Antibiotic Susceptibility Profile and Putative Virulence Genes in Clinical Isolates of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa'nın Klinik İzolatlarında Antibiyotik Duyarlılık Profili ve Varsayılan Virülans Genleri

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ABSTRACT

Background: *Pseudomonas aeruginosa* is a common environmental Gram-negative rod responsible for a wide range of nosocomial infections. The pathogenesis of *P. aeruginosa* is because of the production of various virulence factors. The present study was carried out to determine the presence of virulence factors and the rate of antibiotic resistance among clinical isolates of *P. aeruginosa*.

Methods: This cross-sectional study was performed on 120 clinical isolates of *P. aeruginosa* collected from a teaching hospital in the south of Iran. All isolates were identified with standard biochemical and microbiological tests. Antibacterial susceptibility was determined by disk diffusion method. The presence of virulence genes was screened by PCR.

Results: From 120 *P. aeruginosa* isolates, the most common sites of bacterial isolation were pulmonary with frequency 52 (43.3%), followed by urine 28 (23.3%), blood 17 (14.2%), and wound 14 (11.7%). Antibiotic susceptibility patterns revealed that the highest and lowest resistance rates were against ticarcillin-clavulanate (60%) and amikacin (10%), respectively. The majority of meropenem-resistant isolates had a MIC range >32 µg/mL. Among tested virulence genes, *lasB* gene was detected in the 98.3% of isolates followed by *pvdA* in 68.3%, *pilA* in 8.3% and *pilB* in 5% of isolates.

Conclusion: Results showed a high prevalence of *lasB* in clinical isolates of *P. aeruginosa*. These findings accompanied with remarkable rate of β-lactam-resistance highlights importance of suitable surveillance and control processes to prevent the further spread of these isolates in hospitals.

Key Words: *Pseudomonas aeruginosa*, Virulence genes and Antibiotic resistance

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ÖZET

Amaç: Pseudomonas aeruginosa, çok çeşitli nozokomiyal enfeksiyonlardan sorumlu, yaygın bir çevresel Gram negatif çubuktur. P. aeruginosa'nın patogenezi, çeşitli virülans faktörlerinin üretiminden kaynaklanmaktadır. Bu çalışma, P. aeruginosa'nın klinik izolatlarında virülans faktörlerinin varlığını ve antibiyotik direnç oranını belirlemek amacıyla yapılmıştır.

Yöntem: Bu kesitsel çalışma, İran'ın güneyindeki bir eğitim hastanesinden toplanan 120 klinik P. aeruginosa izolatı üzerinde gerçekleştirildi. Tüm izolatlar standart biyokimyasal ve mikrobiyolojik testlerle tanımlandı. Antibakteriyel duyarlılık disk difüzyon yöntemi ile belirlendi. Virülans genlerinin varlığı PCR ile tarandı.

Bulgular: 120 P. aeruginosa izolatından bakteri izolasyonunun en yaygın yerleri 52 (% 43,3) sıklığı ile pulmoner, bunu idrar 28 (% 23,3), kan 17 (% 14,2) ve yara 14 (% 11,7) izledi. Antibiyotik duyarlılık paternleri, en yüksek ve en düşük direnç oranlarının sırasıyla tikarsilin-klavulanat (% 60) ve amikasine (% 10) karşı olduğunu ortaya koydu. Meropenem dirençli izolatların çoğunun MİK aralığı> 32 µg / mL'dir. Test edilen virülans genleri arasında izolatların% 98,3'ünde lasB geni, ardından% 68,3'ünde pvdA,% 8,3'ünde pilA ve% 5'inde pilB saptanmıştır.

Sonuç: Sonuçlar, P. aeruginosa'nın klinik izolatlarında yüksek bir lasB prevalansı göstermiştir. Bu bulgular, dikkate değer β-laktam direnci oranıyla birlikte, bu izolatların hastanelerde daha fazla yayılmasını önlemek için uygun gözetim ve kontrol süreçlerinin önemini vurgulamaktadır.

