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Detection of *bla*$_{OXA-10}$ and *bla*$_{OXA-48}$ Genes in *Pseudomonas aeruginosa* Clinical Isolates by Multiplex PCR

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ABSTRACT

Introduction: The rapidly increasing extended-spectrum β-lactamase-producing *Pseudomonas aeruginosa* is a threat to health. This study aims to detect the *rpoD* gene and *bla*$_{OXA-10}$ and *bla*$_{OXA-48}$ genes in imipenem-resistant *P. aeruginosa* clinical isolates simultaneously by multiplex polymerase chain reaction. **Methods:** Eighty-five culture plates were collected from patients suspected of *Pseudomonas* spp infection in Ghaem Hospital and Shahid Shourideh Clinic in Mashhad from January to February 2021. After biochemical identification of *P. aeruginosa* isolates and the measurement of antibiotic resistance, *bla*$_{OXA-10}$, *bla*$_{OXA-48}$, and *rpoD* genes were investigated by multiplex polymerase chain reaction in the imipenem-resistant isolates. **Results:** Of 82 *P. aeruginosa* isolates, 38 (46.34%) were resistant to imipenem, with the highest percentage to carbenicillin (69.5%). All imipenem-resistant *P. aeruginosa* isolates were confirmed by multiplex PCR using the primers that targeted the *rpoD* gene. Also, in multiplex PCR, among imipenem-resistant isolates, 10 (26.3%) and 9 (23.6%) had *bla*$_{OXA-10}$ and *bla*$_{OXA-48}$ genes, respectively. **Conclusion:** In addition to molecular identification of *P. aeruginosa*, the present study simultaneously detected *bla*$_{OXA-10}$ and *bla*$_{OXA-48}$ genes by multiplex PCR. Application of this multiplex PCR, rapid identification of patients, and timely treatment can reduce the β-lactamase gene prevalence in *P. aeruginosa* clinical isolates.

INTRODUCTION

Pseudomonas aeruginosa is an aerobic Gram-negative bacillus that causes an extensive range of opportunistic infections in humans. Infections due to this pathogen are frequently intense and health-threatening. *P. aeruginosa* infection is challenging to control and resolve since the causative bacteria are inherently resistant to many antimicrobial agents. The rapidly increasing extended-spectrum β-lactamase (ESBL)-producing *P. aeruginosa* bacteria poses a threat to public health [1]. This bacterium has an innate resistance to most antibiotic classes and can develop resistance against them even throughout treatment. Resistance expansion occurs through chromosomal mutations or horizontal transfer of resistance genes through plasmids, integrons, and transposons. The underlying mechanisms of the resistance are the efflux pump, alterations in outer membrane permeability, and carbapenemase and β-lactamase production. Epidemiological studies show that infections caused by resistant *P. aeruginosa* have increased illness, fatality, duration of stay in the hospital, and treatment cost [2].

Broad-spectrum penicillins, third and fourth descendant carbapenems, cephalosporins, monobactams, aminoglycosides, fluoroquinolones, and different antibiotics such as colistin are effective against *P. aeruginosa*. However, resistance mutations may appear and develop against all the anti-pseudomonal antibiotics [3].

A WHO report has shown vast rates of drug resistance in Gram-negative bacteria, with elevated illness and fatality. The *P. aeruginosa* clinical isolates quickly develop antibiotic resistance and spread in the hospital environment, making them fall under the WHO list of multidrug-resistant (MDR) bacteria [4].

P. aeruginosa has a main σ^{70} called *rpoD* that detects many promoters controlling the expression of housekeeping genes [5]. The OXA-type is one of the significant families of ESBLs. This family, classified into molecular class D and functional group 2d, can hydrolyze oxacillin. Unlike TEM and SHV, they are reported primarily in *P. aeruginosa* of the

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Enterobacteriaceae family. Most of these enzymes are resistant to cephalothin and ampicillin with vast hydrolytic activity versus cloxacillin and oxacillin. However, they are poorly inhibited by clavulanic acid and cannot hydrolyze the fourth and fifth-generation cephalosporins; hence, they are not considered ESBLs [6]. Carbapenems are β-lactam antibiotics that are efficient against Gram-positive and Gram-negative bacteria. These antibiotics are resistant to cephalosporinases and penicillinases. However, some of the β -lactamase groups (A, B, and D) classification by Ambler can hydrolyze carbapenems [7].

Molecular methods can specificity identify the presence of Metallo β-lactamases (MBL) genes. The polymerase chain reaction (PCR) is one of the simplest and fastest methods. Other molecular methods used in MBL recognition are nucleotide sequence analysis, polyacrylamide gel electrophoresis, and isoelectric focusing [3].

