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ORIGINAL ARTICLE

Decreased *carO* gene expression and OXA-type carbapenemases among extensively drug-resistant *Acinetobacter baumannii* strains isolated from burn patients in Tehran, Iran

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ABSTRACT

A major challenge in the treatment of infections has been the rise of extensively drug resistance (XDR) and multidrug resistance (MDR) in *Acinetobacter baumannii*. The goals of this study were to determine the pattern of antimicrobial susceptibility, bla_{OXA} and *carO* genes among burn-isolated *A. baumannii* strains. In this study, 100 *A. baumannii* strains were isolated from burn patients and their susceptibilities to different antibiotics were determined using disc diffusion testing and broth microdilution. Presence of *carO* gene and OXA-type carbapenemase genes was tested by PCR and sequencing. SDS-PAGE was done to survey CarO porin and the expression level of *carO* gene was evaluated by Real-Time PCR. A high rate of resistance to meropenem (98%), imipenem (98%) and doripenem (98%) was detected. All tested *A. baumannii* strains were susceptible to colistin. The results indicated that 84.9% were XDR and 97.9% of strains were MDR. In addition, all strains bore bla_{OXA-51} like and bla_{OXA-23} like and *carO* genes. Nonetheless, bla_{OXA-58} like and bla_{OXA-24} like genes were harbored by 0 percent and 76 percent of strains, respectively. The relative expression levels of the *carO* gene ranged from 0.06 to 35.01 fold lower than that of carbapenem-susceptible *A. baumannii* ATCC19606 and SDS – PAGE analysis of the outer membrane protein showed that all 100 isolates produced CarO. The results of current study revealed prevalence of bla_{OXA} genes and changes in *carO* gene expression in carbapenem resistant *A.baumannii*.

KEYWORDS

Acinetobacter baumannii, *car*O, carbapenemases, bla_{OXA}, multi-drug resistance, extensively drug resistance

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INTRODUCTION

Nowadays, *Acinetobacter baumannii* has become a major hospital-associated pathogen, responsible for 2–10% of all Gram-negative infections in hospitals [1]. *A. baumannii* causes a variety of nosocomial infections, including bloodstream, respiratory tract, surgical site, urinary tract and wound infections, particularly in patients admitted to the intensive care unit

(ICU)[2]. Risk factors can predispose patients to *A. baumannii* infections and they include previous antibiotic therapy, prolonged exposure to antimicrobial agents, burns, hospitalization in ICU, and underlying severe illnesses especially receipt of mechanical ventilation [3]. Because nosocomial isolates of *A. baumannii* are increasingly resistant to commonly used antimicrobial agents, treatment of their infections is extremely difficult [2, 4]. Although carbapenems are the main antibiotics for treating multidrug-resistant *A. baumannii* infections, the emergence of carbapenem resistance may significantly compromise their efficacy [5]. The most significant carbapenem resistance mechanism in *A. baumannii* has been attributed mainly to chromosome and plasmid -encoded carbapenemases, such as OXA carbapenemases and metallo-β-lactamases (MBLs) [6]. In addition, a reduction or lack of outer membrane proteins (OMPs) permeability, excessive efflux pump expression and modified penicillin-binding proteins also have been found to play a role in acquiring carbapenem resistance in *A. baumannii* [5, 7]. At least four main groups of carbapenem-hydrolyzing oxacillinase genes have been described in *A. baumannii*: the intrinsic chromosomal $bla_{\text{OXA-51}}$-like gene and the acquired $bla_{\text{OXA-23}}$-like, $bla_{\text{OXA-58}}$-like and $bla_{\text{OXA-24}}$-like genes [8]. Based on prior studies, acquired OXA enzymes are found both chromosomally and on plasmids [9]. Additionally, reducing the permeability of the outer membrane was correlated with the development of carbapenem-resistant *A. baumannii* strains [10]. A 29 kDa carbapenem-associated outer membrane protein, namely CarO, which is considered as a family member of β-barrel proteins restricted to the Moraxellaceae, was found to have a role in resistance to carbapenems [11]. The previous studies on the MDR strains of *A. baumannii* showing a high MIC level to carbapenems have indicated disruptions of the carO gene by various insertion sequence, leading to the loss of or decrease in CarO [12–14]. Furthermore, other mechanisms associated with carbapenem resistance through affecting the CarO are as follows: i) amino acid alterations that lead to an altered porin with no affinity for the antibiotic and/or; ii) decreased expression of the *carO* gene that resulted in a reduced density of channels [12].

