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RESEARCH ARTICLE

Imipenem resistance associated with amino acid alterations of the OprD porin in *Pseudomonas aeruginosa* clinical isolates

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ABSTRACT

Globally, the spread of carbapenem-resistant strains has limited treatment options for multidrug-resistant (MDR) *Pseudomonas aeruginosa* infections. This study aimed to determine the role of point mutations as well as the expression level of the *oprD* gene in the emergence of imipenem-resistant *P. aeruginosa* strains isolated from patients referred to Ardabil hospitals. A total of 48 imipenem-resistant clinical isolates of *P. aeruginosa* collected between June 2019 and January 2022 were used in this study. Detection of the *oprD* gene and its amino acid alterations was performed using the polymerase chain reaction (PCR) and DNA sequencing techniques. The expression level of the *oprD* gene in imipenem-resistant strains was determined using the real-time quantitative reverse transcription PCR (RT-PCR) method. All imipenem-resistant *P. aeruginosa* strains were positive for the *oprD* gene based on the PCR results, and also five selected isolates indicated one or more amino acid alterations. Detected amino acid alterations in the OprD porin were Ala210Ile, Gln202Glu, Ala189Val, Ala186Pro, Leu170Phe, Leu127Val, Thr115Lys, and Ser103Thr. Based on the RT-PCR results, the *oprD* gene was downregulated in 79.1% of imipenem-resistant *P. aeruginosa* strains. However, 20.9% of strains showed overexpression of the *oprD* gene. Probably, resistance to imipenem in these strains is associated with the presence of carbapenemases, AmpC cephalosporinase, or efflux pumps. Owing to the high prevalence of imipenem-resistant *P. aeruginosa* strains due to various resistance mechanisms in Ardabil hospitals, the implementation of surveillance programs to reduce the spread of these resistant microorganisms along with rational selection and prescription of antibiotics is recommended.

KEYWORDS

Pseudomonas aeruginosa, antibiotic resistance, imipenem, *oprD* gene

INTRODUCTION

Multidrug-resistant (MDR) *Pseudomonas aeruginosa* strains have expanded in hospital environments, mainly in the intensive care units (ICU), all over the world [1–3]. The distribution of MDR *P. aeruginosa* strains in Iran is also high (58%) [4]. Hence, it is not surprising that the prevalence of these resistant bacteria in the hospitals of Ardabil, northwest of Iran, is high (52%) [5]. Recently, MDR *P. aeruginosa* isolates have become a global public

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health problem due to their limited treatment options [1, 3]. The best available antibiotics for the treatment of MDR *P. aeruginosa* infections are carbapenems [6]. However, the World Health Organization (WHO) released a list of antibiotic-resistant bacteria in 2017 in which carbapenem-resistant *P. aeruginosa* was ranked second [7]. This high rate of resistance is compliant with the increasing trend of resistance in different cities of Iran. For example, the overall prevalence of imipenem resistant *P. aeruginosa* strains in Iran is 54%, while the rate has been reported to be higher in Ardabil city (66.7%) [3, 8]. Considering the importance of carbapenem antibiotics in *P. aeruginosa* infection treatment, evaluation of resistance mechanisms to these antibiotics is essential to help better control this opportunistic nosocomial pathogen. In phenotypic methods, there are three antibiotic susceptibility profiles for carbapenem-resistant *P. aeruginosa* clinical isolates including isolates resistant to imipenem (and sensitive to meropenem), sensitive to imipenem (and resistant to meropenem), and resistant to both imipenem and meropenem [9]. This suggests that *P. aeruginosa* resistance to carbapenem antibiotics is multifactorial and can be attributed to the action of carbapenemases (mainly metallo-β-lactamases), increased expression of AmpC cephalosporinase, increased expression of efflux pumps (MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM), and loss or downregulation of the OprD porin [10]. All the above-mentioned carbapenem resistance mechanisms have been assessed in clinical isolates of *P. aeruginosa* collected from Ardabil hospitals except for loss or downregulation of the OprD porin. The loss or downregulation of the OprD porin is the most common resistance mechanism to imipenem, which is also responsible for a low-grade resistance to meropenem in *P. aeruginosa* [9]. Alterations in the *oprD* gene because of point mutations, deletions, or insertions are involved in the loss or reduced expression of the OprD porin in the outer membrane [11].

