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Original Article

Investigating of four main carbapenem-resistance mechanisms in high-level carbapenem resistant *Pseudomonas aeruginosa* isolated from burn patients

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Abstract

Background: *Pseudomonas aeruginosa* is an opportunistic pathogen involved in many infections. Carbapenem-resistant *P. aeruginosa* has emerged as an important cause of infection in different hospitals worldwide. We aimed to determine frequencies of the four main resistance mechanisms [metallo-beta lactamase (MBL) production (bla_{IMP}, bla_{VIM}, bla_{SPM} and bla_{NDM}), overproduction of the MexAB−OprM and MexXY efflux pumps, overproduction of chromosome-encoded AmpC β−lactamase, and reduced *OprD* expression] in high-level carbapenem-resistant *P. aeruginosa* isolated from patients with burns.

Methods: In a descriptive study, 107 *P. aeruginosa* isolates were collected from patients with burn injuries and tested for antibiotic susceptibility, by an E-test for carbapenems, an E-test for metallo-β-lactamase producer isolates, and PCR to detect MBL genes. Furthermore, high-level carbapenem-resistant isolates were tested by real-time PCR for the expression levels of the *mexB*, *mexY*, *ampC*, and *oprD* genes.

Results: Amongst all *P. aeruginosa* isolates, 78.5%, 46.7%, and 15% were imipenem-, meropenem-, and doripenem-resistant, respectively; 72% of isolates were multidrug-resistant. The bla_{IMP} and bla_{VIM} genes were detected in 17.9% and 1.2% of isolates; respectively. The bla_{SPM} and bla_{NDM} genes were not observed. Among the resistant isolates, *mexB* overexpression (63.2%) was the most frequent mechanism, followed by *mexY* overexpression (52.6%), *ampC* overexpression (36.8%), and reduced *oprD* expression (21.1%).

Conclusion: Emerging antimicrobial resistance in burn wound bacterial pathogens is a serious therapeutic challenge for clinicians. In the present study, most of the isolates were MDR. This finding indicated an alarming spread of resistant isolates and suggested that infection control strategies should be considered. Resistance to carbapenems is influenced by several factors, not all of which were evaluated in our study; however, the results showed that production of MBLs and overexpression of the *mexB* gene were the most frequent mechanisms in carbapenem-resistant isolates.

Keywords: Burns; Carbapenems; Efflux pumps; *Pseudomonas aeruginosa*

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

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1. Introduction

Pseudomonas aeruginosa is an opportunistic pathogen involved in many infections, such as respiratory infections, urinary tract infections, wound and soft tissue infections, and bacteremia in immunocompromised patients, including patients with thermal injuries.[1] Despite advances in medicine, *P. aeruginosa* is considered an important infectious threat to patients with burns.[2]

P. aeruginosa displays a primary resistance to many antimicrobial agents because of the outer-membrane barriers, presence of multidrug efflux pumps, and endogenous antimicrobial inactivation.[1] Selection of a suitable antibiotic is complicated by the ability of *P. aeruginosa* to develop resistance to multiple classes of antibacterial agents, and the appropriate choice of antibiotic to begin treatment with is essential to optimizing the clinical outcome.[3]

Carbapenems (e.g., imipenem, meropenem, and doripenem) are members of the β-lactam antibiotic class, and are used commonly to treat infections caused by *P. aeruginosa.*[1] Although carbapenems are the most effective antibiotics for therapy of multidrug-resistant *P. aeruginosa* infections, increased emergence of high-carbapenem resistant isolates has been observed worldwide.[4] Multiple mechanisms are involved in resistance to carbapenems. One of the mechanisms is the acquisition of resistance genes on mobile genetic elements, and another way is through mutations in genes that change the expression and/or function of chromosomally encoded proteins.[3]

Carbapenems are relatively stable to hydrolysis by most β-lactamases[5,6]; however, metallo-β-lactamases (MBL) are able to hydrolyze them efficiently. Genes encoding MBLs are transferred by mobile genetic elements.[7−9]

The three most studied, chromosomally encoded resistance mechanisms against carbapenems in *P. aeruginosa* are: i) inactivation of the outer membrane protein *OprD*; ii) overexpression of chromosome-encoded *ampC* (β−lactamase); and iii) overproduction of multidrug efflux pumps, such as MexAB−OprM and MexXY−OprM.[3]

In our country, studies have been carried out on enzymatic carbapenem resistance mechanisms in *P. aeruginosa* collected from patients with burns[10−12]; however, there is a little information about contribution of different mechanisms to carbapenem resistance in these isolates.[13]

In this study, four main resistance mechanisms (MBLs production, overproduction of the MexAB−OprM and MexXY efflux pumps, overproduction chromosome-encoded AmpC β−lactamase and reducing the *OprD* expression) were examined in high-level carbapenem-resistant *P. aeruginosa* (CRPA) isolated from patients with thermal injury.