OBJECTIVES

In this research, we investigated the carbapenem resistance mechanisms in *A. baumannii* isolated among patients admitted to Shahid Motahari hospital, with asserting the role of carbapenemases and CarO OMP loss.

MATERIALS AND METHODS

Ethics statement

The Ethics Committee of Shahid Beheshti Medical Sciences University Sciences "IR.SBMU.MSP.REC.1395.524″ approved this study.

Clinical samples and bacterial identification

In the present study, 100 *A. baumannii* strains were isolated from the patients admitted to Shahid Motahari Burn Care Cente, Tehran, Iran from May 2016 to April 2017. Sampling of the normal saline-washed wounds was performed from the patients, and then wound swabs were transferred immediately to the microbiology laboratory of the Department of Microbiology of Shahid Beheshti University of Medical Sciences, Tehran, Iran. For the initial recognition of *A. baumannii*, the most important biochemical tests were applied, such as triple sugar iron (TSI), oxidase, motility and O-nitrophenyl-betad-glucopyranose (ONPG), sulfide indole motility (SIM), and 42 ° C growth [15]. The identification of species was confirmed by PCR analysis and sequencing of the 16S rRNA and *rpoB* genes [16]. Isolates recognized as *A.baumannii* were kept at −70 ° C in trypticase soy broth (TSB) (Merck, Germany) filled with 20% glycerol until further processing.

Antimicrobial susceptibility testing

Susceptibility of *A. baumannii* isolates to thirteen following antibiotics (Mast Group Ltd., Bootle, UK) was evaluated by disc diffusion method on Mueller-Hinton agar in accordance with Clinical and Laboratory Standards Institute (CLSI) recommendations [17]: tetracycline (75 μg), piperacillin/tazobactam (100/10 μg), cefotaxime (30 μg), ceftazidime (30 μg), aztreonam (30 μg), cefepime (30 μg), meropenem (10 μg), imipenem (10 μg), doripenem (10 μg), gentamicin (10 μg), amikacin (30 μg), ciprofloxacin (5 μg), and trimetoprim sulfametoxazol(23.75 μg). *Pseudomonas aeruginosa* ATCC 27853 was used as a strain for quality control. Minimum inhibitory concentration (MIC) of five antibiotics, including imipenem, meropenem, ceftazidime, ciprofloxacin, and colistin was defined by broth microdilution method at a final concentration from 0.5 μg/ml to 256 μg/ml for each antimicrobial agent [17]. *Escherichia coli* ATCC 25922 was applied as a strain for quality control.

Polymerase chain reaction (PCR) and sequence analysis

By using the specific primers (Table 1), all *A. baumannii* isolates were screened for the presence of *carO* gene by PCR method. The isolates' DNA was extracted using the DNA Extraction Kit (GeNet Bio Company, Daejeon, Korea; Cat. No, K-3000) in accordance with the manufacturer's guidelines [18]. PCR was performed in a total volume of 25 μL with 12.5 μL of 2 × Master Mix (SinaClon, Tehran, Iran; Cat. no.PR901638), containing 0.4 mmol/L dNTPs, 3 mmol/L MgCl 2, 1 × PCR buffer, and 0.08 IU Taq DNA polymerase, 7.5 μL of sterile distilled water and 1 μL of 10 pmol of each primer. The PCR amplification program repeated in 36 cycles was performed on thermal cycler (Eppendorf, Mastercycler Gradient; Eppendorf, Hamburg, Germany) as follows: initial denaturation at 95°C for 5 min, secondary denaturation at 95 °C for 45 s, annealing at 58 °C for 45 s, extension at 72 °C for 45 s and final extension at 72 °C for 10

