ABSTRACT

Background and Objectives: *Pseudomonas aeruginosa* is an opportunistic pathogen resistant to various antibiotics. The aim of the present study was to study resistant patterns in clinical isolates of *P. aeruginosa*, classify them into pandrug resistance (PDR), extensive drug resistance (XDR) and multidrug resistance (MDR) groups, and identify extended-spectrum β-lactamase (ESBL)-positive isolates using the phenotypic and genotypic methods.

Methods: This cross-sectional study was conducted on 161 *P. aeruginosa* isolates collected from the city of Isfahan, Iran. Antibiotic susceptibility tests were performed using 11 antimicrobial agents. ESBL-positive strains were identified using the phenotypic and genotypic methods.

Results: The highest level of antibiotic resistance was observed against ceftazidime (77.64%). None of the isolates was resistant to polymyxin B. In the phenotypic method, 64 isolates (39.75%) were found as ESLB-positive, whereas 132 isolates (81.98%) were ESBL-positive in the genotypic method. The number of ESBL-positive isolates in the genotypic method was significantly higher than in the phenotypic method. The frequency of XDR and MDR isolates was 50.93% and 27.32%, respectively. None of the isolates was PDR. The frequency of the *blaTEM* gene was significantly higher than other genes (P<0.0001).

Conclusion: It was revealed that the genotypic method was much more accurate in identifying ESBL-positive strains than the phenotypic method. Therefore, use of the molecular method may increase the chance of successful treatment with antibiotics of the β-lactam family.

Keywords: Drug Resistance, β-lactamases, *Pseudomonas aeruginosa*.
INTRODUCTION

*Pseudomonas aeruginosa* is an obligate aerobic, gram-negative and non-spore forming bacillus, which is part of the normal flora of skin and intestines in humans. The bacterium is also found in water and soil. This opportunistic bacterium is one of the most important causes of hospital-acquired infections that can lead to bacteremia, meningitis, urinary tract infection, pulmonary infections, etc., especially in immunocompromised individuals (1, 2). Researchers have found that mortality rates of burn wound infections caused by this bacterium are 40-50% (3). *P. aeruginosa* is also the fourth most frequently isolated pathogen from surgical-site infections (4). Emergence of antibiotic resistance among pathogenic bacteria, especially in health centers and hospitals, has become a major health concern (2). Clinical findings indicate that infections caused by resistant pathogens increase mortality rates and treatment costs (5). It has been found that infections caused by antibiotic-resistant bacteria also increase hospitalization duration by 6.4 to 12.7 days (6). According to the Centers for Disease Control and Prevention (CDC), pattern of resistance of bacteria to multiple antimicrobial agents can be classified into pandrug resistant (PDR), extensive drug resistant (XDR) and multidrug resistant (MDR). Strains resistant to at least one agent from three or more groups of antibiotics were defined as MDR. Those resistant to at least one agent in all but two or fewer antibiotic groups were defined as XDR. Finally, strains resistant to all agents from all antibiotic groups were defined as PDR (7).

One of the important reasons of resistance of *P. aeruginosa* to a wide spectrum of antibiotics is the ability to encode β-lactamase, an enzyme able to destroy the β-lactam ring in the chemical structure of β-lactam antibiotics. Chromosome- and plasmid-borne extended-spectrum β-lactamases (ESBLs) are able to destroy the β-lactam ring in a broad spectrum of β-lactam drugs (4, 8-11). ESBL is produced by various genes including *bla* <sub>ESBL</sub>, *bla* <sub>VERB</sub>, *bla* <sub>PER</sub>, *bla* <sub>OXA</sub>, *bla* <sub>CTX</sub> and *bla* <sub>SHV</sub> and *bla* <sub>GES</sub> genes (12). The possibility of horizontal gene transfer via conjugation, transformation or transduction may increase spread of genes responsible for ESBL production in the bacterial community (12). The purpose of this study was to evaluate resistance patterns of *P. aeruginosa* isolates collected from various hospitals, classify the isolates into the PDR, XDR and MDR groups, and identify ESBL-positive isolates using the phenotypic and genotypic methods.

