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Antibacterial Susceptibility Profiles and the Presence of Beta-Lactamase Resistance Genes and Virulence Factors in *Pseudomonas aeruginosa* Isolates

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Authors
Hajikhani S.[1] *MSc,*
Darban-Sarokhalil D.[2] *PhD,*
Babapour E.*[1] *PhD*

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ABSTRACT

Aims *Pseudomonas aeruginosa* is one of the major causes of nosocomial infections. This study aimed at investigating the antibacterial susceptibility and the prevalence of virulence and resistance genes of P. aeruginosa isolated from patients in Tehran, Iran.

Materials & Methods In this cross-sectional study, 70 *P. aeruginosa isolates* from burn infection and cystic fibrosis patients were collected from Shahid Motahari Hospital and Pediatric Medical Center, Tehran, Iran during 2017-2018. Antibacterial susceptibility against eleven antibiotics was determined based on disk diffusion method. Isolates were, then, screened for the presence of virulence and resistance genes by Polymerase Chain Reaction (PCR).

Findings The highest and lowest antibacterial resistance rates were against ampicillin and meropenem, respectively. The *oprI* and *oprL* genes were present in all *P. aeruginosa* isolates. The prevalence of *toxA*, *exoS*, *exoU*, *exoT*, and *exoY* genes in *P. aeruginosa* isolated from a total of 18 CF patients was 66.6%, 66.6%, 22.2%, 72.2%, and 77.7%; and in isolates from a total of 52 burn patients was 84.7%, 100%, 28.8%, 73.07%, and 64.46%, respectively. *VEB*, *PER*, *TEM*, *SHV*, and *CTX-M* genes were found in 0.0%, 0.0%, 11.1%, 16.6%, and 5.5% *P. aeruginosa* isolated from CF patients; and in 0.0%, 1 1.9%, 50 96.1%, 88.4%, and 40.3%, *P. aeruginosa* isolated from burn patients, respectively.

Conclusion Generally, selective pressure caused by extensive use of antibiotics can be conducive to the selection of MDR bacteria. Therefore, choosing suitable antibiotic based on precise antibiogram tests can prevent the increase of resistance in bacteria.

Keywords *P. aeruginosa*; Virulence Genes; Susceptibility Test; Resistance Genes; PCR

[1]Microbiology Department, Faculty of Science, Alborz Branch, Islamic Azad University, karaj, Iran
[2]Microbiology Department, Medicine Faculty, Iran University of Medical Sciences, Tehran, Iran

Correspondence
Address: Microbiology Department, Faculty of Science, Alborz Branch, Islamic Azad University, Karaj, Iran
Phone: -
Fax: -
e.babapour@kiau.ac.ir

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Introduction

P. aeruginosa is considered as an opportunistic pathogen causing infection in almost all tissues [1]. It has been mostly related to pulmonary infections in cystic fibrosis (CF) patients providing a suitable environment for the growth of this microorganism by sticky and thick mucus in their lungs and airways [2]. Moreover, the isolation of *P. aeruginosa* has been frequently reported from nosocomial infections in burn patients, immunosuppressed individuals, and patients with other malignancies [3].

Being associated with efflux transport systems, I lipoprotein and L lipoprotein are involved in host-bacterium interaction and intrinsic antibacterial resistance. Since these proteins are specifically found in *P. aeruginosa*, they are considered for its molecular identification in clinical samples [4].

P. aeruginosa is believed to be a common pathogen in view of possessing numerous virulence factors, among which are exotoxin A, *exo*S, *exo*T, *exo*U, and *exo*Y [5]. Exotoxin A, which is considered as the most toxic virulence factor, inhibits protein biosynthesis in eukaryotic cells by ADP-ribosylation of elongation factor 2 as a consequence, of which cell lysis can occur [6]. *Exo*S, *exo*T, *ex*oU, and *exo*Y are type III secretion system cytotoxins with different functions [7]. *Exo*S acts by its ADP-ribosylating activity and like *Exo*T, it is involved in persistence and immune evasion by altering the structure of cell cytoskeleton, which subsequently helps the microorganism to reproduce inside host cells. ExoU functions as a phospholipase A2 enzyme and can also disrupt phagocyte attraction. Moreover, it has been suggested that acting together with ExoT triggers pro-apoptotic pathways, by which it can impede wound healing process. With its adenylate cyclase activity, *Exo*Y can disrupt actin cytoskeleton and also can promote the production of cGMP and cUMP in host cells [8, 9].

