Avicenna J Clin Microb Infec. 2016 November; 3(4):e40898. doi: 10.17795/ajcmi-40898.

Published online 2016 October 24. **Research Article**

# AdeABC Efflux Pump Genes in Multidrug Resistant *Acinetobacter baumannii* Isolates

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**Received** 2016 July 17; **Revised** 2016 September 18; **Accepted** 2016 October 06.

## Abstract

**Objectives:** *Acinetobacter baumannii* is an opportunistic nosocomial pathogen and a cause of severe infections in hospitalized patients, owing to its ability to acquire drug resistance by the efflux pump mechanism. The current study investigated the detection and prevalence of efflux pump genes (AdeABC) in multidrug resistant *A. baumannii* isolates and the role of these genes in multidrug and carbapenems resistance.

**Methods:** This study was conducted on 84 multidrug resistant and 13 carbapenem-susceptible *A. baumannii* isolates obtained from burn and wound infections in Baghdad hospitals, Iraq. The AdeABC genes were detected by polymerase chain reaction (PCR) assay. Phe-arg-beta-naphthylamide (PA$\beta$N)-based method was used for determination of efflux pump activity.

**Results:** This study showed high prevalence of efflux pump genes in our local isolates. The AdeB gene was present in all multidrug resistant isolates (100%) while AdeRS gene was found in 95.2%, AdeC gene in 83.3% and AdeA gene in 77.4%. By comparing the prevalence of these gene in carbapenem-susceptible isolates, it was demonstrated that the gene AdeB was absent in all susceptible isolates, also the regulatory gene AdeRS was not found in most of these isolates, while the other genes (AdeA and AdeC) were detected in most carbapenem- susceptible isolates. Susceptibility of isolates to Amikacin, Gentamicin, Ciprofloxacin, Levofloxacin, Tetracycline and Tigecycline was highly increased in the presence of efflux pump inhibitor, so that, PA$\beta$N reduced the minimum inhibitory concentrations (MICs) by 4 to 32 folds. Also, MICs of carbapenems were reduced in the presence of the inhibitor by two to eight folds, while the MICs of colistin were not affected.

**Conclusions:** AdeABC efflux system plays a vital role in multidrug resistance in clinical *A. baumannii* isolates. It was noted that the most important gene responsible for multidrug resistance within this system was the AdeB gene especially in carbapenems resistance.

*Keywords:* *Acinetobacter baumannii*, Multidrug Resistance, Efflux Pumps

## 1. Background

*Acinetobacter baumannii* has become the main cause of nosocomial infections worldwide due to its propensity to rapidly acquire resistance determinants to a wide range of antibiotics (1). The most prevalent *A. baumannii* multidrug resistance (MDR) determinants include efflux pumps genes, beta-lactamases, integrons, and insertion sequence (IS) elements (2). Efflux systems pump several compounds, including antibiotics, out of the bacterial cell and can play a major role in bacterial resistance to different classes of antibiotics (3). In Acinetobacter, the chromosomally encoded pump is a tripartite efflux machinery that belongs to the RND -type superfamily. The AdeABC efflux pump (RND-type superfamily) consists of adeA (membrane fusion), adeB (multidrug transporter) and adeC (outer membrane) genes. These three genes are contiguous and adjacent by two-component regulatory systems; adeR and adeS (4). The major efflux mechanism associated with carbapenem resistance in *A. baumannii* is the chromosomally encoded tripartite efflux pump, AdeABC, found in approximately 80% of clinical isolates. Over-expression of this pump confers resistance to aminoglycosides and decreases susceptibility to fluoroquinolones, tetracycline, chloramphenicol, erythromycin, trimethoprim and ethidium bromide, as well as to netilmicin and meropenem (5). The synergy between acquired oxacillinases and the AdeABC pump has been reported and implicated in higher levels of resistance to $\beta$-lactams, including carbapenems (4).

## 2. Objectives

The aims of this study were to estimate the prevalence of the AdeABC genes in multidrug resistant *A. baumannii*

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isolates and the role of the efflux pump in multidrug and carbapenems resistance of *A. baumannii* strains isolated from burn and wound infections in Baghdad hospitals.

## 3. Methods

### 3.1. Bacterial Isolates and Antimicrobial Susceptibility Tests

From February to July 2015, a total of 96 *A. baumannii* clinical isolates were recovered from 476 burn and wound infections from patients in Baghdad hospitals, Iraq. The clinical isolates were identified according to conventional biochemical tests, API20 E and Vitek2 system. The BlaOXA-51 gene was also detected for confirming the identification (6). Antimicrobial susceptibility tests were performed using Disc diffusion method and minimal inhibitory concentrations (MICs) using the microdilution method in Mueller-Hinton broth based on the results reported by the Clinical and Laboratory Standards Institute (CLSI) guidelines (7). *Pseudomonas aeruginosa* ATCC-27853 was used for quality control in antimicrobial susceptibility testing (8).

