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Molecular investigation of *exoU* and *exoY* virulence genes in *Pseudomonas aeruginosa* collected from hospitalized patients in North of Iran: A descriptive-analytical study

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ABSTRACT

Objective: To investigate the frequency of *exoU* and *exoY* genes in patients with *Pseudomonas aeruginosa* infection.

Methods: In this study, 100 clinical isolates of *Pseudomonas aeruginosa* were collected from patients hospitalized in educational-therapeutic hospitals and were identified using standard microbiological tests. Then, the antibiotic resistance pattern of the isolates was determined by the disk agar diffusion method. The bacterial DNAs were extracted by the alkaline lysis method. Finally, the presence of *exoU* and *exoY* genes was evaluated by the PCR test.

Results: In this study, 47%, 72%, 29%, 39%, 40%, and 44% of the isolates were non-susceptible to piperacillin, aztreonam, ceftazidime, imipenem, tobramycin, and ciprofloxacin, respectively. In addition, 95% and 93% of the clinical isolates carried the *exoU* and *exoY* genes. Blood and fecal isolates had both virulence genes, while only one wound isolate had neither genes. Meanwhile, all urinary isolates contained the *exoY* gene and only one isolate lacked the *exoU* gene. Also, 88 isolates simultaneously had both *exoU* and *exoY* genes.

Conclusions: High prevalence of *exoU* and *exoY* genes in this region indicates a significant role of type Ⅲ secretion system in pathogenesis of *Pseudomonas aeruginosa*. The type Ⅲ secretion system may be a suitable target to reduce the pathogenicity of this bacterium.

KEYWORDS: *Pseudomonas aeruginosa*; *exoU*; *exoY*; Virulence gene; Type Ⅲ secretion system; PCR

1. Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic multidrug-resistant (MDR) pathogen causing life-threatening infections in immunocompromised patients[1]. Growth of *P. aeruginosa* in diverse environments, such as hot tubs, humidifiers, soil, respirators, sinks, contaminated patients, and disinfectants, increases the prevalence of infections[2]. Due to the high intrinsic

Significance

Induction of cytotoxicity by the type Ⅲ secretion system is an important pathogenicity strategy of *Pseudomonas aeruginosa*. ExoS, ExoU, ExoY, and ExoT can induce various forms of programmed death in host cells. This study showed that the high prevalence of ExoU and ExoY in this region can be a target for therapy or prevention of pathogenesis.

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and acquired resistance to current antibiotics and the severity of infections caused by *P. aeruginosa*, this organism is grouped as the critical pathogen[3]. The versatility of this organism to infect a wide range of hosts is due to the production of various virulence factors, mediating host colonization and dissemination, protection against the host immune system, and/or exacerbation of epithelial damage[4]. Inducing cytotoxicity in target cells is one of the most significant strategies of this bacterium in pathogenesis. This organism produces many cytotoxins to induce different forms of programmed cell death[5]. Apoptosis, pyroptosis, and necroptosis are major types of programmed cell death in eukaryotic cells[5]. Apoptosis is an important process for maintaining human health, and its dysregulation leads to dangerous complications such as disorders in the immune system and infection control[6]. *P. aeruginosa* produces some cytotoxins leading to the apoptosis of host cells, which may be beneficial to the bacteria due to their anti-inflammatory and immunosuppressive nature[5]. Exotoxin A is an important toxin secreted by the type Ⅱ secretion system that stops the protein synthesis of the host cells and causes apoptotic cell death[7]. Azorin is another toxin inducing the apoptosis and programmed death of macrophages[5]. Its increased expression has been observed in *P. aeruginosa* isolated from the lungs of cystic fibrosis patients[8]. Type 3 secretion system (T3SS) mediated exotoxins are also associated with the host cell membrane and induce the apoptosis. Four most significant cytotoxins secreted by this system are ExoS, ExoU, ExoY, and ExoT[9]. ExoU is a 74 kDa (687 amino acids) soluble protein and a potent inducer of cytotoxicity in eukaryotic host cells[10]. Entry of ExoU into mammalian cells via the T3SS leads to rapid cell necroptosis due to the phospholipase activity of this toxin[11]. Studies have shown that the ExoU can cause the exacerbation of respiratory distress syndrome[12], suppression of the local immune system[13], and a decrease in the production of inflammatory cytokines[14] in animal models. ExoY is a 42 kDa nucleotidyl cyclase that increases the accumulation of cyclic nucleotide monophosphates in host cells[15]. ExoY reduces the production of inflammatory cytokines[16]. This protein was recently identified in 93% of *P. aeruginosa* clinical isolates in patients with malignant pneumonia and end-organ dysfunction[17]. Another study has shown that infection with ExoY-expressing *P. aeruginosa* can increase apoptosis in the lung cells of infected mice[18]. *P. aeruginosa* is an opportunistic pathogen causing hospital and community-acquired infections with fatal effects on the host by producing T3SS-mediated toxins. We must know the prevalence of the genes encoding these virulence factors. This study aimed to investigate the prevalence *exoU* and *exoY* genes in this region.