MATERIAL AND METHODS

In this cross-sectional study, 161 *P. aeruginosa* isolates referred to laboratory for identification of the bacterial species, were collected from different treatment centers in Isfahan from May 2017 to September 2017. Susceptibility of the isolates to different antimicrobial agents including tobramycin (10 μg), amikacin (30 μg), ciprofloxacin (5 μg), levofloxacin (5 μg), ceftazidime (30 μg), cefepime (30 μg), piperacillin (100 μg), imipenem (10 μg), meropenem (10 μg), azteronam (30 μg) and polymyxin B (10 μg) were assessed by the Kirby-Bauer method.

Data were analyzed according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2017) (13) and *P. aeruginosa* (ATCC 27853) was used as the control. The combined disk method was used to identify ESBL-producing bacteria. Briefly, suspensions of ceftazidime-resistant *P. aeruginosa* equaling 0.5 McFarland turbidity standard were cultured on Mueller-Hinton agar (CONDA, Spain). Then, ceftazidime and ceftazidime-clavulanic acid disks were placed 25 mm apart on the culture medium using sterilized forceps. If the inhibition zone diameter around the ceftazidime-clavulanic acid disks was ≥5 mm than that around the ceftazidime disk, the isolate was considered as ESBL-producing (14). After evaluating the antibiotic resistance patterns, the isolates were classified as PDR, XDR and MDR according to the standards introduced by the CDC (7). For this purpose, whole genomic DNA extraction was performed using a commercial DNA extraction kit (SinaClon, Iran) according to the manufacturer’s protocol. Specific primers for the *bla* <sub>SHV</sub>, *bla* <sub>TEM</sub>, *bla* <sub>CTX</sub>, *bla* <sub>OXA</sub>, *bla* <sub>PER</sub>, *bla* <sub>VERB</sub> and *bla* <sub>GES</sub> genes were used for molecular detection of ESBL-producing bacteria using polymerase chain reaction (PCR) (Table 1). DNA amplification was conducted in a 25 μl reaction mixture containing 1 μl of template DNA, 0.5 μl of each primer (10 pmol), 2.5 μl of 10X buffer, 1 μl of MgCl<sub>2</sub> (50 Mm), 0.5 μl of dNTPs (10 mM) and 1.5 units of Taq DNA polymerase (CinnaGen, Iran) (10). PCR products (5 μl)
were subjected to electrophoresis in 1% agarose gel (SinaClon, Iran). Presence and size of amplicons were analyzed by a Gel documentation system (Bio-Rad, USA). Data were analyzed with GraphPad (GraphPad Software Inc., USA) using the Fisher’s exact test. P-values less than 0.05 were considered statistically significant.

RESULTS

General characteristics and sources of the 161 P. aeruginosa isolates are presented in table 2. As shown in table 3, the highest level of resistance was recorded against ceftazidime (77.64%). In addition, none of the isolates was resistant to polymyxin B. In the phenotypic method, 64 isolates (39.75%) were identified as ESBL-positive, whereas 132 isolates (81.98%) had at least one of the resistant genes in the genotypic method. Moreover, 29 isolates (18.01%) did not contain any of the resistance genes. The resistance genes were not detected in eight isolates (12.5%) found as ESBL-positive in the phenotypic method. The number of ESBL-positive isolates identified in the genotypic method was significantly greater than that of the phenotypic method. The frequency of the blaTEM, blaSHV, blaCTX-M and blaOXA was 60.86%, 29.81%, 24.22% and 14.28%, respectively. BlaPER, blaVEB, and blaGES were not detected in any of the isolates. The frequency of the blaTEM gene was significantly higher than the other genes (P<0.0001) (Table 4).