In this study, imipenem-resistant *P. aeruginosa* isolates from patients referring to Ardabil hospitals were evaluated to determine the role of point mutations as well as the expression level of the *oprD* gene in the emergence of these resistant bacteria.

MATERIALS AND METHODS

Carbapenem-resistant *P. aeruginosa*

A total of 48 imipenem-resistant clinical isolates of *P. aeruginosa* were used in this cross-sectional study. Isolates were obtained from different specimens of patients who were referred to Ardabil hospitals between June 2019 and January 2022 and were identified based on laboratory standard tests. Antimicrobial susceptibility testing of *P. aeruginosa* isolates to imipenem was previously done using the disk diffusion method according to the Clinical and Laboratory Standard Institute (CLSI) guidelines [3]. All imipenem-resistant *P. aeruginosa* strains were MDR.

Detection of the *oprD* gene

The presence of the *oprD* gene in imipenem resistant *P. aeruginosa* was determined using the polymerase chain reaction (PCR) technique (Eppendorf Thermal Cycler, Germany). For this aim, chromosomal DNA was extracted using the boiling method, and its quality was confirmed using a NanoDrop 2000c Spectrophotometer (Thermo Scientific, USA). PCR reactions for the *oprD* gene amplification were performed in a volume of 25 µL (20 µL of Master Mix (Ampliqon, Denmark) with 3 µL of extracted bacterial DNA, and 2 µL of primers (10 µmol L^{-1}) (Metabion, Germany)). The presence/absence of the *oprD* gene was determined in 1% agarose gel electrophoresis and confirmed by nucleotide sequencing. Primers used in the present study were forward: ATGAAAGTGATGAAGTGGAGC and reverse: AGGGAGGCGCTGAGGTT (amplicon size 671 bp) [12]. This primer set was not used for measuring the rate of the *oprD* gene expression. In addition, the PCR program was as follows: initial denaturation at 95 °C for 5 min (1 cycle), followed by three steps of 34 cycles including denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min, and extension at 72 °C for 1 min.

Identification of amino acid alterations in the OprD porin

Molecular identification of amino acid alterations in the OprD porin in clinical isolates of imipenem-resistant *P. aeruginosa* was performed using the PCR and nucleotide sequencing methods. For this purpose, after determining the presence of the *oprD* gene in imipenem-resistant *P. aeruginosa* isolates, five strains were selected randomly and their PCR products were sequenced. An imipenem-sensitive strain was used as a control as well. The results were compared with the reference strain PAO1 and then the main point mutations were determined. Analysis of amino acid changes was done using the BioEdit Sequence Alignment Editor (version 7.2.5).

Analysis of the *oprD* gene expression

The level of the *oprD* gene expression in imipenem-resistant *P. aeruginosa* strains was assessed using the real-time quantitative reverse transcription PCR (RT-PCR) method (LightCycler® System, Roche Diagnostics). For this purpose, total RNA extraction was performed using the RNA extraction kit (Favorgen, Taiwan). Qualified RNAs were identified using a NanoDrop 2000c Spectrophotometer, and were selected for cDNA synthesis using oligo (dT) and random hexamer primers (50 µM) based on the manufacturer's instructions (YTA, Iran). In addition, the primers used for the *oprD* gene transcriptional expression in RT-PCR were forward: CGACCTGCTGCTCCGCAACTA and reverse: TTGCATCTCGCCCCACTTCAG (amplicon size 301 bp) [13]. The housekeeping ribosomal gene *rpsL* was used as the internal control and its primers were forward: GCTGCAAAACTGCCCGCAACG and reverse: ACCGCAGGTGTCCAGCGAACC (amplicon size 250 bp) [13].