2. Patients and methods

2.1. Ethical approval statements

A written informed agreement form was supplied by the contributors. The categorizing data of patients was reserved undisclosed. This study was conducted conferring to the Declarations of Helsinki. Moreover, this study was approved by Iran National Committee for Ethics in Biomedical Research with IR.MAZUMS.REC.1397.368 ethical code.

2.2. Study design

This descriptive-analytical study was carried out on the clinical strains of *P. aeruginosa* isolated from patients admitted to the educational-therapeutic hospitals affiliated with the Mazandaran University of Medical Sciences in North of Iran. The isolates were collected from September 2022 to March 2023. The standard microbiological and biochemical tests, such as gram staining, oxidase test, growth at 42 ˚C, the ability to produce pigment, no fermentation of sugars in Triple Sugar Iron agar (Condalab, Spain), consumption of citrate, oxidation of glucose in Oxidation/Fermentation culture medium (Condalab), growth on cetrimide agar (Condalab), and colony odor, were used to identify the *P. aeruginosa* isolates[19]. To evaluate the antibiotic susceptibility pattern of the isolates, the disk agar diffusion method was used based on the guidelines of the Clinical and Laboratory Standards Institute[20]. This test was performed on Mueller Hinton agar (Condalab) after preparing turbidity equal to 0.5 McFarland (1.5×10^8 cfu/mL) and inoculating it on the surface of the culture medium. The following antibiotic disks (MAST, UK) were used for this test: piperacillin (100 μg), aztreonam (30 μg), ceftazidime (30 μg), imipenem (10 μg), tobramycin (10 μg), and ciprofloxacin (5 μg).

Then, the alkaline lysis method was used to extract the DNAs of the bacteria, as previously reported[21]. First, a cell membrane lysing buffer was obtained by adding 0.5 g of sodium dodecyl sulfate (Sigma, Germany) and 0.4 g of sodium hydroxide in 200 μL of sterile distilled water. Then, some 24-hour pure colonies of the desired bacteria were added to 20 μL of this lysis buffer in a sterile 1.5 mL microtube. In the next step, the microtubes were incubated at 95°C for 10 min and then centrifuged at 13 000 *g* for 3 min. Then, 180 μL of sterile distilled water was added to the microtubes and used as the extracted DNA. Before using the extracted DNAs, their optical density (OD) was measured with a NanoDrop spectrophotometer (ND 1000, USA). Also, the extracted DNAs were electrophoresed on 1.5% agarose gel (Sigma) to observe the DNA bands. Then, the extracted DNAs were stored in a –20 °C freezer to be used for subsequent tests.

