Original Article

Detection of Exotoxins and Antimicrobial Susceptibility Pattern in Clinical *Pseudomonas aeruginosa* Isolates

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Abstract

Background: *Pseudomonas aeruginosa* is a common opportunistic pathogen that causes nosocomial infection in immunocompromised patients. Among different virulence factors, the type III secretion system (TTSS) is an important agent in virulence and development of antimicrobial resistance in *P. aeruginosa*. Previous studies have shown that production of TTSS proteins was correlated with increasing virulence and resistance to several antibiotics. In this study, the exotoxins genes (*exoU* and *exoS*) and pattern of antimicrobial susceptibility in clinical *P. aeruginosa* isolates were determined.

Methods: A total of 175 *P. aeruginosa* isolates were collected from patients hospitalized in Shahrekord and Chamran educational hospitals of Isfahan, Iran (during April to December 2015). Antimicrobial susceptibility test was performed by disk diffusion test. The presence of exotoxin genes was detected using multiplex polymerase chain reaction (PCR) of *exoU* and *exoS* genes.

Results: The antibiotic resistance rate was higher than 70% to many antibiotics. The highest rates of resistance (155 and 148) were related to Levofloxacin (88.6%) and Meropenem (84.6%), respectively. The *exoU* gene was found in 75 (42.9%) isolates and 136 (77.7%) isolates carried the *exoS*. In addition, 36 (20.6%) of the isolates carried both genes. A statistical significance was observed between the presence of *exoU* gene and resistance to piperacillin (*P* = 0.01).

Conclusions: The result of this study showed a high resistance rate to the most antibiotic classes and a specific relationship between the virulence genotype and antimicrobial resistance especially more virulent genotype of *exoU$^+$*. In order to prevent the spread of more virulent strains in health care facilities, molecular methods alongside antimicrobial susceptibility tests are suggested.

Keywords: Virulence factors, Genotype, Type III secretion systems, *Pseudomonas Aeruginosa*

Background

Pseudomonas aeruginosa is a gram-negative and an opportunistic pathogen which grows in minimal nutritional requirements and a wide range of temperature. It can grow on most surfaces, especially moist surfaces such as medical devices and skin (1,2). *P. aeruginosa* in weakened and immunosuppressed patients, who are with third- degree burns, cystic fibrosis (CF), wounds, indwelling catheter, and prolonged duration of ventilation, can cause nosocomial diseases (3). Multiple factors are involved in pathogenicity of *P. aeruginosa*. The type III secretion system (TTSS) which has been known to be a major virulence, is determined in pathogenesis of acute infection, bacteremia, sepsis, and subsequent mortality. The TTSS allows the injection of toxins into the cytosol of target eukaryotic cells, where they destroy host cell defense and signaling systems and subsequently rapid call necrosis or modulating the actin cytoskeleton (4-6). Four effector proteins have been identified: ExoU,

a phospholipase which has been characterized as a major virulence factor in acute lung injury, ExoY, which is an adenylate cyclase, and ExoS as well as ExoT which are bifunctional proteins (5,6). ExoU and ExoS are variably present and are important in pathogenesis, whereas almost all of the isolates encode ExoT and ExoY and have a minor effect on virulence (4,5). Previous studies have shown that production of ExoU was correlated with increasing virulence (7). Similarly, in other studies, it was found that infected patients with TTSS$^+$ isolates show more severe infections and the mortality rate of this patients in first 30 days of infection is high (3,8). The existence of many agents in *P. aeruginosa* leads to intrinsic resistance to many antimicrobials including bacterium's outer membrane barrier, the presence of multi-drug efflux transporters, and endogenous antimicrobial inactivation. All of these agents, as well as inappropriate chemotherapy and lagging in antibiotic discovery caused "antibiotic resistance crisis" (6,9). Previous studies demonstrated

that inappropriate chemotherapy leads to the emergence of multi-drug resistant isolates (10), especially in burn patients; the rate of resistance to most of antibiotics for *P. aeruginosa* isolated was reported higher than 70% (11), however, the ExoU$^+$ isolates were more resistance to fluoroquinolones (12).

Objectives

The aim of this study was to identify antimicrobial susceptibility pattern and to characterize the presence of *exoS* and *exoU* genes in clinically isolated *P. aeruginosa* strains. An improved understanding of these virulence factors is important for selection of cure pathway and suitable antibiotic.

Methods

Isolation and Identification of Bacteria

In this descriptive study which was approved by the ethics committee of Shahrekord University of Medical sciences (research project number 2450), a total of 175 non-replicated *P. aeruginosa* isolates were collected (during April to December 2015) from teaching hospitals of Shahrekord University of Medical Sciences and Chamran hospital of Isfahan, Iran. These isolates were derived from routine diagnostic tasks and different specimens including wound, blood, urine, specimens respiratory, burn wound, cerebrospinal fluid, etc. After transporting the isolates to Shahrekord University of Medical Microbiology Laboratory, they were identified as *P. aeruginosa* using conventional methods (gram staining, catalase, oxidase, non-fermentation in oxidation fermentation (OF) test and pigment production) (13).