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Each RT-PCR reaction for the *oprD* and *rpsL* genes was performed in a volume of 20 µL (SYBR Green PCR Master Mix without ROX™ (10 µL) (Ampliqon, Denmark), primers (2 µL) (10 µmol L^{-1}) (Metabion, Germany), cDNA (1 µL), and DEPC-treated water (7 µL)). RT-PCR programs were as follows: pre-incubation at 95 °C for 600 s (1 cycle), followed by 40 cycles of amplification steps including 95 °C for 20 s, 64 °C for 20 s (both *oprD* and *rpsL* genes), and 72 °C for 30 s. All amplifications were done in duplicate. The expression of the *oprD* gene in each isolate was standardized to the housekeeping *rpsL* gene and then compared with the corresponding mRNA expression by the reference strain *P. aeruginosa* PAO1. The *oprD* mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method and the expression rate ≤30% compared with the reference strain was considered as diminished gene expression [14].

The melting curve of each amplicon was analyzed to evaluate specific cDNA amplification with the following program: 1 cycle at 95 °C for 10 s, 65 °C for 60 s, and 97 °C for 1 s. Data analysis included the cycle threshold (Ct) from primer dimer- and artefact-free amplifications.

Other imipenem resistance mechanisms of *P. aeruginosa* resistance, *i.e.* carbapenemases (IMP, VIM, OXA-23, and OXA-48), AmpC cephalosporinase, and efflux pumps (MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM), were investigated in our previous studies. To have an overview of the presence of various resistance mechanisms, the results of previous studies are compared in Table 1 [15].

RESULTS

All imipenem-resistant *P. aeruginosa* strains harbored the *oprD* gene based on the PCR results. The nucleotide sequence accession number for the *oprD* gene in the GenBank database is ON009070. In addition, all of the imipenem-resistant and *oprD* gene-positive *P. aeruginosa* strains were MDR.

The *oprD* gene of imipenem-resistant *P. aeruginosa* strains was subjected to DNA sequencing to find point mutations associated with resistance to carbapenems. ON986770 to ON986774 are nucleotide sequence accession numbers received from the GenBank database. All five clinical isolates of *P. aeruginosa* indicated one or more amino acid alterations. Amino acid alterations in the OprD porin are shown in Fig. 1. The most common mutations occurred at codons 210 (Ala210Ile) and 202 (Gln202Glu). These alterations were observed in four isolates. At codons 189 and 186, Ala189Val and Ala186Pro amino acid alterations were found, respectively. Amino acid substitutions of Leu170Phe and Leu127Val at positions 170 and 127, respectively, were detected in two different strains. One strain showed amino acid changes at codons 115 (Thr115Lys) and 103 (Ser103Thr).

Based on the RT-PCR results, the *oprD* mRNA expression was downregulated in 79.1% (38 out of 48) of imipenem-resistant *P. aeruginosa* strains (Table 1). As shown in Fig. 2, the expression level of the *oprD* gene was between −716.5 (0.001) and 100.7 folds compared with the reference strain PAO1 (expression rate = 1). In addition, 20.9% of imipenem-resistant *P. aeruginosa* strains showed overexpression of the *oprD* gene. Nevertheless, Table 1 shows that these strains carried genes encoding carbapenemases (for example, carbapenemase (bla_{IMP} and bla_{VIM} genes), oxacillinase-23 (bla_{OXA-23}), and −48 (bla_{OXA-48})), overproduction of the AmpC cephalosporinase or efflux pumps. Table 1 showed all resistance mechanisms to imipenem in *P. aeruginosa* strains isolated from different Ardabil hospitals. As shown in Table 1, the main mechanisms associated with imipenem resistance in *P. aeruginosa* strains were the loss of outer membrane porin OprD and overexpression of the efflux pumps.