The PCR method was used to identify the presence of the *exoU* and *exoY* genes. The primers (*exoY*-forward: 5′-CGGATTCTATGGCAGGGAGG-3′ and *exoY*-reverse:

5′-GCCCTTGATGCACTCGACCA-3′, and *exoU*-forward: 5′-CCGTTGTGGTGCCGTTGAAG-3′, and *exoU*-reverse: 5′-CCAGATGTTCACCGACTCGC-3′) were selected from a previous study[22], blasted on the NCBI website, and sent for synthesis (Metabion, Germany). The initial setup of the PCR test was done using the control strains carrying the desired genes. In this study, the annealing temperature for both *exoU* and *exoY* genes was 60 °C. The final volume for both PCR reactions was set to 15 μL containing 7.5 μL of PCR Master Mix (Ampliqon, Denmark). Also, 5 pmol of each forward and reverse primer, and 300 ng of the template DNA were used in each reaction. The PCR conditions were the same for *exoU* and *exoY* genes. A denaturation step was first performed for 5 min at 94 °C. Then, 36 cycles of PCR were done, including a denaturation step at 94 °C for 30 s, an annealing step at 60 °C for 30 s, and an extension step at 72 °C for 45 s. Then, a final amplification step was performed at 72 °C for 10 min. Then, the PCR products were electrophoresed on a 1% agarose gel (Sigma) containing the Safe Stain (SinaClon, Iran).

2.3. Statistical analysis

Data were analyzed using SPSS 22 software. After checking the data and variables for normality, the frequency of the studied variables was determined using descriptive tests. Also, a Chi-square statistical test was used to compare variables, and a P-value<0.05 was considered statistically significant.

3. Results

In this study, 100 non-replicated clinical isolates of *P. aeruginosa* were collected from 100 patients hospitalized in educational-therapeutic hospitals. All patients were potentially eligible due to the positive results of bacterial culture and detection of *P. aeruginosa* in the laboratory of hospitals. In addition, all bacterial isolates were confirmed again by our team. The studied patients included 60 men and 40 women, and the average age of the patients was 47 years. The strains were isolated from different clinical samples, including

Table 1. Number of *Pseudomonas aeruginosa* clinical isolates carrying virulence genes based on the type of clinical sample (n=100, n, %).

Clinical samples	*exoU+/exoY+* (n=90)	*exoU+/exoY−* (n=5)	*exoU−/exoY+* (n=3)	*exoU−/exoY−* (n=2)
Urine (n=26)	25 (27.8)	-	1	-
Respiratory (n=37)	34 (37.8)	-	1	2
Wound (n=20)	18 (20.0)	1	1	-
Catheter (n=8)	5 (5.6)	3	-	-
Blood (n=5)	5 (5.6)	-	-	-
Stool (n=2)	1 (1.1)	1	-	-
Eye (n=2)	2 (2.2)	-	-	-

Table 2. Frequency of *Pseudomonas aeruginosa* isolates carrying virulence genes in terms of the hospital departments (n=100, n, %).

Departments	*exoU+/exoY+* (n=91)	*exoU+/exoY−* (n=4)	*exoU−/exoY+* (n=2)	*exoU−/exoY−* (n=3)
ICU (n=53)	51 (56.0)	-	-	2
CCU (n=5)	5 (5.5)	-	-	-
Burn (n=6)	6 (6.6)	-	-	-
Internal (n=9)	7 (7.7)	-	2	-
Surgery (n=6)	3 (3.3)	3	-	-
Emergency (n=13)	12 (13.2)	-	-	1
Pediatric (n=5)	5 (5.5)	-	-	-
Oncology (n=1)	-	1	-	-
Neurology (n=2)	2 (2.2)	-	-	-

ICU: intensive care unit; CCU: Cardiac Care Unit.

Table 3. Isolates of *Pseudomonas aeruginosa* carrying virulence genes according to the treatment centers (n=100, n, %).

Treatment centers	*exoU+/exoY+* (n=89)	*exoU+/exoY−* (n=6)	*exoU−/exoY+* (n=4)	*exoU−/exoY−* (n=1)
General hospital (n=40)	35 (39.3)	4	-	1
Infectious hospital (n=22)	21 (23.6)	-	1	-
Pediatric hospital (n=17)	14 (15.7)	-	3	-
Burn hospital (n=11)	11 (12.4)	-	-	-
Heart hospital (n=10)	8 (9.0)	2	-	-

Table 4. Relationship between the presence of *exoU* and *exoY* genes and resistance to antibiotics (n=100, n, %).