Anahtar Sözcükler: Pseudomonas aeruginosa, Virülans genleri ve Antibiyotik direnci

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INTRODUCTION

Pseudomonas aeruginosa is a common environmental Gram-negative rod that is responsible for a wide range of nosocomial infections, including ventilator-associated pneumonia, wound infections and a variety of systemic infections in immunosuppressed patients (1, 2). The spread of this organism in healthcare settings is due to several factors, including its ability to colonize multiple environmental niches and to utilize many environmental compounds as energy sources (1, 3). *P. aeruginosa* infections are mostly associated with the compromise of host defense such as burn wounds or cystic fibrosis (1, 4). These infections could be more severe in cancer and neutropenic patients (4).

The pathogenesis of *P. aeruginosa* is because of the production of various virulence factors which can causes widespread tissue damage, and bloodstream invasion (5). The most common virulence factors are zinc metalloprotease (LasB), pyoverdin and type IV pili (6). The most functions of type IV pili is aggregation, attachment to surfaces, uptake of DNA during transformation and motility. Twitching motility in this organism depends on this factor (7). The pilus filament, encoded by the *pil*A gene and *pil*B gene is putative pilin polymerase (8). *P. aeruginosa* produces several extracellular proteases, including elastase LasB (9). Zinc metalloprotease activity of LasB is on the lung tissue, so that reduces the secretion of extracellular matrix compounds such as collagen 3 and 4, elastin and proteoglycan, as well as host cell-matrix protease inhibition (10, 11). The enzyme can also decompose complement components and inhibit neutrophil action, leading to further tissue damage in acute infections. The other virulence factor is pyoverdin, a siderophore that competes with mammalian transferrin for iron (12, 13) by the specific receptor on the surface of the outer membrane (14, 15). Pyoverdine also regulates the secretion of other virulence factors of bacteria such as exotoxin A and an endoprotease (16).

Empirical antibiotic therapy includes monotherapy and combination therapy for *P. aeruginosa* which reduce the mortality in patients with severe infections (17). However, the ability of this bacterium to resist many of the currently available antibiotics has become a global challenge (17). The occurrence of multidrug-resistant (MDR) *P. aeruginosa* in developing countries has become a serious problem (5). MDR bacteria are related to increased mortality and costs due to prolonged hospitalization, the need for surgery and prolonged treatment with antibiotics (5).

Despite the high incidence of *P. aeruginosa* infections in hospitalized patients and increasing antibiotic resistance, there are low reports about the prevalence of virulence factors and antibiotic resistance of *P. aeruginosa* isolated in the south of Iran. Therefore, the present study aimed to investigate the prevalence of virulence factors and antibiotic resistance in *P. aeruginosa* isolated from different clinical specimens in hospitalized patients.

METHODS

Study population and P. aeruginosa isolates

This cross-sectional study was performed on 120 isolates of *P. aeruginosa* collected from Nemazee teaching hospital in Shiraz, Iran, during the period from June to December 2016. Non-duplicated *P. aeruginosa* were isolated from different body sites, including blood, wound, pulmonary, urine, eye, ear, and cerebrospinal fluid (CSF). Exclusion criteria were not receiving any antibiotic at least one month prior the sampling and unclear medical records. Standard microbiological and biochemical tests, including colony morphology on blood agar (Merck, Germany), Gram staining, the reaction on triple sugar iron agar (Merck, Germany), oxidase reaction, were used for identification of *P. aeruginosa* isolates. Isolates were confirmed by previously described primers targeting the *algD* genes (18). Characterized isolates for further experiments were kept frozen in tryptic soy broth (Merck Co., Germany) containing 20% glycerol (Merck KGaA, Germany) at − 70 °C.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing against antibiotics, including meropenem, aztreonam, imipenem, amikacin, gentamycin, ciprofloxacin, piperacillin /tazobactam, ticarcillin/clavulanate, ceftazidime (Mast Co., UK) was investigated by standard Kirby-Bauer method as described by the Clinical and Laboratory Standards Institute (CLSI) guidelines (19). *P. aeruginosa* ATCC 27853 was used as the quality control strain for antibacterial susceptibility testing. The minimum inhibitory concentrations (MICs) of each isolate was determined using the Epsilometer test (E-test) strips (Liofilchem, Italy) containing meropenem (1 - 32 µg / mL) as recommended by CLSI (19). The MIC values ≤2, and ≥ 8 µg/mL were determined as susceptible, and resistant, respectively.

