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Article

Expression analysis of drug-resistant gene (*blaOXA-51)* in carbapenemases producing *Acinetobacter baumannii* treated with imipenem/sulbactam combination

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Drug-resistant *Acinetobacter baumannii* is a frightening reality. The aim of this study is to examine the expression profiles of *blaOXA-51* gene in carbapenemases producing *A. baumannii* treated with imipenem/sulbactam combination. Carbapenemases producing *A. baumannii* was identified among clinical isolates of *A. baumannii* obtained from patients at Shahid Rajaee hospital, Gachsaran, Iran, from January to June 2018. Synergism testing of imipenem/sulbactam on carbapenemases producing *A. baumannii* was carried out by broth microdilution method. Eventually, the expression of *blaOXA-51* gene was carried out to investigate the inhibitory properties of imipenem/sulbactam combination against carbapenemases producing *A. baumannii* using quantitative real time RT-PCR. Among *A. baumannii* isolates, 24% were carbapenemases producing *A. baumannii*. Imipenem/sulbactam combination revealed synergistic and partial synergistic effect for all tested isolates (FIC= 0.313-0.75). Finally, imipenem/sulbactam combination displayed significant down-regulation of *blaOXA-51* gene in carbapenemases producing *A. baumannii*. Imipenem synergizes with sulbactam against carbapenemases producing *A. baumannii* by targeting of the *blaOXA-51* gene.

Keywords: *Acinetobacter baumannii*. *blaOXA-51.* Carbapenemases. Imipenem. Sulbactam.

INTRODUCTION

Acinetobacter baumannii is an aerobic non-motile Gram-negative, non-fermentative and oxidase-negative bacillus and a frequent cause of life-threatening infections in hospitals. The combination of persistent presence of *A. baumannii* in the environment and its multidrug-resistant to many drugs determinants renders it a common nosocomial pathogen (Hou, Yang, 2015; Lin, Lan, 2014; Manchanda *et al*., 2010; Perez *et al.,* 2007). It is well known that *A. baumannii* is highly resistant to major classes of antibiotics such as aminoglycosides, third generation cephalosporins and fluoroquinolone (Hou, Yang, 2015).

Carbapenems are considered as the gold standard against multidrug-resistant Gram-negative organisms. Carbapenems, including imipenem, meropenem, biapenem, ertapenem, and doripenem, are classified as β-lactam antibiotics. However, the number of carbapenem-resistant *A. baumannii* strains have been increasing rapidly (Da Silva, Domingues, 2016; Fritzenwanker *et al*., 2018; Karam *et al*., 2016; Papp-Wallace *et al*., 2011). Several mechanisms associated with resistance to carbapenems include production of β-lactamases. Carbapenemases are specific β-lactamases that are classified into three functional groups: class A serine carbapenemases (e.g., KPC and GES enzymes), class B metallo-β-lactamases (e.g., VIM, IMP, and NDM β-lactamases), and class D carbapenemases (e.g., OXA-23, −24/40, −48, −51, −55, −58, −143 and −235) through the expression of chromosomal and plasmid genes (Bahador *et al*., 2015; Bush, 2013; Higgins *et al*., 2013; Papp-Wallace *et al*., 2011). Although overproduction of class C β-lactamases (e.g., CMY-10 and PDC β-lactamases) do not involve robust carbapenemases, it may contribute to carbapenem resistance, particularly in combination with other drug-resistance mechanisms (Papp-Wallace *et al*., 2011). Different other mechanisms may be responsible for carbapenem-resistance in *A. baumannii* include loss or alterations of specific outer-membrane proteins, modification of penicillin binding proteins and AdeABC efflux pump (Da Silva, Domingues, 2016).

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Class D carbapenemases, also known as OXA-type enzymes or oxacillinases, are the most clinically problematic enzymes in this regard. The majority of these enzymes have been identified in various *Acinetobacter* clinical isolates (Antunes *et al*., 2014; Diene, Rolain, 2014; Walther-Rasmussen, Hoiby, 2006). Moreover, the class D β-lactamase OXA-51 is ubiquitous and unique carbapenemases to emerge in multi-resistant of *A. baumannii* (Brown *et al*., 2005; Evans *et al*., 2008).

