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CHARACTERIZATION OF CARBAPENEM-RESISTANT BUT CEPHALOSPORIN-SUSCEPTIBLE *PSEUDOMONAS AERUGINOSA*

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In this study, mechanisms of carbapenem resistance in carbapenem-resistant but cephalosporin-susceptible (Car-R/Ceph-S) *Pseudomonas aeruginosa* were investigated. A total of 243 *P. aeruginosa* isolates were studied. The disk diffusion and agar dilution methods were used for determination of antibiotic susceptibility patterns. AmpC and efflux pump overproductions were detected by phenotypic methods. The presence of carbapenemase-encoding genes was detected by polymerase chain reaction (PCR). The expression of OprD, MexAB-OprM, and MexXY-OprM efflux pumps was assessed by real-time PCR. According to disk diffusion method, altogether 116 *P. aeruginosa* isolates (47.7%) were carbapenem-resistant and among them, 23 isolates (19.8%) were cephalosporin-susceptible. Carbapenemase producer was not detected. Overexpression of AmpC was detected in one (4.3%) isolate that was ceftazidime-susceptible but cefepime-resistant. Overexpression of MexAB-OprM and MexXY-OprM efflux pumps was detected in 12 (60.9%) and 16 (68.8%) of isolates, respectively. A total of 16 (68.8%) isolates showed decreased expression of OprD. The Car-R/Ceph-S *P. aeruginosa* did not develop by carbapenemase production. The resistance to carbapenem was mediated in our clinical isolates by decreased expression of OprD and overexpression of MexAB-OprM and MexXY-OprM efflux systems or the combination of these mechanisms.

Keywords: antimicrobial agents, carbapenem, mechanism, *Pseudomonas aeruginosa*

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Introduction

Pseudomonas aeruginosa is a common bacterial pathogen in healthcare-associated infections, particularly in immunocompromised patients, and shows notorious adaptability and capability to acquire resistance mechanisms against antibiotics [1]. The origin of this opportunistic organism can be endogenous or exogenous [2]. Infections due to *P. aeruginosa* are often problematical because of the high level of intrinsic or acquired resistance to different antimicrobial agents [1, 3, 4]. The increasing incidence of hospital infections caused by multidrug-resistant (MDR) *P. aeruginosa* strains with resistance to broad-spectrum β-lactams, aminoglycosides, and fluoroquinolones strictly compromises the choice of an applicable option for the treatment [5, 6]. β-lactam agents are the main choice to treat severe infections caused by *P. aeruginosa*. However, the production of β-lactamases, such as cephalosporinases and carbapenemases, has been frequently described among clinical isolates of *P. aeruginosa* [7]. In *P. aeruginosa*, the reduced susceptibility to carbapenems is due to the acquired carbapenemases in combination with intrinsic mechanisms such as downexpression or absence of OprD porin, efflux pumps system overexpression, chromosomal AmpC β-lactamase production, and target modifications. However, carbapenemases have an extended substrate spectrum to penicillins and cephalosporins, besides carbapenems. Therefore, carbapenemase-producing bacterial strains commonly exhibit resistance to virtually all β-lactams [8]. The carbapenem molecules are more stable against hydrolysis by the most serine-β-lactamases; these drugs have a particular value in the treatment of infections caused by cephalosporinase-producing bacteria, which remain susceptible to carbapenems [9]. Car-R/Ceph-S *P. aeruginosa* have been reported by few studies [9, 10]. Since these *P. aeruginosa* do not produce high levels of AmpC, theoretically ceftazidime or cefepime could be prescribed for treatment of such infections [9, 10]. We aimed to investigate the mechanisms of carbapenem resistance in clinical isolates of Car-R/Ceph-S *P. aeruginosa* from Iran.

Methods

Patients and isolates

A total of 243 *P. aeruginosa* isolates were collected from infected patients in Azerbaijan, Iran, during 2016–2018. The isolates were identified by biochemical and standard methods of microbiology. The disk diffusion method was used for the initial detection of Car-R/Ceph-S (ceftazidime or cefepime) isolates. The demographic and clinical information, such as gender, age, duration of

hospitalization, intensive care unit (ICU) stay, and the treatment outcome, was collected. *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as quality control strains for standard microbiology testing.

This study was approved by the Ethics Committee of Tabriz University of Medical Sciences (IR.TBZMED.REC. 1396.306).