Treatment of infection caused by this microorganism has become complicated owing to the promotion and spread of antibacterial resistance [10]. *P .aeruginosa* strains have been frequently reported to produce different classes of enzymes called extended spectrum beta-lactamases (ESBLs), which facilitate resistance against extended-spectrum cephalosporins [11]. Thus far, more than 300 various ESLB variants with different amino acid sequences have been identified with TEM, SHV, and CTX-M being the prominent types [12]. PER-1 and VEB-1 have also been reported to be widespread in *P. aeruginosa* strains isolated from hospitalized patients [13]. The detection of ESBL and virulence genes by molecular methods in *P. aeruginosa* isolated from cystic fibrosis and burn patients and finding an antimicrobial resistance pattern can help us determine the epidemiology and risk factors of the infections caused by this microorganism. Since antibacterial resistance genes are carried by self-transferring or motile plasmids horizontally, ESBLs distribute largely in different geographical regions. Identification of beta-lactamase enzymes can help the prevention of antibacterial resistance and treatment of infection.

Materials and Methods

Sample Collection: A total of 70 clinical *P. aeruginosa* isolates from burn infection and cystic fibrosis patients were collected from the laboratory of Shahid Motahari Hospital and Pediatric Medical Center, Tehran, Iran during 2017 to 2018. 18 *P. aeruginosa* isolates were from CF patients and 52 isolates were from burn infections.

Identification of *P. aeruginosa* isolates: Isolates were identified based on Gram-staining and colony morphology on Nutrient agar, Blood agar, and MacConkey agar. Biochemical tests were performed for the confirmation of the isolates as being *P. aeruginosa* including catalase test, oxidase test, lactose fermentation, mannitol fermentation, MRVP, motility, OF, nitrate reduction, citrate hydrolysis, gas production, gelatin hydrolysis, and cetrimide test. Isolates were, then, stored at -20ºC in Tryptic Soy Broth (TSB) containing 20% glycerol for further examinations.

Antibiotic Susceptibility Test: Antibiotic susceptibility test was carried out by Kirby-Bauer disk diffusion method on Mueller Hinton Agar with 11 different commercially available antibiotic disks, including imipenem, meropenem, gentamicin, ciprofloxacin, ampicillin, amikacin, ceftazidime, cefepime, tetracycline, kanamycin, and ceftriaxone. The results of susceptibility tests were determined according to the CLSI guidelines. *P. aeruginosa* ATCC 27853 was used for quality control.

Phenotypic detection of ESBLs: In order to identify ESBL-producing isolates, antibiotic disks containing ceftazidim (30 µg), cefotaxime (30 µg), and cefepime (30 µg) were placed on bacterial culture on Mueller Hinton Agar to a distance of 20 mm from combined disks of cefotaxim/clavulanic acid, ceftazidim/clavulanic acid, and cefepime/clavulanic acid. Inhibition zone of 5 mm or more for antibiotics tested in combination with clavulonic acid compared to the inhibition zone for antibiotics tested alone was indicative of ESBL producing isolates. *K. pneumonia* ATCC 700603 and *E. coli* ATCC 25922 were used as standard strains for ESBL and non-ESBL producing strains, respectively.

DNA Extraction: Bacterial genomic DNA was extracted, using Genomic DNA Extraction Kit (CinnaGen, Tehran, Iran) according to the manufacturer's protocol after sub-culturing the stored isolates on blood agar and incubating them at 37ºC for 24 hours. Purity and quality of the extracted DNA were reevaluated by a NanoDrop spectrophotometer (Thermo Fisher, USA) and 1% agarose gel electrophoresis (Sigma, USA), respectively.

Molecular identification of virulence genes, ESBLs, *opr*I, and *opr*L: Specific primers were used for the detection of virulence genes, including *tox*A, *exo*S, *exo*U, *exo*T, and *exo*Y, antibacterial resistance genes including *TEM*, *SHV*, *CTX-M*, *PER*, and *VEB*, and lipoprotein genes including *opr*L and *opr*I according to the references listed in Table1. Polymerase chain reactions (PCR) were carried out in a thermal cycler (Bio-Rad, USA) in a volume of 25µl reaction mixture, which consisted of 2 µl of DNA template, 1 µl of forward and reverse primers (10pmol/µl), 12 µl of Taq PCR Master Mix 2X (Fermentas, Lithuania), and 9 µl of DNase/RNAse free distilled water (Thermo Fisher Scientific). The program for amplification included an initial denaturation step at 95°C for 3 minutes, 35×95°C for 15 seconds; annealing (annealing Tm for each primer is shown in Table 1) for 1 minute; extension at 72°C for 1 minute, and a final extension at 72°C for 5 minutes. Finally, the amplicons were visualized on 1% agarose gel containing safe stain (Greenview Plus, Andy Gold™, USA).