OXA-10 and OXA-48 carbapenemase are crucial because OXA-10 hydrolyze poorly aztreonam and ceftriaxone, giving most microbes less susceptibility to these antimicrobials. Altogether, OXA-type β-lactamases are explosively increasing based on the amino acid sequence alterations; until now, 498 reports are available in the database [6]. The ESBLs OXA-11, OXA-14, OXA-16, OXA-17, OXA-19, and OXA-28 are relevant structurally to OXA-10. All of these ESBLs have been found in *P. aeruginosa* [8]. Also, in some cases, the OXA-48 has migrated into the *Enterobacteriaceae* and is becoming a significant cause of carbapenem resistance. The emergence of OXA enzymes that can confer resistance to carbapenems has converted these β-lactamases from a minor obstacle into a significant problem, reducing the clinical efficacy of carbapenems [9].

For the first time in Iran, the present study investigated the simultaneous presence of the *rpoD* gene and bla_{OXA-10} and bla_{OXA-48} genes in imipenem-resistant *P. aeruginosa* clinical isolates.

MATERIALS AND METHODS

Isolation and biochemical identification *P. aeruginosa*. Eighty-five culture plates from patients in Ghaem Hospital and Shahid Shourideh Clinic in Mashhad, suspected with Pseudomonas spp. infection was collected from January to February 2021 and transferred to the Microbiology Laboratory under sterile conditions on an ice pack. The identity of *P. aeruginosa* was confirmed by culturing the isolates on selective Cetrimide agar medium plates (Merck, Germany) followed by incubation at 42°C for 24 hours. The green-blue colonies were isolated and purified. After Gram staining, biochemical identification of *P. aeruginosa* was performed using biochemical tests, i.e., catalase, oxidase, methyl-red, SIM, Simmons citrate, culture in TSI agar, urea (Merck, Germany), and growth at 42°C and pigment output in Cetrimide agar medium [10].

Determination of antibiotic resistance patterns. The Kirby-Bauer disk diffusion method was performed for antibiotic susceptibility under aerobic conditions and according to the Clinical and Laboratory Standards Institute (CLSI, 2018) [11]. Antibiotic disks used for this purpose included Imipenem (IPM) (10 μg), Ceftazidime (CAZ) (30 μg), and Carbenicillin (CB) (100 μg) (Padtan Teb, Iran) [12-17].

Molecular identification and detection of genes. For this purpose, a multiplex PCR experiment was designed and performed. DNA extraction from IPM-resistant isolates was performed using the boiling technique. For this purpose, 3-4 new colonies from 24-hour old cultures were added to microtubes containing 200 μL of distilled water, followed by incubation at 100 °C for 10 min. The suspensions were centrifuged at 9,500 × g for 5 min in a BRK5424 rotor (Cleaver Scientific, United Kingdom), and the supernatants containing DNA were transferred to the new microtubes [18]. Identification of *P. aeruginosa* isolates was identified by primers complementary to the *rpoD* gene, and primers for bla_{OXA-10} and bla_{OXA-48} genes were used in IPM-resistant strains to identify β-lactamase enzymes producing isolates. The specificity of the primer sequences was checked by the Basic Local Alignment Search Tool (BLAST) at the NCBI site (http://www.ncbi.nlm.nih.gov/) and synthesized at a commercial company (Cinnagen, Iran) (Table 1).

Table 1. The sequence of primers used to amplify the *rpoD*, bla_{OXA-10} and, bla_{OXA-48} genes in multiplex PCR test

Primer pairs	Target	Primer sequence (5'→3')	Amplified product size (bp)	Reference
rpoD – F	rpoD	GGGCGAAGAAGGAAATGGTC	178	[19]
rpoD – R		CAGGTGGCGTAGGTGGAGAA		
OXA – 10 – F	OXA – 10 group	TCAACAAATCGCCAGAGAAG	276	[20]
OXA – 10 – R		TCCCACACCAGAAAAACCAG		
OXA – 48 – F	OXA – 48 group	GCGTGGTTAAGGATGAACAC	438	[21]
OXA – 48 – R		CATCAAGTTCAACCCAACCG		

The multiplex PCR tests were performed in final 25 μL volumes containing 12.5 μL PCR master mix 2X (Pars Tous, Iran), 0.75 μL of each primer with a concentration of 1 mM (CinnaGen, Iran), 2 μL template DNA, and 6 μL sterile distilled water. The optimize thermocycler (Kyratec, Korea) program consisted of 5 min initial denaturation at 95°C, 35 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s,

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extension at 72°C for 30 s and a final extension at 72°C for 5 min., The PCR products were visualized by gel electrophoresis (Interlab, Italy) in 2% (w/v) agarose gel (Dena Gene, Iran) in TBE buffer (Pars Tous, Iran) stained with ethidium bromide (Merck, Germany) and photographed under UV in a Gel Documentation System (Cleaver Scientific, United Kingdom).