DISCUSSION

Choosing effective antibiotics against carbapenem-resistant *P. aeruginosa* infections is difficult because of this microorganism's intrinsic and acquired resistance mechanisms [16]. Outer membrane protein OprD is the principal portal of entry for carbapenems and hence loss or downregulation of the OprD porin plays an important role in the emergence of resistance to imipenem and reduced susceptibility to meropenem [17]. Point mutations that cause the formation of a premature stop codons in the *oprD* gene along with large deletions, which interfere with the expression of the *oprD* gene, are involved in the loss of the OprD porin from the outer membranes of *P. aeruginosa* strains [17]. In the current study, common amino acid alterations found in the OprD porin were Ala210Ile, Gln202Glu, Ala189Val, Ala186Pro, Leu170Phe, Leu127Val, Thr115Lys, and Ser103Thr. Similar results were reported in a previous study in Isfahan by Fazeli et al. [18]. Noteworthy, some of these amino acid alterations were also observed in the imipenem-sensitive strain, which implies that other mechanisms are also involved in the induction of resistance to imipenem. In addition to point mutations in the *oprD* gene, inactivation of this gene through the insertion of mobile genetic elements confers resistance to carbapenems [16]. The role of mobile genetic elements including different insertion sequences (ISs), such as IS*Pa1328*, IS*Pa1635*, IS*Ppu21*, IS*Ppu22*, IS*Pa133*, IS*Pa46*, IS*Pa45*, IS*Pa26*, and IS*Pa8*, in the emergence of carbapenem-resistant *P. aeruginosa* strains in different region of the world has been emphasized [12]. Nevertheless, a study conducted by Nazari et al. indicated that ISs were not involved in the emergence of carbapenem-resistant *P. aeruginosa* strains in Ardabil hospitals [12].

In the current study, we assessed the association between imipenem resistance and diminished gene expression of outer membrane protein OprD using the RT-PCR in imipenem-resistant *P. aeruginosa* strains. Our results showed that the *oprD* gene was downregulated in 79.1% of the clinical strains of imipenem-resistant and MDR *P. aeruginosa*. Rodríguez-Martínez et al. reported loss of the OprD porin in 65% of the imipenem-resistant *P. aeruginosa*

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Table 1. Characteristics of imipenem-resistant *P. aeruginosa* strains

Isolate	Type of specimen	Hospital	*oprD* mRNA expression*	Other imipenem resistance mechanisms		
				Carbapenemase	AmpC overproduction	Efflux pump overproduction
29	Urinary	Sabalan	**0.02**	–	AmpC cephalosporinase	*mexA, mexC, mexY*
32	Urinary	Alavi	0.7	–	AmpC cephalosporinase	*mexA, mexC, mexY*
34	Pulmonary	Alavi	**0.005**	IMP	–	*mexA, mexC*
36	Urinary	Bu-Ali	1.93	–	AmpC cephalosporinase	*mexA, mexC, mexE, mexY*
39	Urinary	Imam Khomeini	4.19	IMP	–	*mexA, mexC, mexE, mexY*
40	Urinary	Imam Khomeini	**0.02**	–	AmpC cephalosporinase	*mexA*
41	Urinary	Imam Khomeini	**0.04**	VIM	AmpC cephalosporinase	*mexA, mexC, mexE, mexY*
43	Urinary	Alavi	**0.1**	IMP	AmpC cephalosporinase	*mexA, mexC, mexY*
49	Wound	Imam Khomeini	**0.05**	–	–	*mexA, mexY*
61	Pulmonary	Alavi	**0.06**	–	–	*mexA*
62	Pulmonary	Alavi	**0.01**	OXA-23	AmpC cephalosporinase	*mexA, mexC, mexY*
63	Pulmonary	Alavi	**0.08**	OXA-23	AmpC cephalosporinase	*mexA, mexC, mexY*
64	Pulmonary	Alavi	**0.01**	–	–	*mexA, mexC, mexY*
65	Pulmonary	Alavi	**0.01**	IMP, OXA-23	AmpC cephalosporinase	*mexA, mexC, mexY*
66	Urinary	Alavi	**0.02**	IMP	–	–
68	Blood	Alavi	**0.03**	OXA-23	–	*mexC*
72	Urinary	Imam Reza	**0.002**	–	–	*mexC*
74	Urinary	Imam Reza	0.6	OXA-23	–	*mexA, mexC*
75	Urinary	Imam Reza	5.3	OXA-23	–	*mexA, mexC, mexY*
77	Wound	Alavi	**0.02**	OXA-23	–	–
79	Urinary	Imam Reza	**0.05**	OXA-23	–	*mexA, mexC, mexY*
80	Wound	Imam Khomeini	0.5	OXA-23	–	*mexA, mexC, mexY*
81	Urinary	Imam Khomeini	0.9	OXA-23	AmpC cephalosporinase	*mexA, mexC*
83	Blood	Alavi	**0.02**	OXA-23	–	*mexC*
84	Pulmonary	Alavi	**0.03**	OXA-23	–	*mexA, mexC*
85	Pulmonary	Imam Khomeini	**0.04**	OXA-23, OXA-48	–	*mexA, mexC*
94	Urinary	Imam Khomeini	**0.001**	OXA-23	AmpC cephalosporinase	*mexA, mexC*
96	Wound	Imam Khomeini	**0.03**	OXA-23	–	*mexA, mexC*
97	Urinary	Imam Khomeini	**0.01**	OXA-23	AmpC cephalosporinase	*mexA, mexC, mexY*
98	Pulmonary	Imam Khomeini	**0.04**	OXA-23, OXA-48	AmpC cephalosporinase	*mexA, mexC, mexY*
100	Urinary	Imam Khomeini	**0.03**	OXA-23	–	*mexA, mexC*
102	Urinary	Imam Khomeini	**0.01**	–	–	–
103	Wound	Imam Khomeini	**0.09**	–	AmpC cephalosporinase	*mexA, mexC, mexY*
110	Blood	Imam Khomeini	**0.02**	–	–	–

