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Multi-drug Resistance Profiles and Expression of *AdeIJK* and *AbeM* in *Acinetobacter baumannii* Collected from Humans by Real-time PCR

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ABSTRACT

Background: Acquiring genetic determinants with antibiotic resistance and mutation in regulatory genes of *Acinetobacter baumannii* can made many problems in treatment of patients. The AdeIJK pump are associated with decrease susceptibility to trimethoprim, fluoroquinolones, β-lactams, novobiocin, tetracycline, lincosamides, erythromycin, chloramphenicol and AbeM pump can decrease the MIC of chloramphenicol, trimethoprim, aminoglycosides and fluoroquinolones. Upregulation of drug transporters systems, modifications in gyrA and parC genes have major role to fluoroquinolones resistance in *A. baumannii*. The aim of this study was investigation the contribution of *adeJ* and *abeM* pumps in extrusion of ciprofloxacin in *A. baumannii*.

Methods: For confirmation of species the *bla*OXA-51 gene was applied. Disk diffusion method was performed for antimicrobial susceptibility test. For illustration of active efflux pumps the CCCP and ciprofloxacin were used to determine MIC. To detect the RNA transcript of AdeJ and AbeM pumps in isolates collected from two hospitals from July 2016 to March 2017 qRT-PCR was carried out.

Results: The MICs of ciprofloxacin decreased 32-fold or more in 7 strains, 16-fold in 2 strains, 8-fold in 10 strains, 4-fold in 25 strains and 2-fold in 6 strains after adding CCCP. Overexpression of the *adeJ* (84%) and *abeM* (88.63%) genes were indicated by qRT-PCR.

Conclusion: Efflux pups inhibitor are new approach for increase susceptibility to many classes of antibiotics. In *A. baumannii* strains transporting system may have contribution in ciprofloxacin resistance as shown with this study.

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Introduction

Acinetobacter baumannii is a non-fastidious, aerobic, coccobacillus, ubiquitous organisms which has been implicated as an opportunistic pathogen causing hospital acquired infections (1, 2). After Pseudomonas, it is the most prevalent isolated non-fermenter encountered in human specimens (2).

Many reporting from high morbidity and mortality of *A. baumannii* has been received because of ability of this pathogen to acquire resistance determinants from other pathogens, living for long times on host and materials and resilience on wide range of environmental conditions (3, 4). The emergence and dissemination of isolates resistant to many classes of antibiotics are a great threat to human lives and health (2, 5). Enzymatic hydrolysis like β–lactamases, overexpression of active drug transporters, aminoglycoside-modifying enzymes, class D oxacillinases, chromosomally encoded cephalosporinase, defects in membrane, integrons and insertion sequence(IS) elements, changing of target sites, result in resistance to multiple classes of antibiotics (3, 6-10).

MDR in bacteria maybe associated with these families of multidrug efflux systems: (1) the multidrug and toxic compound extrusion (MATE) family, the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily, the small multidrug resistance (SMR) family, the major facilitator superfamily (MFS) and the resistance-nodulation-cell division (RND) family (3, 4, 11, 12). Three category of efflux systems exit which efflux drugs with ATP, ion gradients and phosphorylation of the drugs (13).

The proton motive force is the energy source of RND and MATE families (12). This transporter system may be inactivated with blocking the energy source of efflux, blocking interaction of antibiotics with the transporter protein, inhibiting the regulatory genes of efflux pumps and by blocking the interactions of various parts of an efflux (4).

Fluoroquinolones, aminoglycosides, chloramphenicol and trimethoprim are the antibiotics that affected by this pumps (11, 12). To demonstrate the primary or secondary multidrug efflux pumps, carbonyl cyanide-m-chlorophenylhydrazone (CCCP) and other proton conductors have been repeatedly applied (13).

CCCP is a proton-conducting uncoupler that disturbs respiration-generated proton gradient to inhibit efflux pumps that used proton to catalyze their reactions (12, 13). For investigation of efflux systems MICs, CCCP has become a fundamental tool (12).

For detection the level expression of *adeJ* and *abeM* pumps in isolates of *Acinetobacter* species obtained from Shahid Motahari and Milad hospitals from July 2016 to March 2017 real time PCR was used.

Material and methods

Collection of samples and identification

The MacConkey agar and Blood agar were chosen for culturing of the strains. Isolates confirmed by biochemical tests such as SIM, TSI, Oxidation Fermentation, oxidase, catalase, citrate, and growth at 44°C. The isolates were confirmed by amplification of *bla*OXA-51 gene by PCR.

