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Research Article

# Survey on Some Carbapenems and Colistin Resistance Genes Among *Pseudomonas aeruginosa* Isolates from Burn and Cystic Fibrosis Patients, Tehran, Iran

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**Abstract**

**Background:** *Psseudomonas aerugiona* is a major cause of morbidity and mortality among hospitalized patients.
**Objectives:** Because of increasing antibiotic resistance, this study investigated the prevalence of $bla_{IMP}$, $bla_{PER}$, $bla_{VEB}$, $bla_{GES}$, *pmrA*, *pmrB*, and *mcr-1* genes in *P. aeruginosa* isolates among burn and CF (Cystic Fibrosis) patients in Tehran.
**Methods:** During 2016 - 2017, 80 and 41 isolates of *P. aeruginosa* were collected from burn and CF patients, respectively, in Shahid Motahhari and Mofid Children's hospitals. Based on the CLSI protocol, an antibiotic susceptibility test was performed using the disk diffusion method. Then PCR and further sequencing were used to evaluate the frequency of the above-named genes.
**Results:** In both groups, high rates of resistance to amikacin, cefepime, imipenem, ciprofloxacin, ceftazidime, aztreonam, piperacillin, gentamycin and piperacillin-tazobactam were detected. Colistin was determined to be the best choice for treatment. In burn patients, the highest frequency was detected for the $bla_{VEB-1}$ (55%) gene and the frequencies of $bla_{PER-1}$, $bla_{IMP-1}$, and $bla_{GES-1}$ were 27.5%, 25%, and 13.75%, respectively. In CF patients, the $bla_{PER-1}$ gene was more common (12.19%), and the frequency rates of $bla_{VEB-1}$ and $bla_{IMP-1}$ were 2.43% each. The $bla_{GES-1}$ gene was not detected. Despite the fact that all of the isolates in both groups had *pmrA* and *pmrB* genes, different mutations were detected by sequencing. The *mcr-1* gene was not shown in all isolates.
**Conclusions:** Hopefully, by the low frequency of *mcr-1* gene and the rate of mutation in *pmrAB* genes in this study, the rate of resistance can keep low with caution prescription to polymyxins.

*Keywords:* Antibiotic Resistance, Burn, Cystic Fibrosis, *P. aeruginosa*

## 1. Background

Antibiotic resistance is an important warning for infection control and the treatment of infectious disease (1). Multidrug-resistant strains of *Pseudomonas aeruginosa* (MDRP) have been distinguished to be resistant to carbapenems, fluoroquinolones, and aminoglycosides and are common causative nosocomial infections, especially in immune deficient patients such as those with cystic fibrosis (CF) and burn patients (2, 3). In both burn and CF patients, *P. aeruginosa* is a major cause of morbidity and mortality among hospitalized patients (4, 5). Most studies on antibiotic resistance mechanisms in *P. aeruginosa* have investigated modifying enzymes and attaining plasmid resistance genes, the overexpression of efflux pumps, or post-mutational changes in chromosomal and plasmidic genes (6-8). Metallo-beta-lactamases form the most important group of carbapenemases such as $bla_{IMP}$ found in *P. aeruginosa*. This type is mostly located in class 1 integrons and was discovered in Japan in 1991 (9). GES-1, a plasmid-borne integron detected in *P. aeruginosa* (formerly, detected among *Klebsiella pneumoniae* in France in 2000) too (10). The VEB and PER types are the most common ESBLs reported in *P. aeruginosa*. These types belong to class A of ESBLs and illustrate a high rate of resistance to cephems, monobactams, and ceftazidime (11, 12). The $bla_{VEB-1}$ gene was first detected in *Escherichia coli* in a Vietnamese patient and subsequently discovered in *P. aeruginosa* isolates from a patient from Thailand (10). PER-1 was first discovered in *P. aeruginosa* isolated from a Turkish patient in France in 1991. The dissemination of PER-1 among European countries with no close geographical vicinity to Turkey, such as Belgium, Italy, and Poland as well as in Asia, has been reported (13). Molecular classification of betalactamas was shown in table 1.PmrA/PmrB proteins as a two component system (TCS), including the response regulator

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PmrA protein and its consubstantial sensor kinase PmrB, have been distinguished as the main regulatory system in polymyxin B resistance. The activity of this TCS PmrAB in *P. aeruginosa* has been related to resistance to cationic antimicrobial peptides consisting of colistin (14-16).