2. Methods

2.1. Bacterial isolates

This descriptive study was approved by the ethics committee of the Ahvaz Jundishapur University of Medical Sciences (Grant No: CMRC-55). During the period from June 2011 through May 2012, a total of 107 non-duplicate *Pseudomonas* spp. were collected from the microbiology laboratory of a burns teaching hospital (Taleghani hospital) in Ahvaz, in the South west of Iran. Identification of *P. aeruginosa* was performed using previously described standard phenotypic tests, and verified by polymerase chain reaction (PCR), using specific primers for the *P. aeruginosa gyrB* gene.[14]

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing for the bacterial isolates was carried out using the Kirby−Bauer method, as recommended by the Clinical and Laboratory Standards Institute (CLSI).[15] The following antibiotics were tested: imipenem (10 μg), meropenem (10 μg), doripenem (10 μg), ceftazidime (30 μg), cefepime (30 μg), piperacillin (100 μg), piperacillin/tazobactam (100/10 μg), gentamicin (10 μg), amikacin (30 μg), tobramycin (10 μg), ciprofloxacin (5 μg), aztreonam (30 μg), polymyxin B (300 units), and colistin (10 μg) (Mast Group Ltd, UK). The minimum inhibitory concentrations (MICs) of carbapenems (imipenem [IMI], meropenem [MRP], and doripenem [DOR]) were obtained by an E-test (Liofilchem, Italy), as described in the manufacturer's instructions. Carbapenem resistance was determined based on the MIC breakpoints. When an isolate was resistant to three carbapenems (imipenem, meropenem, and doripenem), that isolate was considered high-level carbapenem resistant. If an isolate was resistant to three or more classes of antimicrobial agents (i.e., penicillins/cephalosporins, carbapenems, aminoglycosides, and fluoroquinolones), that isolate was considered multidrug resistant (MDR). In accordance with the CLSI guidelines, *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were used as control strains in all susceptibility assays.

2.3. Phenotypic and molecular detection of MBLs

For the phenotypic detection of metallo-β-lactamase producer isolates, an E-test MBL strip containing a double-sided seven-dilution range of imipenem (4−256 μg/ml; IMI) and imipenem in combination with a fixed concentration of EDTA (1−64 μg/ml; IMD) (Liofilchem, Italy) was used. The test was considered positive when the IMI/IMD ratio was ≥8 μg/ml.

All imipenem-resistant *P. aeruginosa* isolates were screened by PCR for the bla_{IMP}, bla_{VIM}, bla_{SPM}, and bla_{NDM} genes. For the bla_{SPM} and bla_{NDM} genes, the primers used were as reported by Poirel et al.[16] and primers newly designed in this study were used for the bla_{IMP} and bla_{VIM} genes (Table 1). Primers were prepared by TAG Copenhagen A/S, Denmark, and all chemical materials were from SinaClon (Iran). DNA was extracted by the boiling method. Briefly, frozen bacteria were sub-cultured on a Mueller−Hinton's agar plate (Merck, Germany) and incubated at 35 °C overnight. After growth, one to five colonies were suspended in 500 μl of 1 × Tris-EDTA buffer, heated at 95 °C for 10 min, and placed at room

Table 1
List of primers that used in this study.

Primer name	Oligonucleotide sequence	References
VIM-F	5′-GGTCTCATTGTCCGTGATGGTG-3′	This study
VIM-R	5′-GGAATCTCGCTCCCCTCTACCT-3′	
IMP-F	5′-TCCCCACGTATGCATCTGAATTAAC-3′	This study
IMP-R	5′-CGGACTTTGGCCAAGCTTCTATATT-3′	
mexB-F	5′-CAAGGGCGTCGGTGACTTCCAG-3′	[17]
mexB-R	5′-ACCTGGCAACCGTCGGGATTGA-3′	
mexY-F	5′-GGACCACGCCGAAACCGAACG-3′	[17]
mexY-R	5′-CGCCGCAACTGACCCGCTACA-3′	
oprD-F	5′-CGACCTGCTGCTCCGCAACTA-3′	[17]
oprD-R	5′-TTGCATCTCGCCCCACTTCAG-3′	
ampC-F	5′-CGCCGTACAACCGGTGAT-3′	[18]
ampC-R	5′-CGGCCGTCCTCTTTCGA-3′	
rpsL-F	5′-GCTGCAAAACTGCCCGCAACG-3′	[17]
rpsL-R	5′-ACCCGAGGTGTCCAGCGAACC-3′	