Table 1. Primer sequences used in this study for PCR and Real-Time PCR

Gene	Sequence (5′→3′)	Size of amplicon (bp)	Reference
carO	AGCTTTACTTGCTGCTGGTG TCCTGAGCAGATGTTGGGTT	615	This study
carO for RT-PCR	AGCTTTACTTGCTGCTGGTG CGAGCGCCTACTGGAATTA	96	This study
OXA-23 like	GATCGGATTGGAGAACCAGA ATTTCTGACCGCATTTCCAT	501	[22]
OXA-24 like	GGTTAGTTGGCCCCCTTAAA AGTTGAGCGAAAAGGGGATT	246	[22]
OXA-51 like	GCGTGGTTAAGGATGAACAC CATCAAGTTCAACCCACCG	353	[22]
OXA-58 like	AAGTATTGGGGCTTGTGCTG CCCCTCTGCGCTCTACATAC	432	[22]
rpoB	GTGATAARATGGCBGGTCGT CGBGCRTGCATYTTGTCRT	450	[16]

min. PCR products were electrophoresized in a 1–1.51% agarose gel, then visualized by ethidium bromide staining under UV light. Insertion sequences were specified by the online tool IS finder (https://www-is.biotoul.fr/). Additionally, multiplex PCR was performed to detection of $bla_{\text{OXA-58-like}}$, $bla_{\text{OXA-24-like}}$, $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-51-like}}$ genes by using the specific primers against an internal positive control for each gene. Furthermore, PCRs were conducted for detecting the IS*Aba1*/$bla_{\text{OXA-23}}$-like and IS*Aba1*/$bla_{\text{OXA-51}}$-like sequences. The PCR products were analyzed with electrophoresis in a 1–1.51% agarose gel. Purification of PCR products was carried out using PCR Purification Kit (Macrogene Co. Korea) for sequencing, followed by direct amplicon sequencing by an ABI PRISM 3700 sequencer (Macrogen Inc., Seoul, Korea). Using NCBI BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) and FinchTV software, nucleotide sequences survey was performed.

Real-time reverse transcriptase-PCR (RT-PCR)

The levels of expression of the *carO* gene in isolates were determined through real-time RT-PCR using the target- and housekeeping-specific primers shown in Table 1. After an overnight culture of each isolate on Luria–Bertani broth [19], RNA extraction was carried out using the RNX-Plus Kit (Cat. No., RN7713C; SinaClon) as directed by the manufacturer. DNase I (Fermentas, Waltham, MA, USA) was used to remove the contaminating DNA. Determination of total RNA concentration was performed by Nanodrop (DS-11 Spectrophotometer, USA). By the Takara Kit (Shiga, Japan), DNase-treated RNA was reverse-transcribed into cDNA. Real-time RT-PCR assay was performed by the Power SYBR® Green PCR Master Mix (Bioneer, Daejeon, Korea) on a Corbett Rotor-Gene 6000 Real-Time rotary analyzer (Corbett Life Science, Sydney, Australia) with denaturation at 94 °C for 10 min, followed by 40 cycles consisting of 15 s at 94 °C and 1 min at 60 °C. Samples were run in triplicate and included 2 μL of cDNA per reaction. For confirmation the absence of contaminating cDNA, controls were run without reverse transcriptase. The relative expression of the examined genes was normalized against the 16sRNA housekeeping gene and was calculated based on a $2^{-\Delta\Delta CT}$ method. The results obtained as relative mRNA expression were compared with *Acinetobacter baumanii* ATCC 19606. Reduced *carO* differential transcription of strains relative to that of ATCC 19606 was significant when the ratios obtained between relative quantification values (relative quantification value of calibrator/relative quantification value of strains) were ≥ 2.0.

Outer membrane protein (OMP) analysis

As previously described [20], Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) experiments were performed. As a positive control, *A. baumannii* ATCC 19606 was used.

Statistical analysis

By using eather the Pearson χ^2 test or Fisher's exact test, categorical variables were evaluated. The *P*-value and confidence of intervals were < 0.05 and 95%, respectively.

RESULTS

Study population

Over a one-year period, 100 *A. baumannii* isolates were obtained from burn wound of patients hospitalized in Shahid Motahari therapeutic center. All 100 isolates were obtained from female (30%) and male (70%) between the ages of 1 and 85.