Table 1) Characteristics of the primers used in this study

Targeted genes	Sequence (5′ to 3′)	Tm (°C)	Amplicon size (bp)	Reference
***exo*S**	F GCGAGGTCAGCAGAGTATCG R TTCGGCGTCACTGTGGATGC	52	118	[14]
***tox*A**	F GACAACGCCCTCAGCATCACCAGC R CGCTGGCCCATTCGCTCCAGCGCT	57	396	[15]
***opr*L**	F ATGGAAATGCTGAAATTCGGC R CTTCTTCAGCTCGACGCGACG	60	504	[16]
***opr*I**	F ATGAACAACGTTCTGAAATTCTCTGCT R CTTGCGGCTGGCTTTTTCCAG	48	249	[17]
***exo*U**	F CCGTTGTGGTGCCGTTGAAG R CCAGATGTTCACCGACTCGC	54	135	[18]
***exo*T**	F AATCGCCGTCCAACTGCATGCG R TGTTCGCCGAGGTACTGCTC	56	152	[18]
***exo*Y**	F CGGATTCTATGGCAGGGAGG R GCCCTTGATGCACTCGACCA	55	289	[19]
VEB	F ATTTCCCGATGCAAAGCGT R TTATTCCGGAAGTCCCTGT	60	542	[20]
PER	F ATGAATGTCATTATAAAAGC R AATTTGGGCTTAGGGCAGA	50	926	[21]
TEM	F ATAAAATTCTTGAAGAC R TTACCAATGCTTAATCA	62	1075	[22]
SHV	F TGGTTATGCGTTATATTCGCC R GCTTAGCGTTGCCAGTGCT	59	867	[23
CTX-M	F GGTTAAAAAATCACTGCGTC R TTGGTGACGATTTTAGCCGC	55	863	[24]

In order to control the quality of reactions, amplicons were sequenced (Macrogen, South Korea) randomly after purification. Advanced BLAST search program was used to analyze the sequence data. Standard strain *P. aeruginosa* ATCC 27853 was used as positive control.

Statistical analysis: To find the significant difference between the incidence of genes and the source of *P. aeruginosa* isolation, Chi-square test and Fisher's exact test analysis were performed, using SPSS 16.0 software (SPSS Inc., Chicago, IL) a p-value<0.05 was considered as a statistically significant difference.

Findings

Antibacterial susceptibility test: Overall, the highest and lowest antibacterial resistance rates were against ampicillin and meropenem with the prevalence rates of 89.46% and 14.6%, respectively (Table 2). Moreover, the highest and the lowest resistance rates against the studied antibiotics were observed in samples obtained from burn infections and cystic fibrosis patients, respectively (Diagram 1).

Table 2) Antibacterial resistance rate in *P. aeruginosa* isolates from CF and burn patients (The numbers are based on %)

Antibiotic	CF	Burn	Total (n=70)
IPM	7.6	38.88	15.2
MEM	9.61	27.77	14.6
GEN	13.46	61.11	25.56
CIP	40.38	72.22	48.3
AMP	90.38	88.88	89.46
AMK	84.61	61.11	78.1
CAZ	32.69	72.22	42.6
CEP	51.92	66.66	55.38
TET	67.3	72.22	68.16
KAN	46.15	50	46.86
CRO	50	72.22	55.38

Abbreviations: IPM=Imipenem (10 µg/disk); MEM=Meropenem (10 µg/disk); GEN=Gentamicin (30 µg/disk); CIP= Ciprofloxacin (5 µg/disk); AMP= Ampicillin (30 µg/disk); AMK= Amikacin (30 µg/disk); CAZ=Ceftazidime (30 µg/disk); TET=Tetracycline (µg/disk) ; KAN= Kanamycin (30 µg/disk); CRO= Ceftriaxone (30 µg/disk).

