi	1	0	1
	AT, Cp, Gm	0	1	1
Five, MDR	PM, AT, Cp, IP, MP	0	1	1
	TZ, PM, AT, Cp, Gm	0	5	5
	Ak, AT, Cp, IP, Gm	0	1	1
	Ak, AT, Cp, Gm, Pi	0	1	1
Six, MDR	Ak, AT, Cp, IP, MP, Gm	0	1	1
	Ak, PM, AT, Cp, IP, Gm	0	1	1
	TZ, Ak, PM, AT, Cp, Gm	0	1	1
	TZ, PM, AT, Cp, IP, Gm	0	1	1

Seven, MDR	TZ, Ak, PM, AT, Cp, IP, Gm	0	1	1
	TZ, Ak, PM, AT, Cp, Gm, Pi	0	2	2
	TZ, Ak, AT, Cp, IP, Gm, Pi	0	1	1
Eight, MDR	Ak, PM, AT, Cp, IP, MP, Gm, Pi	0	1	1
	TZ, PM, AT, Cp, IP, MP, Gm, Pi	0	2	2
	TZ, Ak, PM, AT, Cp, IP, MP, Gm	0	1	1
	TZ, Ak, PM, AT, Cp, IP, Gm, Pi	0	1	1
Nine, MDR	TZ, Ak, PM, AT, Cp, IP, MP, Gm, Pi	0	2	2

*: Ciprofloxacin Cp, Piperacillin Pi , Aztreonam AT; Imipenem IP, Meropenem MP; Ceftazidime TZ ; Cefepime PM, Gentamicin Gm, Amikacin Ak. **: MDR: multi drug resistant.

Table 3. Virulence patterns of 70 clinical *Pseudomonas aeruginosa* isolates.

Virulence pattern	**Isolates, N (%)**
exoS+, *exoY*-, *exoT*-, *exoU*-, *algD*-	15 (21.4)
exoS+, *exoY*+, *exoT*-, *exoU*-, *algD*-	5 (7.1)
exoS-, *exoY*-, *exoT*-, *exoU*+, *algD*-	4 (5.7)
exoS+, *exoY*+, *exoT*+, *exoU*-, *algD*-	2 (2.85)
exoS+, *exoY*-, *exoT*+, *exoU*-, *algD*-	2 (2.85)
exoS+, *exoY*-, *exoT*-, *exoU*-, *algD*+	2 (2.85)
exoS-, *exoY*-, *exoT*-, *exoU*-, *algD*+	2 (2.85)
exoS-, *exoY*-, *exoT*-, *exoU*+, *algD*+	1 (1.4)
exoS-, *exoY*+, *exoT*-, *exoU*-, *algD*-	1 (1.4)
exoS-, *exoY*+, *exoT*-, *exoU*+, *algD*-	1 (1.4)
exoS-, *exoY*-, *exoT*-, *exoU*-, *algD*-	35 (50)

METADATA

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TITLE PAGE

Title: Relationship between type III secretion toxins, biofilm formation, and antibiotic resistance in clinical *Pseudomonas aeruginosa* isolates

Running title: Resistance and virulence in *Pseudomonas aeruginosa*

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Declarations

List of abbreviations: T3SS: Type III secretion system; ExoS: Exoenzyme S; ExoU: Exoenzyme U; ExoT: Exoenzyme T; ExoY: Exoenzyme Y; PCR: Polymerase chain reaction; TSB: Trypticase soy broth; MDR: Multidrug-resistant; bp: Base pairs.

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Authors' contributions: SD designed the search and prepared the manuscript. SM analyzed the data. AR performed microbiological and molecular studies.