Statistical analysis. Statistical analysis of data was performed using SPSS software version 23 and comparing the proportions with the Fisher's exact test at a probability level of five percent (P<0.05).

RESULTS

Biochemical identification of *P. aeruginosa*. In this study, based on biochemical tests (Table 2), 82 *P. aeruginosa* isolates were identified among the 85 collected specimens .

Table 2. Results of biochemical tests for detecting *P. aeruginosa* isolates.

Test	Gram stain	Citrate	Urease	Methyl Red	Grow at 42°C	Motility	Indole	Sugar fermentation	Catalase	Oxidase
Result	Negative	+	-	-	+	+	-	-	+	+

Antibiotic susceptibility pattern. Out of 82 *P. aeruginosa* isolates, 38 (46.34%) were resistant to IPM. The isolates were categorized as "susceptible," "intermediate," or "resistant" to the antibiotics IPM, CAZ, and CB (Table 3).

Table 3. Antibiotic susceptibility pattern among 82 *P. aeruginosa* isolates

Antibiotic	Susceptibility (%)		
	Susceptible	Intermediate	Resistant
IPM	47.56	6.09	46.34
CAZ	21.9	17.7	60.9
CB	20.7	9.7	69.5

IPM, Imipenem; CAZ, Ceftazidime; CB, Carbenicillin

Figure 1 shows the resistance to CB and CAZ antibiotics among the susceptible and resistant isolates to imipenem. As shown in the diagram, based on the statistical analysis performed, the resistance rates to CB and CAZ antibiotics among imipenem-resistant isolates were significantly higher than imipenem-susceptible isolates (p<0.0001), indicating the possibility of simultaneous transfer of antibiotic resistance genes. The resistance rates to CB and CAZ among imipenem-resistant isolates were 100% (Fig.1).

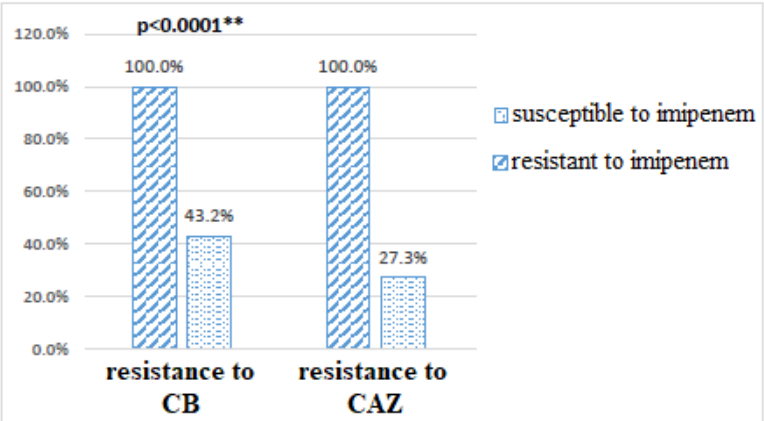

Fig. 1. Resistance pattern to CB and CAZ among susceptible and resistant isolates to imipenem.
CAZ, Ceftazidime; CB, Carbenicillin

Molecular identification of isolates and genes. All imipenem-resistant *P. aeruginosa* isolates (n=38) were confirmed by multiplex PCR amplifying the *rpoD* gene. Also, in multiplex PCR, among imipenem-resistant isolates had 10 (26.3%) bla$_{OXA-10}$ gene and 9 (23.6%) bla$_{OXA-48}$ gene. The amplicons size yielded by the multiplex PCR for the *rpoD*, bla$_{OXA-10}$, and bla$_{OXA-48}$ genes were similar to the standard samples, i.e., 178, 276, and 438 bp, respectively (Fig. 2).

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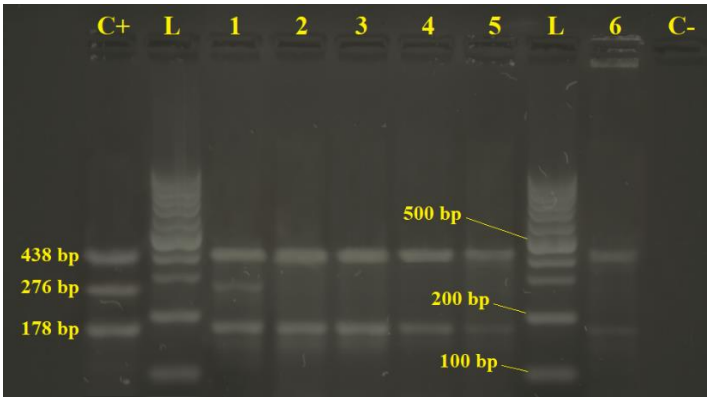

Fig 2. Gel electrophoresis of multiplex PCR products after amplification of *rpoD*, *bla*$_{OXA-10}$, and *bla*$_{OXA-48}$ genes for imipenem-resistant *P. aeruginosa* isolates along with the 100-bp DNA size marker. Lanes 1-6, specimens; L, 100-bp DNA Ladder- 100-1000 bp (Sinaclone, Iran); C+, positive control; C-, negative control.

