Examining Exotoxin Virulence Factor and Fosfomycin Antibiotic Resistance Genes in *Pseudomonas aeruginosa* Causing Eye Infections

**ABSTRACT**

**Background:** *Pseudomonas aeruginosa* is considered as an important opportunistic bacterial pathogen associated with nosocomial infections. Therefore, it is important to identify this bacterium in clinical samples and report the results to health authorities. The aim of this study was the molecular identification of some virulence factors and fosfomycin resistance genes in *P. aeruginosa* strains.

**Materials & Methods:** A total of 100 *P. aeruginosa* strains were isolated from clinical samples of patients with eye infections in three distinct laboratories in Tehran hospitals (Pars, Milad, and Motahari). The antibiogram of all isolates against eight antibiotics was determined by standard Kirby-Bauer disk diffusion method. Then DNA was extracted from the isolates, and the frequency of *exoY*, *exoT*, *exoU*, *exoS*, *fosC*, *fosB*, and *fosA* genes was evaluated by multiplex PCR (polymerase chain reaction).

**Findings:** The highest resistance was observed to cotrimoxazole (85%), cefazidime (83%), cefotaxime (79%), and cefepime (72%), and the highest sensitivity was observed to ciprofloxacin (55%), gentamicin (52%), and piperacillin (41%), respectively. Out of 60 investigated isolates, 58 isolates were positive for *exoY*, *exoT*, and *exoU*, while only four isolates were *exoS* positive. In addition, one strain (1.66%) had the *fosC* gene, two strains (3.33%) had the *fosB* gene, and 12 strains (20.02%) had the *fosA* gene.

**Conclusion:** The results showed that the frequency of fosfomycin resistance genes, whose protein product modifies the epoxide group of fosfomycin and reduces the effectiveness of this antibiotic, was significantly low in the investigated strains.

**Keywords:** *Pseudomonas aeruginosa*, Antibiotic resistance, Fosfomycin, Exotoxin

**CITATION LINKS**

Introduction

*Pseudomonas aeruginosa* is considered as an important opportunistic bacterial pathogen commonly associated with nosocomial One of the most important human infections. features of *P. aeruginosa* is its low sensitivity to antibiotics, this feature could be attributed as well as to the function of efflux pumps the low permeability of the cell membrane in this bacterium. In addition, the inherent antibiotic resistance of *P. aeruginosa* strains could be due to the acquisition of antibiotic resistance genes through horizontal gene transfer or gene mutations (chromosomally-encoded genes) [1]. Studies have shown that the prevalence of antimicrobial-resistant *P. aeruginosa* strains is increasing globally [2]. Additionally, some isolates are resistant to several antimicrobial agents, which could be attributed to various resistance mechanisms, production of hydrolyzing enzymes, such as loss of outer membrane proteins, output systems, and target ONS [3]. According to the level of antibiotic resistance, strains could be classified as multidrug-resistant (MDR), extensively drug-resistant (XDR), and pandrug-resistant (PDR). Infections caused by these resistant isolates may be associated with increased morbidity and mortality rates, which could be due to limited effective antimicrobial options. A literature review of multidrug-resistant *P. aeruginosa* showed a wide variety of descriptions for MDR. The lack of a particular description for multidrug-resistance in comparison of clinical studies makes data problematic [4]. Furthermore, the true incidence rate of multidrug resistant strains could not be well characterized. Nonetheless, in most research studies, MDR has been described as resistance to at least three antimicrobial agents belonging to different antibiotic classes, including glycosides, antipseudomonal penicillins, cephalosporins, especially fluoroquinolones and amino carbapenems [5, 11].