Table 1 - Sequences of the specific primers and size of amplicons

| Primer | Sequence (5’ to 3’) | Amplicon Size (bp) | Reference |
|--------|---------------------|--------------------|-----------|
| blaTEM | ATGAGTATTCAACATTTCCG | 867 (25)           |          |
|        | CTGACATGTCCTGTTTCA   |                    |          |
| blaSHV | GATGAACTGTTTCCCCGATG | 214 (26)           |          |
|        | CGCTGTTATGCCTAGT     |                    |          |
| blaCTX-M| TTTGCCGATGTCGATACCAT | 590 (27)           |          |
|        | GAATCTGTTGTTGCCATCA  |                    |          |
| blaOXA | GGCAGTTTGAGATGAAAC   | 438 (28)           |          |
|        | CATCAAGTTTCAACCCACCG |                    |          |
| blaPER | ATGAAATGTCAATTTAAAAA | 925 (29)           |          |
|        | GTTCGTTTGGTGCCGACAA  |                    |          |
| blaVEB | CGGACTTCCATTCTCGATGC | 642 (30)           |          |
|        | GGCACCTGCAACAAAAATACCG|                   |          |
| blaGES | ATGGCGTTATTACCCGAC   | 844 (28)           |          |
|        | CTATTGTTCGTCGCAAGG   |                    |          |

Table 2 - General characteristics of the 161 P. aeruginosa isolates used in the study

| Sex | Female | Male | Urine | Wound/Abscess | Respiratory | Dialysis-related fluids | Acetic fluid | Blood | Cerebrospinal fluid | Plural fluid |
|-----|--------|------|-------|--------------|-------------|-------------------------|--------------|-------|---------------------|-------------|
|     | 90     | 71   | 31    | 8            | 85          | 4                       | 3            | 22    | 5                   | 3           |

Table 3- Antibiotic resistance pattern of P. aeruginosa isolates based on the phenotypic method

| Antibiotic         | Sensitive | Intermediate | Resistant |                  |
|--------------------|-----------|--------------|-----------|------------------|
|                    | Number (%)| Number (%)   | Number (%)|                  |
| Aminoglycosides    |           |              |           |                  |
| Tobramycin         | 53 (32.91)| 17 (10.56)   | 91 (56.52)|                  |
| Amikacin           | 119 (73.91)| 0 (0.0)     | 42 (26.09)|                  |
| Quinolone and Fluoroquinolones | | | | |
| Ciprofloxacin      | 77 (47.83)| 32 (19.88)  | 52 (32.30)|                  |
| Levofloxacin       | 83 (51.55)| 0 (0.0)     | 78 (48.45)|                  |
| B-Lactams          |           |              |           |                  |
| Ceftazidime        | 36 (22.36)| 0 (0.0)     | 125 (77.64)|                  |
| Cefepime           | 74 (45.96)| 0 (0.0)     | 87 (54.04)|                  |
| Piperacillin       | 42 (26.09)| 4 (2.48)    | 115 (71.43)|                  |
| Carbapenems        |           |              |           |                  |
| Imipenem           | 37 (22.98)| 0 (0.0)     | 124 (77.02)|                  |
| Meropenem          | 73 (45.34)| 3 (1.90)    | 85 (52.80)|                  |
| Monobactams        |           |              |           |                  |
| Azteronam          | 103 (63.97)| 0 (0.0)    | 58 (36.03)|                  |
| Polymyxin B        | 161 (100.0)| 0 (0.0)    | 0 (0.0)  |                  |
Table 4: Resistance phenotypes and presence of resistance genes in ESBL-positive *P. aeruginosa*