### 3.2. Detection of the Efflux Pump-Mediated Antibiotic Resistance

Fifty microliters of Mueller Hinton broth was added to the wells of a sterile microdilution plate. By adding 50 $\mu$L of suitable concentrations of antibiotic to the first line of wells, serial dilutions were performed. Furthermore, 20 $\mu$g/mL constant P$\beta$NA (Phe-arg-beta-naphthylamide) concentration was obtained by adding 40 $\mu$L of the bacterial suspension and 10 $\mu$L of the stock P$\beta$NA (200 $\mu$g/mL) solution to each well. Fold decrease in the MIC values of each antibiotic was evaluated as the effect of the efflux pump inhibitor (9).

### 3.3. Detection of AdeABC Efflux Pump System by the Polymerase Chain Reaction

*Acinetobacter baumannii* isolates were assayed for AdeABC genes by PCR with primers previously described (Table 1). The amplification conditions of AdeA gene were as follows: initial denaturation at 94°C for five minutes, 30 cycles of 94°C for one minute, 56°C for one minute, 72°C for one minute and final elongation at 72°C for seven minutes (10). The amplification conditions of AdeB gene were as follows: initial denaturation at 94°C for two minutes, 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for two minutes and final elongation at 72°C for two minutes (11). The AdeC and AdeRS genes were amplified using the following conditions: initial denaturation at 94°C for five minutes, 35 cycles of 94°C for 30 seconds, 57°C for 40 seconds and 72°C for 50 seconds and a final elongation at 72°C for six minutes (12).

### 3.4. Agarose Gel Electrophoresis

Gel electrophoresis was used for the detection of PCR products, visualized with the aid of ethidium bromide and UV transilluminator documentation system. The size of PCR products was compared with a 100-bp DNA Ladder (Bioneer, Korea).

### 3.5. Statistical Analysis

SPSS software version 16 was used for analysis by conducting Chi-square test. Differences between values were considered significant at $P \leq 0.01$.

## 4. Results and Discussion

### 4.1. Detection and Distribution of AdeABC Efflux Pump Genes

In our previous reports, 96 isolates were identified as *A. baumannii*, 84 of these isolates were multidrug resistant and 13 isolates were carbapenems susceptible (6, 8). Genes encoding efflux pumps in all 84 multidrug resistant *A. baumannii* isolates were determined by PCR assay, efflux pump adeA, adeB and adeC genes, and their regulator adeRS gene was detected in these isolate (Figure 1). These genes were also detected in 13 carbapenem-susceptible A. baumannii isolates.

Polymerase Chain Reaction assay was performed with specific primers detection genes of AdeABC system. Amplification fragments with sizes of approximately 513 bp, 981 bp, 560 bp and 790 bp were detected on agarose gel that corresponded to adeA, adeB, adeC and adeRS genes, respectively.

The distribution of efflux pump in multidrug-resistant isolates (n = 84) is shown in Table 2.

The results of this study revealed the dominance of adeB gene among multidrug resistant *A. baumannii* strains (MDRAB) isolated from burn and wound infections, and prevalence of this gene in these isolates was 100%. Also the results demonstrated the presence of regulator gene adeRS in 95.2% of the MDRAB isolates. From a total of 84 MDRAB isolates, the number of isolates, which carried the gene adeC were 70 isolates (83.3%), while the lowest percentage of these genes was recorded for adeA gene (77.4%), (high significant differences at $P < 0.01$).