Disc diffusion agar

Antimicrobial susceptibility testing was performed on Mueller Hinton agar (Merck, Germany) against: piperacillin/tazobactam (PTZ: 100/10 μg), ceftazidime (CAZ: 30 μg), amikacin (AK: 30 μg), meropenem (MEM: 10 μg), trimethoprim-sulfamethoxazole (TS, 1.25/23.75 μg), minocycline (MIN, 30 μg), imipenem (IPM: 10 μg), cefotaxime (CTX: 30 μg), ciprofloxacin (CIP: 5 μg), cefepime (FEP: 30 μg) and gentamicin (GEN: 10 μg) by the Kirby-Bauer disk diffusion method based on CLSI Guidelines 2016.

Detection of active drug efflux

Briefly, 100 µl Mueller-Hinton broths were dispensed in every well of sterile 96-well microtitration plate wells. Then 100 µl of ciprofloxacin were pipetted in first wells of every column of plates and then with two-fold serial dilutions to other wells at the final concentration 256 µg/ml then 10 µL of diluted (1:20 ratio) bacterial suspension adjusted to 0.5 McFarland scale was added to each well. CCCP was added in wells at the final concentration 25 µg/ml.

Polymerase chain reaction (PCR)

Genomic DNA Purification Kit (Thermo, USA, Cat. No. K0512) was applied for extraction of DNA. Taq DNA Polymerase Master Mix Red-$Mgcl_2$, forward/reverse primers, DNA template mix with other for performing the PCR reaction. The primers sequence used for detection of active drug efflux pumps genes are shown in Table 1.

RT-PCR

RNX –PLUS solution (Cinaclon, Iran, Cat No. RN7713C) was applied for extraction of RNA. DNase1-RNase free was used for removing of DNA. By measuring their absorbance at 260 nm and 280 nm the concentrations of RNA in samples were determined. For cDNA synthesis, Accu Power Rocket Script RT Premix (Bioneer, China, Cat No. K-2101), RNA and random hexamers mix with other. RT-PCR was performed in a Corbett with SYBR Green qPCR Master Mix (Yekta Tajhiz Azma, Iran, Cat No: YT2551) to detect transcripts of genes. Amplification was done in a 20 µl final volume containing SYBR Green qPCR Master Mix, primers and cDNA. The reaction conditions were initial denaturation 95°C for 5 min; 40 cycles of denaturation 95°C for 20 sec, annealing 57°C for *adeJ* and 56°C for *abeM* for 30 sec, and 72°C for 30 sec. Table 1 shown the primers sequence used for detection of expression of active drug efflux pumps.

Results

The rate of antimicrobial resistance results of 51 isolates of *A. baumannii* are shown in table 2. The results of MIC for ciprofloxacin showed that 32 µg/mL in 5 isolates, 64 µg/mL in 23 isolates, 128 µg/mL in 13 isolates and 256 µg/mL in 9 isolates. After adding CCCP the MICs of ciprofloxacin decreased to 32-fold or more in 7 strains, 16-fold in 2 strains, 8-fold in 10 strains, 4-fold in 25 strains and 2-fold in 6 strains. The results showed that in the 88% of the isolates MICs decreased at least 4 fold.

All of the isolates amplified these genes by PCR. Overexpression means that the isolates have 4 fold increases in expression level of genes compared with reference strain (ATCC 19606). The results of qRT-PCR showed that 37 and 39 isolates had overexpression of the *adeJ* and *abeM* genes. Amplification plot and melting curve of *abeM* gene in strains are shown in Figure 1 and 2.

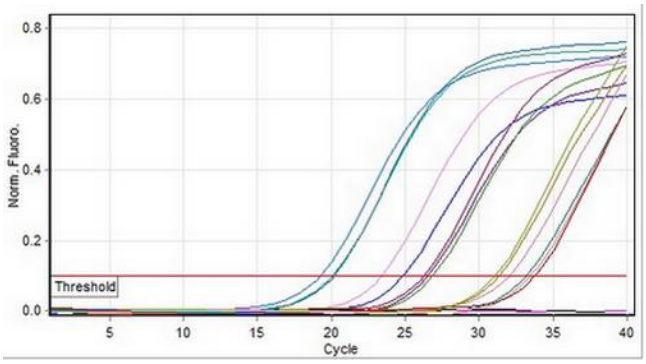

Figure 1. Amplification plot of *abeM* gene in clinical isolates of *A. baumannii*.

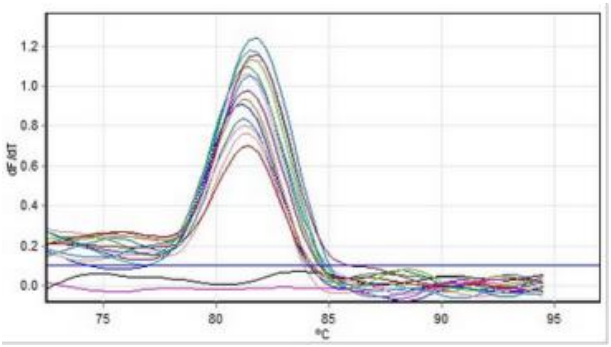

Figure 2. Melting curve of *abeM* gene in clinical isolates of *A. baumannii*.

