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ORIGINAL CONTRIBUTION

In vitro Reducing Effect of Cloxacillin on Minimum Inhibitory Concentrations to Imipenem, Meropenem, Ceftazidime, and Cefepime in Carbapenem-resistant *Pseudomonas aeruginosa* Isolates

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Today, resistance to antibacterial agents is the most important problem facing public health. *Pseudomonas aeruginosa* is a common gram-negative bacterium and an important cause of nosocomial infections. Resistance to many antibiotics in strains of *P. aeruginosa* isolated from hospital settings such as cephalosporins and carbapenems have been recently reported. Therefore, the introduction of a new strategy to treat the infection of these organisms will be beneficial. In this study we determined the ability of cloxacillin to reduce Minimum Inhibitory Concentrations (MICs) of carbapenem-resistant *P. aeruginosa* to imipenem (IMI), meropenem (MEM), ceftazidime (CAZ), and cefepime (FEP). From 2015 to 2017, 61 non-duplicates of carbapenem-resistant *P. aeruginosa* were collected from clinical samples of hospitalized patients in Kerman, Iran. The MICs of the isolates to IMI, MEM, CAZ, and FEP with/without cloxacillin were determined by microbroth dilution method. The level of MIC of isolates to carbapenems (IMI and MEM) and cephalosporins (CAZ and FEP) ranged from 1-256 µg/mL and 4-1024 µg/mL alone and from 1-32 µg/mL and 1-512 µg/mL in combination with cloxacillin, respectively. The MIC showed a significant difference reduction after the addition of cloxacillin ($P \leq 0.05$). Our results showed *in vitro* potentially of cloxacillin in reduction of MIC to IMI, MEM, CAZ, and FEP in multi-drug resistant *P. aeruginosa,* therefore combination of these antibiotics with cloxacillin could be beneficial for treatment of infections caused by multi-drug resistant *P. aeruginosa.*

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Abbreviations: IMI, Imipenem; MEM, Meropenem; CAZ, Ceftazidime; FEP, Cefepime; COL, Cloxacillin; MIC, Minimum Inhibitory Concentration; *P. aeruginosa*, *Pseudomonas aeruginosa*; OprD, Name of porin; *bla*, beta-lactamase gene; *ampC*, *ampC gene*; ESBL, Extended spectrum beta-lactamase; MBL, Metallo-beta-lactamase.

Keywords: *P. aeruginosa*, Carbapenem-Resistance, Cloxacillin, Minimum Inhibitory Concentration

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INTRODUCTION

Pseudomonas aeruginosa is the most common cause of life-threatening nosocomial infections that can be particularly serious among immunocompromised and severely ill patients. This pathogen is a prevalent agent causing pneumonia, bacteremia, urinary tract, skin, and soft tissue infections. *P. aeruginosa* can be isolated from a variety of environments such as soil, water, and a variety of hospital surfaces [1,2]. This bacterium is considered to be a serious challenge to treat in nosocomial and community acquired infections and choosing the right antibiotic to initiate therapy is very important to optimize the clinical results. The increasing isolation of non-susceptible *P. aeruginosa* strains in medical settings and development of resistance through the course of therapy is due to a number of factors, including acquisition of resistance genes (plasmid mediated) or through mutations that change expression and/or function of chromosomally encoded mechanisms [2,3].

Carbapenems and cephalosporins have a wide range of antimicrobial activities and are being utilized as the last choice for the treatment of infections caused by multidrug resistant *P. aeruginosa* isolates, however, resistance to this drug is rising [4]. One of the most important causes of resistance to carbapenems is the production of a variety of plasmid mediated hydrolyzing enzymes such as metallo-beta-lactamases (MBL) and extended-spectrum beta-lactamase (ESBL) to inactivate the drugs [5]. In the absence of MBLs and ESBLs, resistance to carbapenems can be due to other mechanisms such as increased production of chromosomally-encoded AmpC cephalosporinase, reduced outer membrane porins expression, and overexpression of the efflux systems. *P. aeruginosa* carries an inducible extended-spectrum AmpC (ESAC) cephalosporinase which is related to the chromosomally encoded AmpC found in *Enterobacteriaceae* and other nonfermenting gram-negative bacilli [6-8]. This enzyme can be plasmid encoded, however, most plasmid-borne *ampC* genes are not inducible [9,10]. These β-lactamase enzymes demonstrate activity against many beta-lactams but even more active on cephalosporins, including cephamycins, monobactams, and in some cases carbapenems, third and fourth generation cephalosporins [9,11].