temperature for 5 min. Samples were then placed at −20 °C for 10 min and after centrifugation at 14,000 rpm for 10 min at 4 °C, 2 µl of the supernatant was used as the template in a 50-µl PCR reaction. The master mixture for the detection of all genes was: 5 µl of 10 × reaction buffer; 2 µl of 50 mM $MgCl_2$; 1 µl of 2.5 mM dNTPs; 2 µl of each primer (20 pmol/µL); 0.4 µl Taq polymerase 5 U/µl; and 35.6 µl distilled water. DNA template was amplified in a Master cycler Eppendorf (Eppendorf, Germany) under the following conditions: initial denaturation for 3 min at 94 °C; followed by 35 cycles at 94 °C for 45 s, at specific annealing temperatures for 45 s, then at 72 °C for 45 s; a final extension for 5 min at 72 °C; and then maintenance at 4 °C. Amplicons were electrophoresed through a 1.5% agarose gel with 0.5 µg/ml ethidium bromide in 1 × Tris Borate EDTA buffer. The gels were visualized and photographed under ultraviolet illumination. Amplified products of the bla_{IMP} and bla_{VIM} genes underwent bidirectional sequencing using an ABI 3730XL DNA Analyzer (Applied Biosystems, USA).

2.4. Gene expression analysis by Real-time reverse transcription (RT)-PCR

Real-time RT-PCR was performed on high-level carbapenem-resistant *P. aeruginosa* isolates. The expressions of genes encoding the efflux proteins MexB and MexY, as well as *OprD* and chromosomal *AmpC*, were determined by real-time RT-PCR, as described previously,[17,18] using the primers detailed in Table 1. First, total RNA was extracted using a High Pure RNA Isolation kit (Roche, Germany) and cDNA was then synthesized using a Transcriptor First Strand cDNA Synthesis kit (Roche, Germany), according to the manufacturer's recommendations. Real-time PCR was performed using a StepOne™ Real-Time PCR System (Applied Biosystems, USA). Quantification of the gene transcript was done using SYBR® Premix Ex TaqTM (TLi RNaseH Plus), ROX plus (Takara, Japan). A gene coding for a ribosomal protein, *rpsL*, was assessed in parallel to normalize the transcription levels of the target genes and then calibrated relative to *P. aeruginosa* PTCC 1430 (ATCC 27853), which was assigned a value of 1.0.[18] The relative gene expressions were calculated by the standard curve method. Reduced *oprD* expression was considered relevant when it was ≤30% compared with that in *P. aeruginosa* PTCC 1430 (ATCC 27853).[4] If in one isolate, the expression levels of the *ampC* and *mexY* genes were ≥10-fold higher than that in *P. aeruginosa* PTCC 1430 (ATCC 27853), that isolate was considered an AmpC and MexXY overproducer. Moreover, in accordance with previously defined criteria, an isolate were categorized as a MexAB−OprM overproducer if the level of *mexB* expression was ≥3-fold higher than that in strain *P. aeruginosa* PTCC 1430 (ATCC 27853).[4]

2.5. Statistical analysis

The results were analyzed using the SPSS software (Version. 19 IBM, Chicago, IL, USA). Categorical variables are reported as the number and percentage. Descriptive statistics were used for data analysis.

3. Results

The sources of *P. aeruginosa* isolates according to specimens and wards are shown in Table 2. Among these isolates, 75 (70.1%) were resistant to imipenem, 51 (47.7%) to meropenem, and 34 (31.8%) to doripenem by the Kirby−Bauer method. The results of susceptibility tests for other antimicrobial agents are summarized in Table 3. Among the isolates,

Table 2
Sources of *P. aeruginosa* clinical isolates (N = 107).