Detection of virulence genes by PCR

The bacterial whole genome was extracted from *P. aeruginosa* isolates using the boiling method as previously described (20). Simplex PCR reaction was carried out for evaluation of four major virulence factors (*PilA, PilB, pvdA,* and *lasB* genes) with specific primers (Table1). PCR amplification was performed using T100™ thermal cycler (Bio-Radd, Hercules, CA, USA) in a final volume of 25 µl containing 2 µL template DNA, 0.2 mM of each deoxynucleoside triphosphate, 10 pmol of each primer, 10 mM Tris-HCl, 1.5 mM MgCl2, 50 mM KCl, and 1.5 U of Taq DNA polymerase. The cycling conditions were set up as follows: initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 60 s, primer annealing at 57°C for 60 s, extension at 72°C for 2 min and a final extension at 72°C for 10 min. The amplifications were loaded into the wells of agarose gel (1.5%) containing safe DNA stain carefully and electrophoresed at 75 V for 90 min and observed under the UV trans-illuminator. *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were used as positive and negative control, respectively for *algD* gene.

Table1: Primers of virulence factor genes used in PCR

Target gene	Primer sequence (5'-3')	PCR product (bp)	References
pilB-F	ACAGCATCCAACTGAGCG	1675	4
pilB-R	TTGACTTCCTCCAGGCTG		
pilA-F	TCGAACTGATGATCGTGG	408	4
pilA-R	CTTTCGGAGTGAACATCG		
pvdA-F	GACTCAGGCAACTGCAAC	1281	32
pvdA-R	TTCAGGTGCTGGTACAGG		
lasB-F	GGAATGAACGAAGCGTTCTC	300	32
lasB-R	GGTCCAGTAGTAGCGGTTGG		

Data analysis

Statistical analysis, including frequencies, cross-tabulation of microbiological, clinical and demographic data was performed through the use of SPSS™ software, version 22.0 (IBM Corp., USA). The results were presented as descriptive statistics in terms of relative frequency. Values were expressed as the mean ± standard deviation (continuous variables) or percentages of the group (categorical variables).

RESULTS

In this study, among 120 *P. aeruginosa* isolates, 73 (61%) and 47 (39%) were from male and female patients, respectively, aged from 1 month to 86 years old. The most common sites of bacterial isolation were from pulmonary with frequency 52 (43.3%) followed by urine 28 (23.3%), blood 17 (14.2%), wound 14 (11.7%), eye 7 (5.8%), ear 1 (0.8%), and CSF 1 (0.8%). Antibiotic susceptibility patterns revealed that the highest and lowest resistance rates were against ticarcillin-clavulanate (60%) and amikacin (10%), respectively (Table2). The highest antibiotic resistance was observed in blood culture isolates. Totally, 28.3% of isolates were considered as MDR phenotype. Totally, the MIC range of >90% all meropenem-resistant isolates was >32 µg/mL, and both MIC50 and MIC90 were assessed > 32 µg/mL.

Table2. Antimicrobial susceptibility of *P. aeruginosa* isolates

Antibiotic	Total (N = 120) No. (%)		
	R	I	S
Meropenem	30 (25)	2 (1.7)	88 (73.3)
Imipenem	29 (24.2)	6 (5)	85 (70.8)
Aztreonam	13 (10.9)	19 (15.8)	88 (73.3)
Piperacillin-tazobactam	14 (11.7)	14 (11.7)	92 (76.6)
Ticarcillin-clavulanate	72 (60)	8 (6.7)	40 (33.3)
Ceftazidime	23 (19.2)	1 (0.8)	96 (80)
Gentamicin	24 (20)	6 (5)	90 (75)
Amikacin	12 (10)	11 (9.2)	97 (80.8)
Ciprofloxacin	27 (22.5)	0	93 (77.5)