Antibiotic Susceptibility Test

Antibiotic susceptibility testing of the isolates was performed using Kirby-Bauer disk diffusion method according to Clinical and Laboratory Standard Institute guideline (CLSI) (14). The tested antimicrobial agents were as follows: Imipenem (10 μg), meropenem (10 μg), doripenem (10 μg), levofloxacin (5 μg), ciprofloxacin (5 μg), ceftazidime (30 μg), cefepime (30 μg), colistin (10 μg), polymyxin B (30 μg), gentamicin (10 μg), amikacin (30 μg), tobramycin (10 μg), piperacillin (100 μg), piperacillin /tazobactam (100/10 μg), and aztreonam (30 μg) (MAST, Group Ltd, Merseyside, UK). *Escherichia coli*:

ATCC25922 and *Pseudomonas aeruginosa*: ATCC27853 were used as quality control (CLSI, 2015).

DNA Extraction

DNA extraction was carried out through boiling method with some modifications. An aliquot of 1 mL of the culture grown overnight at 37°C in 100 μL of TEA (Tris EDTA Acid Acetic) buffer was boiled for 10 minutes at 100°C and immediately placed in -20˚C for 10 minutes. Following the centrifugation of bacterial suspensions at 13000× g at 4°C for 10 minutes, the supernatant was collected and DNA template was used for polymerase chain reaction (PCR) (15).

Virulence Genotyping Test Using Multiplex PCR

In order to identify *exoU* and *exoS* genes in *P. aeruginosa* isolates, multiplex PCR (MPCR) was performed. Two sets of primers were used the details of which are provided in Table 1 (synthesized by Bioneer, Inc. Seoul, South Korea). MPCR mixture consisted of 1X reaction buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0]), 2 mM MgCl2, 200 μM concentration of each of 4 deoxyribonucleoside triphosphates (dNTPs) (Sina Clon Bio Science Co. Tehran, Iran), 0.4 μM primers, 5 U of Taq DNA-polymerase (Sina Clon Bio Science Co. Tehran, Iran), and 50 ng DNA. Amplification was carried out in a T100^{TM} Thermal Cycler (Bio-Rad Laboratory, Inc. USA) (16). After an initial denaturation step for 5 minutes at 94°C, 30 cycles of amplification were performed as follows: 94°C for 30 seconds, 59°C for 45 seconds, and 72°C for 45 seconds. The reaction was completed with a final extension at 72°C for 10 minutes. The amplified products were analyzed by 1.5% agarose gel electrophoresis which was visualized on an ultraviolet illumination.

Statistical Package for Social Sciences (SPSS) software, version 22 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Fisher exact test or chi-squared test was performed in order to analyze categorical data. A *P* value of <0.05 was considered statistically significant.

Results

The results of antimicrobial susceptibility testing are shown in Table 2. All of the isolates were susceptible to colistin and polymyxin B and most of them were resistant to levofloxacin (155, 88.6%) and Meropenem

Table 1. Oligonucleotide Primers

Name	Primer Sequence	References	Size of the Amplicon, bp
ExoU-F[a]	5′-AGC GTT AGC GAC GTG CGT TTC A-3′	This study	404[c]
ExoU-R[b]	5′-GCG CCG ATC TCG CTG CTA ATG T-3′		
ExoS-F	5′-TTC GGG CAG GGC ACG ATA TCC A-3′	This study	240
ExoS-R	5′-TTC CGG TTT GCT TGC CAG GTC G-3′		

[a] Forward primer.
[b] Reverse primer.
[c] Base pair.

(148, 84.6%). The highest susceptibility was related to piperacillin/tazobactam (59, 33.7%) and amikacin (54, 30.9), respectively. In order to detect *exoS* and *exoU* genes, MPCR was run for all isolates. Gel electrophoresis results of a number of isolates are shown in Figure 1. All of the 175 clinical isolates carried *exoS*, *exoU*, and *exoU/exoS*. The *exoU* gene was found in 75 (42.9%) isolates and 136 (77.7%) of isolates had *exoS* gene. Furthermore, 36 (20.6%) of the isolates carried both of the genes. Statistical analysis showed a significant difference between the presence of *exoU* gene and resistance to piperacillin ($P=0.01$). However, no significant difference was observed between the presence of exotoxin genes and other antibiotics' resistance. Frequency of $exoU^+$ and $exoU^-$ among piperacillin-resistant and non-resistant isolates is shown in Figure 2.