Discussion

Nowadays there are few antibiotic for treatment of *A. baumannii* due to low susceptibility to many classes of antibiotics (15, 17-18). Serious infections that are caused by *A. baumannii* include pneumonia, keratitis, necrotizing fasciitis, meningitis, sepsis, UTI, bacteremia and endocarditis in immunocompromised patients (3, 7, 18). Gram-negative and Gram-positive bacteria have drug efflux pumps, structure of cell envelope in Gram-negative bacteria made the resistance of this type more important subject. Efflux pumps and permeability defect associated with each other for antibiotic resistance in many cases (4).

Disk diffusion results showed maximum resistance to cefotaxime, piperacillin-tazobactam, cefepime, ciprofloxacin, ceftazidime, imipenem, meropenem and amikacin which were similar with other studies (20-22).

To find the contribution of active drug efflux in resistance pathway to ciprofloxacin the inhibitory effect of CCCP was applied in this study. The MIC value of ciprofloxacin was decreased in plate containing CCCP. The role of efflux pumps in ciprofloxacin resistance was further illustrated with observation that MICs decreased 4-fold after adding CCCP (23). In some isolates the susceptibility was still low after adding CCCP. This indicates that various mechanisms are associated for antibiotic resistance in MDRAB. Other resistance pathways alternatives the efflux pumps; therefore, the resistance rate was not reduced. Tetracycline MICs with using CCCP were measured in burn infections and ventilator-associated pneumonia and the results indicated 2-4 fold reduction in 32.14% isolates, 8 fold reductions in 46.42% isolates, 16 fold reductions in 1.78% isolates and 32 fold reductions in the 1.78% isolate (24). The differences in the reported values between the present study and those reported may be due to differences in antibiotic selections.

In one study, PCR screening revealed high distributions of *adeJ* (100%) and *abeM* (100%) genes but authors could not find correlation between antibiotics and active drug efflux pumps (25). Yoon et al. collected 14 MDR isolates of *A. baumannii* and investigated the presence of the *abeJ* transporters were in 100% of MDR isolates of *A. baumannii* (26). Among 50 resistant isolates collected in this study, overexpression of *adeJ* (84%) and *abeM* (88.63%) were detected. Positive *adeJ* and *abeM* were detected in 45 (90%) and 40 (80%) of the 50 of imipenem resistance isolates was detected (7). In study with Pagdepanichkit et al. on MDRAB the expression rate of *adeJ* were 97% (23). Fernando et al. collected 16 MDR isolates and concluded regarding other resistance pathways present in these isolates, the expression of efflux pumps genes were not associated exactly with antibiotic resistance (27). Overexpression of *adeJ* was observed higher in tigecycline-nonsusceptible *A. baumannii* isolates (18).

Sun et al. studied on 81 TGC-resistant XDRAB and found the overexpression of *adeI* in 21% of the isolates (28). Lin et al. selected MDR A. baumannii isolates and the presence *adeJ* and *abeM* was determined. They showed that in 22.2% and 66.6% of strains had overexpression of *adeJ* and *abeM* gene (9). This dissimilarity may be due to different drug treatment in various countries, different antibiotic that has been used for screening and due to the numbers of the strains.

Table 1. Sequences of primer sets for amplifying genes

Primer	Sequence	Target gene and purpose	Reference
*abeM*_ F *abeM*_ R	AAGTCTTTATTGCCGCACAC ATCGGTGCCTGAGTATCTTG	*abeM*, PCR	This study
*abeJ*_ F *abeJ*_ R	ATTGCACCACCAACCGTAAC TAGCTGGATCAAGCCAGATA	*adeJ*, PCR	14
*abeM*_RT_F *abeM*_RT_R	TGCCAATTGGTTTAGCTGTG TACTTGGTGTGCGGCAATAA	*abeM*, gene expression	15
*abeJ*_RT_F *abeJ*_RT_R	GCGAATGGACGTATGGTTCT CATTGCTTTCATGGCATCAC	*adeJ*, gene expression	16

Table 2. Antimicrobial susceptibility test results of *A. baumannii*

Antibiotic	IPM	MEM	CAZ	CTX	AK	PTZ	MIN	CIP	FEP	TS	GEN
Resistant %	96	96	96	100	96	98	86	98	98	92	90

Conclusion

Active drug export and genetic determinants like transposon, integrons and plasmids may lead to low susceptibility of this opportunistic pathogen to many drugs. Hence, monitoring the MDR isolates can be done with using standard tests for determining the resistance rate and MIC breakpoint of them. A new strategy for control the antibiotic resistance in bacteria is efflux inhibitors. Among ciprofloxacin -resistant MDRAB isolates, CCCP is a perfect tool for reversing ciprofloxacin resistance.

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Conflict of interest

There is no conflict of interest.

Financial disclosure

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