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REFERENCES

Reference sequence number	Authors. Title	Source	URL
1.	Strateva T, Mitov I. Contribution of an arsenal of virulence factors to pathogenesis of *Pseudomonas aeruginosa* infections	Annals of microbiology. 2011;61(4):717-32.	10.1007/s13213-011-0273-y.
2.	Hsu DI, Okamoto MP, Murthy R, Wong-Beringer A. Fluoroquinolone-resistant *Pseudomonas aeruginosa*: risk factors for acquisition and impact on outcomes.	Journal of Antimicrobial Chemotherapy. 2005;55(4):535-41.	10.1093/jac/dki026
3.	Moradali MF, Ghods S, Rehm BH. *Pseudomonas aeruginosa* lifestyle: a paradigm for adaptation, survival, and persistence.	Frontiers in cellular and infection microbiology. 2017;7:39.	10.3389/fcimb.2017.00039.
4.	Engel J, Balachandran P. Role of *Pseudomonas aeruginosa* type III effectors in disease.	Current opinion in microbiology. 2009;12(1):61-6.	10.1016/j.mib.2008.12.007.
5.	Subedi D, Vijay AK, Kohli GS, Rice SA, Willcox M. Association between possession of ExoU and antibiotic resistance in *Pseudomonas aeruginosa*.	PLoS One. 2018;13(9):e0204936.	10.1371/journal.pone.0204936.
6.	Tille P. Bailey & Scott's diagnostic microbiology.	Elsevier Health Sciences; 2015.	-
7.	Saleem S, Bokhari H. Resistance profile of genetically distinct clinical	Journal of infection and public health. 2020;13(4):598-605.	10.1016/j.jiph.2019.08.019

	Pseudomonas aeruginosa isolates from public hospitals in central Pakistan.		
8	Wayne P. Clinical and laboratory standards institute. Performance standards for antimicrobial susceptibility testing.	2019.	-
9	Khare P, Raj V, Chandra S, Agarwal S. Quantitative and qualitative assessment of DNA extracted from saliva for its use in forensic identification.	Journal of forensic dental sciences. 2014;6(2):81-5.	10.4103/0975-1475.132529
10.	O'Toole GA. Microtiter dish biofilm formation assay.	Journal of Visualized Experiments. 2011;(47):e2437.	10.3791/2437.
11.	Stepanović S, Vuković D, Hola V, Bonaventura GD, Djukić S, Ćirković I, Ruzicka F. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci.	Apmis. 2007;115(8):891-9.	10.1111/j.1600-0463.2007.apm_630.x.
12.	Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liljequist B, Paterson DL. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired	Clinical microbiology and infection. 2012;18(3):268-81.	10.1111/j.1469-0691.2011.03570.x.

	resistance.		
13.	Horna G, Quezada K, Ramos S, Mosqueda N, Rubio M, Guerra H, Ruiz J. Specific type IV pili groups in clinical isolates of *Pseudomonas aeruginosa*.	International Microbiology. 2019;22(1):131-41.	10.1007/s10123-018-00035-3
14.	Al-Wrafy F, Brzozowska E, Górska S, Gamian A. Pathogenic factors of *Pseudomonas aeruginosa*-the role of biofilm in pathogenicity and as a target for phage therapy.	Advances in Hygiene & Experimental Medicine/Postepy Hig Med Med Dosw 2017;71:78-91.	10.5604/01.3001.0010.3792.
15.	Samad A, Ahmed T, Rahim A, Khalil A, Ali I. Antimicrobial susceptibility patterns of clinical isolates of *Pseudomonas aeruginosa* isolated from patients of respiratory tract infections in a Tertiary Care Hospital, Peshawar.	Pak J Med sciences. 2017;33(3):670-4.	10.12669/pjms.333.12416
16.	Al Dawodeyah HY, Obeidat N, Abu-Qatouseh LF, Shehabi AA. Antimicrobial resistance and putative virulence genes of *Pseudomonas aeruginosa* isolates from patients with respiratory tract infection.	Germs. 2018;8(1):31-40.	10.18683/germs.2018.1130.
17.	Khoramrooz SS, Rahbari N, Parhizgari N, Sharifi A, Yazdanpanah M, Gharibpour F, Rabani SM, Malekhosseini SA, Marashifard M. Frequency of type III secretion system cytotoxins-encoding genes among *Pseudomonas aeruginosa*	J Adv Med Biomed Res. 2015;23(99):52-63.	-