Abbreviations: R: resistant; I: intermediate-resistant; S: susceptible

Specific primers were used for the detection of virulence genes, including *pvdA, lasB, pilA, and pilB* according to the references listed in Table1. The most prevalent virulence gene was *lasB* (98.3%), followed by *pvdA* (68.3%) (Figure 1). The frequency of *pilA* and *pilB* genes that is related to type IV pili, was lower than other genes of study (Table3). The *pilB* gene did not determine from blood and wound specimens. However, it seems that *lasB* and *pvdA* genes are less likely to occur in these samples. Coexistence of virulence factors was observed among majority of isolates. There were 7 different virulence profiles of *P. aeruginosa* isolates with *lasB-pvdA* and *lasB* being the most common, while the rarest were *pilA-lasB*, and *pvdA* (Table 4).

Table3: Distribution of genotypic virulence determinants of *P. aeruginosa* according to the type of specimens

Samples (N)	*pilA* No. (%)	*pilB* No. (%)	*pvdA* No. (%)	*lasB* No. (%)
Pulmonary (52)	3 (5.8)	4 (7.7)	35 (67.3)	51 (98.1)
Urine (28)	2 (7.1)	2 (7.1)	21 (75)	28 (100)
Blood (17)	2 (11.8)	0	12 (70.6)	17 (100)
Wound (14)	3 (21.4)	0	6 (42.9)	14 (100)
CSF (1)	0	0	1 (100)	1 (100)
Ear (1)	0	0	1 (100)	1 (100)
Eye (7)	0	0	6 (85.7)	6 (85.7)
Total (120)	10 (8.3)	6 (5)	82 (68.3)	118 (98.3)

Table 4. The coexistence pattern of virulence genes

Pattern	Frequency	Percent
lasB	35	29.2
lasB-pvdA	67	55.8
pilA-lasB	2	1.7
pilA-lasB-pvdA	8	6.7
pilB-lasB	1	.8
pilB-lasB-pvdA	5	4.2
pvdA	2	1.7
Total	120	100.0

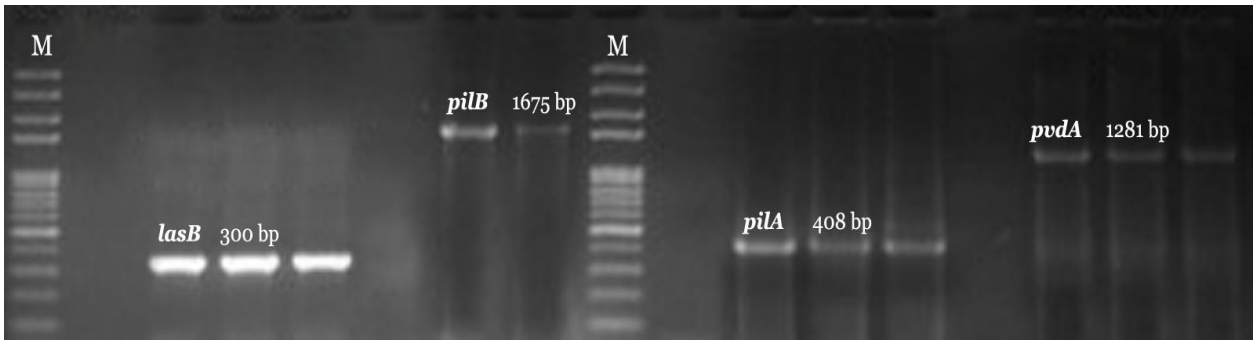

Figure1: Agarose gel electrophoresis of PCR products for *pilA, pilB, pvdA,* and *lasB* genes. M: 100 bp DNA size marker

DISCUSSION

Healthcare-associated infections (HAIs) are the worldwide public health problem that medical centers usually struggle with both in developing and industrial countries (21, 22). *P. aeruginosa* is frequently responsible for a wide variety of nosocomial infections (23, 24). Nosocomial infections are often difficult to treat due to naturally resistant to many antibiotics and have a remarkable capacity for acquiring new resistance mechanisms under selective pressures from antibiotics, creating increased therapeutic problems (22, 25, 26). In according to studies in different provinces of Iran the prevalence of MDR *P. aeruginosa* is high (58%) that is of paramount concern because these isolates are simultaneously resistant against multiple antibiotics (27). We carried this study because the knowledge of the current antimicrobial profile is necessary for the selection of appropriate empirical treatment of these infections and control of *P. aeruginosa* in hospitals is essential.