(continued)

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Table 1. Continued

Isolate	Type of specimen	Hospital	*oprD* mRNA expression*	Other imipenem resistance mechanisms		
				Carbapenemase	AmpC overproduction	Efflux pump overproduction
118	Pulmonary	Alavi	**0.02**	–	–	*mexA, mexC*
120	Pulmonary	Alavi	**0.01**	–	–	*mexC*
124	Pulmonary	Alavi	**0.03**	–	–	*mexA, mexC*
126	Urinary	Imam Reza	**0.01**	–	–	*mexA, mexC*
127	Urinary	Imam Reza	1.1	–	AmpC cephalosporinase	*mexA, mexC, mexY*
130	Urinary	Imam Khomeini	100.7	–	AmpC cephalosporinase	*mexA, mexC, mexE, mexY*
137	Pulmonary	Imam Reza	**0.02**	–	–	*mexA, mexC, mexE*
138	Urinary	Imam Reza	**0.009**	–	–	*mexC*
140	Pulmonary	Imam Reza	**0.01**	–	–	*mexY*
141	Pulmonary	Imam Reza	**0.1**	–	–	*mexA, mexC*
142	Pulmonary	Imam Khomeini	**0.02**	–	–	*mexA, mexC*
144	Wound	Imam Khomeini	0.4	–	–	*mexC*
146	Urinary	Imam Khomeini	**0.2**	–	–	*mexA, mexC*
147	Urinary	Imam Khomeini	**0.01**	–	–	*mexC*
148**	Urinary	Imam Khomeini	0.5	–	–	–
PAO-1***			1	–	–	–

*Highlighted numbers indicate decreased gene expression.
**Imipenem-sensitive *P. aeruginosa.*
***Standard strain.
(–) indicates not detected.