DISCUSSION

P. aeruginosa is an opportunistic and nosocomial pathogen that can cause several infections with high fatality in patients, especially in immunocompromised individuals hospitalized in hospitals [22-24]. Therefore, a reliable method is required to detect this pathogen as rapidly as possible and track the genes involved in antibiotic resistance.

MBLs are significant because of conferring resistance against carbapenems (imipenem, meropenem, biapenem, and ertapenem), the most efficacious antibiotics used against *P. aeruginosa*. The prevalence of carbapenem resistance in *P. aeruginosa*, which plays a crucial role in nosocomial infections, is a severe threat in treating conditions caused by this bacterium [25, 26].

This study aimed to appraise the antimicrobial resistance pattern and detect the frequency of *bla*$_{OXA-10}$ and *bla*$_{OXA-48}$ genes alongside the *rpoD* gene in *P. aeruginosa* clinical isolates.

Most isolates in this study showed multiple resistance to various antibiotics, including IMP (46.34%), CAZ (60.9%), and CB (69.5%), with rates higher than those reported in China [27] but lower than in India [28]. These variations might be due to differences in antibiotic use in different countries (29). A survey on *P. aeruginosa* isolates from respiratory and urinary secretions reported the highest resistance to aztreonam and imipenem [30]. In another survey on *P. aeruginosa* blood isolates, the frequency of imipenem-resistant strains was 34% [31]. The achievements of the present study are similar to the findings of the above studies. In a study, the frequency of carbapenem-resistant in *P. aeruginosa* clinical isolates was 24% [32]. A 10-year longitudinal surveillance study in Taiwan, among 78 non-duplicated imipenem-resistant *P. aeruginosa* isolates collected from patients with blood infections, reported an imipenem resistance of 5.9% [33]. A possible cause of the difference in the results of these studies with the present study might be extended hospitalization at the intensive care unit (≥29 days) that can lead to the acquisition of carbapenem resistance [34].

In Our study, all imipenem-resistant *P. aeruginosa* isolates (n=38) were confirmed by amplifying the *rpoD* gene in multiplex PCR. Also, in multiplex PCR, among imipenem-resistant isolates, 10 (26.3%) and 9 (23.6%) had *bla*$_{OXA-10}$ and *bla*$_{OXA-48}$ genes, respectively. In conclusion, a high prevalence of *bla*$_{OXA-10}$ and *bla*$_{OXA-48}$ genes was observed in this study, and imipenem was the effective agent against *P. aeruginosa* isolates.

Similar studies like the present have used *rpoD* gene amplification as a rapid, inexpensive, and reliable method for the molecular identification of *P. aeruginosa* [35-37]. In a similar study, of 50 imipenem-resistant isolates, 18 (36%) had the *bla*$_{OXA-10}$ gene [12]. In two other studies, *bla*$_{OXA-10}$ was detected in 22 (32.3 %) and 12 (27.27%) imipenem-resistant isolates [13, 14]. In Tehran, 83.95% of *P. aeruginosa* isolates in the Burn Unit of Shahid Motahari Hospital showed resistance to imipenem, and 83.70% contained *bla*$_{OXA-48}$ [15]; the imipenem resistance rate and isolates percentage containing the *bla*$_{OXA-48}$ gene was more than the present study. This discrepancy may be due to different clinical specimens. Although the resistance mechanisms to imipenem in isolates are different, the results of the present study and similar researches can reverberate that the OXA-type β-lactamases can efficiently hydrolyze carbapenem.

Recent reports indicate a high prevalence of ESBLs, especially OXA-types, in hospitalized patients in various cities of Iran [17, 38, 39]. Therefore, surveillance programs must be adopted to prevent the spread of these worrisome resistance genes [26]. Application of this multiplex PCR, rapid identification of patients, and timely treatment can reduce the β-lactamase gene prevalence in *P. aeruginosa* clinical isolates.

Further studies are required to test the specificity of this multiplex PCR with DNA from other *Pseudomonas* species and other bacteria.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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