



# Distribution and Expression of Efflux Pump Gene and Antibiotic Resistance in *Acinetobacter baumannii*

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## Abstract

**Background:** Multidrug efflux pump is a ubiquitous mechanism of drug resistance in bacterial pathogens, such as *Acinetobacter baumannii*, which is mediated by integral membrane transport proteins. The purpose of the current study was to analyse the distribution of *adeG* and its role in resistance to ciprofloxacin in *A. baumannii* isolates from two hospitals of Tehran.

**Methods:** Fifty-one isolates of *A. baumannii* were recovered from Shahid Motahari and Milad hospitals of Tehran, from July 2016 to March 2017. Identification of *A. baumannii* was confirmed by phenotypically and molecular methods. Antibiotic susceptibility testing was prepared by the Kirby-Bauer method. Resistance to ciprofloxacin was confirmed by determination of minimum inhibitory concentration (MIC). Efflux pumps inhibitor was used for phenotypic detection of active efflux pumps. Real-time polymerase chain reaction (PCR) was used to analyze *adeG* gene over expression.

**Results:** Fifty-one isolates of *A. baumannii* were confirmed by phenotypic and molecular methods. Results of antibiotic susceptibility testing confirmed resistance to ciprofloxacin in all isolates. Results of conventional PCR showed that all of the isolates had the *adeG* gene. The MIC of ciprofloxacin was decreased by 4 to 32 folds or more in 88% of resistant isolates after adding the efflux pump inhibitor. Over expression of AdeG efflux pump gene was confirmed by real-time PCR in 34 strains.

**Conclusions:** In conclusion, multidrug-resistant *A. baumannii* were prevalent in the studied isolates. Carbonyl cyanide-3-chlorophenylhydrazone could reverse the susceptibility to ciprofloxacin in *A. baumannii* and was associated with overexpression of the *adeG* gene.

**Keywords:** Multidrug Efflux Pump Genes, Real-Time Polymerase Chain Reaction, *Acinetobacter baumannii*, *adeG* Gene

## 1. Background

*Acinetobacter baumannii* is an oxidase-negative and catalase-positive non-motile gram-negative pathogen. It is responsible for infections in skin and soft tissue, bloodstream, meningitis, urinary tract infection, pneumonia, ventilator-associated pneumonia and endocarditis, and patients in intensive care units and immunocompromised patients are at high risk for acquiring this pathogen (1, 2). Large genomic plasticity and mutation of endogenous structural or regulatory genes, overexpression of  $\beta$ -lactamase (AmpC) and OXA-51-like  $\beta$ -lactamase, mutation in the *gyrA* and *parC* genes, chromosomally encoded efflux systems, alterations of the drug target, decreased expression of the outer membrane proteins, enzymatic modification of antibiotics, target gene mutation, and horizontal acquisition of genetic elements may be responsible for increased antimicrobial resistance in *Acinetobacter* species

(3-7). Chromosome, transposons, and plasmids encode for efflux pump transporters and up- or down-regulated in regulatory genes can change the expression of these pumps (8). *Acinetobacter baumannii* has very low susceptibility to several unrelated drug classes, including penicillins, cephalosporins, carbapenems, fluoroquinolones, aminoglycosides, trimethoprim, tetracyclines, and chloramphenicol (1, 9-13). Efflux pumps of the resistance-nodulation-division superfamily (RND) like MexB, AcrB and AdeABC, AdeDE, AdeIJK, and AdeFGH, are associated with high resistance to antimicrobial agents in *Escherichia coli*, *Pseudomonas aeruginosa*, and *A. baumannii* (14). Mutations in the targets of these drugs, topoisomerase IV and DNA gyrase, primarily are responsible for resistance to fluoroquinolones. Active efflux AdeABC, AdeIJK, and AdeFGH are a secondary route to fluoroquinolone resistance (15, 16).

Classification of efflux pumps has resulted in five distinct groups: Multidrug and toxic compound extrusion

(MATE) family; the major facilitator superfamily (MFS); resistance-nodulation-division (RND) superfamily; adenosine triphosphate (ATP)-binding cassette (ABC) superfamily; and small multidrug resistance (SMR) family (14, 17-20). A transporter (efflux) protein, a periplasmic accessory protein, and an outer membrane channel protein are the three components of RND (12, 17).

Almost 90% of clinical isolates have AdeFGH, and show multidrug resistance when overexpressed (21). This contributes to high-level resistance to sulfonamides, trimethoprim, tigecycline, chloramphenicol, tetracyclines, sulfamethoxazole, fluoroquinolones, and clindamycin (14, 21, 22). The present study was performed to investigate ciprofloxacin resistance with AdeG in *A. baumannii* strains collected from two hospitals.