Discussion

Among all of the pathogenic bacteria, *P. aeruginosa* contributes to 11%of all nosocomial infections (17). The clinical *P. aeruginosa* isolates that secret type III proteins (ExoU and ExoS) show more antimicrobial resistance and associate with increased rates of mortality and morbidity (3).

In the present study, all of the isolates demonstrated at least one of the exotoxin and the prevalence of $exoU^+/exoS^-$, $exoU^-/exoS^+$ and $exoU^+/exoS^+$ clinical isolates were totally 42.9%, 77.7% and 20.6%; respectively. In several studies having been conducted on *P. aeruginosa* isolates, the presence of exotoxin gene has been reported differently. In a study by Cho et al, 66 carbapenem-
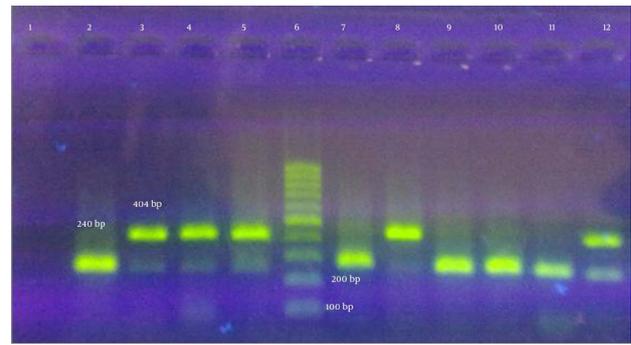

Figure 1. Gel Electrophoresis Results of *exoU* Gene (404 bp) and *exoS* Gene (240 bp). Lane 1: Negative control. Lane 6: 100 bp DNA ladder. Lanes 2, 7, 9, 10 and 11: Isolates with *exoS* gene. Lanes 3, 4, 5 and 8: Isolates with *exoU* gene. Lane 12: Positive control.
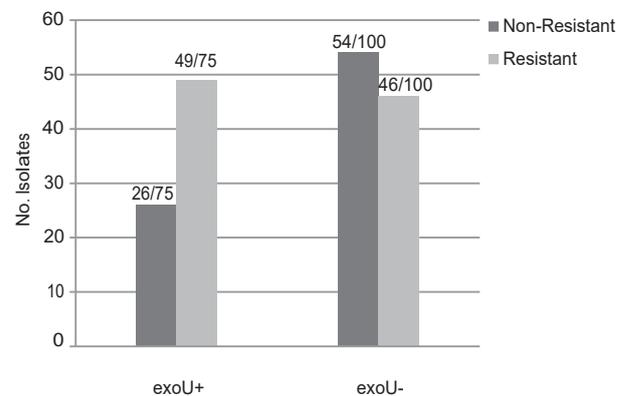

Figure 2. Frequency of $exoU^+$ and $exoU^-$ Among Piperacillin-Resistant and Non-resistant Isolates.

resistant *P. aeruginosa* isolates from different clinical source were investigated and the prevalence of *exoU*, *exoS*, and *exoU/exoS* genes were reported 66.7%, 30.3%, and 3%; respectively (5). These findings are contrary to our results. Similarly, in a study conducted by Ferreira et al, all of the 32 *P. aeroginosa* isolates from patients with bacteremia and ventilator associated pneumonia (VAP) carried *exoS* gene. The *exoU* gene was observed only in 9.4% of strains and three isolates were positive for the two effector genes of *exoU* and *exoS* (9.4 %). (10). In another study by Mitove and colleagues on *P. aeruginosa* isolated from patients with CF and nosocomial infections, the prevalence of *exoS* and *exoU* have been reported 62.4 and 30.2%; respectively (18). Similar to our results, in this study the prevalence of exoU-/exoS+ isolates was more than the $exoU^+/exoS^-$ isolates. However, in our study the prevalence of $exoU^+/exoS^-$ was higher than those of the above-mentioned study. In the study by Firuzi-Dalvand et al, the prevalence of *exoU* and *exoS* genes in burn wound *P. aeruginosa* isolates were reported 76% and 68%; respectively (19). In another study carried out by Agnello et al, the prevalence of *exoU*, *exoS* , *exoU/exoS* or other exotoxin genes were 38%, 56% and 8%, respectively (4).