	isolated from burn patients.		
18.	Strateva T, Markova B, Ivanova D, Mitov I. Distribution of the type III effector proteins-encoding genes among nosocomial *Pseudomonas aeruginosa* isolates from Bulgaria.	Annals of microbiology. 2010;60(3):503-9.	10.1007/s13213-010-0079- 3.
19.	Mohamad M, Rostami S, Zamanzad B, Gholipour A, Deris F. Detection of exotoxins and antimicrobial susceptibility pattern in clinical *Pseudomonas aeruginosa* Isolates.	Avicenna Journal of Clinical Microbiology and Infection. 2018;5(2): 36-40.	10.34172/ajcmi.2018.07
20.	Pobiega M, Chmielarczyk A, Kozioł J, Pomorska-Wesołowska M, Ziolkowski G, Romaniszyn D, Bulanda M, Wojkowska-Mach J. Virulence factors genes and drug resistance in *Pseudomonas aeruginosa* strains derived from different forms of community and healthcare associated infections.	Advances in Hygiene & Experimental Medicine/Postepy Hig Med Dosw , 2018; 72: 751-759.	10.5604/01.3001.0012.2426
21.	Azimi S, Kafil HS, Baghi HB, Shokrian S, Najaf K, Asgharzadeh M, Yousefi M, Shahrivar F, Aghazadeh M. Presence of exoY, exoS, exoU and exoT genes, antibiotic resistance and biofilm production among *Pseudomonas aeruginosa* isolates in Northwest Iran.	GMS Hyg Infect Control. 2016; 11.	10.3205/dgkh000264
22.	Haghi F, Zeighami H, Monazami A, Toutouchi F, Nazaralian S, Naderi G.	Microbial pathogenesis. 2018;115:251-6.	10.1016/j.micpath.2017.12.

	Diversity of virulence genes in multidrug resistant *Pseudomonas aeruginosa* isolated from burn wound infections.		052
23.	Shariff M, Chhabra SK, Rahman M-u. Similar virulence properties of infection and colonization associated *Pseudomonas aeruginosa*.	Journal of medical microbiology. 2017;66(10):1489- 98.	10.1099/jmm.0.000569.
24.	Choy MH, Stapleton F, Willcox MD, Zhu H. Comparison of virulence factors in *Pseudomonas aeruginosa* strains isolated from contact lens-and non-contact lens-related keratitis.	Journal of medical microbiology. 2008;57(12):1539-46.	10.1099/jmm.0.2008/003723-0
25.	Kulasekara BR, Kulasekara HD, Wolfgang MC, Stevens L, Frank DW, Lory S. Acquisition and evolution of the exoU locus in *Pseudomonas aeruginosa*.	Journal of bacteriology. 2006;188(11):4037-50.	10.1128/JB.02000-05
26.	Agnello M, Finkel SE, Wong-Beringer A. Fitness cost of fluoroquinolone resistance in clinical isolates of *Pseudomonas aeruginosa* differs by type III secretion genotype.	Frontiers in microbiology. 2016;7:1591.	10.3389/fmicb.2016.01591
27.	Badamchi A, Masoumi H, Javadinia S, Asgarian R, Tabatabaee A. Molecular detection of six virulence genes in	Microbial pathogenesis. 2017;107:44-7.	10.1016/j.micpath.2017.03. 009

	Pseudomonas aeruginosa isolates detected in children with urinary tract infection.		
28.	Fazeli N, Momtaz H. Virulence gene profiles of multidrug-resistant *Pseudomonas aeruginosa* isolated from Iranian hospital infections.	Iran Red Crescent Med J. 2014; 16(10): e15722.	10.5812/ircmj.15722
29.	Newman JW, Floyd RV, Fothergill JL. The contribution of *Pseudomonas aeruginosa* virulence factors and host factors in the establishment of urinary tract infections.	FEMS Microbiology Letters. 2017;364(15).	10.1093/femsle/fnx124
30.	Tielen P, Narten M, Rosin N, Biegler I, Haddad I, Hogardt M, Neubauer R, Schobert M, Wiehlmann L, Jahn D. Genotypic and phenotypic characterization of *Pseudomonas aeruginosa* isolates from urinary tract infections.	International Journal of Medical Microbiology. 2011;301(4):282-92.	10.1016/j.ijmm.2010.10.005
31.	Bogiel T, Depka D, Rzepka M, Kwiecińska-Piróg J, Gospodarek-Komkowska E. Prevalence of the Genes Associated with Biofilm and Toxins Synthesis amongst the *Pseudomonas aeruginosa* Clinical Strains.	Antibiotics. 2021;10(3):241.	10.3390/antibiotics10030241
32.	Alonso B, Fernández-Barat L, Di Domenico EG, Marín M, Cercenado E, Merino I, de Pablos M, Muñoz P, Guembe M.	BMC infectious diseases. 2020;20(1):1-8.	10.1186/s12879-020-05534-1

	Characterization of the virulence of *Pseudomonas aeruginosa* strains causing ventilator-associated pneumonia.		