The diversity of class B and class D carbapenemases in clinical *Acinetobacter baumannii* isolates

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SUMMARY

Wide distribution of multidrug-resistant *Acinetobacter baumannii* strains has become a foremost concern in hospital environments. Treatment of infections caused by multidrug resistant strains has conventionally involved the use of ß-lactams such as carbapenems. In this study, we report the distribution of carbapenemase genes in *A. baumannii* isolated from hospitalized patients. The study was conducted on 110 non-repetitive *A. baumannii* isolates collected from hospitalized patients, over a nine-month period. Clinical isolates were examined by conventional susceptibility testing, using the disk-diffusion method and multiplex polymerase chain reaction to detect acquired carbapenemase genes. All of the isolates were completely resistant to TOB, SXT, IPM, MEM, CTX, CRO, FEP, CAZ, CIP, PTZ, PIP and were susceptible to colistin, but moderately susceptible TET (2.72%), AK (4.54%) and GEN (3.63%). The prevalence of $bla_{OXA-51-like}$, $bla_{OXA-23-like}$, $bla_{OXA-24-like}$, $bla_{OXA-58-like}$, bla_{SIM} and bla_{SPM} genes was 100%, 96.36%, 35.45%, 7.27%, 7.27% and 3.63%, respectively. bla_{GIM} and bla_{VIM} genes were not detected among the strains. Our results suggest that OXA-type carbapenemase genes plus class B β-lactamases contribute to carbapenem resistance in the collected isolates. Therefore, quick identification of these resistant genes using molecular approaches is critical in limiting the spread of infections caused by *A. baumannii*. Drug administration correction of the physicians, based on antibiotic susceptibility testing and more knowledge on the nosocomial infection control policies as essential need.

Keywords: *Acinetobacter baumannii*, MDR, carbapenemases, OXA, Iran

■ INTRODUCTION

Acinetobacter baumannii (*A. baumannii*) is one of the most important opportunistic pathogens causing nosocomial infections or healthcare associated infections [1, 2]. It is well known that

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Acinetobacter is widely found in intensive care units (ICUs) and can cause life threatening diseases including septicemia, meningitis, pneumonia and burn wound bacterial infections [3]. Multidrug-resistant *A. baumannii* (MDRAB) strains possess a significant capacity to obtain mobile genetic elements, which disseminate antibiotic resistance, predominantly between Gram-negative bacterial pathogens [4]. On the other hand, the absence of novel therapeutic agents and lack of proper management of infections related with MDR strains led to a major concerns worldwide

[5,6]. Carbapenems (imipenem, meropenem, doripenem, ertapenem) are a class of β-lactam drugs that are conventionally used to treat difficult Gram-negative infections [7], but the reports of resistance to carbapenems have emerged in clinically relevant *A. baumannii* strains. Carbapenem resistance in *Acinetobacter* spp is associated with one or several of these mechanisms: hydrolyzing β-lactamases of metallo-β-lactamases (ambler class B) and oxacillinases (ambler class D), decreased outer membrane permeability, target-site modification, and up-regulation of multidrug efflux pumps [8]. Oxacillinases (OXA) and less frequently metallo-β-lactamases producing strains cause difficulties in antibiotic treatment. On the other hand, clinically important MBL families have been located in gene cassettes and can spread among Gram-negative bacteria [9]. Until now, eleven types of MBLs have been described throughout the world, six of them including; imipenemase (IMP), Verona imipenemase (VIM), Sao Paulo metallo-β-lactamase (SPM), German imipenemase (GIM), SIM (Seoul imipenemase) and New Delhi metallo beta-lactamase (NDM) being reported in *A. baumannii* [10].

Carbapenem-hydrolyzing class D β-lactamases (CHDLs) have been reported exclusively among this pathogen. The CHDLs in *Acinetobacter* strains can be divided into four clusters, including OXA-23-like, OXA-24-like, OXA-58-like and intrinsic OXA-51-like enzymes [11, 12]. Previous work has described that OXA group genes are the common carbapenemase in *A. baumannii* and bla_{OXA-23} or bla_{OXA-51} are the most common detected genes in Iran [13]. In order to comprehensively control the infections produced by this organism, understanding the molecular basis of the infection is necessary. Here, we investigated the antibiotic susceptibility and presence of genes encoding carbapenemases in the clinically-related *A. baumannii* that were collected from two hospitals in Tehran, Iran.