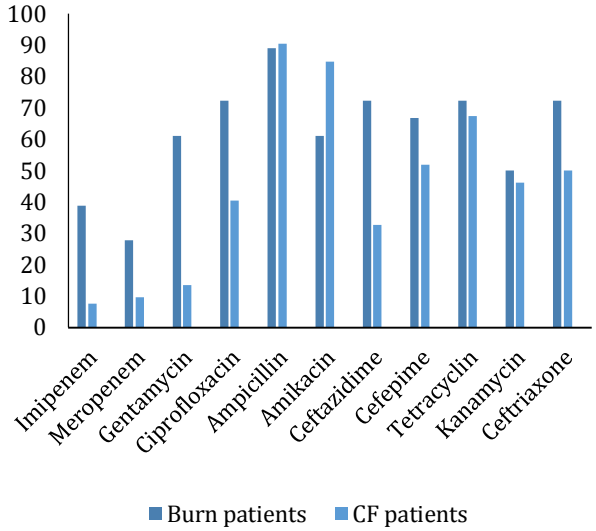

Diagram 1) Antibacterial resistance rate in burn and CF patients

Detection of virulence genes: The *oprI* and *oprL* genes were present in all 70 *P. aeruginosa* isolates collected from CF and burn patients. Nevertheless, the prevalence of *tox*A, *exo*S, *exo*U, *exo*T,and *exo*Y genes in *P. aeruginosa* isolated from a total of 18 CF patients was 12 (66.6%), 12 (66.6%), 4 (22.2%), 13 (72.2%), and 14 (77.7%); and in *P. aeruginosa* isolated from a total of 52 burn patients was 44 (84.7%), 52 (100%), 15 (28.8%), 38 (73.07%), and 33 (64.46%), respectively (p>0.05; Figure 1).

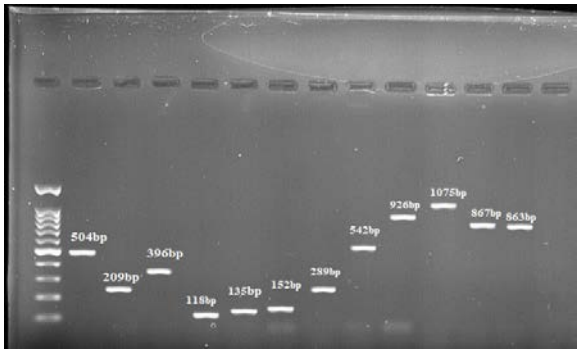

Figure 1) Agarose gel electrophoresis showing band patterns of the studied genes. Lane 1: 100 bp ladder; Lane2-13: *opr*L; *opr*I; *tox*A; *exo*S; *exo*U; *exo*T; *exo*Y; *VEB*; *PER*; *TEM*; *SHV*; *CTX-M*

Detection of ESBL genes: According to our results, *VEB, PER, TEM, SHV,* and *CTX-M* genes were found in 0 (0.0%), 0 (0.0%), 2 (11.1%), 3 (16.6%), and 1 (5.5%) *P. aeruginosa* isolated from CF patients; and in 0 (0.0%), 1 (1.9%), 50 (96.1%), 46 (88.4%), and 21 (40.3%) *P. aeruginosa* isolated from burn patients, respectively (Figure 1).

Discussion

Nosocomial infection is considered as one of the major problems that medical centers usually struggle with both in developing and developed countries. Nosocomial infection in burn wards is usually caused by opportunistic bacteria and broad wounds after burns facilitate the entrance of *P. aeruginosa* to the injured tissues [25].

Infection with this microorganism can lead to various complications including septicemia, pneumoni,a and meningitis [26]. Additionally, *P. aeruginosa* is prevalently isolated from CF patients. Cystic fibrosis is a chronic disease in children with the implication of chronic mucopurulent coughs from their first years of life Most CF patients suffer from weight loss, extensive perspiration, and fatty stool [27].

*opr*L and *opr*I are lipoprotein constituents of efflux pump, which are considered as suitable markers for the molecular identification of *P. aeruginosa*. According to the current study, all isolates showed the presence of *opr*I and *opr*L genes that was consistent with the study of Hocquet *et al*., who identified *P. aeruginosa* isolates from pulmonary infections based on *opr*I and *opr*L genes, indicating a high sensitivity of using these genes as an identification tool [21].

P. aeruginosa harbors a variety of virulence factors, including exotoxin A, *Exo*S, *Exo*U, *Exo*T, and *Exo*Y. This microorganism has also other pathogenicity determinants such as alkaline protease, elastase, alginate, and biofilm formation, which are not studied in this investigation [8].

Exotoxin A is chromosomally encoded and is the major toxin produced by *P. aeruginosa*. In this investigation, the prevalence of *tox*A gene in burn and CF samples by PCR method was found to be 12 (66.6%) and 44 (84.7%), respectively (p>0.05). These values were lower compared to the study conducted by Amini *et al*. [28]; however, it is still indicative of the high prevalence of this gene in *P. aeruginosa* isolated from clinical samples.