Sources	No. (%) of strains
wards	
ICU	26 (24.3)
Pediatric	8 (7.5)
Burn (men)	35 (32.7)
Burn (Women)	35 (32.7)
Repair	3 (2.8)
Specimens	
Burn wound	93 (86.9)
Blood	13 (12.1)
Urine	1 (0.9)

Table 3
Antimicrobial susceptibility in *P. aeruginosa* clinical isolates.

Antimicrobial agents	Sensitive N (%)	Intermediate N (%)	Resistant N (%)
Ceftazidime	8 (7.5)	1 (0.9)	98 (91.6)
Cefepime	9 (8.4)	3 (2.8)	95 (88.8)
Piperacillin	17 (15.9)	0 (0)	90 (84.1)
Piperacillin/tazobactam	36 (33.6)	0 (0)	71 (66.4)
Gentamicin	13 (12.1)	0 (0)	94 (87.9)
Amikacin	14 (13.1)	4 (3.7)	89 (83.2)
Tobramycin	13 (12.1)	0 (0)	94 (87.9)
Ciprofloxacin	15 (14)	1 (0.9)	91 (85)
Aztreonam	11 (10.3)	2 (1.9)	94 (87.9)
Polymyxin B	107 (100)	0 (0)	0 (0)
Colistin	107 (100)	0 (0)	0 (0)

Table 4
MIC profiles of carbapenems.

Carbapenems	MIC (μg/ml)		
	≤4 (S) N (%)	8 (I) N (%)	≥16 (R) N (%)
Imipenem	23 (21.5)	0 (0)	84 (78.5)
Meropenem	27 (25.2)	30 (28)	50 (46.7)
Doripenem	66 (61.7)	25 (23.4)	16 (15)

72% were MDR. The MICs for carbapenems were determined by an E-test and the results are shown in Table 4 and Chart 1.

PCR analysis of MBL genes was performed for all carbapenem-resistant *P. aeruginosa* isolates. The bla_{IMP} and bla_{VIM} genes were detected in 15 (17.9%) and one (1.2%) of the isolates, respectively. These isolates were resistant to all of the carbapenems and were positive in the MBL E-test. No amplicons were detected for the bla_{SPM} and bla_{NDM} genes.

According to the MIC results for the carbapenems, 19 (17.76%) isolates were imipenem/meropenem resistant, had intermediate susceptibility to doripenem, and were considered as high-level carbapenem-resistant isolates. These isolates were multidrug resistant and were not MBL-producers. Among them, *mexB* overexpression (12/19, 63.2%) was the most frequent mechanism, followed by *mexY* overexpression (10/19, 52.6%), *ampC* overexpression (7/19, 36.8%), and reduced expression of *oprD* (4/19, 21.1%). Table 5 shows the MICs and gene expression levels of resistant isolates. All 19 isolates demonstrated at least one of the resistance mechanisms. Based on the overexpression of *mexB* and *mexY*, isolates could be divided into four groups (1–4). Most of the isolates were placed in group 1 (7/19, 36.8%), which represented overexpression of both *mexB* and *mexY*. One isolate in this group concomitantly showed *ampC* overexpression and downregulation of *oprD*. The isolates in group 2 (5/19, 26.3%) showed overexpression of *mexB* only and did not show any other mechanisms. Among the isolates belonging to group 3, which represented *mexY* overexpression only, two isolates were indicated as overproducers of AmpC. Isolates in group 4 (3/19, 15.8%) did not show overexpression of *mexB* and *mexY*.

Table 5
MIC and gene expression levels of the high level carbapenem-resistant *P. aeruginosa* isolates.