In many strains of *P. aeruginosa*, *ampC* expression is low but during treatment with carbapenems including IMI which is strong inducer for AmpC β-lactamase, the production of AmpC increased, leading to failure of treatment [12]. In contrast to extended-spectrum beta-lactamases (ESBLs) which can be inactivated by the β-lactamase inhibitors, such as clavulanic acid, sulbactam, and tazobactam—AmpC β-lactamases are not inhibited by these agents [9]. However, AmpC β-lactamases can be inhibited by boronic acid and cloxacillin [13,14]. Cloxacillin is an antibiotic used for the treatment of several bacterial infections including impetigo, cellulitis, pneumonia, septic arthritis, and otitis externa [15]. This antibacterial agent is a semisynthetic β-lactamase resistant penicillin which binds to penicillin-binding proteins (PBPs) located on the inner membrane of the bacterial cell wall and inactivates them, resulting in the inhibition of the cross-linkage in peptidoglycans. This leads to the disruption of the cell wall, and eventually results in cell lysis. Cell lysis then activates autolytic enzymes of the cell wall; it is probable that cloxacillin interferes with an autolysin inhibitor [9,15]. In this study we investigate the MIC of carbapenem resistance isolates of *P. aeruginosa* to different carbapenem and cephalosporins and the reducing effects of cloxacillin in combination of the corresponding antibiotics.

METHODS

A total of 61 non-duplicated carbapenem-resistant *P. aeruginosa* were collected from blood 13(21.3%), urinary tract infections 22(36.1%), wound of burn patients 12(19.7%) and other miscellaneous samples, 14(22.8%). The samples were collected from infected hospitalized patients from three major hospitals (Shafa, Afzalipour, and Bahonar) located in different regions of Kerman, Iran. Bacterial identification was performed using standard bacteriological methods [16].

The DNA templates from all the isolates for detection of carbapenemase, metallo-beta-lactamase (MBL), and extended spectrum beta-lactamase (ESBL) genes were extracted by boiling (10 minutes in 95°C) and PCR was carried out in a thermal cycler (Bio Rad, USA) and bla_{IMP}, bla_{VIM}, bla_{SIM}, bla_{SPM}, bla_{GIM}, bla_{AIM}, bla_{KPC}, bla_{GES}, bla_{NDM}, bla_{CTX-M} genes were detected based on previous studies [7,17].

Minimum Inhibitory Concentration (MICs) of the isolates to imipenem (IMI), meropenem (MEM), ceftazidime (CAZ), and cefepime (FEP) (Jaber Ebne Hayyan Pharmaceutical Co., Iran) were determined alone and with combination of 250μg/mL of cloxacillin (COL) (Sigma-Aldrich, Product Number: 27555) using microbroth dilution methods according to CLSI recommendations [18]. Isolates were considered to be an AmpC overproducer when a two-fold or more dilution difference (at its minimum) was detected between the MICs of the IMI, MEM, CAZ, and FEP in presence or absence of COL [10,19]. *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as quality control strains.

Statistical analysis of data was carried out using the SPSS Statistics v17.0 software. The χ^2 and T-test was used for comparison of data. A difference was considered statistically significant at *P*-value of ≤ 0.05.

RESULTS

All of the isolates were resistant to IMI and MEM. The MIC to IMI, MEM, CAZ, and FEP ranged from 2-≥1024 µg/mL (Table 1). Combination of COL with these agents reduced the range MIC to 1-512 µg/mL. In the current study, four samples total were positive for MBL genes, comprising one bla_{IMP} (1.6%), one bla_{VIM} (1.6%), one bla_{SIM} (1.6%), and one bla_{NDM} (1.6%) (Table 1). The genes were confirmed by sequencing and submitted in GenBank with accession numbers bla_{IMP} (MG589419), bla_{VIM} (MG589421), bla_{SIM} (MG589420), and bla_{NDM} (MG589422). Fifty-six percent of the isolates overproduced the *ampC* β-lactamase and reduced the MICs to IMI, MEM, CAZ, and FEP when the agents were tested with COL (Table 1). The distribution of the MIC range in presence and absence of cloxacillin is presented in Table 1. The mean MIC to all agents except for MEM was significantly reduced in the presence of cloxacillin (Table 2). In the case of IMI, the reduction in the MIC was mostly seen in the lower range, and the two isolates with MIC higher than 256 µg/mL were not affected by combination with COL. However, the MIC to CAZ and FEP were markedly reduced over the high MIC levels. Our findings showed that the MIC_{50} for IMI, MEM, CAZ, and FEP was reduced 2-, 4-, and 8-fold in combination with COL. The MIC_{90} for IMI and PEP was reduced by 8-fold, CAZ by 2-fold and no reduction in the MIC_{90} of MEM was observed in the presence of COL (Table 1).