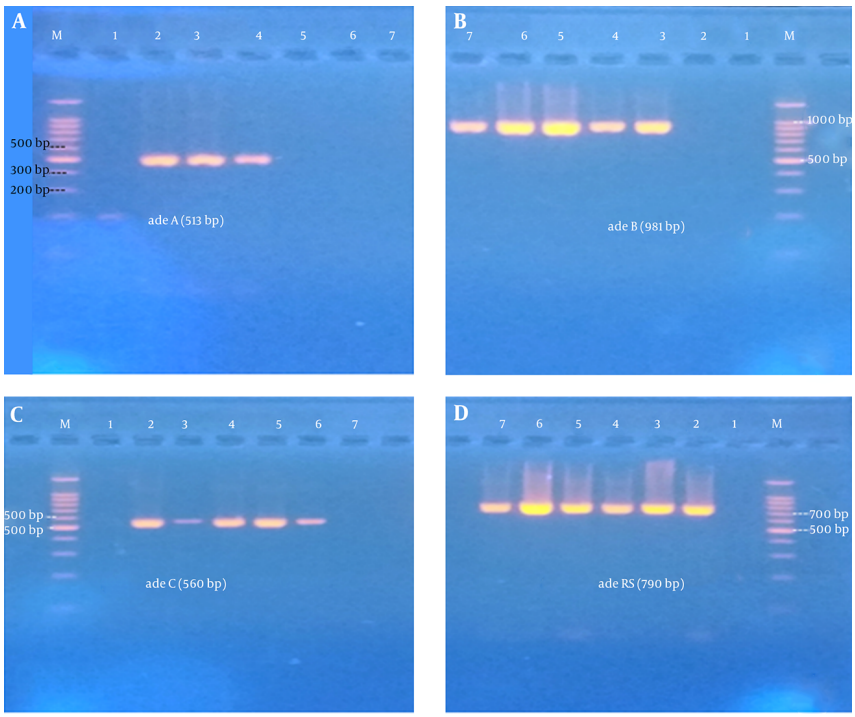
The novelty of the current study was to detect and determine the prevalence of efflux pump genes in MDR *A. baumannii* in Baghdad hospitals.

Previous study by Japoni Nejad et al. (13) from Iran reported that from a total of 56 multidrug resistant isolates of *A. baumannii*, the adeA, adeB and adeC genes were detected in 100%, 100% and 96.5% of isolates, respectively and all isolates were resistant to cefotaxim, ceftazidim, cefepim, cefoxitin, azteronam, ciprofloxacin and imipenem.

**Table 1.** The Sequences of Primers used for Polymerase Chain Reaction to Detect Acinetobacter baumannii Efflux Pump Genes

| Gene name | Primer Name | Sequence | Reference |
|---|---|---|---|
| **AdeA** | F | 5'-ATC TTC CTG CAC GTG TAC AT-3' | Lin et al., 2009 (10) |
| | R | 5'-GGC GTT CAT ACT CAC TAA CC-3' | |
| **AdeB** | F | 5'-GTATGAATTGATGCTGC-3' | Ahmad et al., 2011 (11) |
| | R | 5'-CACTCGTAGCCAATACC-3' | |
| **AdeC** | F | 5'-AGCCTGCAATTACATCTCAT-3' | Lopes, 2011 (12) |
| | R | 5'-TGGCACTTCACTATCAATAC-3' | |
| **AdeRS** | adeR F | 5'-GCA TTA CGC ATA GGT GCA GA-3' | Lopes, 2011 (12) |
| | adeS R | 5'-GAG GTC GCC GTG ACT AAT TT-3' | |

**Figure 1.** Polymerase Chain Reaction Amplification of adeA, adeB, adeC and adeRS Genes in Six Selected Isolates of *Acinetobacter baumannii*




Lane M, 100-bp DNA ladder; lane 1, negative control; (a) lane 2 - 4, PCR product of adeA gene (513 bp); (b) lane 3 - 7, PCR product for adeB gene (981 bp).(c) lane2 - 6, PCR product of adeC gene (560 bp); (d) Lane 2 - 7, PCR product of adeRS gene (790 bp) (70V for two hours).

In another conducted study from Iran on 60 *A. baumannii* isolates from burn units, the results revealed the detection of adeA and adeB genes in all isolates (100%) while AdeC was present in 51 isolates (85%) (14). In another study by Hou et al. (15) it was found that adeB, adeR, adeS, adeJ and abeM were found in the majority of imipenem resistant *A. baumannii* (> 80%). A study by Wong et al. (16) from Malaysia investigated 39 isolates of carbapenem resistant Acinetobacter spp. isolated at University Malaya Medical Centre, Malaysia, in 2004, and showed the presence of adeA, adeB, adeR and adeS genes in 36 isolates while 34 of them carried the adeC gene. Another study demonstrated the high prevalence of efflux pump genes of AdeABC system in multidrug resistant isolates of *A. baumannii* isolated from hospitals in China; hence 88.2% of isolates carried genes of AdeABC efflux system (17). In a similar study conducted by Lin et al. (18), it was found that the efflux pump gene AdeB existed in all MDR A. bau-