MATERIALS AND METHODS

Clinical isolation and laboratory identification
This cross-sectional study was performed over a 9-month period (November 2016 till July 2017) on clinical samples collected from patients admitted to the Shahid Motahari and Milad Hospitals of Tehran, Iran. Overall, we studied on 419 consecutively clinical specimens collected from wound swabs (n=204), urine (n=78), tracheal tube (n=55), blood (n=48) and sputum (n=34). All the specimens were transferred to the microbiology laboratory of the department of microbiology of IUMS and were processed for culture and identification using standard procedures. In summary, each sample was cultured on the MacConkey agar (Merck Co., Germany) and incubated at 37°C for 24 h. Then, all suspected grown colonies were initially recognized using standard biochemical and microbiological tests like, Gram staining, oxidase, catalase, motility, citrate utilization, oxidative/fermentative (O/F) glucose tests and growth ability at 44°C [14]. The identification of isolates was confirmed using a Microgen identification kit (Microgen TM, UK).

Susceptibility testing
Susceptibility test to antimicrobial agents were conducted using a Kirby–Bauer disk diffusion assay according to the standards and interpretive criteria described by the Clinical and Laboratory Standards Institute (CLSI). The antibiotics included tetracycline (TE: 10 µg), meropenem (MEM: 10 µg), amikacin (AK: 30 µg), imipenem (IPM: 10 µg), cefotaxime (CTX: 30 µg), ceftriaxone (CRO: 30 µg), piperacillin/tazobactam (PTZ: 100/10 µg), piperacillin (PIP: 100 µg), ceftazidime (CAZ: 30 µg), ciprofloxacin (CIP: 5 µg), cefepime (FEP: 30 µg), trimethoprim-sulfamethoxazole (SXT: 2.5 µg), tobramycin (TOB: 10 µg) and gentamicin (GEN: 10 µg) all purchased from Mast, UK. The size of the zone of inhibition was measured after 20-24 h and interpreted as susceptible (S), intermediate (I) and resistant (R) [15]. Intermediate sensitivity was considered as resistance in our survey. Resistance to more than 3 antibiotics (>3) was defined as multi-drug-resistance (MDR) index of the isolates [14]. Also, minimal inhibitory concentrations (MICs) for colistin (range 0.25-256 µg/mL), IPM (range 4-512 µg/mL) and MEM (range 4-512 µg/mL) were measured by broth microdilution according to CLSI 2014. To quality control (QC), *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used.

Template DNA preparation
After growth of bacteria on the Mueller-Hinton agar plates, DNA was extracted from pure colo-

Table 1 - Primers used in present study.

Primer	Sequence (5'→3')	Gene	Product size (bp)	Reference
OXA$_{23}$-F OXA$_{23}$-R	GATCGGATTGGAGAACCAGA ATTTCTGACCGCATTTCCAT	blaOXA-23	1058	[19]
OXA$_{24}$-F OXA$_{24}$-R	GGTTAGTTGGCCCCCTTAAA AGTTGAGCGAAAAGGGGATT	blaOXA-24	246	[20]
OXA$_{51}$-F OXA$_{51}$-R	TAATGCTTTGATCGGCCTTG TGGATTGCACTTCATCTTGG	blaOXA-51	353	[21]
OXA$_{58}$-F OXA$_{58}$-R	AAGTATTGGGGCTTGTGCTG CCCCTCTGCGCTCTACATAC	blaOXA-58	599	[22]
VIM-F VIM-R	GATGGTGTTTGGTCGCATA CGAATGCGCAGCACCAG	blaVIM	309	[22]
GIM-F GIM-R	TCGACACACCTTGGTCTGAA AACTTCCAACTTTGCCATGC	blaGIM	271	[23]
SPM-F SPM-R	AAAATCTGGGTACGCAAACG ACATTATCCGCTGGAACAGG	blaSPM	477	[22]
SIM-F SIM-R	TACAAGGGATTCGGCATCG TACAAGGGATTCGGCATCG	blaSIM	570	[24]

nies. The boiling method described by Higgins et al. was used for genomic extraction. The DNA template was stored at -20°C until use. The quality and quantity of DNA were analyzed using a NanoDrop [16].