| Isolate | Resistance phenotypes a | Resistance genes | Sex b | Source c |
|---------|-------------------------|------------------|-------|----------|
| Pae-1   | TOB, PIP, CAZ, IMP     | *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub> | M     | U        |
| Pae-2   | TOB, PIP, LOM, CAZ, FEP, IMP, MEN | *bla*<sub>TEM</sub>, *bla*<sub>CTX</sub>, *bla*<sub>OXA</sub> | M     | PF       |
| Pae-3   | AN, PIP, CAZ, FEP, IMP | *bla*<sub>TEM</sub> | M     | BL       |
| Pae-5   | TOB, PIP, LOM, CAZ, FEP, IMP, MEN | *bla*<sub>TEM</sub> | M     | R        |
| Pae-8   | TOB, PIP, CP, LOM, CAZ, FEP, IMP, MEN, AZT | *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX</sub>, *bla*<sub>OXA</sub> | F     | CSF      |
| Pae-9   | AN, PIP, CAZ, FEP, IMP | *bla*<sub>TEM</sub> | M     | U        |
| Pae-15  | TOB, AN, PIP, CAZ, FEP, IMP | *bla*<sub>TEM</sub> | M     | R        |
| Pae-16  | PIP, LOM, CAZ, IMP     | *bla*<sub>TEM</sub> | M     | R        |
| Pae-19  | TOB, PIP, CP, LOM, CAZ, FEP, IMP, MEN, AZT | *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX</sub>, *bla*<sub>OXA</sub> | F     | W/A      |
| Pae-20  | TOB, AN, PIP, CAZ, FEP, IMP | *bla*<sub>TEM</sub> | F     | R        |
| Pae-21  | TOB, PIP, LOM, CAZ, FEP, IMP, MEN | *bla*<sub>TEM</sub> | F     | R        |
| Pae-25  | CP, CAZ, FEP, MEN     | *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub> | F     | U        |
| Pae-28  | TOB, PIP, CP, LOM, CAZ, FEP, IMP, MEN, AZT | *bla*<sub>TEM</sub> | F     | R        |
| Pae-30  | TOB, PIP, LOM, CAZ, FEP, IMP, MEN | --- | M     | BL       |
| Pae-31  | TOB, PIP, LOM, CAZ, FEP, IMP, MEN | *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX</sub> | F     | BL       |
| Pae-32  | TOB, AN, PIP, CAZ, FEP, IMP, AZT | *bla*<sub>TEM</sub> | F     | R        |
| Pae-33  | TOB, AN, PIP, CAZ, FEP, IMP | *bla*<sub>TEM</sub> | F     | R        |
| Pae-36  | TOB, PIP, CP, LOM, CAZ, FEP, IMP, MEN | *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>OXA</sub> | M     | R        |
| Pae-40  | TOB, AN, PIP, CAZ, FEP, IMP | *bla*<sub>SHV</sub> | M     | R        |
| Pae-45  | TOB, PIP, CP, LOM, CAZ, FEP, IMP, MEN | *bla*<sub>TEM</sub> | F     | U        |
| Pae-47  | TOB, PIP, LOM, CAZ, FEP, IMP, MEN, AZT | *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>OXA</sub> | M     | R        |
| Pae-48  | TOB, AN, PIP, CAZ, IMP | --- | F     | R        |
| Pae-53  | TOB, PIP, LOM, CAZ, FEP, IMP, MEN | *bla*<sub>SHV</sub> | M     | WA       |
| Pae-54  | TOB, AN, PIP, CAZ, FEP, IMP | *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX</sub> | M     | D/F      |
| Pae-57  | PIP, CP, LOM, CAZ, FEP, IMP, MEN, AZT | *bla*<sub>TEM</sub> | F     | CSF      |
| Pae-58  | TOB, PIP, CP, LOM, CAZ, FEP, IMP, MEN | *bla*<sub>TEM</sub> | M     | BL       |
| Pae-62  | TOB, AN, PIP, CAZ, IMP | --- | F     | U        |
| Pae-63  | TOB, PIP, CAZ, IMP | *bla*<sub>TEM</sub> | F     | R        |
| Pae-64  | TOB, PIP, LOM, CAZ, FEP, IMP, MEN | *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub> | M     | R        |
| Pae-70  | TOB, PIP, LOM, CAZ, FEP, IMP | *bla*<sub>CTX</sub> | F     | R        |
| Pae-72  | TOB, PIP, LOM, CAZ, FEP, IMP, MEN | --- | F     | R        |
| Pae-74  | TOB, PIP, CP, LOM, CAZ, FEP, IMP, MEN, AZT | *bla*<sub>OXA</sub> | F     | U        |
| Pae-76  | TOB, PIP, LOM, CAZ, FEP, IMP, MEN | *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX</sub>, *bla*<sub>OXA</sub> | M     | BL       |
| Pae-81  | AN, PIP, CAZ, FEP, IMP, AZT | *bla*<sub>OXA</sub> | M     | U        |
| Pae-82  | TOB, PIP, CP, LOM, CAZ, FEP, IMP, MEN, AZT | --- | F     | BL       |