The *mcr-1* gene was first reported in animal and human isolates of *Escherichia coli* and *Klebsiella pneumoniae* in China. An uncommon enzymatic phosphoethanolamine transferase activity (*mcr-1* gene product) was detected as a mechanism of colistin resistance related to the enzymes (17).

## 2. Objectives

Due to increasing of microbial resistance, this study was performed to investigate the frequency of $bla_{IMP}$, $bla_{GES}$, $bla_{VEB}$, $bla_{PER}$ and *mcr-1* genes and mutations in *pmrA* and *pmrB* genes in *P. aeruginosa* isolates among CF and burn patients in two educational hospitals in Tehran, Iran.

## 3. Methods

### 3.1. Bacterial Identification

This descriptive study was approved by the Research and Ethics Committee of the Science and Research Branch of Islamic Azad University in, Tehran, Iran. According to the physician's requests, samples collection was done by hospitals laboratories staffs and transferred to the Microbiology laboratory of Shahid Beheshti University of Medical Sciences. From July 2016 to July 2017, 41 isolates of *P. aeruginosa* from 50 sputum samples of CF patients of Mofid Children's Hospital (Tehran, Iran) and simultaneously 80 isolates of *P. aeruginosa* from 250 wound samples of burn patients admitted to the Shahid Motahhari Hospital (Tehran, Iran) were collected. The wound exudates and sputum samples were cultured on blood agar, chocolate agar, and MacConkey agar (Merck, Germany). The quality of the sputum samples was checked by gram stain (18). After 24 hours at 37°C, biochemical tests such as catalase and oxidase tests, triple sugar iron agar (TSI) (Merck, Germany), oxidative fermentation (OF) (Merck, Germany), and other identification tests based on standard diagnostic protocol were performed on grown colonies (18). The identified and confirmed *P. aeruginosa* isolates were stored in TSB (tryptic soy broth) medium with 20% glycerol at -75°C for further evaluation.

### 3.2. Antimicrobial Susceptibility Test (AST)

According to Clinical Laboratory Standards Institute (CLSI) guidelines 2016 (19), the Kirby-Bauer disk diffusion method on Mueller Hinton agar (Merck, Germany) was performed. The antimicrobial susceptibility test was applied on ceftazidime 30 $\mu$g, piperacillin-tazobactam 100/10 $\mu$g, gentamycin 10 $\mu$g, cefepime 30 $\mu$g, aztreonam 30 $\mu$g, imipenem 10 $\mu$g, amikacin 30 $\mu$g, ciprofloxacin 5 $\mu$g, and piperacillin 100 $\mu$g. All were purchased from the Rosco Company (Taastrup, Denmark), except piperacillin which was purchased from the Mast Group (Merseyside, UK). *P. aeruginosa* ATCC27853 was used as a control strain.

### 3.3. DNA Extraction and Molecular Characterization

DNA was extracted using the boiling method (20). The PCR method was used to detect the presence of the $bla_{IMP}$, $bla_{PER}$, $bla_{VEB}$, $bla_{GES}$, *pmrA*, *pmrB*, and *mcr-1* genes. The sequences of PCR primers used in this study are shown in Table 1.

The PCR mixture included the DNA template, forward and reverse primers, and the master mix (Taq 2X Master Mix- Ampliqon Co.). The PCR programs are listed in Table 2. The PCR products were analyzed by electrophoresis on 1% agarose gel at 100 V for 45 minutes in 1X TBE (Tris-HCl Boric Acid EDTA), and the results were checked by UV irradiation in the gel documentation system. The sequencing of the genes showing the accuracy of the PCR products was performed by the MacroGen Company (South Korea). The sequence analysis was done with Chromas 1.45 software and BLAST in the NCBI (National Center for Biotechnology Information) website. Amino acid sequences were detected using the MultAlin (multiple sequence alignment with hierarchical clustering, F. CORPET, 1988, Nucl. Acids Res., 16 (22), 10881-10890) program and compared with PAO1 as the *P. aeruginosa* standard strain.

### 3.4. Statistical Analysis

Chi-square statistical analysis test was used to determine whether or not there is any significant difference in the antibiotic resistance rate between *P. aeruginosa* isolated from CF and burn wound infections.

## 4. Results

### 4.1. Bacterial Identification

All gram-negative bacilli, oxidase positive, TSI(K/K), OF+/-, motility positive, and cetrimide agar growth positive colonies were identified as *P. aeruginosa*.