Detection of carbapenemase-encoding genes
All interested genes and corresponding specific primers used for PCR amplification are listed in Table 1, and their specificities were checked using primer-BLAST (NCBI, Bethesda, USA; http://blast.ncbi.nlm.nih.gov/Blast.cgi). Overall, two separate M-PCRs assays were performed to screen the presence of OXA genes (*OXA-23, OXA-24, OXA-51, OXA-51*) and MBL genes (*VIM, SIM, SPM* and *GIM*) in the *A. baumannii* isolates according to the method described previously [17, 18]. Briefly, two microliters of extracted genomic DNA was subjected to M-PCRs in a 50-µL reaction mixture. The first M-PCR assay was performed for the detection of OXA group genes. The mix contains 1× PCR buffer, 2.5 mM of MgCl$_2$, 2 mM of each dNTPs, 10 µmol/L of each oxacillinase primers and 1 U of Taq DNA polymerase (Amplicon Co., Denmark). Amplification was carried out with the following conditions; denaturation at 94°C for 10 minutes, 35 cycles with denaturation at 94°C for 30 seconds, annealing at 52°C for 40 seconds, extension at 72°C for 50 seconds with a final extension at 72°C

for 6 minutes. To detect the MBL genes, a PCR mixtures (50 µL final volumes) containing 1× PCR buffer, 2.5 mM of MgCl$_2$, 1.5 mM deoxynucleoside triphosphates, each of the forward and reverse primers (10 µmol/L) and Taq DNA polymerase (5 U/µl) (Amplicon Co., Denmark) were used. The reaction mixture was completed in a PCR system (Eppendorf Co., Germany) with the following protocol: initial denaturation at 94°C for 5 minutes, followed by 33 cycles of 45 seconds at 94°C, annealing at 52°C for 45 seconds, extension at 72°C for 55 seconds and final extension at 72°C for 6 minutes. The amplified products were visualized by electrophoresis on 1.5% agarose gels stained with EtBr and compared a molecular-weight size marker (Fermentas, St. Leon-Rot, Germany). Positive and negative controls were included with each reaction. In addition, negative strains were repeated twice for accuracy. Two strains with positive PCR amplification were selected, and were sequenced (Bioneer Company, Korea) and searched using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The nucleotide sequences were evaluated with Chromas 1.45 software and BLAST in NCBI.

Statistical Analysis
The MINITAB16 software was used for data analyses. The value and confidence of intervals were <0.05 and 95%, respectively.

Ethics statement

Ethical approval was not needed for this work since there was no direct patient involvement and only bacterial isolates were retrospectively studied.

■ RESULTS

Bacterial collection

A total of 110 non-duplicated clinical *A. baumannii* isolates were obtained from hospitalized patients, during the period of this study. These strains were mostly isolated from wound samples (n=51, 46.36%), followed by tracheal tube (n=37, 33.63). Overall, 51 (46.36%) and 59 (53.63%) strains were collected from Shahid Motahari and Milad hospitals respectively.

Antimicrobial susceptibility profile

All the isolates were tested for their susceptibility against 14 commercially available antibiotics using disk diffusion method. The obtained result revealed high level of multi antibiotic resistance among the studied isolates. All the isolates were completely (100%) resistant to TOB, SXT, IPM, MEM, CTX, CRO, FEP, CAZ, CIP, PTZ and PIP, respectively. It was observed that 4 (2.72%), 5 (4.54%) and 3 (3.63%) *A. baumannii*. were sensitive to TET, AK and GEN respectively. While all isolates 110 (100%) were sensitive to COL. Rate of resistance to antibiotics among these strains are reported in Figure 1. The frequency of MDR *A. baumannii* among our isolates was found to be 82%. Additionally, MIC50 and MIC90 of both an-

tibiotics (IMP and MEM) was determined as 64 and 128 µg/ml, which reveals a lack of clinical efficacy of these antibiotics against infections produced by MDR isolates.

