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The Involvement of Drug Efflux System in Amikacin Resistance of Multiple Drug Resistant *Acinetobacter baumannii* Isolates in Isfahan, Iran

Yalda Ostadi, Ali Akbar Rezai, Mahdi Moghadampour, Jamshid Faghri *

Department of Microbiology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran.

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ABSTRACT

Background: Due to the extensive usage of antibiotics in recent decades, the emergence of multiple drug-resistant (MDR) strains has dramatically increased. In the present study, we studied the distribution and involvement of drug efflux system in conferring resistance to amikacin in MDR isolates of *Acinetobacter baumannii* isolated from hospitalized patients.

Methods: In this cross-sectional study 80 MDR *A. baumannii* were isolated from hospitalized patients in Alzahra hospital, Isfahan, Iran. *A. baumannii* isolates were identified by standard microbiologic procedure and were confirmed by specific PCR primers. Minimum inhibitory concentration (MIC) was determined by agar dilution method according to CLSI guidelines. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used as an efflux pump inhibitor for amikacin susceptibility. The presence of efflux genes was detected by PCR method.

Results: Antibiotic susceptibility results showed that 39 of isolates had MIC ≥ 32 $\mu\text{g/mL}$ and were amikacin-resistant. Totally, 41 isolates which had an amikacin MIC ≥ 2 $\mu\text{g/mL}$ were tested for reduction of MIC in presence of 25 $\mu\text{g/mL}$ efflux pumps inhibitor. After the treatment, 25 (61%) isolates had ≥ 2 fold and 15 (36.6%) isolates had 4 fold reduction in amikacin MIC. The results of PCR-amplifications indicated that the presence rate of AdeA, AdeB, AdeC, AbeM and AdeS genes were 100%, 96.3%, 95%, 98.8%, and 95%, respectively.

Conclusion: In summary, significant involvement of drug efflux system in conferring resistance to amikacin along with high distribution of efflux genes suggests an alternative therapy using antibiotics in combination with efflux inhibitors in the fight against MDR isolates of *A. baumannii*.

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Introduction

Acinetobacter baumannii is a ubiquitous, non-fermenting, Gram-negative, rod-shaped bacterial species which is considered as an opportunistic pathogen (1). This bacterium is one of the most commonly isolated pathogens in nosocomial infections and hospital outbreaks such as sepsis, pneumonia, urinary tract infection, meningitis and wound infection (2). In recent years, due to the extensive use of antibiotics, the emergence of multiple drug resistant (MDR) strains has dramatically increased that led to limited efficiency of many commonly used antimicrobial agents (3). Resistance to the different classes of antibiotics in *A. baumannii* is often due to several mechanisms such as β -lactamases production, aminoglycoside-modifying enzymes, permeability defects, alteration of target sites and changes in the expression of efflux pumps (4).

Recently, the role of bacterial efflux pumps in multidrug resistance has been demonstrated which may be related to the overexpression of efflux pumps. Expression of these efflux pumps are generally linked to mutations in their regulatory genes (5). To date, several categories of efflux pumps in clinical isolates of *A. baumannii* have been identified, including the resistance-nodulation-division (RND) family, major facilitator superfamily (MFS), multidrug and toxic compound extrusion (MATE) family, and small multidrug resistance (SMR) family of transporters (6). Among these groups, RND family has the most important role in multidrug resistance of isolates. The AdeABC efflux pump system is the main RND-type system in *Acinetobacter* strains (6). The AdeABC efflux pump (RND type superfamily) consists of *adeA* (membrane fusion), *adeB* (multidrug transporter) and *adeC* (outer membrane) genes. Furthermore, there are two regulatory genes, *adeS* (sensor kinase) and *adeR* (regulator), which are associated with controlling the expression of the AdeABC pump (6, 7). There are a few data available on the genotypic characterization of

RND efflux pumps and the role of these elements in MDR *A. baumannii* isolates in our region.