DISCUSSION

Microbial resistance has increased prominently in recent years around the world [19]. MDR bacteria like *P. aeruginosa* is one of the most common pathogens involved in severe nosocomial infections and treatment of hospitalized patients often represents a challenge to clinicians [20]. Carbapenems are a proper choice for the treatment of infections with these bacteria. Various mechanisms such as MBL production, mutation in outer membrane protein such as OprD, chromosomally-mediated β-lactamase (AmpC) and efflux pumps overexpression are involved in carbapenems resistance among *P. aeruginosa* strains [21,22]. Upon understanding the main mechanisms involved in β-lactam resistance prevalent in a hospital, an appropriate therapy for nosocomial infections can be developed rationally [9].

In this study the rate of resistance among *P. aeruginosa* to IMI, MEM, and CAZ were respectively high (above 70.5%). It should be considered that 93.4% of carbapenem resistant *P. aeruginosa* in our study were MBL negative and only four isolates were positive for MBL.

In the absence of MBLs enzymes, carbapenem resistance is mostly multilateral and including increased production of AmpC cephalosporinase, efflux pump overexpression and inactivation of OprD. AmpC β-lactamases are also responsible for resistances to aminopenicillins, cephalosporins, oxyimino-cephalosporins, cephamycins, carbapenems, and monobactams [2,23]. Our study showed that 91.8% of isolates were AmpC overproducers. Rodríguez *et al.* reported that 21 of their isolates overexpressed the AmpC β-lactamase and had decreased MICs of CAZ, IMI, and FEP after COL addition, suggesting the presence of an extended-spectrum cephalosporinases (ESACs) in clinical *P. aeruginosa* isolates [10]. In a study in Iran, Mirsalehian *et al*. reported that MICs of IMI and CAZ among 52 isolates of *P. aeruginosa* was reduced after adding COL which suggests that the main mechanism associated with susceptibility reduction or resistance to IMI was probably overproduction of AmpC and it can play a supplementary role in susceptibility reduction or resistance to IMI [24]. According to the results of Polsfuss *et al*., detection of AmpC production in bacterial pathogens might be of importance for ensuring that the antibiotic therapy is effective, since the presence of an AmpC beta-lactamase frequently leads to failure of treatment when broad-spectrum cephalosporins are used [15]. In accordance with Rodríguez-Martínez *et al*., we demonstrate that COL had a lesser impact on resistance to MEM, therefore the mechanisms leading to MEM resistance seem to be multifactorial among the isolates, such as overexpression of the efflux pumps [10]. The result of the research by Tam *et al*. shows that β-lactam ⁄ β-lactamase inhibitor combinations may not be helpful as empirical therapy in clinical settings where *ampC* over-expression is common, since the hydrolytic activity of AmpC is not controlled by inhibitor such as clavulanic acid. *ampC* over-expression appears to be a considerable mechanism of β-lactam resistance in *P. aeruginosa* [25]. In conclusion, regarding the increasing drug resistance with multiple mechanisms and based on the inhibitory potential of COL and its repressing impact on AmpC β-lactamase, administration of antipseudomonal antibacterial agents with COL may be advantageous so as to prevent bacterial resistance throughout the course of treatment in serious infections with *P. aeruginosa*, however this reducing effect should be also evaluated *in vivo*.

Table 1. Minimum Inbibitory concentrations (MIC) of 61 carbapenem-resistant *P. aeruginosa* to imipenem (IMI), meropenem (MEM), ceftazidime (CAZ) and cefepime (FEP) in presence or absence of cloxacillin (CLO). a: AmpC non producers