Antibiotic susceptibility patterns

The disk diffusion method. The disk diffusion method was performed on Mueller–Hinton agar according to Clinical and Laboratory Standards Institute (CLSI) guidelines [11]. Antibiotic disks in this study included ceftazidime, colistin, levofloxacin, polymyxin B, aztreonam, ciprofloxacin, amikacin, cefepime, and piperacillin/tazobactam. In this study, MDR was considered as acquired non-susceptibility to at least one agent in three or more antimicrobial classes [12]. *P. aeruginosa* ATCC 27853 was used as quality control strain for susceptibility testing according to CLSI guidelines.

Minimum inhibitory concentration (MIC)

The agar dilution method was performed on Mueller–Hinton agar as recommended by CLSI guidelines for determination of MIC. The included antibiotics were ceftazidime, imipenem, cefepime, and meropenem. The results were interpreted according to the breakpoints of CLSI [11].

Detection of AmpC overproduction

AmpC overproduction was detected by agar plate supplemented with cloxacillin (250 μg/ml), because cloxacillin inhibits AmpC β-lactamase effects. At least a twofold decreased concentration of ceftazidime MIC in the presence of cloxacillin compared to MIC of ceftazidime without cloxacillin was considered as an AmpC overproduction [7].

Phenotypic detection of efflux pumps

The inhibitory effect of phenylalanine-arginine beta-naphthylamide (PaβN; as an efflux pump inhibitor) at a concentration of 40 μg/ml on the MIC of imipenem and meropenem was detected according to previous studies. At least twofold decreased MIC in the presence of PAβN compared to MIC value without inhibitor was considered as an overexpression of efflux pumps [13].

Polymerase chain reaction (PCR)

Total DNA of *P. aeruginosa* isolates was extracted by CTAB Proteinase K method [14]. Eleven pairs of primers were used to amplify fragments with sizes from 232 to 798 bp those as previously described by Poirel et al. [15]. Three multiplex reactions were defined including set 1 for detection of bla_{IMP}, bla_{VIM}, and bla_{SPM}; set 2 for detection of bla_{NDM}, bla_{KPC}, $bla_{\mathrm{OXA\text{-}48}}$, and bla_{BIC}; and set 3 for detection of bla_{AIM}, bla_{GIM}, bla_{SIM}, and bla_{DIM}. One microliter of total DNA was subjected to the multiplex PCR in a 25-µl reaction mixture. Reactions were carried out at 94 °C for 10 min followed by 36 cycles of amplification involving of 30 s at 94 °C, 40 s at 54 °C, and 50 s at 72 °C, with 5 min at 72 °C for the final extension. The PCR products were detected by electrophoresis in a 2% agarose gel containing 0.05 mg/L ethidium bromide [15].

Real-time PCR

RNA extraction kit (SinaClon Co., Tehran, Iran) was used for total RNA extraction from bacterial isolates according to the manufacturer's instruction. The extracted RNA was treated with RNase-free DNase I (SinaClon) to remove the residual DNA. The quantity and quality of the extracted RNA were measured by NanoDrop spectrophotometer (ND-1000, Wilmington, USA) and the absence of genomic DNA residuals was detected by PCR using primers of efflux genes. Five micrograms of DNA-free RNA were used for the synthesis of cDNA by reverse transcription using M-mulv reverse transcriptase and random hexamer as a primer (SinaClon), according to the manufacturer's instructions. Reverse transcriptase was inactivated by incubation at 70 °C for 10 min. The cDNAs were stored at −20 °C until use [7]. The expression levels of *oprD*, *mexAB*, and *mexXY* were measured by one-step real-time quantitative reverse transcription PCR (RT-PCR) and specific primers as recommended previously. The PCR was carried out in Rotor-Gene Real-time PCR device (Model RG 3000; Corbett Research, Sydney, Australia) in duplicate runs by SYBR premix EX TaqII, Tli RNaseH plus (Takara Bio Inc., Otsu, Shiga, Japan). The transcription level of the constitutively expressed *rpsL* housekeeping gene was considered as standardized expression levels. Gene expression was considered as ratios of the target gene and housekeeping gene (*rpsL*) according to a relative quantification determination as described previously. Reduced *oprD* expression was considered relevant when its level was more than twofold lower compared to that of *P. aeruginosa* PAO1 reference strain [7]. The results represent comparative expression levels for target genes in isolates compared to the PAO1 wild-type strain. Hyperproduction of

mRNA for *mexB* was considered as the cDNA level more than threefold and for mRNA of *mexY* tenfold higher than this level in the *P. aeruginosa* [16].