Therefore, in the present study, we investigated the distribution and involvement of drug efflux system in conferring resistance to amikacin in MDR isolates of *A. baumannii* isolated from hospitalized patients in Isfahan, Iran.

Materials and Methods

Study design and sample collection

This cross-sectional study was carried out between March 2017 and June 2017 among 80 MDR *A. baumannii* isolates which were isolated from clinical specimens of hospitalized patients in ICU department of Alzahra hospital, Isfahan, Iran. This study was in accordance with the Declaration of Helsinki and approved by the ethics committee of the Isfahan University of Medical Sciences. All clinical isolates of *A. baumannii* were identified based on colony morphology, Gram staining, oxidative or fermentative metabolism, catalase and oxidase reaction, and finally confirmed by the amplification of bla_{OXA-51}-like gene as previously described (8). The confirmed isolates were stored at -80 °C for further analysis.

Inhibitory effects of CCCP on efflux pumps

Minimum inhibitory concentrations (MICs) of the MDR *A. baumannii* isolates to amikacin (Sigma-Aldrich, Germany) were determined by agar dilution method at 5 different concentration (0.5, 2, 8, 32 and 128 $\mu\text{g}/\text{mL}$) as described by the Clinical and Laboratory Standards Institute (CLSI) guidelines (9). According to CLSI breakpoints, isolates with a MIC $\geq 32 \mu\text{g}/\text{mL}$ considered as intermediate-resistant or resistant isolates. To assay the effect of efflux mechanisms toward amikacin resistance, the MIC reduction assay was performed using carbonyl cyanide 3-chlorophenyl hydrazine (CCCP) (HiMedia, India) as an efflux pump inhibitor. In view of the

bactericidal effect of CCCP, a plate containing 25 µg/mL CCCP without antibiotic was used as control. Finally, the MIC of the *A. baumannii* isolates was evaluated by the agar dilution method on Mueller-Hinton agar containing 25 µg/mL of CCCP with different concentrations of amikacin. A positive criterion for the contribution of efflux pumps to resistance was a two to four fold reduction in MIC of the antibiotic in the presence of the efflux inhibitor (10).

DNA extraction and molecular assay

The small-scale phenol-chloroform method was used to extract genomic DNA as described previously (11). The extracted DNA was dissolved in 100 µl sterile distilled water and stored in -20 °C. The standard PCR assay was performed using specific primers for the presence of *adeA*, *adeB*, *adeC*, *abeM* genes and a regulatory gene) *adeS* (in clinical isolates of *A. baumannii* previously described (Table 1) (12). The reactions were performed with initial denaturation at 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 55 °C for 45 s and 72 °C for 1 min, and final elongation at 72 °C for 5 min. PCR products were separated on 1.5% agarose gel prepared in 1X TAE (Tris/ Acetate/EDTA) buffer and visualized using an ultraviolet light after staining with safe stain load dye (CinnaGen Co., Tehran, Iran).

Results

During the study period, a total of 80 non-duplicate *A. baumannii* isolates were collected from different clinical samples of Alzahra hospital. Overall, 60 (75%) *A. baumannii* isolates were obtained from male and 20 (25%) from female with a mean age of (\pm SD) 55 \pm 20, ranging from 9 to 88 years old. Sixty three (78.8%) isolates of *A. baumannii* were from the intensive care units (ICUs), 11 (13.8%) from the internal medicine wards and 6 (7.5%) from the surgery ward. The most frequent *A. baumannii* infection was associated with respiratory infections (RTIs)

(57/80), followed by bloodstream infections (BSIs) (7/80), urinary tract infections (UTIs) (6/80), skin and soft tissue infections (SSTIs), (5/80), meningitis (4/80), and eye infection (1/80). Amikacin susceptibility of *A. baumannii* isolates showed the MIC₅₀, MIC₉₀ and MIC range (MIC at which 50% and 90% of isolates were inhibited) were 8 µg/mL, 128 µg/mL, and 0.5-128 µg/mL, respectively. Collectively, 48.8% (39/80), 48.7% (39/80), 2.5% (2/80), 7.5% (6/80), and 41.3% (33/80) of isolates had a MIC equal to 0.5 µg/mL, 2 µg/ml, 8 µg/mL, 32 µg/mL and 128 µg/mL, respectively. Moreover, 39 (48.8%) of isolates had a MIC \geq 32 µg/mL that accounted as amikacin-resistant isolates.