Molecular detection of carbapenemase-encoding genes

The bla_{OXA-51}-like carbapenemase gene was detected in 100% of strains. The positive rates of bla_{OXA23}, bla_{OXA24} and bla_{OXA58} were 96.63% (103/110), 35.45% (39/110) and 7.27% (8/110), respectively (Figure 2). Screening of the MBL genes in all strains showed that 7.27% (8/110) and 3.63% (4/110) of the isolates have bla_{SIM} and bla_{SPM} genes respectively. No other carbapenemase gene, in-
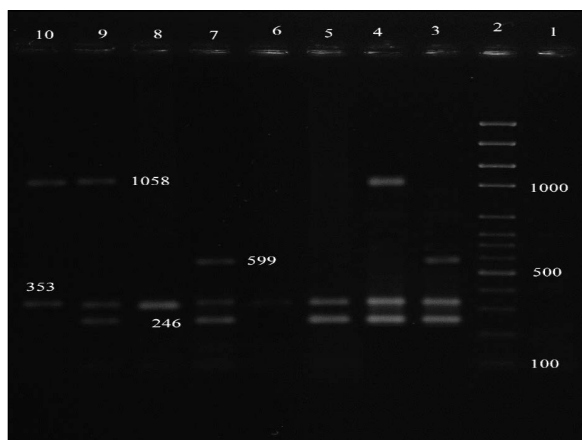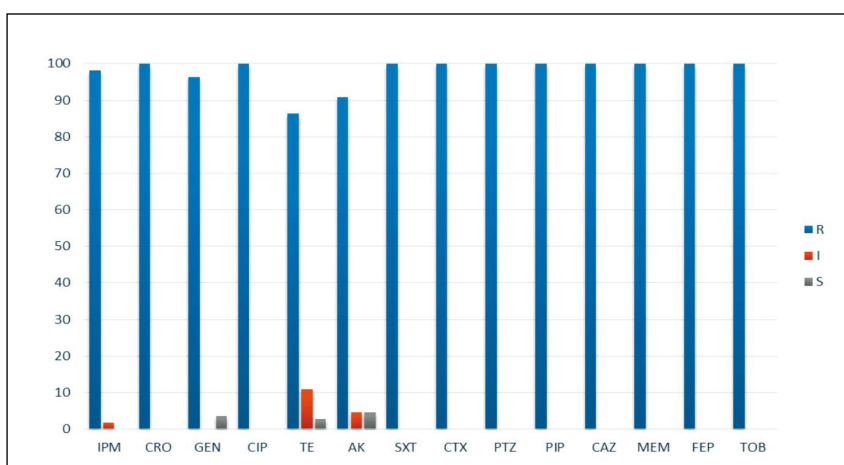
Figure 2 - M-PCR gel showing of studied OXA genes in *A. baumannii* isolates, Lane 2: DNA ladder 100 bp, Lanes 3-10: clinical samples, Lane 1: Negative control.

Figure 1 - The antimicrobial susceptibility of 110 *A. baumannii* isolates using disk diffusion.
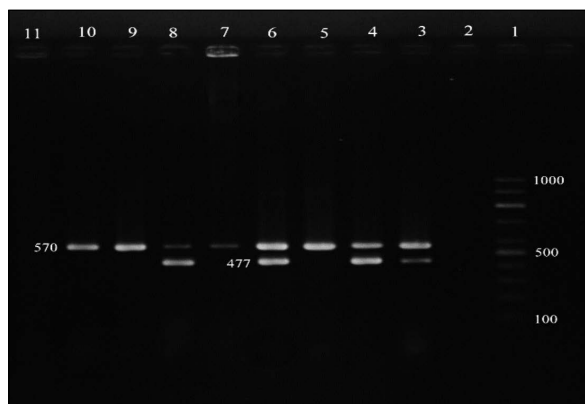
Figure 3 - M-PCR gel showing of studied MBL genes in *A. baumannii* isolates, Lane 1: DNA ladder 100 bp, Lane 2: Negative control, Lanes 3-11: clinical samples.

cluding bla_{VIM} and bla_{GIM}, was detected (Figure 3). Sequencing of PCR products showed conserved regions for the restriction sequence and genes which was confirmed by BLAST.