Statistical analysis

The data were analyzed using the Statistical Package for the Social Sciences (SPSS, Inc., Chicago, IL) software, version 20. Comparison of the data among various groups was performed by χ^2 and Fisher's exact tests. The values of $p \leq 0.05$ were considered to be statistically significant.

Results

Patients and isolates

According to the disk diffusion results, 116 *P. aeruginosa* isolates (47.7%) were carbapenem-resistant. Among the carbapenem-resistant, 23 non-duplicated isolates (19.8%) were cephalosporin-susceptible that were collected from wound (43.5%), blood (17.4%), urine (21.7%), respiratory specimen (13%), and middle-ear discharge (4.3%). The mean age of patients was 38 ± 24 years and the most frequent age range of patients was 31–50 years. These patients [13 males (56.6%) and 10 females (43.5%)] were hospitalized in burn (34.8%), ICU (43.5%) and internal (8.7%), transplantation (4.3%), surgery (4.3%), and urology (4.3%) wards. The mean duration of hospitalization was 36 ± 22 days. The most patients (25.8%) had 11–20 days of hospitalization, whereas one patient (4.2%) was hospitalized more than 100 days. Thirteen (56.5%) patients were observed during the stay in ICU. The death occurred in 7 (30.1%) patients, whereas 5 (21.7%) of them have stayed in ICU. Cephalosporin consumption was observed in 13 patients (56.5%). There was no statistically significant association between cephalosporin consumption and patient outcome or ICU stay. Table I summarizes the clinical features of patients with Car-R/Ceph-S *P. aeruginosa* infections.

Antibiotics resistance patterns

All isolates were colistin- and polymyxin B-susceptible; however, different frequency of resistance was found against other antimicrobial agents including levofloxacin (87%), ciprofloxacin (87%), aztreonam (87%), cefepime (69.6%), piperacillin–tazobactam (69.6%), amikacin (65.2%), and ceftazidime (7.4%). According to the results of disk diffusion method, 20 isolates (87%) were MDR. MIC_{50} and MIC_{90} of imipenem, meropenem, ceftazidime, and cefepime were 16

Table I. Clinical features of patients with Carb-R/Ceph-S *P. aeruginosa* infections

Patient	Age (years)	Ward	Specimen	ICU stay	Cephalosporin consumption	Hospitalization day	Outcome
1	19	Burn	Wound	−	+	35	Cure
2	54	Burn	Wound	+	−	20	Cure
3	59	Internal	Urine	+	−	16	Cure
4	47	Burn	Wound	−	−	10	Death
5	61	ICU	Urine	+	+	95	Death
6	53	ICU	Respiratory	+	+	51	Cure
7	60	Urology	Urine	−	−	8	Cure
8	1	ICU	Blood	+	+	59	Death
9	10	Burn	Blood	−	−	21	Cure
10	8	ICU	Blood	+	+	26	Cure
11	24	ICU	Blood	+	−	23	Cure
12	42	ICU	Wound	+	−	36	Cure
13	49	Burn	Wound	−	+	33	Death
14	18	Internal	Middle ear	+	+	13	Cure
15	5	ICU	Wound	+	−	4	Death
16	82	ICU	Respiratory	+	−	56	Cure
17	37	Surgery	Urine	−	+	24	Cure
18	37	ICU	Urine	+	+	143	Death
19	68	Burn	Wound	−	−	54	Cure
20	26	Burn	Wound	−	−	37	Cure
21	83	ICU	Respiratory	+	−	39	Cure
22	35	Surgery	Wound	−	+	15	Cure
23	5	Burn	Wound	−	−	15	Death

Note: ICU: intensive care unit.

and 32, 32 and 64, 4 and 8, 32 and 64, respectively. According to MIC results, all isolates were ceftazidime-susceptible (MIC $\leq$ 8) and 9 isolates (38.7%) were cefepime-susceptible (MIC $\leq$ 8). All isolates were meropenem-resistant (MIC $\geq$ 8) and 21 isolates (90.3%) were imipenem-resistant (MIC $\geq$ 8).