A number of international authorities in 2011 decided to develop a standard definition for acquired antibiotic resistance profiles of bacterial agents causing multidrug-resistant diseases in healthcare systems. Accordingly, MDR is defined as non-susceptibility to at least three antibiotics belonging to different antibiotic categories, XDR is defined as non-susceptibility to at least one antibiotic in all but ≤2 antibiotic categories, and PDR is described as non-sensitivity to all antibiotics in all antibiotic classes [6]. Fosfomycin is an antimicrobial agent inhibiting cell wall bio-synthesis through inhibiting the MurA enzyme (UDP-N-acetylgalactosamine-1-carboxyvinyltransferase), this enzyme is known to catalyze the first phase of bacterial cell wall peptidoglycan bio-synthesis [7]. The main mechanism described for in *P. aeruginosa* fosfomycin resistance strains is loss-of-function mutations in glpT (glycerol-3-phosphate transporter), which is known as the only fosfomycin transporter in these bacteria [8]. Moreover, an increase in the expression level of MurA-encoding gene, of fosfomycin, and its mutations as the target have been reported to be responsible for fosfomycin resistance in some species like *Chlamydia* spp., *Borrelia burgdorferi*, and *Mycobacterium tuberculosis* [9]. Furthermore, the expression of *FosA*, which encodes an enzyme inactivating fosfomycin, along with the activity of an alternative peptidoglycan-recycling pathway is able to bypass MurA in the conversion of cell wall products. Moreover, *FosA* plays an important role in the inherent fosfomycin resistance of *P. aeruginosa* strains [9-10]. Also, elimination of genes involved in the peptidoglycan recycling pathway has been reported to improve sensitivity of *P. aeruginosa* strains to fosfomycin [12].

**Objectives:** Since most of the studies conducted on multidrug resistant *P. aeruginosa*...
aeruginosa in Iran have not investigated these mechanisms, and there is no study on extensively drug-resistant P. aeruginosa isolates, the present study aimed to identify these phenotypes in P. aeruginosa isolates collected from people with eye infections in Tehran.

**Material and Method**

**Collection of P. aeruginosa:** In this study, a total of 100 P. aeruginosa isolates were recovered from clinical specimens of patients with eye infections in three hospitals from April to June 2022. (Pars, Milad, and Motahri) in Tehran and transferred P. to the laboratory in sterile containers. Industrial aeruginosa ATCC27853 (Scientific Collection Center, Tehran, Iran) was used according to the recommended instructions. After breaking the lyophilized ampoule, its content was poured into Mueller-Hinton broth (QLab, Canada) medium and kept at 37 °C for 24 hrs. Then a culture medium with bacterial culture on Mueller-Hinton Streak agar (QLab -Canada) was prepared, and the bacterial colony was used as a control in subsequent tests [13].

**Purification of P. aeruginosa:** In order to purify the collected samples, a streak culture was prepared on Mueller-Hinton agar medium and to provide a proper environment, the plates were incubated in an incubator (Shimaz- BIN 55- Iran) at 37 °C for 24 hrs. This process was repeated three times until the colonies of one species were isolated from the purified colonies. After preparing the microbial spread on the slide and fixing the sample, warm staining was done, and the samples were checked for purity with a light microscope (Luoxin Lx-China) and a lens with-6745TV- Shanghai 100 magnification [14].

**Confirmation of P. aeruginosa by biochemical tests:** Biochemical tests were performed to confirm P. aeruginosa isolates, including oxidase, catalase, urease, movement screening, sugar fermentation, citrate consumption, oxidation fermentation, growth on MacConkey agar at 42 °C, and pyocyanin pigment production tests [15].

**Determination of antibiotic resistance of P. aeruginosa:** Bacterial suspension lawn (0.5 McFarland) was prepared, and of the bacterial suspension was done culture on Mueller Hinton agar (MHA) medium and allowed to absorb the moisture of the bacterial suspension. Clinical Laboratory Standards Institute (CLSI) guidelines 2022. were used for antimicrobial sensitivity testing, and antibiotics were provided from Padtan Teb Co., Tehran, Iran. Antibiotic disks used in this study included amoxyclavulanic acid (30 μg), imipenem (10 μg), ciprofloxacin (5 μg), piperacilin-tazobactam (100/10 μg), ticarcillin (75 μg), piperacillin (100 μg), cefoxitin (30 μg) ceftazidime (30 μg), aztreonam (30 μg), colistin (10 μg), gentamycin (10 μg), tobramycin (10 μg), amikacin (30μg), and fosfomycin (5 μg). They were placed on the surface of agar plates with a standard distance (24 mm) from each other. The plates were incubated at 37 °C for 24 hrs. P. aeruginosa ATCC27853 was used as a control for all antibiotic disks, except for penicillin/beta-lactamase inhibitors, for which Escherichia coli was used [16].