◼ DISCUSSION

The emergence and spread of MDR *Acinetobacter baumannii* is worldwide, especially in developing countries, therefore the epidemiological approaches are essential to develop active tactics to efficient checking, control and management of this ubiquitous pathogen [5]. This work provides important outcomes regarding the antimicrobial susceptibility, dissemination and diversity of carbapenem resistance encoding genes among MDRAB in Iran. The frequency of MDR among *A. baumannii* isolates was found to be 100% among the 110 strains tested. Two recent studies, from our geographic area had shown results similar to ours but with a lower prevalence of MDR strains (about 56% and 66%) [25-29]. This difference may be due to the total number of *A. baumannii* isolates in the mentioned studies than our study, region or period of study that can play a key role in reports of increase and/or decrease in the prevalence of MDRAB. Hence, according to the results colistin had good *in vitro* activity against the MDRAB and that it may be considered as a choice for the treatment of infections caused by this organism. It seems that lipopeptides antibiotics including colistin and polymixin B are the only restricted drugs which are effective for treatment

of MDRAB infections during the recent years in Iran though its nephrotoxicity is a disadvantage to its usage [30]. As mentioned above the evaluation of the most common types of carbapenemase genes in clinical isolates is crucial to control the spread of resistance in medical centers and community. In the present study, a high prevalence of bla_{OXA-51} gene was detected in MDRAB isolates; while 96.63%, 35.45% and 7.27% of isolates were positive for bla_{OXA-23}, bla_{OXA-24} and bla_{OXA-58} genes respectively. Another study in Iran has demonstrated that the most prevalent carbapenem hydrolyzing β-lactamases genes in *A. baumannii* included bla_{OXA-51} (100%) and bla_{OXA-23} (83%) followed by bla_{VIM} (12.5%) [32]. A parallel high percentage of resistance has been stated in many developing countries. A study from South Africa identified 59%, 83% and 3% of *A. baumannii* isolates were positive for bla_{OXA-23}, bla_{OXA-51} and bla_{OXA-58} genes respectively, but none of the isolates was positive for bla_{OXA-24} [22]. Gao et al. reported that the bla_{OXA-51} gene was detected in all examined isolates and 94% of strains carried bla_{OXA-23} gene, which is in accordance with present study [31, 32]. The second most common carbapenemase in *A. baumannii* are MBLs. In our study, 7.27% and 3.63% of isolates carried bla_{SIM} and bla_{SPM} genes respectively and no bla_{VIM} and bla_{GIM} were detected in the isolates. Peymani et al reported that 29% and 61% of isolates carried bla_{VIM} and bla_{IMP} [33]. In another study on MBL-producing strains of *A. baumannii*, 10.3% of the isolates carried bla_{IMP} and, no bla_{VIM} gene was detected [34]. Safari and colleagues also showed 99% of *A. baumannii* isolates were MBL producers by the phenotypic method [35]. In Turkey, Aksoy et al. concluded that only $bla_{OXA\ 23^{-}like}$ carbapenemase was responsible for carbapenem resistance in carbapenem-resistant *Acinetobacter* strains [36]. Our study had several limitations: first of all, the small sample size and inadequate demographic data about the patients. Multicenter surveys involving more patients are needed to validate our results. Secondly, no molecular typing between the isolates causing MDRAB infections was investigated.

Taken together, early and rapid detection of carbapenemases encoding genes among MDR clinical isolates may be critical, because can help to prevent the spread of resistance genes to other bacteria. The results of this study demonstrate that OXA group genes were predominant in

our clinical isolates of *A. baumannii* compared to MBLs genes.

In conclusion, new therapeutic regimes and use of appropriate susceptibility testing before empirical therapy are clearly needed. Further studies are needed to specify the other mechanisms of antibiotic resistance to carbapenems in *A. baumannii* strains.

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Competing interests

No potential conflict of interest related to this article was reported.

Ethical approval

Not required.

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