Outbreaks of antibiotic resistance *A. baumannii* have become increasingly common and have been recognized as a tremendous challenge worldwide (Bahador *et al*., 2015; Manchanda *et al*., 2010; Safari *et al*., 2013). Combination therapies are urgently needed for the treatment of *A. baumannii* infection. Despite the report of combination therapies with imipenem (Fishbain, Peleg, 2010; Lin, Lan, 2014; Spellberg, Bonomo, 2015), it is little known about the efficacy of imipenem/sulbactam on drug-resistant genes of *A. baumannii* (Montero *et al*., 2004). Therefore, the present study was designed to evaluate the imipenem/sulbactam combination effects on clinical isolates of carbapenemases producing *A. baumannii.* The study examined expression profiles of *blaOXA-51* gene involved in the carbapenemases producing *A. baumannii* treated with imipenem/sulbactam combination.

MATERIAL AND METHODS

Antibiotics

Amikacin (AMK; 30 µg/disk), ampicillin/sulbactam (SAM, 10/10 µg/disc), cefixime (CFM; 5 µg/disc), cefotaxime (CTX; 30 µg/disc), ceftazidime (CAZ; 30 µg/disc), ceftriaxone (CRO; 30 µg/disc), ciprofloxacin (CIP, 5 µg/disc), gentamicin (GEN, 10 µg/disc), imipenem (IPM, 10 µg/disc), meropenem (MEM; 10 µg/disc), nitrofurantoin (NIT; 300 µg/disc), sulbactam (SUL; 10 µg/disc), trimethoprim/sulfamethoxazole (SXT; 25 µg/disc) (HiMedia Laboratories, Mumbai,. India), imipenem and sulbactam powder (Sigma-Aldrich Co. St. Louis, MO, USA). The antifungal powders were dissolved in dimethyl sulfoxide (DMSO) and stock solutions diluted based on Clinical and Laboratory Standard Institute (CLSI) guidelines (CLSI M07-A10).

Source of bacteria

Microbiological study was performed on 50 samples of *A. baumannii* isolated from blood (6%, n=3), urine (32%, n=16), wound (42%, n=21) and respiratory secretions (20%, n=10) of 258 patients admitted to the intensive care unit of Shahid Rajaee hospital, Gachsaran, Iran, within a 6-month period from January to June 2018. Clinical samples were inoculated onto blood agar (Merck) and MacConkey agar (Merck) medium and incubated at 37 ºC for 24 h at microbiology laboratory (Ghajavand *et al*., 2015). *Acinetobacter baumannii* ATCC BAA-747 was acquired from Iranian Research Organization for Science and Technology and stored in trypticase soy broth (TBS, Merck Research Laboratories, Darmstadt, Germany) supplemented with glycerol (30% v/v) at -70 ºC.

Bacterial identification

The clinical isolates were identified according to Gram reaction, morphological, cultural, and physiological properties. The chromogenic agar CHROMagar *Acinetobacter* (CHROMagar, Paris, France) was used for selective and rapid identification of *Acinetobacter* species. The isolates were further identified as *A. baumannii* by the amplification 16S rRNA gene. The amplified PCR products was confirmed by DNA sequencing. The sequence homology was detected using the nucleotide BLAST program (Biglari *et al*., 2017; Chen *et al*., 2017; Ghajavand *et al*., 2015).

Antibiotic susceptibility test

Antibiotic susceptibility of the clinical isolates was determined using disk diffusion method on Mueller-Hinton agar (Merck), referred to the standard operating procedures for disk diffusion (CLSI M02-A12, 2015). The susceptibility patterns of clinical isolates were analysed using software program WHONET 2017.

Detection of carbapenemases producing *A. baumannii*

All *A. baumannii* clinical isolates were tested for carbapenemases production using the phenotypic confirmatory test (Carba NP test), performed and interpreted according to CLSI standards (CLSI M100-S27, 2017; van der Zwaluw *et al*., 2015).