The presence of *exo*S, *exo*U, *exo*T, and *exo*Y toxins are generally correlated with the expression of cytotoxic or invasive phenotypes. In the current study, difference in the prevalence of their encoding genes among isolates from 18 CF and 52 burn patients was not significant (p>0.05). In general, it has been suggested that different virulence genes expression may be observed based on the site of infection [29]. Feltman *et al*. studied *exo*U and *exo*S genes in 51 *P. aeruginosa* isolates from CF patients and reported the prevalence of 78.4% and 25.5%, respectively [30]. Berthelot *et al*. also studied the presence of these genes in environmental and clinical isolates of *P. aeruginosa*, according to which 71% of clinical burn samples and 85% of CF samples harbored *exo*S gene, while this prevalence for *exo*U gene was 42% and 35%, respectively [31].

These results were almost in agreement with the results of the current study. Similar to other studies conducted in Bulgaria and the USA, our results showed the prevalence of 67.1%. Also, the prevalence of *exo*T (72.8%) was in agreement with

previous studies [32]. Differences in prevalence of virulence genes can be explained by lower clonal diversity of the isolates.

P. aeruginosa is inherently resistant to a wide range of antimicrobial agents such as ammonia, hexachlorophene, soups, and iodine solutions. Moreover, the widespread use of antibiotics in recent years has led to the emergence of resistance to different classes of extended spectrum antibiotics such that MDR isolates are considered as major problems in treatment of this microorganism in burn wards and intensive care units [33].

In the current study, the profile of antibacterial resistance of *P. aeruginosa* isolates showed a high resistance. The resistance rate order from the highest to the lowest was as follows: AMP, AMK TET, CEF=CRO, CIP, KAN, CAZ, GEN, IPM, MEM. The lowest resistance rate was against meropenem (14.6%), which was shown mostly in isolates from burn patients.

In a similar study in Iran, Mihani *et al*. found the resistance rate of 23% that was in agreement with the current study [34], while in another study by Doosti *et al.,* the prevalence was reported to be 98.6%. These differences may be due to differences in isolation sites and geographical regions [35]. The highest resistance rate in this study was for ampicillin, which was similar to previous studies [36]. In general, the results of antimicrobial susceptibility testing show that resistance rates against different antibiotics are increasing owing to the spread and transfer of metallo-beta-lactamase and beta-lactamase genes.

In this study, the difference in the prevalence rate of *SHV*, *TEM*, and *CTX-M* genes in isolates from burn infection and CF patients was significant ($p<0.05$) and their prevalence was similar to previous studies. The burn wound is regarded as a site susceptible to the colonization of opportunistic bacteria. Regular bacterial species were isolated from burn patient wounds regularly alter during the course of disease: At first, the burn wound is sterile, but after a while, it becomes gram-positive bacteria such as β-haemolytic *Streptococcus* and colonizes the area. After surgical debridement and skin grafting in early surgery, in addition to boundless use of systemic antibiotics and other treatment strategies, various gram-negative bacteria can be detected. These all could lead to the higher prevalence rate of *SHV*, *TEM*, and *CTX-M* genes in burn patients compared to the CF patients since the bacterium isolated from burn patients may have acquired these genes by horizontal gene transfer [34]. However, interestingly, VEB and PER genes were detected in none and one of the isolates, respectively, which was completely different from the results of Shahcheraghi *et al.*'s study (24% and 17%, respectively) [37]. This difference could be attributed to difference in sampling sources.

Conclusion

Antimicrobial susceptibility tests for *P. aeruginosa* isolated from burn wound infection show that treatment of this infection is difficult due to the high prevalence of antibacterial resistance in these isolates, while in isolates from CF are not confronted with a selective pressure owing to biofilm formation and alginate and, therefore, the lack of antibiotic penetration. However, this lack of drug penetration is one of the major reasons for failure in treatment. Although drug resistance has been mostly attributed to the horizontal gene transfer, it should be considered that selective pressure caused by extensive use of antibiotics lead to the selection of MDR bacteria. Hence, choosing the most suitable antibiotic based on precise antibiogram tests can help prevent the increase in bacterial drug resistance.

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Ethical Permissions: Since we did not use any animal models and we used isolates, which were previously obtained from clinical samples, we have no ethical code for our study.

Conflict of Interests: The author declared that there is no conflict of interest.

Authors' Contribution: Hajikhani S (First author), Introduction author/ Original researcher/ Statistical analyst/ Discussion author (10%); Darban-Sarokhalil D (Second author), Original researcher/ Methodologist/ Discussion author (35%); Babapour E (Third author), Statistical analyst/ Discussion author (55%).

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