All isolated bacteria grew on the plates containing 25 µg/mL CCCP without amikacin that indicates non-antibiotic effect of CCCP. Totally, 41 isolates which had an amikacin MIC \geq 2 µg/mL were tested for MIC reduction in presence of 25 µg/mL CCCP. All results of the CCCP effect on amikacin susceptibility are presented in Table 2. After bacterial treatment with 25 µg/mL CCCP; 56% (23/41), 4.9% (2/41), and 39% (16/41) of isolates had a MIC equal to 0.5 µg/mL, 8 µg/mL and 32 µg/mL, respectively. Moreover, 25 (61%) isolates had \geq 2 fold and 15 (36.6%) isolates had 4 fold reduction in amikacin MIC.

The molecular assay of efflux pumps encoding genes revealed that of 80 MDR *A. baumannii* isolates, the presence rate of *AdeA*, *AdeB*, *AdeC*, *AbeM* and *AdeS* genes were 100%, 96.3%, 95%, 98.8%, and 95%, respectively (Figure 1).

Discussion

Treatment failure of MDR *A. baumannii* infections causes serious morbidity and mortality in the hospitalized patient (3). Drug resistance in many cases is attributable to over expression of efflux pumps which are frequently found in Gram-negative bacteria (16). This study provided an insight into the contribution of efflux pumps to increased MIC among MDR isolates of *A.*

baumannii in Isfahan hospitals, the central part of Iran.

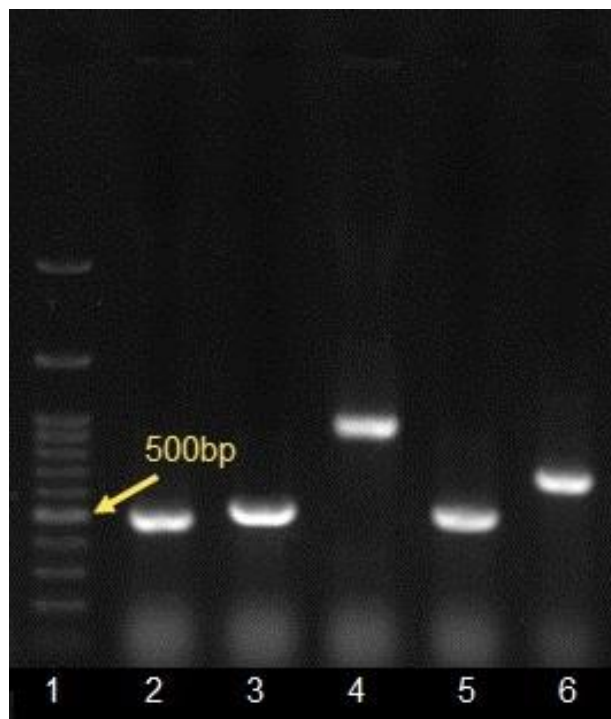


Figure 1. PCR of *adeA*, *adeB*, *adeC*, *adeS* and *AbeM* genes in *Acinetobacter baumannii* isolated. Lane 1, 100bp DNA ladder; lane 2, *adeA* gene (513bp); lane 3, *adeC* gene (560bp); lane 4, *adeB* gene (981bp); lane 5, *adeS* (544bp); lane 6, *AbeM* (703bp).