**Molecular identification of P. aeruginosa**

**Genomic DNA extraction:** DNA extraction Tehran, Iran) was kit (Yekta Tajhiz Azma, used to extract DNA from P. aeruginosa isolates according to the manufacturer’s DNA, instructions [17]. To assess the extracted 1.5% agarose gel electrophoresis (Sigma-was performed. After Aldrich, Germany) performing electrophoresis for 45 min at -95 V, the identity of the bands was determined. The quality of the bands was checked using a transilluminator device (Cleaver Scientific-proBLUEVIEW, England) [18].

**Multiplex PCR reaction for exo genes:**
Polymerase chain reaction was performed using specific primers for exo (exoY, exoT, exoU, exoS) and fos (fosA, fosB, fosC) genes of *P. aeruginosa* (synthesized by Biotechnology Company), whose sequences are shown in Table 1 \[19\].

Forward and reverse primers were added to a volume of 100 μL (1:10 ratio) and kept as mother stock. The samples were poured into 0.2-mL PCR tubes (master mix 5.12 μL, forward primer 0.5 μL at a concentration of 25 μM, reverse primer 0.5 μL at a concentration of 25 μM, DNA sample 2 μL, and deionized distilled water 5.9 μL) and transferred to a thermocycler. The PCR (BIO-RAD -CFX Opus96-USA) reaction was performed in a total volume of 25 μL in 35 cycles. Distilled water was used as a negative control, and a positive control was obtained from patients with positive examined strains. The temperature program considered for the PCR reaction is showed in Table 2.

To identify PCR products, 3 μL of PCR solution along with 1.5 μL of loading buffer were loaded on 1.5% agarose gel. After performing electrophoresis for 45 min at -95 V, the bands were identified by comparison with a molecular weight marker (Ladder) of 100 bp (Sigma-Aldrich, Germany). After the samples reached one quarter of the

### Table 1) Characterization of exotoxin virulence gene primers (exoY, exoT, exoU, exoS)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence</th>
<th>PCR Product Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>exoY-F</td>
<td>CGGATTCTATGGCAGGGAGG</td>
<td>282</td>
</tr>
<tr>
<td>exoY-R</td>
<td>GCCCTTGATGCACTCGACA</td>
<td></td>
</tr>
<tr>
<td>exoT-F</td>
<td>AATCGCCGTCTCAACTGCATGCG</td>
<td>152</td>
</tr>
<tr>
<td>exoT-R</td>
<td>TGTTCCGGGAGGTACTGCTC</td>
<td></td>
</tr>
<tr>
<td>exoU-F</td>
<td>CGGTTGTGGTGCCGTTGAAG</td>
<td>134</td>
</tr>
<tr>
<td>exoU-R</td>
<td>CCAGATGTTCACCGACTCGC</td>
<td></td>
</tr>
<tr>
<td>exoS-F</td>
<td>CTTGAAGGGACTCGACAAGG</td>
<td>118</td>
</tr>
<tr>
<td>exoS-R</td>
<td>TTCAGGTTCCGGTGATGAAT</td>
<td></td>
</tr>
<tr>
<td>fosA-F</td>
<td>ATCTGTGGGTCTGCTGTGCTT</td>
<td>217</td>
</tr>
<tr>
<td>fosA-R</td>
<td>ATGCCGCATAGGGCTTTCTC</td>
<td></td>
</tr>
<tr>
<td>fosB-F</td>
<td>CCTGCGCATTATATCAGCAGT</td>
<td>312</td>
</tr>
<tr>
<td>fosB-R</td>
<td>CGGTATCTTTCCATACCTCAG</td>
<td></td>
</tr>
<tr>
<td>fosC-F</td>
<td>CTGGCGTATTATCAGCGGTTT</td>
<td>354</td>
</tr>
<tr>
<td>fosC-R</td>
<td>CTGGCGGTTTTTATCCGAGGTTT</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2) PCR temperature program

<table>
<thead>
<tr>
<th>Step</th>
<th>Section</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
<th>Repetition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Primary denaturation</td>
<td>95</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Secondary denaturation</td>
<td>95</td>
<td>1</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Connection</td>
<td>60</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>expansion</td>
<td>72</td>
<td>30s</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>The final expansion</td>
<td>72</td>
<td>10</td>
<td>1</td>
</tr>
</tbody>
</table>
end of the gel, the electric current was cut off, and the gel was photographed by the transilluminator device (Cleaver Scientific-proBLUEVIEW, England) in the vicinity of ultraviolet light \[18\].