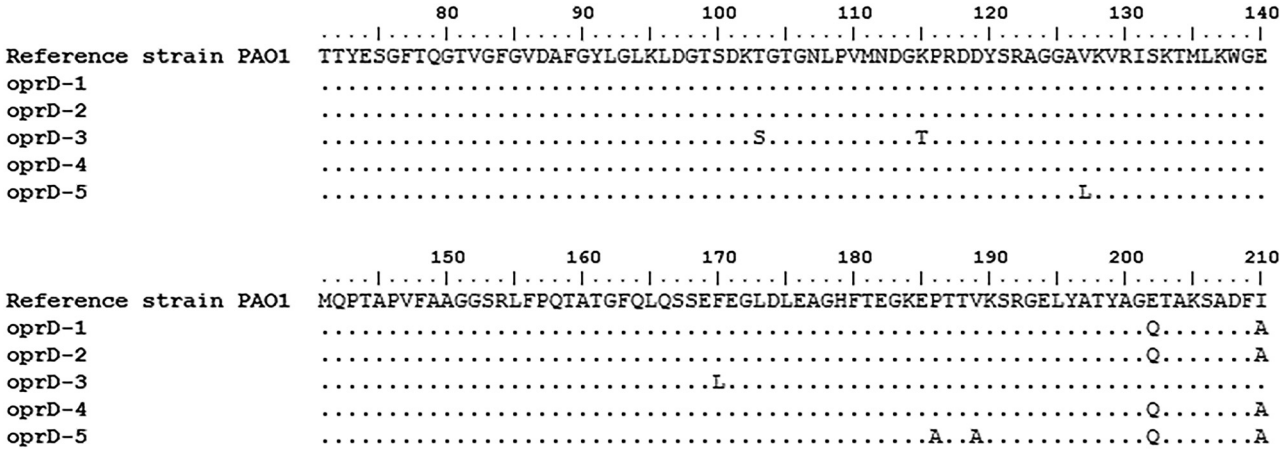

Fig. 1. Amino acid alterations in the OprD porin

isolates [10]. Quale et al. reported a decreased expression of the *oprD* gene in all imipenem-resistant *P. aeruginosa* strains [19]. Arabestani et al. reported the presence of the *oprD* gene in all clinical strains using the PCR, but the gene expression rate was different [20]. Aghazadeh et al. showed downregulation of the *oprD* gene was 14.8% in non-metallo-β-lactamase producing *P. aeruginosa* [21]. Based on the study by Mirsalehian et al., the *oprD* gene expression was downregulated in 56.6% of MDR *P. aeruginosa* strains [22]. Differences in the results of various studies can be attributed to the existence of several carbapenem resistance mechanisms in *P. aeruginosa* strains, and different interpretations of gene expression results. In the current study, 20.9% of imipenem-resistant *P. aeruginosa* strains showed

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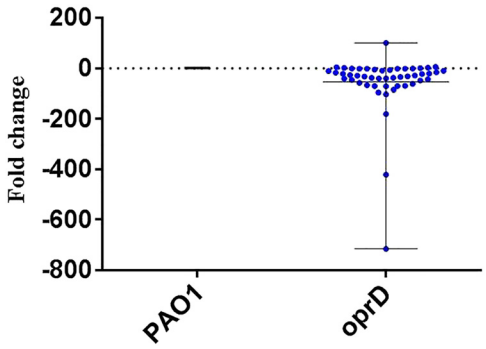

Fig. 2. Downregulation of the OprD porin in 48 imipenem-resistant *P. aeruginosa* clinical isolates. A decreased level of the OprD transcription was observed in the majority of *P. aeruginosa* isolates compared with the reference strain PAO1. Each blue circle represents a strain

overexpression of the *oprD* gene. It seems that resistance to imipenem in these *P. aeruginosa* strains is associated with the presence of genes encoding carbapenemases, overexpression of the AmpC cephalosporinase, or overexpression of the efflux pumps. Based on our previous study on 48 imipenem-resistant *P. aeruginosa* strains, overexpression of the MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM was observed in 75%, 83.3%, 10.4%, and 41.6% of isolates, respectively [15]. Furthermore, overexpression of the AmpC cephalosporinase and carbapenemase-encoding genes was observed in 33.3% and 47.9% of imipenem-resistant *P. aeruginosa* strains, respectively [unpublished data].

Considering the high prevalence of imipenem-resistant and MDR *P. aeruginosa* strains in Ardabil hospitals, which is associated with the presence of different resistance mechanisms, further research on the efficacy of other available antibiotics such as polymyxins is recommended.

The main limitation of the current study was the lack of assessment of alterations in the *oprD* gene through point mutations in all imipenem-resistant *P. aeruginosa* clinical isolates.

CONCLUSIONS

The present results revealed diverse mechanisms involved in the resistance of *P. aeruginosa* clinical isolates to carbapenems. One major resistance mechanism was decreased permeability to carbapenems due to the downregulation of OprD porin. On the other hand, our previous studies showed that the prevalence of carbapenem-resistant *P. aeruginosa* is high in Ardabil hospitals. Hence, implementation of surveillance programs to reduce the spread of these resistant microorganisms in hospitals along with rational selection and prescription of antibiotics are key measures to reduce the spread of carbapenem-resistant *P. aeruginosa*.

Conflict of interest: The authors declare that there is no conflict of interest.

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