Mechanisms of resistance to carbapenems

Out of 23 Car-R/Ceph-S, none of the isolates were positive for carbapenemase genes. Overexpression of AmpC was detected in one (4.3%) isolate that was ceftazidime-susceptible but cefepime-resistant. In this isolate, a decrease of ceftazidime MIC was observed in the presence of cloxacillin. One isolate was negative for all detected mechanisms of carbapenem resistance. Phenotypic assay for overexpressed efflux pumps was positive in 14 (60.9%) isolates. Among them, 11 isolates (47.3%) showed at least two times decrease in both meropenem and imipenem MICs in the presence of PAβN, whereas this characteristic was found in

one isolate (4.3%) only by imipenem and in two isolates (8.6%) only by meropenem in the presence of PAβN. Overexpression of MexAB-OprM and MexXY-OprM efflux pumps was detected in 12 (52.1%) and 16 (68.8%) of isolates by the real-time PCR, respectively. Overexpression of both MexAB-OprM and MexXY-OprM was detected in 11 isolates (47.3%). A total of 16 (68.8%) isolates showed decreased expression of OprD compared to the housekeeping gene (*rpsL*). Table II shows the various mechanisms of carbapenem resistance among our isolates. Our data show that 11 isolates (47.3%) of Car-R/Ceph-S *P. aeruginosa* have multifactorial carbapenem resistance mechanisms including both overexpression of efflux pumps and decreased OprD expression. Monofactorial mechanisms of carbapenem resistance were detected among 11 isolates (41.7%), which were associated with overexpression of efflux pumps (6 isolates: 25.8%) and decreased expression of OprD (5 isolates: 21.5%).

Discussion

Carbapenems are the last choice for treatment of many infections caused by drug-resistant bacterial pathogens. Unfortunately, carbapenem-resistant *P. aeruginosa* are on the rise. Resistance to carbapenem in *P. aeruginosa* may be due to a combination of β-lactamases (especially AmpC) production, porin mutations, efflux pump systems overexpression, and/or penicillin-binding protein modifications [17]. Multifactorial mechanisms confer increased resistance to carbapenems, but some β-lactam agents and aminoglycosides may retain *in vitro* anti-bacterial effects [7, 17]. This study demonstrated clinical isolates of *P. aeruginosa* exhibiting decreased susceptibility to carbapenems but remain cephalosporin-susceptible from Iranian clinical settings. These phenotypes have been reported from countries such as China and Brazil [9, 10, 18]. Similar to other studies, high frequency of resistance was observed in different group of antibiotics including aminoglycosides, quinolones, and penicillins [19]. The treatment of infections caused by *P. aeruginosa* is commonly problematic due to the high levels of resistance to antibiotics and the emergence of resistance during therapy [20]. Similar to other studies, all *P. aeruginosa* isolates were polymyxin-susceptible [21, 22]. Colistin, also referred as polymyxin E, is an old, cationic polypeptide antimicrobial agent with significant *in vitro* activity against *P. aeruginosa*, for which it is currently the only available active antibiotic against MDR isolates. Growing usage of colistin for antibiotic therapy of infections caused by MDR bacteria may lead to the development of colistin-resistant strains in some regions. The worldwide frequency of colistin-resistant *P. aeruginosa* is low and may be different between countries and over time [1]. The high frequency of resistance may limit the option for antibiotic therapy

Table II. The frequency of different mechanisms of carbapenem-resistant among *P. aeruginosa* in this study

Isolate	IMI MIC (μg/ml)	MRO MIC (μg/ml)	CAZ MIC (μg/ml)	FEP MIC (μg/ml)	OprD decreased expression	MexAB-OprM overexpression	MexXY-OprM overexpression	AmpC
1	4	8	4	8	−	−	−	−
2	8	64	1	32	+	+	+	−
3	16	64	8	64	+	+	+	−
4	8	16	4	16	+	−	−	−
5	16	16	1	32	+	−	−	−
6	16	8	8	128	+	+	+	−
7	16	64	8	64	−	+	+	−
8	16	8	4	4	+	−	−	−
9	16	32	4	64	+	−	+	−
10	8	8	1	8	−	+	+	−
11	32	16	4	32	−	+	+	−
12	16	32	1	32	+	−	−	−
13	32	32	4	64	+	+	+	−
14	16	32	1	32	−	+	+	−
15	16	32	4	64	−	+	+	−
16	16	8	1	1	+	−	+	−
17	0.5	8	1	1	−	−	+	−
18	32	32	1	8	+	+	+	−
19	16	32	4	32	+	−	+	−
20	8	32	8	64	+	−	+	−
21	1	16	1	16	+	+	−	−
22	8	16	1	16	+	−	−	−
23	32	32	4	64	+	+	+	+

Note: CAZ: ceftazidime; FEP: cefepime; IMI: imipenem; MRO: meropenem; MIC: minimum inhibitory concentration.