### 4.2. AST Results

By the AST test, it was shown that among the *P. aeruginosa* isolates from burn patients, the resistance rate to gentamycin, piperacillin, and ceftazidime was 95.1%, to imipenem was 93.5%, to ciprofloxacin, aztreonam, and piperacillin-tazobactam was 88.7%, to cefepime was 85.5%,

**Table 1.** The List and Sequences of Primers Used in This Study

| Primer | Primer Sequence | Product Size, bp | Reference |
|---|---|---|---|
| **PER** | | 340 | (21) |
| F | 5'-GCAACTGCTGCAATACTCGG-3' | | |
| R | 5'-ATGTGCGACCACAGTACCAG-3' | | |
| **VEB** | | 643 | (15) |
| F | 5'-CGACTTCCATTTCCCGATGC-3' | | |
| R | 5'-GGACTCTGCAACAAATACGC-3' | | |
| **GES** | | 864 | (22) |
| F | 5'-ATGCGCTTCATTCACGCAC-3' | | |
| R | 5'-CTATTTGTCCGTGCTCAGG-3' | | |
| **IMP** | | 587 | (4) |
| F | 5'-GAAGGCGTTTATGTTCATAC-3' | | |
| R | 5'-GTAAGTTTCAAGAGTGATGC-3' | | |
| **PmrA** | | 301 | This study |
| F | 5'-CGACGACTACCTGACCAAG-3' | | |
| R | 5'-GTGGACGTGGACTTCGATG-3' | | |
| **PmrB** | | 1211 | This study |
| F | 5'-CCTACCACCTCTCGCTGAAG-3' | | |
| R | 5'-GAAGTGCAGTTCGACGATGC-3' | | |
| **Mcr-1** | | 309 | (23) |
| F | 5'-CGGTCAGTCCGTTTGTTC-3' | | |
| R | 5'-CTTGGTCGGTCTGTAGGG-3' | | |

**Table 2.** DNA Amplification (PCR) Programs

| Gene | Initial Denaturation | | Denaturation | | Annealing | | Extension | | Final Extension | | Cycle |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Temperature, °C | Time | Temperature, °C | Time | Temperature, °C | Time | Temperature, °C | Time | Temperature, °C | Time | |
| ***bla*$_{PER}$** | 94 | 5' | 94 | 45" | 59 | 45" | 72 | 45" | 72 | 5' | 36 |
| ***bla*$_{VEB}$** | 94 | 5' | 94 | 45" | 57 | 45" | 72 | 45" | 72 | 5' | 36 |
| ***bla*$_{GES}$** | 94 | 5' | 94 | 45" | 58 | 45" | 72 | 1' | 72 | 5' | 36 |
| ***bla*$_{IMP}$** | 94 | 5' | 94 | 45" | 51 | 45" | 72 | 45" | 72 | 5' | 36 |
| ***pmrA*** | 94 | 5' | 94 | 45" | 59 | 45" | 72 | 45" | 72 | 5' | 36 |
| ***pmrB*** | 94 | 5' | 94 | 30" | 64 | 30" | 72 | 30" | 72 | 5' | 30 |
| ***mcr-1*** | 94 | 5' | 94 | 45" | 54 | 45" | 72 | 45" | 72 | 5' | 36 |

and to amikacin was 83.8%. In CF patients, the resistance rate to amikacin was 53.65%, to cefepime was 51.21%, to ciprofloxacin, imipenem, and ceftazidime was 46.34%, to aztreonam was 43.9%, to piperacillin was 41.46%, to gentamycin was 39.02%, to piperacillin-tazobactam was 34.14. The comparison of resistance rates between the two groups of *P. aeruginosa* isolates is shown in Figure 1. The rate of resistance in burns were higher than CF patients ($P \leq 0.05$).