Antimicrobial Susceptibility test

Rate of resistance to all antibiotics tested among the *A. baumannii* isolates was as follows: piperacillin/tazobactam (99%), piperacillin (100%), cefotaxime (100%), ceftazidime (100%), ceftriaxone (100%), cefepime (100%), imipenem (98%), meropenem (98%), doripenem (98%), amikacin

Table 2. The Disc Diffusion Values of the Antibiotics against *A. baumannii*

Antimicrobials	Susceptible No. (%)	Intermediate No. (%)	Resistant No. (%)
Imipenem	0	2 (2)	98 (98%)
Meropenem	1 (1)	1 (1)	98 (98%)
Doripenem	0	2	98(98%)
Ceftriaxone	0	0	100 (100%)
Cefotaxime	0	0	100 (100%)
Ceftazidime	0	0	100 (100%)
Cefepime	0	0	100 (100%)
Ciprofloxacin	0	0	100 (100%)
Trimetoprim-sulfametoxazole	4 (4)	2 (2)	94 (94%)
Amikacin	5 (5)	5 (5)	90 (90%)
Tetracycline	10 (10)	8 (8)	82 (82%)
Gentamycin	5 (5)	2 (2)	93 (93%)
Piperacillin	0	0	100 (100%)
Piperacillin Tazobactam	0	1 (1)	99 (99%)

(90%), gentamicin (93%),ciprofloxacin (100%), trimethoprim/sulfamethoxazole (94%) and tetracycline (87%) (Table 2). Generally, 91% of isolates were resistant to all antibiotics, and also, 98% showed resistance to all three carbapenems tested. The results showed that 98% and 85% of isolates were MDR and XDR, respectively. Table 3 indicates the meropenem, imipenem, colistin, ciprofloxacin and ceftazidime MIC50 and MIC90 against the *A. baumannii* isolates. Colistin was found to be the most effective antibiotic with the highest susceptibility rate against *A. baumannii* strains (100%) (MIC range = 0.25–2 μg/ml).

PCR amplification

PCR assay revealed the presence of *carO* gene in 100% (100/100) of *A. baumannii* isolates. Based on sequencing data, *carO* gene sequences of 6 XDR isolates were different from those of *A. baumanii* ATCC 19606: NCBI protein database showed an amino acid alteration serine to threonine at position 182 in all 6 carbapenem-resistant isolates Multiple-sequence alignment of CarO from clinical isolates in comparison to *A. baumannii* ATCC 19606 standard strain. The result showed that the six isolates carO gene expression was significantly decreased ($P<0.01$). In addition, PCR of bla_{OXA} determined that all *A. baumannii* isolates carried bla_{OXA-23}-like and bla_{OXA-51}-like genes, 77% were positive for bla_{OXA-24}-like, whereas, none of the isolates harbored bla_{OXA-58}-like. Additionally, all isolates which harbored bla_{OXA} genes were carbapenem-resistant. The ISAba1 element was found in all but one of the isolates. For 80 percent and 65 percent of mutants, respectively, the co-existence of IS*Aba1*/bla_{OXA-23}-like and IS*Aba1*/bla_{OXA-51}-like genes were observed. The levels of imipenem and meropenem resistance were associated with OXA-like genes. OXA-23 carbapenemase production was more frequently associated (100 out of 100 isolates; $P < 0.001$) with high-level imipenem resistance (MICs ≥ 32 mg/L).

Table 3. The MIC Values of the Antibiotics against *A. baumannii*

Antibiotic	Range	MIC50	MIC90
Imipenem	2–256	64	128
Meropenem	2–256	32	128
Ceftazidime	2–512	256	512
Ciprofloxacin	2–512	256	512
Colistin	0.25–2	<1	2

Expression of *carO* gene

Real-time PCR analysis revealed that expression level of the carO gene in 48% ($n = 48$) of all isolates were 0.06 to 35.01 fold lower than that of carbapenem-susceptible ATCC 19606. Reduced *carO* differential transcription of strains relative to that of *A. baumannii* ATCC 19606 was considered significant when the ratios obtained between relative quantification values (relative quantification value of calibrator/relative quantification value of strains) were ≥ 2.0. Results indicated that *carO* gene was decreased (≥ 2.0 fold) in 10 out of 100 isolates (10%) in comparison to the *A. baumannii* ATCC 19606 standard strain.