In the present study, the majority of *A. baumannii* isolates (71.3%) were recovered from RTIs. This result highlighted the importance of *A. baumannii* in causing nosocomial RTIs among hospitalized patients, particularly in ICUs. In accordance to our findings, several studies have reported non-fermenting Gram-negative bacilli (NFGNB), including *Pseudomonas aeruginosa* and *A. baumannii* as frequent pathogens isolated from respiratory tract specimens (1, 17-21). Moreover, as a general concept from previous studies, the antibiotics resistance ability of *A. baumannii* resulting in a selective advantage in settings such as ICUs (22), which is consistent

with our findings regarding high isolation rate from ICUs.

In the present study, 48.8% of *A. baumannii* isolates were amikacin-resistant. Although aminoglycosides not often used as single agents for the treatment of *A. baumannii* infections, amikacin and tobramycin showed promising activity against *A. baumannii* isolates (3). Unfortunately, resistance to this class of antibiotic is now increasing in many parts of the world (23, 24). In this regard, a systemic review by Moradi et al. showed that trends of amikacin-resistant *A. baumannii* isolates have increased from 58.4% to 95% in Iran within 12-years period (2001 to 2013) (25).

In majority of the isolates (61%), amikacin susceptibility was increased two fold or more in presence of efflux inhibitors. Moreover, 53.9% of amikacin-resistant isolates have completely become susceptible. These susceptibility changes and reduction in MIC suggest the involvement of the efflux pumps in conferring resistance to amikacin in our isolates. Previous studies indicated that several mechanisms could be involved in resistance to aminoglycosides in *A. baumannii* (26, 27); however, we showed the contribution of efflux systems in amikacin resistance in MDR *A. baumannii* isolated from our region. Previously, in agreement with our finding, several reports showed the important role of drug efflux system among Gram-negative bacteria in conferring resistance to fluoroquinolones, carbapenem and biocides (10, 28-32).

The presence of AdeABC genes could be used as an indicator for emerging diagnosis of MDR strains. The distribution of these genes was extremely high in our tested MRD isolates. Same as our findings, different studies have shown the high prevalence of AdeABC genes in clinical isolates of *A. baumannii* in Iran and other country (32-36).

Table 1. The sequences of primers used for polymerase chain reaction to detect *Acinetobacter baumannii* efflux pump genes.

Genes	Sequence	Reference
AdeA	F: 5'-ATC TTC CTG CAC GTG TAC AT -3' R: 5'-GGC GTT CAT ACT CAC TAA CC-3'	(13)
AdeB	F: 5'-GTATGAATTGATGCTGC-3' R: 5'-CACTCGTAGCCAATACC-3'	(13)
AdeC	F :5'-AGCCTGCAATTACATCTCAT-3' R: 5'-TGGCACTTCACTATCAATAC-3	(13)
AbeM	F :5' GTAGGTGTAGGCTTATGGA-3' F :5'- GTACCGAAGTGACTGAAAT-3'	(14)
AdeS	F:5'- TGTGGGTTATGCAGTTGCTTTT -3' R:5'- GGCATAGGCAATCCCGATT-3'	(15)

Table 2. The results of the CCCP effect on Amikacin susceptibility in clinical isolates of *A. baumannii*.

MIC of amikacin (Total No).	Fold reduction in amikacin MIC + CCCP*	Amikacin + CCCP MIC50/90 µg/mL	Change from resistant to susceptible phenotype No. (%)
8 µg/mL (2)	2	1/1	-
32 µg/mL (6)	3	1/1	6 (100)
128 µg/mL (33)	1-4	8/32	15 (45.5)

Conclusion

It was shown that the significant involvement of drug efflux system in conferring resistance to amikacin in MDR *A. baumannii* isolates. These

findings along with the high distribution of the AdeABC encoding genes suggest an alternative therapy using antibiotics in combination with efflux inhibitors in the fight against MDR isolates. However, future efforts are necessitate to

investigating the role of drug efflux in conferring resistance to other antibiotics and pathogens.

Conflict of interest

The authors declare that there are no conflicts of interest.

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