a: TOB: tobramycin, AN: amikacin, CP: ciprofloxacin, LOM: levofloxacin, CAZ: ceftazidime, FEP: cefepime, IMP: imipenem, MEN: meropenem, AZT: azteronam
b: F: Female, M: Male
c: U: urine, W/A: wound/abscess, R: respiratory, D/F: dialysis-related fluids, AF: ascitic fluid, BL: blood, PF: plural fluid
Continue of table 4- Resistance phenotypes and presence of resistance genes in ESBL-positive *P. aeruginosa*

| Isolate | Resistance phenotypes | Resistance genes | Sex | Source |
|---------|-----------------------|------------------|-----|--------|
| Pae-84  | TOB, PIP, CP, LOM, CAZ, FEP, IMP, MEN, AZT | --- | M | U |
| Pae-86  | LOM, CAZ, FEP, IMP, MEN | **blaTEM** | M | R |
| Pae-87  | TOB, PIP, LOM, CAZ, FEP, IMP, MEN | **blaTEM** | F | R |
| Pae-90  | TOB, PIP, CP, LOM, CAZ, FEP, IMP, MEN, AZT | --- | F | BL |
| Pae-92  | TOB, PIP, CP, LOM, CAZ, FEP, IMP, MEN, AZT | **blaTEM**, **blaCTX** | M | R |
| Pae-94  | TOB, PIP, LOM, CAZ, FEP, IMP, MEN | **blaSHV** | F | U |
| Pae-95  | PIP, LOM, CAZ, IMP, MEN | --- | M | U |
| Pae-96  | TOB, AN, PIP, sCAZ, FEP, IMP, MEN, AZT | **blaTEM** | M | BL |
| Pae-97  | TOB, PIP, CAZ,IMP, MEN, AZT | **blaTEM**, **blaSHV**, **blaOXA** | F | PF |
| Pae-98  | PIP, CP, LOM, CAZ, IMP, MEN | **blaTEM**, **blaCTX**, **blaOXA** | M | R |
| Pae-108 | CP, LOM,CAZ, IMP, MEN | **blaTEM** | M | BL |
| Pae-110 | TOB, PIP, CP, LOM, CAZ, IMP, MEN, AZT | **blaSHV** | M | U |
| Pae-115 | TOB, PIP, CP, LOM, CAZ, IMP, MEN, AZT | **blaTEM**, **blaSHV**, **blaCTX**, **blaOXA** | M | U |
| Pae-118 | TOB, AN, PIP, CAZ, IMP, AZT | **blaTEM**, **blaCTX** | M | W/A |
| Pae-121 | CP, LOM, CAZ, MEN | **blaTEM**, **blaSHV** | F | R |
| Pae-123 | TOB, AN, PIP, CAZ, IMP, MEN, AZT | **blaTEM** | F | R |
| Pae-125 | FEP, MEN | **blaTEM**, **blaCTX** | M | R |
| Pae-127 | TOB, PIP, CP, LOM, CAZ, IMP, MEN | **blaTEM**, **blaSHV**, **blaCTX** | F | U |
| Pae-130 | TOB, AN, PIP, CAZ, IMP, AZT | **blaSHV**, **blaOXA** | F | U |
| Pae-133 | TOB, AN, PIP, CAZ, IMP, AZT | **blaTEM**, **blaCTX**, **blaOXA** | F | A/F |
| Pae-137 | TOB, PIP, CP, LOM, CAZ, IMP, MEN, AZT | --- | M | R |
| Pae-138 | TOB, AN, PIP, CAZ, IMP, AZT | **blaOXA** | F | U |
| Pae-140 | CAZ,FEp, MEN | **blaSHV** | M | BL |
| Pae-143 | PIP, CP, LOM, CAZ, IMP, MEN | **blaTEM**, **blaSHV** | M | R |
| Pae-150 | CAZ,FEp | **blaTEM**, **blaSHV**, **blaCTX** | F | BL |
| Pae-155 | CAZ,FEp | **blaTEM**, **blaSHV** | F | R |
| Pae-156 | CP, LOM,CAZ, IMP, MEN | **blaTEM** | M | U |
| Pae-160 | TOB, PIP, CP, LOM, CAZ, IMP, MEN, AZT | **blaTEM**, **blaSHV** | F | R |
| Pae-161 | CAZ,FEp, MEN | **blaTEM**, **blaCTX**, **blaOXA** | F | CSF |