Synergism testing

In order to determine synergism between imipenem and sulbactam, antibacterial susceptibility of imipenem

and sulbactam alone and in combination against carbapenemases producing *A. baumannii* isolates were determined via broth microdilution method (CLSI M07-A10). Hundred µL of the two-fold dilution of imipenem and sulbactam (range 0.125–512 µg/mL) alone or in combination were aliquoted in the wells of 96-well U-bottom microtiter plates in the presence of 100 µL of 2-8 × 10^4 colony-forming units (CFU)/mL of carbapenemases producing *A. baumannii* isolates and incubated at 35 ºC. The absorbance was measured at 630 nm using a Stat Fax 303 Reader (Awareness Technology, Inc., USA) after 24 h of incubation. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of the antibacterial agents that reduced ≥50% or 90% of absorbance compared to the positive control. The synergistic activity of the imipenem/sulbactam combination was determined based on the fractional inhibitory concentration (FIC) index (Khodavandi *et al*., 2010).

Quantitative analysis of *blaOXA-51* gene expression in carbapenemases producing *A. baumannii*

Expression of *blaOXA-51* in carbapenemases producing *A. baumannii* isolate treated with imipenem and sulbactam alone and in combination was analysed by Quantitative Real-Time PCR. Briefly, total RNA was extracted from carbapenemases producing *A. baumannii* isolate according to the manufacturer's operating instructions of RNeasy Mini Kit (Qiagen, Hilden, Germany) with slight modifications. The extracted RNA was treated with DNase (Fermentas, USA) to remove genomic DNA. RNA quality was checked by 1.2% (w/v) formaldehyde-denaturing agarose gel electrophoresis. The concentrations and absorbance ratio of RNA at A260/A280 and A260/A230 were measured using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). According to the manufacturer's protocol, total RNA (0.5 µg) was copied into single-stranded cDNA using Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase and random hexamer oligonucleotides (Fermentas, USA). *A. baumannii blaOXA-51* gene was amplified from the synthesized cDNA with primers designed via Primer3 program. The following sequences of primers were used for *blaOXA-51* gene: 5' AATGATCTTGCTCGTGCTTC 3′ (forward), 5′ CATGTCCTTTTCCCATTCTG 3′ (reverse) and 16S rRNA (house-keeping gene): 5' GTGGACGTTACTCGCAGAAT 3' (forward), 5' TCACGCTACACTGGATGCTA 3' (reverse). Real-time PCR was performed using ™SYBR Green qPCR Master Mix (Fermentas, USA) on a Bio-Rad MiniOpticonTM system (USA). Amplification conditions were as follows: 5 min at 95 ºC; 40 cycles of 15 s at 95 ºC, 20 s at 55 ºC and 15 s at 72 ºC. Melting curve analysis was performed in the range of 65 ºC to 95 ºC, 0.5 ºC per 5 s increments. The expression levels of *blaOXA-51* gene were calculated by comparative Ct method ($2^{-\Delta Ct}$ formula) after normalization with 16S rRNA gene (Khodavandi *et al*., 2011; Schmittgen, Livak, 2008).

Ethics

This study was approved by Research Ethics Committee of our institute (Ethical code 14930554962002). The study protocol conformed to the ethical guidelines of the 2008 Declaration of Helsinki.

Statistical analysis

Results are expressed as mean value ± standard error of three independent replicate experiments. All experiments were repeated three times to compensate for possible errors. Statistical analysis was performed using analysis of variance (ANOVA). The comparison two means were calculated using the Tukey's Post hoc test. Values of $p < 0.05$ were considered significant. Statistical analysis was performed using the SPSS software (version 22; SPSS Inc., Chicago, IL).

RESULTS

A. baumannii clinical isolates were identified by morphological and biochemical methods. Of 258 patients from whom we obtained samples, 56.20% (n=145) were male and 43.80% (n=113) were female. Patients' age ranged from 14 to 86 years old with a mean of 40.00 ± 0.10 years old. In this study, 50 (19.38%) *A. baumannii* isolates were identified. Table I shows the results of antibiotic susceptibility of the *A. baumannii* isolates. All clinical isolates of *A. baumannii* showed multidrug-resistance (MDR) patterns. Among isolates identified as MDR *A. baumannii,* 98% (n=49) possible extensively drug-resistant (XDR) and 12% (n=6) possible pandrug-resistant (PDR) were detected. An isolation rate of 24% (n=12) carbapenemases producing *A. baumannii* were obtained.