Empirical therapy protocol for *P. aeruginosa* infections in our region is combination of this antibiotics: piperacillin, imipenem, meropenem, ciprofloxacin, ceftazidime, aztreonam. With attention to antibiotic resistance pattern in present study, this protocol can still be used to treat of patients but should be used with precaution. The highest resistance rate in the present study was like to the previous reports from Iran and other countries (28-33) were seen against β-lactam antibiotics. These high rates in our study can be described by the fact that acquisition of resistance genes [e.g., those encoding β-lactamases (34)] via horizontal gene transfer can drive antimicrobial/multidrug resistance development in *P. aeruginosa* (35). Imipenem and meropenem as carbapenems are the most effective antibiotics for the treatment of infections caused by MDR strains of *P. aeruginosa*, but extensive clinical use of carbapenems has caused an increase in carbapenem resistance (36). In our study the remarkable rate of resistant to carbapenem was observed. The results of our study showed that considerable numbers of isolates were susceptible to aminoglycosides.

The same findings have been reported previously in different parts of country that showed aminoglycosides as one the most effective antibiotics against clinical isolates of *P. aeruginosa* (30, 31, 37).

P. aeruginosa harbors a diversity of virulence factors that help in invasion and damage to host tissues including type IV pili, elastase, and pyoverdin (21, 23). In agreement with other reports (28, 32, 38, 39), the prevalence of *lasB* gene in present study was very high (98.3%). This enzyme was found in isolates from all infectious sites with variable rates. Also, the high rate of this virulence factor at the pulmonary samples (98.1%) may be an indication of this enzyme in the progress of lung diseases.

A study from Romania (40) reported a 55% prevalence of *pvdA* gene in isolates from blood and wound samples whereas in our study the highest prevalence of this gene regard to sample size was from urine, blood, and pulmonary. PvdA, an important enzyme involved in the synthesis of siderophore pyoverdine, which is a valuable molecule to enhance iron uptake in poor or no available iron environment, as mammalian serum (40). Also, pyoverdine-mediated iron transport be important for biofilm development and bacterial virulence of respiratory isolates (41). Other studies from Iran reported a low prevalence (24.5%, 9.8%) of *pvdA* gene from *P. aeruginosa* samples (42, 43). In agreement with our study, two previous studies reported a low prevalence of *pilA* (34.3%, 16.1%) and *pilB* (17.6%, 9.1%) genes among Iranian patients (42, 43). PilA (the major pilin subunit) and PilB (type IV fimbrial biogenesis protein) are the most important components of type IV pili in *P. aeruginosa* that involved in adherence, motility, competence for DNA uptake, and pathogenesis (44). In contrast to our findings, the frequency of these genes was higher in two studies from Africa and Iraq with frequencies 70.2% and 91.8%, respectively (38, 45) indicating the pili genes differ depending on geographic regions, isolate source and patient setting. In Elogne and our studies**,** the high frequency of *las*B gene was observed in isolates from pulmonary samples which represents the importance of this factor in development of pneumonia and increasing the risk of mortality (38).

CONCLUSION

Based on the results, it can be concluded that the most tested isolates possessed *lasB* and *pvdA* genes as a virulence factor. The pathogenicity of *P. aeruginosa* is multifactorial involving both secreted and cell-associated bacterial products such as elastase, type IV pili and pyoverdin. These bacteria express different virulence genes from different infection sites that might be the cause of the development of the resistance pattern in *P. aeruginosa*. Thus, suitable surveillance and control processes are critical to prevent the further spread of these isolates in hospitals.

Conflict of interest

The authors declare that they have no conflicts of interest.

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