**DISCUSSION**

Production of broad-spectrum β-lactamases is one of the ways through which *P. aeruginosa* becomes resistant to antibiotics of the β-lactam family. Since *P. aeruginosa* is an important cause of hospital-acquired infections, spread of ESBL-producing genes among *P. aeruginosa* strains can make treatment of infections more challenging (12). Therefore, periodic studies on the extent of resistance shown by this bacterium are of great importance. Based on the results, the highest rate of resistance was observed against ceftazidime, while none of the *P. aeruginosa* isolates was resistant to polymyxin B. Moreover, 39.75% of the isolates were ESBL-producing. A study by Ullah et al. showed that the lowest and highest level of antibiotic resistance was against meropenem (5.66%) and ampicillin (94.34%), respectively. Furthermore, 25.47% of the isolates were resistant to amikacin (15). Tavajjohi and Moniri reported that 9.2% of the *P. aeruginosa* isolates were ESBL-positive. Furthermore, more than 30% of the isolates were MDR, 13% of which were resistant to all studied antibiotics. The highest and lowest...
Employment of the phenotypic method could produce false negative results and disrupt the treatment process since antibiotics of the β-lactam family may be utilized to treat infections caused by ESBL-positive bacteria. Therefore, it is recommended to use the genotypic method for the detection of ESBL-positive isolates. In the present study, 12.5% of the ESBL-producing isolates had none of the studied resistance genes, probably because the enzyme was produced by genes (such as bla<sub>TEM</sub>, bla<sub>SHV</sub>, etc.) other than those investigated in the present study. Bacteriophages can be considered as alternatives to antibiotics considering the emergence and rising incidence of multi-drug resistance (21). The natural potential of virulent phages to infect and destroy specific bacterial host cell makes them safe antibacterial agents for treatment of various infections caused by <i>Shigella</i> spp. (21, 22), <i>Salmonella</i> spp. (23), <i>Proteus mirabilis</i> (24), <i>Klebsiella pneumoniae</i> (14), etc. Since most <i>P. aeruginosa</i> strains isolated in the present study were MDR, they can be utilized as host for phage isolation and phage cocktail preparation against <i>P. aeruginosa</i>.

**CONCLUSION**

The resistance patterns in bacteria should be periodically and systematically studied. Reporting the results of such studies to physicians could help specify resistance frequencies and bacterial susceptibility to various antibiotics, increasing the chance of successful treatment of infections. Furthermore, it is highly recommended to use the genotypic method along with the phenotypic method for the detection of ESBL-positive <i>P. aeruginosa</i> strains because of the better accuracy. This can greatly influence the effectiveness of the treatment process. Moreover, we suggest using the CDC protocol for identification of MDR, PDR and XDR isolates.

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**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.
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