Table II summarizes the MIC and FIC values of imipenem and sulbactam alone and in combination

against carbapenemases producing *A. baumannii* isolates. Imipenem/sulbactam combination against carbapenemases producing *A. baumannii* isolates displayed synergy and partial synergy effects (FIC= 0.313-0.75). All carbapenemases producing *A. baumannii* isolates *were shown to be* resistant to imipenem alone with MICs range from 8-32 µg/mL. Sulbactam alone revealed the inhibitory activity against all carbapenemases producing *A. baumannii* isolates. Imipenem/sulbactam combination significantly ($p \leq 0.001$) reduced the MICs of imipenem and sulbactam against carbapenemases producing *A. baumannii* isolates.

TABLE I – Antibiotic susceptibility testing results for clinical isolates of *A. baumannii*

Code	Antibiotic Name	Antibiotic Class	Antibiotic Subclass	Breakpoints	%R	%I	%S	%R 95%C.I.
AMK_ND30	Amikacin	Aminoglycosides		15 - 16	76	16	8	61.5-86.5
CAZ_ND30	Ceftazidime	Cephems	Cephalosporins III	15 - 17	94	2	4	82.5-98.4
CFM_ND5	Cefixime	Cephems-Oral	Cephalosporins	16 - 18	48	40	12	33.9-62.4
CIP_ND5	Ciprofloxacin	Quinolones	Fluoroquinolones	16 - 20	34	44	22	21.6-48.9
CRO_ND30	Ceftriaxone	Cephems	Cephalosporins III	14 - 20	68	32	0	53.2-80.1
CTX_ND30	Cefotaxime	Cephems	Cephalosporins III	15 - 22	98	2	0	88.0-99.9
GEN_ND10	Gentamicin	Aminoglycosides		13 - 14	48	18	34	33.9-62.4
IPM_ND10	Imipenem	Penems	Carbapenems	19 - 21	82	10	8	68.1-91.0
MEM_ND10	Meropenem	Penems	Carbapenems	15-17	82	8	10	68.1-91.0
NIT_ND300	Nitrofurantoin	Nitrofurans		15 - 16	72	16	12	57.3-83.3
SAM_ND10	Ampicillin/ Sulbactam	Beta-lactam+Inhibitors		12 - 14	36	34	30	23.3-50.9
SUL_ND	Sulbactam	Beta-lactamase inhibitors		15 - 16	6	0	94	82.5-98.4
SXT_ND1.2	Trimethoprim/ Sulfamethoxazole	Folate pathway inhibitors		11 - 15	30	42	28	18.3-44.8

TABLE II – MIC (μg/mL) and FIC values of imipenem and sulbactam alone and in combination against carbapenemases producing *A. baumannii* isolates

Isolates/Antibacterial	Imipenem		Sulbactam		Imipenem/Sulbactam			
	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	FIC	Interpretation
A. baumannii ATCC BAA-747	2	0.125	2	0.5	0.5	0.0625	0.5	Synergism
CI- 9	16	4	8	4	4	1	0.75	Partial Synergism
CI- 12	16	8	8	4	2	1	0.375	Synergism
CI- 14	32	8	8	4	2	1	0.313	Synergism
CI- 18	32	16	8	4	4	2	0.625	Partial Synergism
CI- 19	16	4	8	4	4	1	0.75	Partial Synergism
CI- 34	8	4	4	4	2	2	0.75	Partial Synergism
CI- 35	32	8	8	4	2	1	0.313	Synergism
CI- 36	16	8	8	4	2	1	0.375	Synergism
CI- 39	32	8	8	4	2	1	0.313	Synergism
CI- 40	32	16	8	4	4	2	0.625	Partial Synergism
CI- 42	32	8	8	4	2	1	0.313	Synergism
CI- 43	32	16	4	4	2	2	0.563	Partial Synergism

CI: Clinical isolates of carbapenemases producing *A. baumannii*.