Antibiotics	*exoU*+ (n=95)	*exoU*− (n=5)	χ^2	*P*	*exoY*+ (n=93)	*exoY*− (n=7)	χ^2	*P*
Piperacillin								
Susceptible	49 (51.6)	4 (80)	0.006	0.35	50 (53.8)	3 (43)	0.008	0.32
Non-susceptible	26 (27.4)	1 (20)			43 (46.2)	4 (57)		
Aztreonam								
Susceptible	26 (27.4)	2 (40)	0.001	0.65	26 (28.0)	2 (29)	0.003	0.48
Non-susceptible	69 (72.6)	3 (60)			67 (72.0)	5 (71)		
Ceftazidime								
Susceptible	66 (69.5)	5 (100)	0.006	0.34	65 (69.9)	6 (86)	0.001	0.66
Non-susceptible	29 (30.5)	0			28 (30.1)	1 (14)		
Imipenem								
Susceptible	57 (60.0)	4 (80)	0.001	0.62	55 (59.1)	6 (86)	0.006	0.36
Non-susceptible	38 (40.0)	1 (20)			38 (40.9)	1 (14)		
Tobramycin								
Susceptible	55 (57.9)	5 (100)	0.002	0.17	56 (60.2)	4 (57)	0.015	0.18
Non-susceptible	40 (42.1)	0			37 (39.8)	3 (43)		
Ciprofloxacin								
Susceptible	52 (54.7)	4 (80)	0.002	0.53	52 (55.9)	4 (57)	0.002	0.17
Non-susceptible	43 (45.3)	1 (20)			41 (44.1)	3 (43)		

respiratory (37%), urine (26%), wound (20%), catheter (8%), blood (5%), stool (2%), and eye (2%). Also, the clinical isolates were collected from different hospital wards, including Intensive Care Units (n=53), Emergency (n=13), Internal (n=9), Burn (n=6), Surgery (n=6), Cardiac care Units (n=5), Pediatric (n=5), Neurology (n=2), and Oncology (n=1).

According to the molecular analysis, 95% and 93% of the *P. aeruginosa* clinical isolates in this study carried the *exoU* and *exoY* genes, respectively (Supplementary Figure 1).

Among the 5 isolates without the *exoU* gene, 3 isolates were collected from a pediatric hospital, one from a general hospital, and one from an infectious center. In addition, 2 *exoU*-negative isolates were obtained from the internal ward, 2 from the intensive care unit (ICU), and one from the emergency department. Also, these 5 isolates were collected from 3 respiratory samples, one urine, and one wound sample. None of these 5 isolates were resistant to piperacillin, ceftazidime, and tobramycin, while only one isolate was resistant to aztreonam, imipenem, and ciprofloxacin. On the other hand, among the 7 isolates without the *exoY* gene, 5 and 2 isolates were collected from the general hospital and a heart center, respectively. In addition, 3, 2, 1, and 1 isolates were obtained from the surgery, ICU, emergency, and oncology departments, respectively. Among these 7 clinical isolates, 3, 2, 1, and 1 isolates were collected from catheter, respiratory, stool, and wound samples, respectively. Among them, 4 isolates were resistant to aztreonam, while 2 isolates were resistant to ciprofloxacin and tobramycin, and one was resistant to the rest of the tested antibiotics.

Table 1 shows the frequency of virulence genes in clinical isolates of *P. aeruginosa* collected from different clinical samples. The isolates collected from blood and stool samples had all investigated virulence genes, while among the wound isolates, only one lacked both genes. Meanwhile, all urinary isolates carried the *exoY* gene and only one isolate lacked the *exoU* gene. In general, except for the presence of *exo*Y gene in catheter isolates, no statistically significant difference was observed between the presence of desired genes and the type of clinical samples (P>0.05).