## 2. Methods

### 2.1. Bacterial Strains and Growth Conditions

Isolates were obtained from Shahid Motahari and Milad Hospitals of Tehran, Iran, from July 2016 to March 2017. MacConkey agar was selected for culture of the samples and their recognition was examined by both biochemical methods, such as oxidase, triple sugar iron (TSI), SIM and oxidation-fermentation (OF), growth on cetrimide agar, growth at 42°C, decarboxylation of lysine and arginine dihydrolase test. Molecular assay by specific primers for presence of *blaOXA-51* gene was investigated. Primer and PCR condition were described previously (23).

### 2.2. Antimicrobial Susceptibility Test

Antibiotic susceptibility testing was prepared by disc diffusion agar on Mueller-Hinton for routine antibiotics and the interpretation of the results was done according to the pattern of the Clinical and Laboratory Standards Institute protocol (CLSI) guide line (24) against: amikacin (30 µg), cefepime (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), imipenem (10 µg), meropenem (10 µg), minocycline (30 µg), trimethoprim-sulfamethoxazole (25 µg), piperacillin-tazobactam (110 µg), and colistin.

### 2.3. Efflux Pumps Gene Detection

Genomic DNA Purification Kit (Thermo Company- USA, Cat. No. K0512) was applied for extraction of bacterial DNA. The DNA template, Taq DNA Polymerase Master Mix Red-MgCl<sub>2</sub> (Ampliqon- Denmark, Cat. No. A180301) and forward/reverse primers were mixed for the PCR reaction. Conditions of PCR reaction for detection of *adeG* gene are shown in Table 2. Electrophoresis system at 100 V for 60

Table 1. Primer for *adeG* Gene Excretions

Primer	Sequence (5' - 3')	Reference
<i>adeG- F</i>	CTCCGTCCATTCCATAAACC	This study
<i>adeG- R</i>	AGATGGCAGACAGTGCAAAC	

minutes composed of 1% agarose gel in 1X TBE and was applied and stained with red safe. Amplified DNA product was visualized under UV light through the Gel Doc instrument.

### 2.4. Inhibitory Role of Carbonyl Cyanide 3-Chlorophenyl Hydrazone

To investigate the role of efflux pump in ciprofloxacin resistance, carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (Sigma, C2759) as an efflux pump inhibitor at final concentration of 25 µg/mL was added to micro titer plates, including Mueller-Hinton (M-H) broth, and compared with plates without CCCP. Control in this test was the plate with CCCP but without ciprofloxacin. The contributor of efflux pump in antibiotic resistance was confirmed if the plate with CCCP showed a four-fold decrease in the MIC compared with the plate without CCCP.

### 2.5. Reverse Transcription (RT)-Polymerase Chain Reaction

RNXTM-PLUS solution (Cinaclon Company, Iran, Cat No. RN7713C) was applied for extraction of total RNA from *A. baumannii* strains, according to instructions of the manufacturer. The RNase-free DNase I (Cinaclon Company, Iran, Cat No. PR891627) was used for removing additional DNA from RNA. cDNA synthesis was prepared by using 1 µg RNA samples, 1 µL random hexamer primers, and AccuPower RocketScript RT Premix (Bioneer, china, Cat No. K-2101).

### 2.6. Semi quantitative Real-Time RT-PCR

To quantify the expression rates of the *adeG* gene, reverse transcription-PCR was examined. Real-time PCR assays were performed using a real-time PCR detection system (Corbett) with the SYBR Green qPCR MasterMix (Yekta Tajhiz Azma, Iran, Cat No: YT2551). The level of gene expression of efflux pump was normalized with the 16S ribosomal RNA gene as a housekeeping gene. Comparing the gene expression in isolates with reference stain (*A. baumannii* ATCC 19606) could determine the expression of the *adeG* gene. Amplification was designed including 10 of SYBR Green qPCR MasterMix, 0.5 µL of primers, 8 µL water, and 1 µL cDNA in a 20 µL total volume. The reaction conditions were 95°C for 5 minutes for the first denaturation; 95°C for 20 seconds for denaturation, 60°C for one minute for annealing for 40 cycles, and melt curve at 72 to 95°C.

**Table 2.** Amplification Condition for Detection of *adeG* Gene

Gene	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension	No. Cycles
<i>adeG</i>	95°C - 5 min	95°C - 20 s	60°C - 30 s	72°C - 30 s	72°C - 5 min	30

### 3. Results

The breakpoint pattern of the 51 isolates of *A. baumannii* to tested antibiotic with disk diffusion was cefotaxime (100%), piperacillin-tazobactam, cefepime, ciprofloxacin (98%), ceftazidime, imipenem, meropenem, amikacin (96%), gentamicin (90%), minocycline (86%), trimethoprim-sulfamethoxazole (92%), and colistin (0%). The PCR results showed that all of the isolates had the *adeG* gene.