Gene expression analysis

The expression levels of *blaOXA-51* were evaluated to investigate the effect of imipenem/sulbactam combination on the carbapenemases producing *A. baumannii* isolates. The carbapenemases producing *A. baumannii* isolates treated with imipenem alone did not induce any significant changes in the expression levels of *blaOXA-51* in comparison to untreated control ($p = 0.123$). The expression levels of *blaOXA-51* were down-regulated ($p \leq 0.01$) in the carbapenemases producing *A. baumannii* isolate treated with sulbactam alone and imipenem/sulbactam combination. The *blaOXA-51* gene was found significantly down-regulated by 3.03- and 3.70-fold after treated

with MIC and 2× MIC of sulbactam alone, respectively (Tukey's HSD, $p \leq 0.01$). The carbapenemases producing *A. baumannii* isolate treated with imipenem/sulbactam combination showed a significant down-regulation in the expression levels of *blaOXA-51* by 6.67- and 10.00-fold at concentrations of MIC and 2× MIC, respectively (Tukey's HSD, $p \leq 0.01$). The box plots allow comparison of *blaOXA-51*/16S rRNA ratio at different concentrations of imipenem and sulbactam alone and in combination based on MIC (Figure 1).

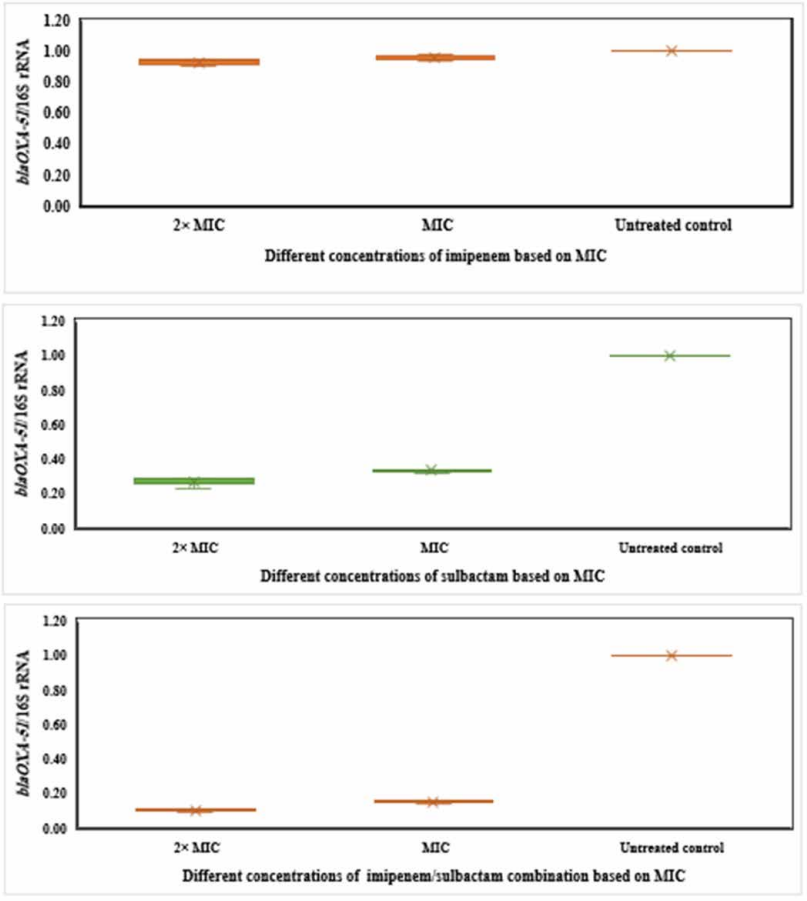

FIGURE 1 – Box plots of *blaOXA-51*/16S rRNA ratio at different concentrations of imipenem and sulbactam alone and in combination based on MIC in carbapenemases producing *A. baumannii* isolate.

DISCUSSION

Infections caused by antibiotic-resistant *A. baumannii* are associated with serious mortality and morbidity (Bahador *et al*., 2015; Manchanda *et al*., 2010; Safari *et al*., 2013). The treatment options available for this antibiotic-resistant pathogen are limited. Previous studies demonstrated the efficacy of sulbactam in combination with antibiotics against antibiotic-resistant *A. baumannii* (Choi *et al*., 2004; 2006; Fishbain, Peleg, 2010; Viehman *et al*., 2014). The present study demonstrated that the effectiveness of imipenem/sulbactam combination against carbapenemases producing *A. baumannii*. Imipenem/sulbactam combination showed synergism and partial synergism with carbapenemases producing *A. baumannii*.