SDS-PAGE

All 98 carbapenem-resistant *A.baumanii* isolates were analyzed by SDS-PAGE. Outer membrane profiles acquired through SDS–PAGE demonstrated that 98% (98/98) isolates had a corresponding band presented apparent molecular masses of 29-kDa on the gel.

Nucleotide sequence accession numbers

The data reported in this analysis on the nucleotide sequence was submitted to the GenBank sequence database and allocated the accession number: MG727557 for the *car*O gene.

DISCUSSION

As an opportunistic pathogen, *A. baumannii* is accountable for 10 % of all nosocomial infections caused by Gram-negative bacilli [14, 21]. The importance of this organism and the need for a qualified infection-preventative plan, especially for antibiotic-resistant strains, is emphasized by many sudden outbreaks of *A. baumanii* reported yearly from various hospitals across the world. In most cases, hospital environments have been identified as the source of infection, according to several studies conducted in hospitals during the outbreaks [22]. Such conditions have been associated with the development of different resistant phenotypes, including carbapenem-resistant *A. baumannii*, an issue raising serious concerns in the light of the limited antimicrobial treatment options [23]. In this study, a significant

percentage of *A. baumannii* isolates (91% and 98%) showed resistance to all antibiotics except for colistin, and all three carbapenems tested, respectively. In contrast, colistin was found to be the most effective antimicrobial agent tested. Similarly, Bahador et al. showed approximately 90% of *A. baumanii* isolates were resistant to at least 10 tested antimicrobial agents [24]. Colistin demonstrated the lowest rate of resistance (1%), followed by tigecycline (19%), however, minocycline (42%) had moderate resistance. Rastegar Lari et al. showed that high rates of resistance to ciprofloxacin (88%), ceftazidime (88%), cefepime (74%) and imipenem (72%) were observed. However, 64% of isolates showed less resistance to gentamicin. Production of various carbapenemases, such as OXA-235, OXA-23, OXA-143, OXA-24, OXA-58, VIM, NDM, and IMP have been introduced as the main mechanism of carbapenem resistance in many countries, including Iran [25, 26]. Production of OXA-23 as a prevalent mechanism of carbapenem resistance in A. baumannii in worldwide was reported formerly [27]. The reduction in expression of outer membrane proteins (OMPs) (CarO, 33–36-kDa OMP, and 43-kDa OMP) is another mechanism associated with resistance to carbapenem in *A. baumannii* isolates worldwide [28]. PCR of bla_{OXA} revealed that all *A. baumannii* isolates carried $bla_{\text{OXA-23}}$-like and $bla_{\text{OXA-51}}$-like genes, 77% were positive for $bla_{\text{OXA-24}}$-like, whereas, none of the isolates harbored $bla_{\text{OXA-58}}$-like. Mirshekar et al. [29] revealed that $bla_{\text{OXA-24}}$-like, and $bla_{\text{OXA-23}}$-like genes existed in 84.72% (61/72), and 30.55% (22/72) of the strains respectively, whereas $bla_{\text{OXA-58}}$-like was not found in any of the *A. baumannii* strains. Xiao et al. found that all isolates possessed $bla_{\text{OXA-51}}$-like gene, 95% had $bla_{\text{OXA-23}}$-like gene and no isolates bore $bla_{\text{OXA-58}}$-like as well as $bla_{\text{OXA-24}}$-like genes. Azizi et al. findings indicated that all of the isolates carried $bla_{\text{OXA-23}}$-like, while $bla_{\text{OXA-24}}$-like was only observed in the isolates showing high MIC value to imipenem and meropenem. The $bla_{\text{OXA-58}}$-like gene was not found in *A. baumannii* population. The present study also describes carbapenem-resistant *A. baumanii* isolates with changes in porin. The insertional disruption of *carO* gene, which is mediated by ISAba825 and ISAba125 elements, has been introduced formerly as the only carbapenem resistance mechanism in Acinetobacter isolates [5, 30]. In a study had been represented that resistance to carbapenems can be developed through either loss of outer membrane *carO* because of insertions, mutations and/or deletions or production of metallo-β-lactamase [23, 31]. In current experiment, *carO* gene was present in 100% (100/100) of the strains. We identified the *carO* nucleotide and deduced sequences of amino acids in the tested strains and detected point mutation in 6 isolates in the same position but disruption of *carO* gene with the insertion sequences was not detected among studied isolates. Pajand and et al. reported that 4 isolates (5.3%) were negative for *carO*. Po-Liang Lu et al. [23] showed that 92 (100%) isolates carried *carO* gene in PCR assay, of which five gave a 1900 bp amplicon instead of the predicted size of 750 bp and none consisted of detectable point mutation. The *car*O gene sequence was identified in 2 isolates with nucleotide insertions, deletions and point mutations as opposed to normal strains and discrimination in their nucleotide and amino acid sequences was also reported by Shu-Zhen Xiao et al. [32]. In present study, we observed reduction in *carO* gene expression in 48 isolates compared to carbapenem-susceptible *A. baumannii* ATCC 19606 strain. A previous study in Iran demonstrated the reduction in expression of *carO* among the carbapenem-non-susceptible isolates in comparison to susceptible isolate. Fonseca et al. [12] reported basal and reduced levels of *carO* transcription between the OXA-23 isolates provided by *A. baumannii*, emphasizing the slight portion of CarO alteration for resistance to carbapenems. Cardoso et al. [33] found overexpressed *carO* gene in some OXA-23–producing clones of *A. baumannii*. They concluded that excessive expression of *carO* could contribute to the successful deployment of the main clones which were producing OXA-23 in Brazil. Generally, combination of resistance mechanisms may play an additional role to confer high-level carbapenem resistance in *A. baumannii* [34–37]. Due to producing of carbapenemase in all *Acinetobacter* isolates tested in this experiment, we concluded that *carO* had a negligible impact on the carbapenem-resistance. In this study, most OXA-23-producing isolates showed imipenem MIC of higher than 128 μg/ml. Six OXA-23-producers with mutation in *carO* had imipenem MICs of 128–256 μg/ml. [23, 38]. Po-Liang Lu reported isolates without CarO protein were reported to have imipenem MICs of 16 mg/L (9). Nevertheless, most of the isolates which produce OXA-72 had a MIC more than 32 mg/L. CarO porin loss in these carbapenemase-positive isolates does not result in a higher MIC value. In accordance with the current study, Yongsoon lee et al. found that the carO-positive isolates exhibited similar MIC values against to both imipenem and meropenem compared to the isolates with no 29-kDa CarO-like protein. Their results suggest that lack of the CarO OMP only had a less impact on carbapenem resistance in *A. baumannii* [6]. Similarly, a high MIC for carbapenems among three *A. baumannii* isolates without the 29 kDa protein has been described by Mostachio et al. [39]. In this study, all isolates had a corresponding band presented apparent molecular masses of 29 kDa on the gel for CarO porin. Yangsoon Lee et al. reported that OMP such as CarO (29 kDa) porin did not show absent or greatly diminished expression in SDS-PAGE. Moreover, in Chang-Ki Kim study, 13 isolate with identical SmaI macrorestriction patterns through SDS-PAGE were represented, of which 12 lacked (missed) the 29-kDa CarO-like OMP unlike the remaining one (isolate SC0701). In the 12 isolates missing the 29-kDa CarO-like protein, PCR assays for the *carO* gene resulted in a PCR product of approximately 1.7 kb in length, which was larger than expected (741 bp), indicating additional DNA being inserted. Carbapenem resistance mediated by CarO deficiency and bla_{OXA} genes in *A. baumannii* clinical isolates in Iranian hospitals may be more common. In this study, we reported the emergence of bla_{OXA} genes and changes in *carO* gene in carbapenem-resistant *A. baumannii* strains isolated from burn patients in Iran.

Conflict of interest: The authors declare that they have no conflict of interests.

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