When resistant isolates were tested in 25 µg/L CCCP concentration, the MICs of ciprofloxacin was decreased by 32 folds or more in seven strains, 16 folds in two strains, eight folds in 10 strains, four folds in 25 strains, and two folds in six strains. Therefore, 44 of the isolates showed a four-fold of more decrease of MICs after addition of CCCP. Thirty-four of 44 overexpressed the AdeG efflux pump. Seven strains had elevated levels (between 1001- to 3000-fold increase) compared to the reference strain, seven isolates had intermediate rates (approximately 501- to 1000-fold), 14 isolates had low (approximately 101- to 500-fold) levels of overexpression, and six strains had very low (approximately 2- to 100-fold) levels of overexpression.

### 4. Discussion

*Acinetobacter baumannii* have evolved to a clinically important nosocomial pathogen because of the increasing frequency of pan drug-resistant (PDR), extensively-drug resistant (XDR), and multidrug-resistant (MDR) strains (25, 26). In some studies, maximum resistance was seen against cefotaxime, cefepime, ciprofloxacin, piperacillin-tazobactam, ceftazidime, imipenem and meropenem, which was similar to other studies (27-30).

Efflux pump inhibitors (EPIs) are agents that can increase resistance to common drugs by preventing energy source of efflux pumps and preventing the interactions of various parts (31). Ciprofloxacin could not be expelled from the cell by action of CCCP in MDR isolates and increases its concentration. The contributor of CCCP in quinolone resistance was further illustrated with observing the reduction of the MIC for ciprofloxacin in all MDR isolates after adding CCCP, which is consistent with other results (32). In the isolates of the current study, 88% of isolates displayed response to CCCP and 12% of the isolates displayed negligible response to CCCP, which illustrated the up-regulation of transporting systems and demonstrated that additional

resistance pathways compensate the loss of transporting systems activity, therefore, the susceptibility of isolates to ciprofloxacin was still low. The current study also showed that the MIC for 62% was reduced significantly by two to four folds when the EPI was added. It was illustrated that CCCP decreased the MIC by two to four folds in *A. baumannii* strains (6, 12, 33). In one study, CCCP partially restored susceptibility in tigecycline resistant *A. baumannii* isolates (31). The differences in the reported values between the present study and those reported previously may be due to differences in antibiotic selections.

It was indicated that in the *A. baumannii* isolated from humans, the *adeF*, *adeG* and *adeH* drug efflux pumps genes were very prevalent (34). Approximately 90% of the *A. baumannii* isolates had the *adeFGH* operon (22), which was similar to the current study.

Resistance to several antimicrobial agents may be partially associated with the *adeFGH* operon. Resistance to fluoroquinolones and tigecycline probably mediated up-regulation of AdeFGH in *A. baumannii* (22). The current researchers found that there was a correlation between overexpression of AdeG and increased ciprofloxacin resistance. Fernando et al. found that the prevalence of RNA transcript of RND efflux pump gene among clinical isolates of *Acinetobacter* species was as follows: *adeG*, *adeJ*, and *adeB*. (9). Emergence and development of multiple antibiotic resistance can be mediated with up-regulation of efflux pumps. It is likely that topoisomerase mutations and efflux phenotype were the result of ciprofloxacin pressure (16). Deng et al. detected that *adeG* expression was not associated with tigecycline resistance in *A. baumannii* isolates (31).

Among 50 resistant isolates collected in this study, 77.27% showed increased expression of the *adeG*. It was shown that 57.14% of 14 MDR clinical isolates of *A. baumannii* had increases in *adeG* expression. It was reported that overexpression of *adeG* was associated with pathogenicity of *A. baumannii* (7). All of the *Acinetobacter* isolates overexpressed the *adeG* (9). In one study, 77.77% of fluoroquinolone *A. baumannii* strains showed overexpression of *adeF* (3). It seems that the expression rate of *adeF* gene compared with the *adeA* and *adeI* genes was very lower in all XDR *A. baumannii* isolates and no isolate overexpressed the *adeF* gene (34). Rumbo et al. isolated four-hundred and forty-four strains of *A. baumannii* and found that expression of *adeG* was not increased (4).

Increases of 357 folds in *adeG* expression in isolates was

observed. Increases of more than 250 folds in adeFGH expression in mutants was observed (22). The control strain had higher level of expression in the adeFGH gene than XDR *A. baumannii* isolates (34). Yoon et al. showed that 57.14% of MDR clinical isolates of *A. baumannii* showed four- to 15-fold greater expression of the *adeG* gene (7). The dissimilarity between the current results and other studies may be because of the dissimilarity of drug regiment in various countries, different figures of the isolates, different antibiotics used for screening, and different detection of genes in efflux pumps used in these studies.

Overexpression of efflux pumps may be associated with increased ciprofloxacin resistance. Therefore, determining the genes that have a role in drug resistance in clinical settings is necessary to inhibit the outbreak of nosocomial infections caused by MDR *A. baumannii*.

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