Sample (Type of MBL genes)	MIC (µg/mL)			
	IMI (IMI/CLO)	MEM (MEM/CLO)	CAZ (CAZ/CLO)	FEP (FEP/CLO)
Ulcer	2(1)	2(1)	512(512)	128(32)
BAL	4(4)	1(1)	512(128)	32(8)
CSF [a]	4(2)	4(4)	1(1)	1(1)
Urine	4(1)	8(4)	1(1)	1(1)
Burn exudate	4(1)	1(1)	512(128)	64(16)
Blood	4(1)	16(8)	512(256)	64(64)
Urine	4(1)	8(2)	1(1)	1(1)
Ulcer	4(1)	2(1)	1(1)	8(1)
Burn exudate	4(1)	2(1)	1024(512)	64(16)
BAL	8(1)	4(2)	256(256)	32(32)
Urine	8(1)	8(4)	16(8)	1(1)
Ulcer	8(1)	1(1)	1(1)	128(1)
Urine	8(1)	4(2)	8(1)	128(1)
Urine	8(1)	1(1)	16(1)	16(1)
Urine 2 isolates/Blood 1 isolate	8(1)	1(1)	1(1)	1(1)
BAL	8(1)	8(8)	256(256)	64(32)
Urine	8(1)	4(2)	1024(512)	256(32)
Urine (*bla*$_{SIM}$)	8(1)	4(2)	1024(512)	128(32)
BAL	8(1)	8(4)	256(256)	64(32)
Urine	8(1)	4(1)	1024(64)	256(16)
Urine	8(1)	4(1)	512(32)	64(1)
Ulcer	8(1)	2(1)	1024(512)	128(32)
Blood	8(1)	16(16)	512(512)	64(64)
Blood	8(2)	16(8)	256(256)	32(32)
BAL	8(2)	8(8)	256(256)	64(32)
BAL	8(2)	32(16)	256(256)	64(64)
BAL	8(2)	8(4)	256(256)	64(32)
Urine	8(2)	8(4)	256(128)	8(1)
Urine [a]	8(4)	8(8)	256(256)	16(16)
Blood [a]	8(4)	16(8)	256(128)	32(32)
Urine	8(4)	4(1)	16(1)	8(8)
Urine 1 isolate/Blood 2 isolates	16(1)	1(1)	1(1)	1(1)
Urine	16(1)	1(1)	256(64)	512(256)
Pharynx	16(1)	1(1)	256(64)	32(16)
BAL	16(1)	2(1)	256(1)	64(1)
Abscess fluid	16(1)	2(1)	8(1)	128(32)
Burn exudate	16(1)	2(2)	512(64)	128(64)
Blood	16(1)	4(1)	512(32)	128(1)
Ulcer	16(1)	4(1)	512(32)	64(1)
Urine	16(1)	4(1)	512(16)	64(1)
Urine	16(1)	4(1)	512(8)	64(1)
Blood	16(1)	4(1)	1024(256)	512(16)

Table 1 cont'd

Urine	16(1)	4(1)	512(16)	128(1)
Sputum	16(1)	8(1)	2048(64)	512(8)
Blood	16(1)	8(2)	1024(64)	512(8)
Ulcer	16(1)	8(4)	256(64)	8(8)
Ulcer	16(2)	4(2)	1(1)	8(1)
Blood	16(2)	4(2)	512(32)	128(1)
Urine	16(2)	2(1)	1024(128)	512(16)
Ulcer	16(4)	32(16)	512(256)	1024(64)
BAL	32(4)	16(8)	32(1)	1(1)
Urine	32(4)	16(8)	1024(128)	128(1)
Blood	128(2)	64(64)	256(256)	64(32)
Urine (*bla*$_{IMP}$)	128(2)	128(128)	512(512)	256(128)
BAL	128(32)	16(8)	256(256)	256(64)
Burn exudate (*bla*$_{VIM}$) [a]	256(256)	128(128)	64(64)	16(16)
Blood (*bla*$_{NDM}$) [a]	1024(1024)	2048(2048)	4096(2048)	1024(1024)

Table 2. Distribution and mean MICs to imipenem (IMI), meropenem (MEM), ceftazidime (CAZ) and cefepim (FEP) against 61 carbapenem-resistant *P. aeruginosa* isolates included in the study.

	MIC value(µg/mL)													
	1	2	4	8	16	32	64	128	256	512	1024	2048	4096	Mean
IMI	-	1	8	24	21	2	-	3	1	-	1	-	-	32.6
IMI+CLO	40	11	7	-	-	1	-	-	1	-	1	-	-	6.21
MEM	12	8	16	12	7	2	1	2	-	-	-	1	-	44.8
MEM+CLO	29	9	7	9	3	-	1	2	-	-	-	1	-	42
CAZ	12	-	-	2	3	1	1	-	16	15	9	1	1	447.6
CAZ+CLO	18	-	-	2	2	4	8	6	13	7	-	1	-	171
FEP	11	-	-	5	3	5	15	11	4	5	2	-	-	135.4
FEP+CLO	26	-	-	5	8	13	6	1	1	-	1	-	-	37.3

Author Contributions: FP: conceived the study. DK-N and SM: participated in the design of the study and performed the statistical analysis. DK-N, SM, and MM: interpreted the data. DK-N and SM: obtained ethical clearance and permission for study. FP: Supervised data collectors. FP, DK-N, MM, and SM: Drafting the article or revisiting. All authors read and approved the final manuscript.

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