### 4.3. Molecular Characterization

PCR results indicated that among burn patients, 55% of isolates had *bla*$_{VEB-1}$, 27.5% had *bla*$_{PER-1}$, 25% had *bla*$_{IMP-1}$, and 13.75% had *bla*$_{GES-1}$. All of the isolates had *pmrA* and *pmrB* genes. Interestingly, based on sequencing, no mutation in the *pmrA* gene was detected in any *P. aeruginosa* isolates from a CF patient. However, mutations in the *pmrB* gene in different domains such as (Arg 366 to Gly), (Thr 368 to Gly), and (Pro 369 to Arg) were observed. Mutations detected af-

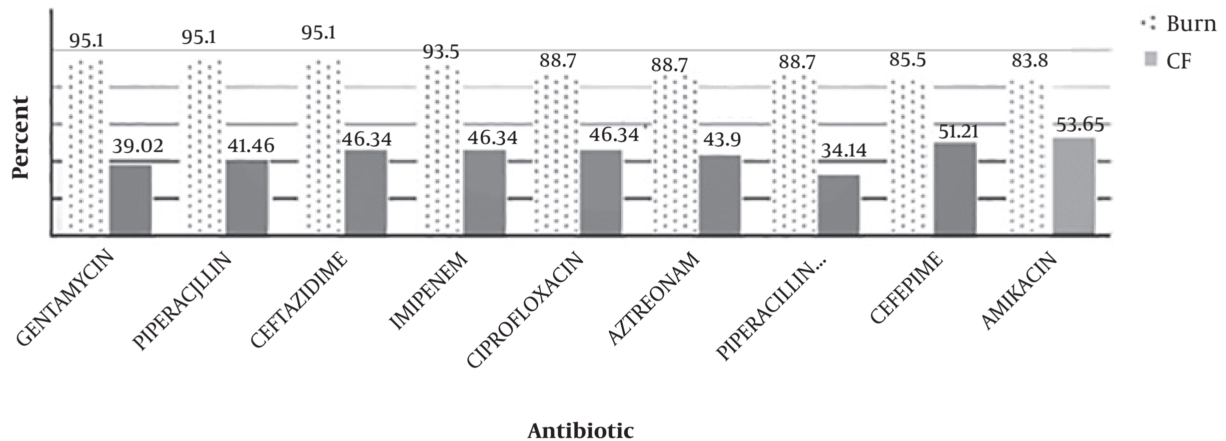


**Figure 1.** Comparison of resistance rate among burn and CF patients

ter the sequence alignment results in *pmrA* and *pmrB* genes in burn patients were as follows: (Ala137 to Glu, Gln 146 His, Arg 153 to Pro.) and (Gln 120 Thr, Gly 121 to Ala, Pro 122 to Ser and Leu 137 to Cys).

In CF patients, among 41 isolates of *P. aeruginosa*, the frequency rates of the *pmrA*, *pmrB*, $bla_{PER-1}$, $bla_{VEB-1}$, $bla_{IMP-1}$, and $bla_{GES-1}$ genes were 100%, 100%, 12.19%, 2.43%, 2.43%, and 0%, respectively.

No *mcr-1* gene was detected in any isolate. The comparison of resistant genes between burn and CF isolates is shown in Figure 2. The sequences of $bla_{PER-1}$ and *pmrB* were blasted in NCBI and submitted with the accession numbers MF671764 and MF671765 respectively (the remained genes were previously submitted in NCBI). The submission of the *pmrA* gene and its mutations in NCBI is being processed.

## 5. Discussion

Recently, *P. aeruginosa* was identified as the most common pathogen among burn and CF patients who were admitted to hospitals in Tehran, Iran. These opportunistic bacteria can be found in ICU, NICU and other hospital wards. In this study the results of AST showed that the rate of resistance to the tested antibiotics was higher among burn patients than CF patients (Figure 1).

A. Kanayama et al. conducted a study from Jan 2013 to 2014 in Takatsuki, Japan. They determined that *P. aeruginosa* isolates from different clinical specimens were resistant to imipenem, gentamycin, amikacin, ciprofloxacin, and ceftazidime (minimum inhibitory concentration 4 - 32 $\mu$g/mL), and they were susceptible to aztreonam. Similarly, in a recent study, AST was done with similar selected antibiotics based on CLSI 2016. The highest resistance rate was related to gentamycin, ceftazidime, and imipenem in burn patients and to amikacin and cefepime in CF patients (2).

In a 2004 - 2015 study in Latin America, among 3613 *P. aeruginosa* isolates, the highest susceptibility (72.8%) was seen to amikacin, and 56.8% of the isolates were susceptible to ceftazidime (24). In the current study, however, high levels of resistance were seen to amikacin in burn and CF patients (83.8% and 53.65%, respectively), and it was not a qualified choice for treatment, at least in Iran.