**Statistical analysis:** In order to analyze the data, SPSS version 26 software was used. For qualitative variables, frequency and percentage were reported, and for quantitative characteristics, if the data distribution was normal, the mean and deviation, and if the data distribution was not normal, the median and interquartile range were reported. The Shapiro-Wilk test was also used to check the normality of the data. The significance level for all tests used is 0.05 (p-value <.005).

**Findings**

**Antibiogram results:** Antibiogram profile of bacterial isolates is shown in Figure 1. According to the results, the highest resistance was observed to cotrimoxazole (85%), ceftazidime (83%), cefotaxime (79%), and cefepime (72%), and the highest sensitivity was observed to ciprofloxacin (55%), gentamicin (52%), and piperacillin (41%), respectively.

**Presence of exotoxin virulence genes:** The presence of exotoxin virulence genes including \( \text{exoY, exoT, exoU, and exoS} \) in 60 \( P. \ aeruginosa \) isolates selected in this study was investigated using multiplex PCR, and the gel electrophoresis results of PCR products are shown in Figure 2. The first well from the left side was considered as a positive control. The results of this study showed that among the 60 investigated strains, 58 strains (96.8%) harbored the \( \text{exoY} \) gene (289 bp band), 58 strains (96.8%) harbored the \( \text{exoT} \) gene (152 bp band), 58 strains (96.8%) had the \( \text{exoU} \) gene (134 bp band), and 4 strains (6.67%) had the \( \text{exoS} \) gene (118 bp band). In other words, the three exotoxins Y, T, and U were present with the same frequency in most of the samples, while the frequency of exotoxin S was much lower, it was observed only in four strains (Figure 3).

**Presence of fosfomycin resistance genes:** The presence of fosfomycin resistance genes including \( \text{fosC, fosB, and fosA} \) in 60 selected \( P. \ aeruginosa \) isolates was investigated using multiplex PCR technique, and the gel electrophoresis results of PCR products are
The results showed that one isolate (1.66%) had the fosC gene (354 bp band), two isolates (3.33%) had the fosB gene (312 bp band), and 12 isolates (20.02%) had the

Figure 2) Gel electrophoresis for samples (a):12-1 exoY, exoT, exoU and exoS genes/ (b):36-13 exoY, exoT, exoU and exoS genes / (c): 60-37 exoY, exoT, exoU and exoS genes.

Figure 3) Distribution of exotoxin genes in *Pseudomonas aeruginosa* isolates.

Figure 4) Gel electrophoresis for samples (a):24-1 fosA, fosB and fosC genes/ (b):48-25 fosA, fosB and fosC genes / (c): 60-49 fosA, fosB and fosC.

Figure 5) Distribution of exotoxin fosfomycin in *Pseudomonas aeruginosa* isolates.
shown in Figure 4. The results showed that one isolate (1.66%) had the $\text{fosC}$ gene (354 bp band), two isolates (3.33%) had the $\text{fosB}$ gene (312 bp band), and 12 isolates (20.02%) had the $\text{fosA}$ gene (217 bp band) (Figure 5). The results indicated a low frequency of fosfomycin resistance genes among the isolates studied in this research.