Based on our results, the *A. baumannii* presented multidrug-resistant to several different types of antibacterial agents especially to cefotaxime, ceftazidime, imipenem and meropenem. Our study and others have demonstrated carbapenems-resistant in *A. baumannii* (Bahador *et al*., 2015; Choi *et al*., 2004). To determine carbapenemases production in *A. baumannii,* the phenotypic confirmatory test was performed and interpreted as previously described by CLSI guidelines. Since the enzymatic degradation by β-lactamases is the most common resistance mechanisms of *A. baumannii*,

bacterial cells are physiologically resistance to the β-lactam antibiotics (Handal *et al*., 2017; Poirel, Nordmann, 2006).

Sulbactam is a β-lactamase inhibitor with antibacterial activity against *Acinetobacter* species. Sulbactam binds to penicillin-binding proteins (PBPs) of *Acinetobacter* species, probably leading to bacterial death. However, sulbactam is degraded by the β-lactamases in *A. baumannii*. In addition, the activity of imipenem is attributed to binding to PBPs (Choi *et al*., 2004; 2006; McLeod *et al*., 2018; Penwell *et al*., 2015). These results suggest that the potent synergic effect of imipenem/sulbactam combination against carbapenemases producing *A. baumannii*. The affinity of sulbactam for PBP2 and also imipenem for PBP2 of Gram-negative bacteria may be the cause of such synergic effects in carbapenemases producing *A. baumannii* (Choi *et al*., 2004; Penwell *et al*., 2015). Montero *et al*. (2004) demonstrated that imipenem/sulbactam combination showed the strong bactericidal efficacy in pneumonia infections caused by moderately carbapenem-resistant *A. baumannii*. Wang *et al*. (2016) investigated the effect of meropenem, imipenem, sulbactam, colistin and tigecycline alone or in combination on biofilm-embedded carbapenem-resistant and carbapenem-susceptible *A. baumannii*. In their study, the combination of imipenem/sulbactam exhibited a killing effect against biofilm-embedded carbapenem-resistant and carbapenem-susceptible *A. baumannii*.

In our study, carbapenemases producing *A. baumannii* showed obvious expression level changes of *blaOXA-51* after exposure to imipenem/sulbactam combination. This variation may be explained by the sulbactam promoting the effects of imipenem, mainly on the PBP2 of *A. baumannii* (Choi *et al*., 2004; 2006; McLeod *et al*., 2018; Penwell *et al*., 2015). Additionally, sulbactam inhibit *A. baumannii* carbapenemases may facilitate the binding of imipenem to the PBP2, thereby leading to down-regulation of *blaOXA-51* in the carbapenemases producing *A. baumannii* treated with imipenem/sulbactam combination. In previous studies, *blaOXA-51* was found to elucidate their carbapenemase activities in *A. baumannii* (Handal *et al*., 2017; Poirel, Nordmann, 2006). Hou, Yang, (2015) found that *OXA-51* and *OXA-23* were the main multidrug-resistant molecular target genes in *A. baumannii*. Hu *et al*. (2007) reported an *OXA-66*/*OXA-51*-like carbapenemase that confers imipenem resistance in *A. baumannii*. Transcriptional profile analysis of the *blaOXA-51* in two clonally related isolates of *A. baumannii* indicated an eight-fold increased expression of *blaOXA-51* in the genetic structure containing insertion sequence *ISAba1* and *ISAba9* compared with *ISAba1* alone upstream of this gene (Figueiredo *et al*., 2009).

CONCLUSION

In summary, this *in vitro* study found that imipenem/sulbactam combination could be the most effective against carbapenemases producing *A. baumannii*. Whether these events reflect the potential of imipenem/sulbactam combination for inhibition of *blaOXA-51* gene in carbapenemases producing *A. baumannii*, which differentially expresses specific gene, requires further investigations to identify other probable molecular targets to this drug combination.

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