In a study by Hakemi Vala et al. during 2014 in Tehran, Iran, *P. aeruginosa* isolates from burn patients showed the highest rate of resistance to aztreonam, ceftriaxone, and cefotaxim (82.98%) and to gentamycin, ceftazidime, and piperacillin (95.1%). Such difference is related to the increased use and/or prescription of antibiotics. Also, the other ESBL genes such as $bla_{DIM, SPM, GIM, NDM, VIM, BIC, CTX-M-15}$ and $bla_{OXA-48}$ were determined in this study that the frequency of $bla_{IMP}$ was 2.1% (3).

In the 2017 study conducted by Hashem et al. in Ismailia, Egypt, only one (4%) isolate of 147 isolates of *P. aeruginosa* from different sources had $bla_{IMP}$, but in the current study, the frequency of this gene was higher among burn patients (25%) (25).

In the study of Kanayama et al. the multiplex PCR results revealed that all isolates had the GES-type $\beta$-lactamase gene; in a recent study, however, 13.75% of *P. aeruginosa* isolates from burn patients had the $bla_{GES}$ gene, but it was not detected in any of the *P. aeruginosa* isolates from CF

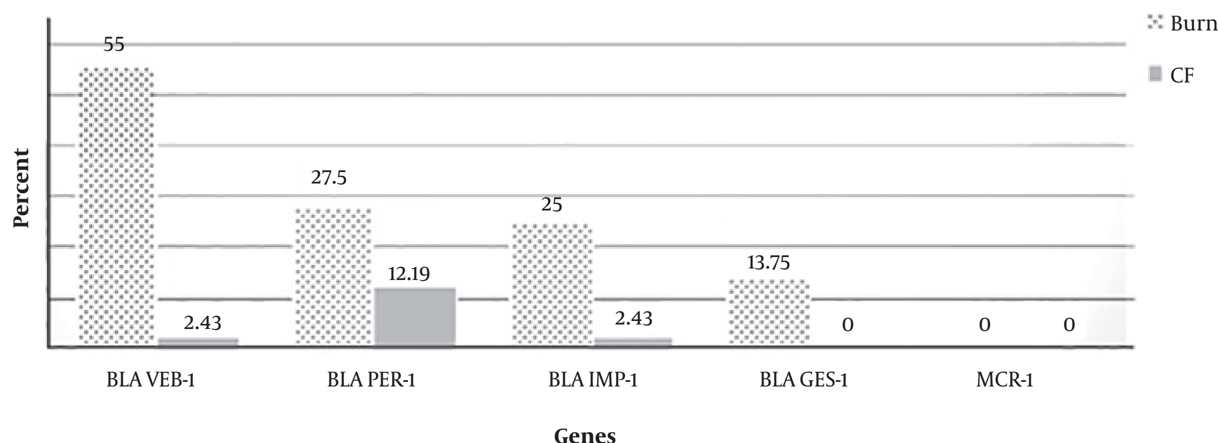


**Figure 2.** Comparison of resistance genes among burn and CF patients

patients (2). These differences can be caused by variations in geographical area or the diversity in both countries' health systems.

Akhi et al. conducted an experiment from July 2008 - 2009 in Tabriz, Iran. Among 56 isolates investigated in their study, 27.5% had the $bla_{PER-1}$ gene. In the current study, 27.5% (burn patients) and 12.19% (CF patients) of *P. aeruginosa* isolates contained the $bla_{PER-1}$ gene. Despite the difference in time, the frequency of this gene has not changed among *P. aeruginosa* isolates from burn patients. However, CF patients are usually young, and the bacterial isolates from such patients had less resistance because of less antibiotic contact during the patient's life (26). Thus, a low antibiotic resistance rate among CF patients is predictable.

In their 2012 - 2013 study in Zahedan, Iran, Bokaeian et al. indicated that among 116 *P. aeruginosa* isolates from different specimens, 13.3% had $bla_{VEB-1}$. In the current study, however, 55% and 2.43% of *P. aeruginosa* isolates from burn and CF patients, respectively, showed this gene (27). The reason for the diversity in the results may be explained by not only the difference in the time frame in which these studies were performed, but also by the wide range of patients who referred to Motahari Hospital in Tehran as a reference burn hospital compared to those who referred to the local hospital in Zahedan.