Strain ID	Group	MIC(μg/ml)			Gene expression			
		IMI	MRP	DOR	*mex*B	*mex*Y	*amp*C	*opr*D
1 (9)	1	96	≥32	8	**3.54**	**85.12**	0.51	2.46
2 (91)	1	192	16	8	**7.05**	**96.22**	0.32	1.99
3 (143)	1	96	≥32	6	**7.25**	**85.15**	7.15	1.97
4 (207)	1	≥256	≥32	8	**10.91**	**30.51**	**25.67**	1.02
5 (213)	1	96	≥32	8	**4.94**	**37.03**	1.07	**0.32**
6 (228)	1	192	16	6	**19.41**	**448.70**	**30.46**	**0.10**
7 (245)	1	96	≥32	6	**5.62**	**25.36**	0.42	**0.13**
8 (31)	2	96	≥32	8	**6.32**	9.54	7.23	0.46
9 (107)	2	128	≥32	6	**5.68**	5.12	4.25	0.45
10 (176)	2	96	24	8	**10.25**	4.57	8.22	0.75
11 (197)	2	128	16	6	**6.55**	5.13	8.74	1.50
12 (244)	2	64	24	8	**9.54**	6.45	0.14	0.64
13 (21)	3	64	16	6	1.33	**54.25**	**53.90**	1.25
14 (38)	3	≥256	≥32	8	1.26	**128.85**	4.24	0.52
15 (199)	3	128	16	8	0.12	**25.55**	**20.62**	1.22
16 (6)	4	42	≥32	12	0.42	6.13	**24.40**	**0.23**
17 (118)	4	96	16	6	0.28	2.12	**89.34**	0.73
18 (259)	4	64	≥32	6	1.25	4.37	**145.32**	0.76
19 (304)	4	32	≥32	12	1.16	6.56	1.05	**0.16**

ID = identification number; MIC = minimum inhibitory concentration; IMI = imipenem; MRP = meropenem; DOR = doripenem; bold items, overexpression and reduced expression of genes.

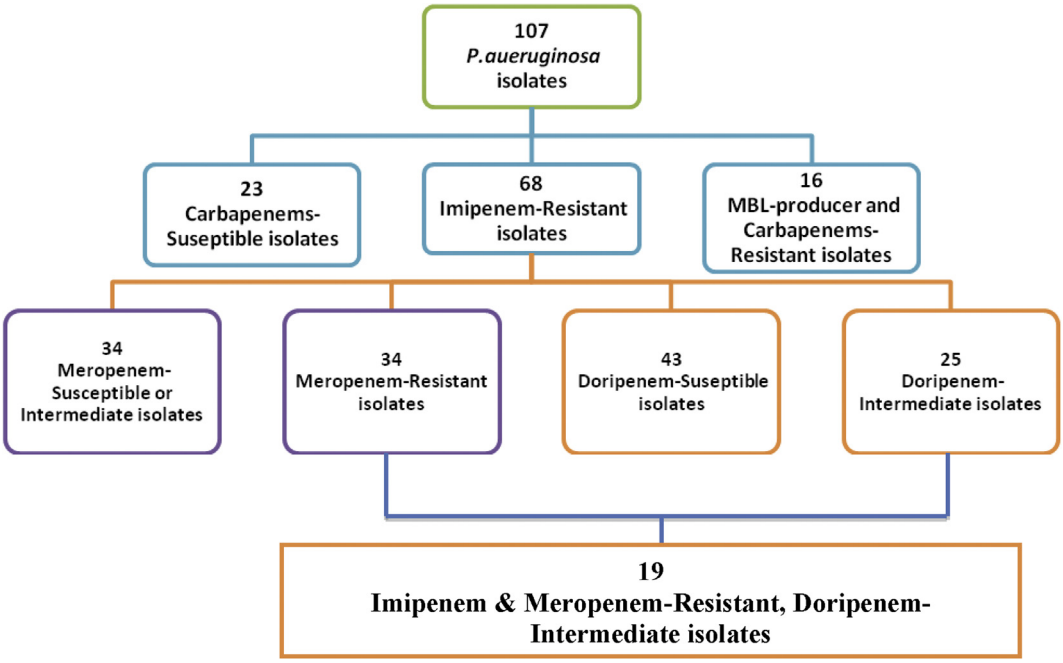

Chart 1. Carbapenem susceptibility pattern of *P. aeruginosa* isolates.

Among them, one isolate represented overexpression of *ampC* associated with reduced expression of *oprD* and two isolates were *ampC* overproducers. Interestingly, one isolate [No. 6 (228)] showed all four resistance mechanisms.

4. Discussion

In this study, we assessed the most significant resistance mechanisms in high-level carbapenem resistant *P. aeruginosa* isolated from patients with thermal injuries.

Most of the isolates (72%) were MDR and this is may be explained by the heavy use of antibiotics in patients with burns, which may have led to the increased emergence of the extensive drug resistance phenotype. In the present study, piperacillin-tazobactam was the most effective antibiotic and carbapenems were useful in the second line. However, 15% of isolates were resistant to three carbapenems and all of them were MBL producers. MBL-producing *P. aeruginosa* (MBLPA) strains are distributed worldwide and can serve as a source of infection for other patients.[19] Previous studies in our country reported different frequencies of MBLPA isolated from patients with burns,[20–23] which could be caused by referral to a hospital with different caring regimens and settings. Carbapenems are important drugs in the treatment of the *P. aeruginosa* infections and MBLs are characterized by the ability to hydrolyze carbapenems[24]; therefore, the detection of MBLPA strains should be conducted in routine laboratory work, and CLSI documents and other guidelines should be considered for MBL detection methods, such as extended spectrum beta-lactamases detection.