of infections caused by *P. aeruginosa*. Therefore, a pattern shift is essential for the control strategy of *P. aeruginosa* infections in our setting. In this study, all Car-R/Ceph-S *P. aeruginosa* were meropenem-resistant, whereas one isolate was imipenem-susceptible. *P. aeruginosa* may be non-susceptible to carbapenems by different mechanisms. The mechanism of meropenem resistance has been complex and multifactorial [7, 17]. The frequency of MBL production was reported among carbapenem-resistant *P. aeruginosa* from 7% to 100% [23, 24]. The MBLs identified in *P. aeruginosa* were often the imipenemase and Verona integron-borne metallo-β-lactamase, and rarely the Sao Paulo metallo-β-lactamase, and German imipenemase types [24]. In this study, MBL genes were not observed among carbapenem-resistant but cephalosporin-susceptible (Car-R/Ceph-S) isolates. MBL enzymes hydrolyze all β-lactams except aztreonam [25]. Similar to other studies, our data showed that the 23 Car-R/Ceph-S clinical isolates were all non-carbapenemase producers. The lack of carbapenemase may be the reason of sensitivity to cephalosporin in this phenotype. In this study, all Car-R/Ceph-S isolates were meropenem-resistant and multifactorial mechanisms were observed in the frequency similar to carbapenem-resistant caused by one mechanism. In this study, the most common mechanism of decreased susceptibility to carbapenems was overexpression of efflux pumps in 17 (73.91) followed by decreased expression of OprD porin detected in 16 (68.8%) isolates. OprD inactivation alone is the source of intermediate susceptibility or resistance to imipenem. Some researchers reported a decrease of OprD as the most common mechanism of resistance to carbapenems [17, 26]. A study from Thailand reported the decreased expression of OprD and increased expression of MexAB-OprM in 93.65% and 92.06% of carbapenem-resistant *P. aeruginosa*, respectively [26]. Another study from China reported that mutational-inactivated *oprD* genes might be the chief mechanism of resistance to carbapenem; however, the expression of efflux pumps was not detected in their study [27]. Campana et al. [9] from Brazil reported a significantly reduced expression or lack of OprD porin among all (100%) Car-R/Ceph-S *P. aeruginosa.* Zeng et al. [10] from China reported reduced expression or lack of OprD porin in 12 of 29 isolates of Car-R/Ceph-S *P. aeruginosa*. The development of carbapenem-resistant *P. aeruginosa* due to the loss of porin has been reported in clinical settings and also after *in vitro* carbapenem exposure. The capability of OprD-mutant choice by carbapenem exposure may play a significant role in OprD-mutant emergence and it should be considered regarding empirical clinical usage of carbapenems [9]. Some studies have reported the expression of efflux pumps as the most common mechanism of resistance to carbapenems especially meropenem among carbapenem-resistant *P. aeruginosa* [7, 28]. In this study, overexpression of MexAB-OprM and MexXY-OprM efflux pumps was observed in 11 (47.3%) and 16 (68.8%) of isolates by real-time PCR, respectively. Overexpression of both MexAB-OprM and

MexXY-OprM was detected in 11 isolates (47.3%). Zeng et al. [10] from China reported overexpression of the MexAB-OprM, MexCD-OprJ, and MexXY-OprM efflux systems in 66.67%, 33.33%, and 91.67% of Car-R/Ceph-S isolates, respectively. Overexpression of these efflux systems mediates resistance to meropenem. We also observed that resistance to cefepime is higher than ceftazidime [all isolates were ceftazidime-susceptible but 9 isolates (38.7%) were cefepime susceptible]. Other studies have reported the role of efflux pumps in the selectively reduced susceptibility to cefepime in *P. aeruginosa* strains [29, 30]. In this study, overexpression of AmpC, decreased expression of OprD, and overexpression of MexAB-OprM and MexXY-OprM efflux systems were not detected for one Car-R/Ceph-S isolate. The resistance to carbapenem in this isolate may be due to the overexpression of other efflux system such as MexCD-OprJ, which has been reported in other study [10]. There was no statistically significant association between cephalosporin consumption and patient outcome or ICU stay. Li et al. [18] from China reported that several factors such as 30-day readmission, central venous catheters, and exposure to carbapenems may influence on acquiring Car-R/Ceph-S *P. aeruginosa* bacteremia.

In conclusion, we isolated Car-R/Ceph-S *P. aeruginosa*, which did not develop resistance by the production of carbapenemase. This phenotype was first reported from our country. The resistance to imipenem and meropenem in our clinical isolates was mediated by the decreased expression of OprD and overexpression of MexAB-OprM and MexXY-OprM efflux systems or the combination of these mechanisms.

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Conflict of Interest

No competing financial interests exist.

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