In the study by Moskowitz et al., in 3 alleles, only in a single base (equal to 1-bp) as a transition or transverse mutation was detected. In addition, 6 alleles showed double mutations in 2 missense mutations in the same PmrB molecule. Deletion of a 3-bp fragment including Asp was deleted in locus 45 of *pmrB* gene. In accordance with the recent study by Moskowitz et al., different mutations were detected in the *pmrB* gene. However, the similarity between the mutated loci was not confirmed. Moreover, no mutation in the *PmrA* gene was detected in the recent study among CF patients similar with the Moskowitz et al.'s study (15).

In a 2016 study by Thi Khanh Nhu et al. published in Nature Journal, the PmrAB two component system is recommended as a first genetic mechanism of colistin resistance, pursuant to the results of the current study and other experiments, different mutations can occur in the *pmrA* and *pmrB* genes which cause resistance to colistin (28).

In the 2009 study by Barrow and Kwon, after comparing resistant isolates with the PAO1 standard strain, the results revealed that nucleotide substitutions in the *PmrB* gene were related to two amino acid changes (Ala 247 to Thr and Tyr 345 to His) for the sequence in one of their clinical isolates which showed polymyxin B resistance. They demonstrated that polymyxin B resistance is generally caused by mutations in PhoQ or PmrB (29). In the current study, the *pmrB* gene showed different amino acid changes as follows: (Ala 1000 to Gly, Gly 1098 to Ala, Ala 1230 to Gly, and Cys 1341 to Gly) in burn patients and (Arg 366 to Gly, Thr 368 to Gly, and Pro 369 to Arg) in CF patients. Also, in a 2012 case report by Lee et al. in *P. aeruginosa* isolates with a urine source, 3 amino acid substitutions were identified in the pmrB gene (Ala 247 to Thr, Met 292 to Thr, and Tyr 345 to His) (30).

Such variations between the results of the above-mentioned studies and the recent study may be due to differences in the type of antibiotics prescribed, the source of bacterial isolation, or the time difference among their experiment and that of the recent study.

Based on the AST results in the current study, all the *P. aeruginosa* isolates in burn patients were susceptible to colistin (polymyxin E), but after molecular tests and sequence analysis, the results showed different mutations which represented colistin resistance in these isolates.

The *mcr-1* gene as a plasmid mediated gene has been detected mostly in *Escherichia coli*, but has also been found in Salmonella spp. and *Klebsiella pneumonia* (31). According to the 2018 study by MacNair et al. which was published in Nature Journal, the *mcr-1* gene causes resistance to colistin (32). To the best of the authors' knowledge, this study is the first of its kind to examine *P. aeruginosa* isolates for the *mcr-1* gene. However, all of the *P. aeruginosa* isolates were negative for the *mcr-1* gene, which indicates that this gene is not common in *P. aeruginosa* isolates in this geographic region.

Based on Thi Khanh Nhu's paper, different mutations in *pmrAB* genes are related to colistin resistance in bacterial isolates (28). Hopefully, by the low frequency of mcr-1 gene and the rate of mutation in *pmrAB* genes in this study, the rate of resistance to colistin is low here. In conclusion, it can keep low with caution prescription to polymixins.

These finding help to physicians that *mcr-1* and pmrAB mutations are not common in *P. aeruginosa* which indicates that resistance to colistin has not been established yet. So they can control it by correct drug prescription. It is recommended that in clinical laboratories after phenotypic diagnoses, molecular tests such as PCR be used to confirm the results. Molecular tests help physicians be more confident in administering the most effective antibiotic. Unfortunately, most of the laboratories and physicians are not familiar with ESBLs and the standard protocols of detection. Also, patients should desist from using antibiotics arbitrarily and complete their treatment courses. Therefore, the management and treatment strategies should be revised. Due to the importance of the MLST (multi locus sequence typing) or PFGE (pulsed field gel electrophoresis) techniques for determining of the clonality of strains, typing of recent isolates is our future plan.

## Footnotes

**Authors' Contribution:** Mojdeh Hakemi-Vala design of study and data analysis. Ghazaleh Talebi data collecting and writing paper.

**Conflict of Interests:** The authors have no conflict of interests relevant to this paper.

**Ethical Approval:** This descriptive study was approved by the Research and Ethics Committee of the Science and Research Branch of Islamic Azad University, Tehran, Iran. In order to maintain patients' confidentiality participants were anonymous and no personal information was collected or included in the study.

**Financial Disclosure:** There was no financial disclosure.

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