Table 2. The Results of Antimicrobial Susceptibility Testing in *Pseudomonas aeruginosa* Clinical Isolates[a]

Antibiotics	Sensitive	Intermediate	Resistance
Imipenem, 10 μg	25 (14.3)	7 (4.0)	143 (81.7)
Meropenem, 10 μg	21 (12.0)	6 (3.4)	148 (84.6)
Doripenem, 10 μg	29 (16.6)	10 (5.7)	136 (77.7)
Levofloxacin, 5 μg	16 (9.1)	4 (2.3)	155 (88.6)
Ciprofloxacin, 5 μg	26 (14.9)	6 (3.4)	143 (81.7)
Ceftazidime, 30 μg	35 (20.0)	1 (0.6)	139 (79.4)
Cefepime, 30 μg	27 (15.4)	9 (5.1)	139 (79.4)
Colistin, 10 μg	175 (100)	0 (0.0)	0 (0.0)
Polymyxin B, 30 μg	175 (100)	0 (0.0)	0 (0.0)
Gentamicin, 10μg	30 (17.2)	6 (3.4)	139 (79.4)
Amikacin, 30 μg	54 (30.9)	13 (7.4)	108 (61.7)
Tobramycin, 10 μg	33 (18.9)	1 (0.6)	141 (80.6)
Piperacillin, 100 μg	29 (16.6)	51 (29.1)	95 (54.3)
Piperacillin /Tazobactam, 100/10 μg	59 (33.7)	46 (26.3)	70 (40.0)
Aztreonam, 30 μg	28 (16.0)	18 (10.3)	129 (73.7)

[a] Values are expressed as No. (%).

Jabalameli et al reported that the frequency of *exoS*, *exoU* and *exoU/exoS* in burn isolates was 29%, 64.5% and 1%, respectively (20), which their findings were in accordance with ours. In a survey carried out by Lakshmi Priya et al, all of the isolates carried one of the *exoU*, *exoS* or *exoU/exoS* genes. The frequency of *exoS* and *exoU* genes has been detected 64% and 23%; respectively (21). Garey et al in a survey on *P. aeruginosa* isolates from patients with bacteremia reported that the prevalence of *exoS*, *exoU* and *exoU/exoS* were 70.5%, 24.5% and 1.6%, respectively (22). Wong-Beringer et al found that the prevalence of *exoS* and *exoU* genes in clinical isolates were 62% and 27%, respectively. These findings were similar to our results (12). According to the mentioned studies and compared to the results of the present study, the observed differences in the prevalence of *exoS* and *exoU* genes may be due to the diversity in the type of specimen, strain types, and geographical area of survey. Notably, in our study the percentage of *exoU$^+$/exoS$^+$* isolates were relatively high (20.6%) compared to other studies. Even though *exoS* and *exoU* genes were located in different location in *P. aeruginosa* genome, the simultaneous carriage of both genes has been reported less (23). A possible explanation is that both *ExoU* and *ExoS* proteins are important for *P. aeruginosa* survival. However, each of them shows different activity in a specific environment and situation (23,24). Therefore, the exotoxins genotype of a clinical isolate may be an indicator of particular environmental reservoir of that isolate.

Many cases of infections resulted from *P. aeruginosa* were hardly cured; besides, inappropriate chemotherapy caused resistance to many of the antibiotics (24,25). The extensive use of antibiotics such as fluoroquinolones can be effective in creating the multi-drug resistant isolates. In addition, previous studies indicated that the presence of the TTSS effector genotypes, especially *exoU* genotype, correlate with increasing the virulence and resistance to one or more antibiotics. In a study by Agnello et al, it was revealed that the presence of *exoU* gene was significantly associated with fluoroquinolones-resistance (4). Wong-Beringer et al also found that 92% of *exoU$^+$* and 61% of *exoS$^+$* isolates were resistance to fluoroquinolones (12). In another study by Cho et al, a higher ratio of *exoU$^+$* strains was fluoroquinolones-resistant than *exoS$^+$* strains ($P \leq 0.0001$) (5). In surveys conducted by Maatallah et al and Pena et al, the presence of *exoU* gene was significantly correlated with multidrug resistance and ciprofloxacin resistance (23, 26). In present study, 81.7% and 88.6% of isolates were resistant to ciprofloxacin and levofloxacin; respectively. On the contrary to other studies, there was no correlation between the resistance to fluoroquinolones and specific virulence genotype. Although, resistance to piperacillin significantly was associated with presence of *exoU* gene ($P = 0.01$). One reason for this difference may be the variation of sample type but further studies should

be conducted in order to find out such a difference.

The present study has some limitations such as single center study with small sample and lack of funds for more experiments including clonality analysis. However, since the study on distribution of TTSS effector genotypes and antibiotic resistance in our region is very few, further studies, like our study, are required in this respect.

Conclusion
Generally, the recent overuse of effective antipseudomonal antibiotics has led to increased resistance in clinical *P. aeruginosa* isolates. The present results indicate a specific relationship between the presence of exotoxin genes and antibiotic resistance. In order to prevent the spread of more virulent strains in health care facilities, molecular assessment in addition to antimicrobial susceptibility tests are suggested.

Authors' Contribution
SMM contributed to sample collection and experiments. SR contributed to study design. FD analyzed the data. SMM and SR wrote the paper.

Conflicting Interests
The authors have no conflict of interest.

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