Table 2 shows the prevalence of virulence genes in terms of the hospital wards. Both studied genes were identified in all isolates collected from patients hospitalized in the Cardiac Care Unit, burn, pediatric, and neurology departments. However, only 3.77% of the ICU isolates lacked these genes. Except for the presence of the *exoY* gene in the surgery section and the *exoU* gene in the internal ward, there was no statistically significant relationship between the presence of these genes and the hospital wards (P>0.05).

In this study, 88 isolates simultaneously carried both *exoU* and *exoY* genes, while 7 isolates had only the *exoU* gene and 5 isolates were carrying only the *exoY* gene. No isolate was observed without the *exoU* or *exoY* gene. In addition, all isolates collected from the burn hospital (Zare) carried both genes, and all isolates obtained from the Infectious Disease Treatment Center (Razi) and the Pediatric Center (Bu-Ali Sina) were carrying the exoY gene. Also, all the isolates collected from the Fatemeh Al-Zahra hospital carried the *exoU* gene (Table 3). However, there was no statistically significant relationship between the frequency of the virulence genes and hospital (P>0.05).

In antibiotic susceptibility testing, 47%, 72%, 29%, 39%, 40%, and 44% of *P. aeruginosa* isolates were non-susceptible (resistant or intermediate resistant) to piperacillin, aztreonam, ceftazidime, imipenem, tobramycin, and ciprofloxacin, respectively. Also, the presence of the virulence genes was not significantly related to the resistance against the tested antibiotics. The highest frequency of the *exoU* and *exoY* genes was observed among aztreonam-resistant isolates, while the lowest frequency of the *exoU* and *exoY* genes was

observed among the piperacillin- and ceftazidime-resistant isolates, respectively (Table 4).

4. Discussion

Due to the ability to control and express a diverse range of virulence genes, *P. aeruginosa* is one of the most important opportunistic pathogens causing life-threatening infections in humans[5]. The virulence factors of this bacterium cause invasion of the host cells and interfere with the intracellular signals of the host, and as a result, serious damage is done to the cell and will cause the bacterium to escape from the host's immune responses[23]. T3SS is significant in the development of infections caused by *P. aeruginosa*[24]. However, some studies exhibited that the expression of T3SS-related toxins in isolates from chronic cystic fibrosis patients is lower than in the isolates collected from acute patients. Jain *et al*. showed that only 12% of cystic fibrosis isolates secreted at least two exotoxins, including ExoS, ExoT, and ExoU[25]. This is supported by another study, which showed that strains change from a T3SS-positive to a T3SS-negative phenotype in chronic cystic fibrosis patients[26]. The high prevalence of the studied genes in our study may be due to the sources of the isolates that were collected from hospitalized patients with acute nosocomial infections. Meanwhile, another Iranian study conducted on different clinical samples prepared from hospitalized patients reported a 38.57% frequency of the *exoU* gene[27]. In addition, the rate of antibiotic resistance in the aforementioned study was also lower than in our research[27]. Another Iranian research in Tehran showed that 73% of the isolates collected from urine samples carried the *exoU* gene[28]. However, 96.15% of the urinary isolates in our study carried this gene.