In the present study, in addition to MBLPA strains, almost 18% of isolates were high-level carbapenem-resistant. These isolates were completely resistant to imipenem and meropenem, but had intermediate susceptibility to doripenem; doripenem-resistant isolates were not detected. The affinity of doripenem and meropenem for penicillin-binding protein (PBP) 2, PBP3, and PBP4 is higher than for other PBPs in *P. aeruginosa* and differs from the affinity profile of imipenem. Accordingly, the activity of doripenem resembles that of meropenem.[25] On the other hand, doripenem is not used in our country and *P. aeruginosa* isolates have not been exposed to doripenem. In addition, meropenem is more susceptible to efflux pumps than doripenem.[25] Accordingly, doripenem could be an effective drug to treat *P. aeruginosa* infection, but should be used properly.

Among the high-level carbapenem-resistant isolates, we detected at least one of the resistance mechanisms. Overexpression of *mexB* (63.2%) was the most frequent mechanism. The MexAB–OprM efflux pump is able to export several different classes of drugs, and has the broadest substrate profile for the β-lactam class, such as carboxypenicillins, aztreonam, extended-spectrum cephalosporins, penems, and carbapenems (meropenem but not imipenem).[3,26] We could not find any relation between the MICs of carbapenems and efflux pump overexpression or downregulation of porin in any of the isolates. However, one isolate with reduced expression of *oprD* had an MIC for doripenem that was greater than that of the other isolates (12 μg/ml). In this isolate, the MIC for meropenem was ≥32 μg/ml and the MIC for imipenem was less than other isolates (32 μg/ml). This resistance profile might relate to a decrease in the porin expression. However, for the isolates that showed only *mexB* overexpression, there is no such explanation.

AmpC β-lactamase is located in the bacterial periplasm. One of the important determinants of the resistance spectrum is the rate of substrate that is delivered to the enzyme. The concentration of carbapenems in the periplasm is dependent on permeability of the cell's outer membrane and the presence of efflux pumps.[27] We found another isolate with a high MIC for doripenem (12 μg/ml) that simultaneously overexpressed AmpC and downregulated *OprD*. Perhaps, in this isolate, the drug concentration has been reduced because of the decreased expression of the *oprD*. Moreover, we found two isolates that overexpressed *ampC* only. Carbapenem resistance in clinical isolates of *P. aeruginosa* involves various combinations of mechanisms.[3] In this study, a few resistance mechanisms have been investigated and in these isolates, other mechanisms may be present that were not evaluated. To clarify these other mechanisms, further investigations are needed.

Among three isolates that overexpressed *mexY*, two isolates simultaneously showed overexpression of *ampC*, and one isolate overexpressed *mexY* alone, which was associated with high MICs for imipenem and meropenem (≥256 and ≥32 respectively). According to a previous study, meropenem is more sensitive than imipenem to overexpression of efflux pumps such as MexXY–OprM.[27] In this isolate, the high MIC for imipenem might be related to the other mechanisms that were not evaluated in this study.

Unfortunately, in the present study, the level of gene expression of efflux pumps and *OprD* porin was not investigated in the carbapenem-susceptible isolates; therefore, we could not compare these two groups of isolates. This comparison could provide more information about the reviewed mechanisms.

In conclusion, emerging antimicrobial resistance trends in burn wound bacterial pathogens is a serious therapeutic challenge for clinicians. Carbapenems are the drug of choice to treat *P. aeruginosa* infection; therefore, detection of carbapenem-resistant *P. aeruginosa* and the mechanisms involved are necessary. Resistance to carbapenems is influenced by several factors, not all of which were evaluated in our study; however, the results showed that mechanisms involving the production of MBLs and overexpression of the *mexB* gene were observed more frequently and also play an important role in the emergence of the high-level carbapenem-resistant phenotype among *P. aeruginosa* isolates. Most of the isolates were MDR, a finding that indicates an alarming spread of resistant isolates. To control the dissemination of resistant isolates, infection control strategies should be considered.

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