**Discussion**

*P. aeruginosa* is considered as an important opportunistic bacterial pathogen and one of the four hospital pathogens responsible for 1.10% of all hospital-acquired infections \[19\]. This bacterium has many pathogenic factors. OPRI and OPRL virulence factors are lipoproteins that form efflux pumps. OPRI is used to identify the family *Pseudomonadaceae*, and OPRL is used to identify the species *P. aeruginosa* genes encode the main lipoproteins of the outer membrane and play a major role in the antibiotic resistance of this bacterium \[20\]. In addition, due to the existence of numerous extracellular and cell-bound virulence factors, the organism could cause a wide range of severe infections. For example, the $\text{toxA}$ gene encodes exotoxin A, which inhibits protein biosynthesis in eukaryotic cells. Enzyme $S$ ($\text{exoS}$) is a bifunctional protein that prevents phagocytosis of bacteria by phagocytes. This enzyme plays a role in bacterial invasion of non-phagocytic cells \[21\], and induces rapid apoptosis in host cells. According to the results, the highest antibiotic resistance was observed to cotrimoxazole, ceftazidime, and cefotaxime, and the highest sensitivity was observed to ciprofloxacin, gentamicin, and piperacillin (Figure 1). Many studies have been done on the resistance of this bacterium to cephalosporins. Saeed and Awad (2009) \[22\] isolated 293 isolates from clinical samples, of which 86% were sensitive to cefotaxime, and 78% were Mir sensitive to ceftriaxone. Anjum and (2010) \[23\] investigated the sensitivity pattern of *P. aeruginosa* isolates to different types of antibiotics, among 100 isolates, 60% were susceptible to cefopirazone, 62% were sensitive to ceftazidime, and 14% were sensitive to cefotaxime. Karami et al. (2019) \[24\] determined the antibiotic resistance and susceptibility patterns of *P. aeruginosa* isolates collected from clinical specimens of people in Kermanshah educational centers. The results showed that imipenem was the most effective drug against clinical strains of *P. aeruginosa*.

*P. aeruginosa* is considered as an important opportunistic bacterial pathogen in hospitals. Therefore, it is important to identify this bacterium in clinical samples and report the results to health authorities. The presence of exotoxin genes including $\text{exoY}$, $\text{exoT}$, $\text{exoU}$, and $\text{exoS}$ in 60 selected *P. aeruginosa* isolates was investigated using multiplex PCR technique, and the gel electrophoresis results of PCR products are shown in Figure 2(a-c). In this study, during the examination of 60 clinical isolates, it was found that the frequency of the studied exoenzymes ($S$, $T$, $U$, and $Y$) was different. Accordingly, among the 60 investigated isolates, 58 isolates were positive for $\text{exoY}$, $\text{exoT}$, and $\text{exoU}$ production, while only four isolates were $\text{exoS}$ producers. It should be noted that the isolates producing $\text{exoY}$, $\text{exoT}$ and $\text{exoU}$ were not the same. Khan and Cerniglia (1994) \[25\] examined the exotoxin A gene in clinical and environmental samples of *P. aeruginosa* and said that this work could be useful for epidemiological studies, they identified *Pseudomonas* by detecting this gene in the samples using PCR. Wolska and colleagues (2012) \[26\] studied the genetic characteristics of clinical isolates of *P. aeruginosa* in terms of the prevalence of six virulence genes and showed that out of 49 isolates, 46% had the $\text{exoS}$ gene, and 76% had the $\text{exoA}$ gene.
is commonly utilized to effectively cure uncomplicated gastrointestinal and urinary tract infections. One of the most desirable features of fosfomycin is that despite the high abundance of antibiotic-resistant mutants, mutation-associated biological costs could effectively control bacterial growth rates, and bacterial agents could not compensate for the host's defense barriers or compete with susceptible bacteria. Given the lack and effective antimicrobials, the of novel use of fosfomycin has been suggested as an alternative therapeutic approach for illnesses induced by different bacterial pathogens, especially *P. aeruginosa*. Nevertheless, whether resistance to fosfomycin in *P. aeruginosa* isolates confers a fitness cost is unclear.

The results indicated a low frequency of fosfomycin resistance genes among the isolates studied in this research. In other words, these results indicated that the frequency of the above genes, whose protein product modifies the epoxide group of fosfomycin and reduces the effectiveness of the antibiotic, was significantly low among the examined strains. Studies have reported that this antibiotic resistance mechanism has a lower distribution than other types of fosfomycin resistance mechanisms [12].

**Conclusion**

This study results showed significant antibiotic resistance of the studied isolates. Also, the presence and abundance of exotoxin virulence genes in these isolates indicated the pathogenicity of the isolates. However, the frequency of fosfomycin resistance genes in these isolates was almost low; thus, the observed resistance could be due to other fosfomycin resistance mechanisms that should be investigated in future studies.

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**Authors’ contributions:** Study conceptualization and design: ZSH and NAS; data analysis and interpretation: ZSH; drafting of the manuscript: AHH; critical revision of the manuscript for important intellectual content: ZSH, NAS; statistical analysis of data: ZSH.

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