It has been detected that the production of ExoU can increase *P. aeruginosa* virulence in the murine model of acute pneumonia[29]. Interestingly, the largest number of our clinical isolates were collected from the respiratory samples, of which 91.89% carried the *exoU* gene and 94.59% contained the *exoY* gene. Of course, only the presence of the genes encoding these enzymes cannot be decisive in pathogenesis of *P. aeruginosa*. Silistre *et al*. observed that among 40 isolates carrying the *exoY* gene, 25 (62.5%) secreted active ExoY[30]. They found that the presence of ExoY can counteract the cytotoxicity of *P. aeruginosa* caused by other virulence factors, and the deletion of *exoY* can lead to increased cytotoxicity for human lung epithelial cells[30]. We also observed in our study that more than 90% of urinary, respiratory, wound, and blood isolates carried both virulence genes at the same time. On the other hand, 53% of the clinical isolates in the present study were collected from patients hospitalized in the ICU with acute infections. However, 22.96% of ICU isolates carried both genes. Yousefi-avarvand *et al*. reported a 66.7% prevalence of the *exoU* gene in clinical isolates of *P. aeruginosa*, while most of the isolates were obtained from respiratory and urine samples, and aztreonam was the least effective antibiotic[31]. Prevalence of the *exoU* and *exoY* genes in another study conducted in the northwest of Iran was 51.8% and 54.3%, respectively, while 12% of the isolates carried both genes simultaneously[32]. Also, they showed that all burn isolates contained the *exoU* gene, but 56% of them had the *exoY* gene[32]. However, all burn isolates in our study carry both *exoU* and *exoY* genes. ExoY has an adenylate cyclase activity[15], and ExoU has a phospholipase activity and disrupts eukaryotic membranes[33]. Therefore, the presence of these genes in the isolates obtained from burn patients can indicate their importance in the pathogenicity of the burn isolates.

The difference in the frequency of these genes in various studies could reflect the fact that the presence of genes encoding ExoY and ExoU cytotoxins in *P. aeruginosa* depends on the site of infection or the type of clinical sample. In another study conducted in Zanjan on burn wounds, 93.1% of the isolates carried the *exoY* gene, while none of the isolates had the *exoU* gene[34]. This result was inconsistent with our study, as all of our burn isolates carried both genes. A study conducted in Peru reported 100% and 22.8% prevalence of the *exoY* and *exoU* genes, respectively. They also observed a significant relationship between the presence of *exoY* and antibiotic resistance of the isolates[35]. Another study also reported the relationship between the presence of the *exoU* gene and antibiotic resistance[36]. We did not find a statistically significant relationship between antibiotic resistance and the presence of these genes, which could be due to the high prevalence of these virulence genes in our study. Although the presence of the *exoU* gene has been implicated in several infections, other studies have reported that this gene is present in 28%-42% of *P. aeruginosa* isolates causing acute infections, especially in the respiratory system[35]. Song *et al*. from China reported a 29.43% prevalence of the *exoU* gene in *P. aeruginosa* clinical isolates and found that the simultaneous presence of the *exoS* and *exoU* genes can increase the pathogenicity of *P. aeruginosa* in animal models[37]. Also, a study by Ullah *et al*. in Pakistan showed that 88.46% and 57.69% of *P. aeruginosa* collected from burn samples were carrying the *exoY* and *exoU* genes, respectively[38]. Zarei *et al*. exhibited that 27.5% of clinical and 15% of environmental *P. aeruginosa* isolates carried the *exoU* gene[39]. The pathogenesis of *P. aeruginosa* is related to the production of cellular and extracellular virulence factors, while proteins secreted by the T3SS are in the group of cell-associated factors[40].

Multiple virulence factors of *P. aeruginosa* help this organism to escape the host's immune system and cause more severe pathogenicity, especially in immunocompromised patients. T3SS enables the bacterium to inject its important exotoxins into the host cells to enhance the pathogenicity. Therefore, this organism can survive even in unfavorable hospital environments. High presence of genes encoding the exotoxins U and Y in this area is worrying.

This may be due to the exposure of bacteria under hostile hospital conditions. The lungs of infected patients, the skin of patients with burns, and the high consumption of antibiotics in medical centers can be irritants. It may be possible to reduce the pathogenicity of this organism by targeting the production of these exotoxins.

Conflict of interest statement

The authors report no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

Authors' contributions

Conceptualization: HRG; Data curation: HRG, ARM, MG, NH; Formal analysis: ARM, MG, LD; Investigation: HRG, ARM, MG, NH, LD; Methodology: HRG, ARM, NH; Project administration: HRG; Software: HRG, MG; Supervision: HRG; Validation: HRG; Visualization: HRG, ARM, MG, LD; Writing and original draft: ARM; Writing, review, and editing: HRG, ARM, MG, LD, NH.

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