**Table 2.** The Distribution of Efflux Pump Genes Among Multidrug Resistant *Acinetobacter baumannii* Isolates

| Efflux Pump Gene Type | *A. baumannii* Isolates MDR | | Chi-Square-$\chi^2$ | P Value |
|---|---|---|---|---|
| | No.( %) of Positive Isolates | No. (%) of Negative Isolates | | |
| **AdeA** | 65 (77.4) | 19 (22.6) | 12.95[a] | 0.0001 |
| **AdeB** | 84 (100) | 0 (0) | 15.00[a] | 0.0001 |
| **AdeC** | 70 (83. 3) | 14 (16.7) | 13.632[a] | 0.0001 |
| **AdeRS** | 80 (95.2) | 4 (4. 8) | 14.873[a] | 0.0001 |

[a] ($P < 0.01$).

mannii isolated from five hospitals in Taiwan. Insertional inactivation of adeB in *A. baumannii* strain BM4454 indicated the role of this efflux pump protein in aminoglycoside resistance and its involvement in reducing the level of susceptibility to other drugs including tetracyclines, fluoroquinolones, erythromycin, trimethoprim and ethidium bromide (19). Additionally, Coyne et al. (20) demonstrated that the overexpression of AdeABC and mutations in the adeRS genes encoding a two-component regulatory system constitutes a major mechanism of multidrug resistance in nosocomial strains of *A. baumannii*.

The main role of AdeB gene resulted from its function; AdeB captures its substrates either from the cytoplasm or within the phospholipids bilayer of the inner membrane in *A. baumannii* while adeA and adeC act as assistance (4, 19).

### *4.2. The role of AdeABC Efflux Pump Genes in Multidrug and Carbapenems Resistance*

It was found from our results of detection of AdeABC efflux system that the most multidrug resistant isolates possessed all genes of the system but it was obvious that adeB gene had the main role in the resistance mechanism. To determinate the role of efflux pump in the multidrug resistant phenotypes in *A. baumannii* isolates, the experiment included detection of the MICs of 12 antibiotics in the presence of 20 $\mu$g/mL of P$\beta$NA, and then, compared the MICs with and without P$\beta$NA (Table 3).

The effect of Phe-Arg-Beta-Naphthylamide (PA$\beta$N) on MICs of *A. baumannii* is summarized in Table 3; it was found that the addition of the PA$\beta$N at a final concentration of 20 $\mu$g/mL greatly reduced the MICs of amikacin from four to eight folds and gentamicin from 8 to 32 folds. Also the effect of PA$\beta$N on MICs of fluoroquinolone (ciprofloxacin and levofloxacin) and tetracycline antibiotics was obvious with reduction in MICs from 4 to 16 folds. It was noted that after exposure to the efflux pump inhibitor, two to eight fold reduction in the MICs of carbapenems was observed. The minimal effect of the inhibitor was correlated with antibiotics colistin, ceftazidime and cefotaxime.

The effects of efflux pump inhibitors such as carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and Phe-Arg-Beta-Naphthylamide (PA$\beta$N) on antimicrobial susceptibility were investigated in some studies (4, 21). One of the reports revealed that the addition of the Phe-arg-beta-naphthylamide at different concentrations reduced the MICs of different antibiotics such as ertapenem (22). Ardebili et al. (21) found that the addition of efflux pump inhibitor CCCP caused an increase in the susceptibility to ciprofloxacin by 4 to 64 folds. Our results suggested that drug efflux pumps contribute to the resistance to fluoroquinolone in *A. baumannii* clinical isolates.

It was reported that the imipenem susceptibility of most *A. baumannii* isolated from burns was increased in the presence of the PA$\beta$N by 4 to 64 folds (14). Our results indicated the role of efflux pump mechanism in imipenem and meropenem resistance in *A. baumannii* isolates. Several studies indicated the role of the AdeABC system in the resistance of *A. baumannii* to many classes of antibiotics such as beta-lactams, aminoglycosides, quinolones and tetracycline (23).

The prevalence of efflux pump genes among 13 carbapenem-susceptible *A. baumannii* isolates is summarized in Table 4, in order to study the role of efflux pump genes in the resistance of carbapenems. It was found that all 13 isolates lacked the AdeB gene while AdeRS gene was present only in five isolates. AdeA and AdeC genes were found in most sensitive isolates.

An efflux pump system AdeABC seems to play an important role in the resistance to imipenem of *A. baumannii*. After exposure to the efflux pump inhibitor, Phe-Arg-$\beta$-naphthylamide, a significant reduction of imipenem MICs was observed in 33 (66%) isolates of IRAB, while no significant decrease occurred in Imipenem-Susceptible *A. baumannii* (ISAB) (15). The results conducted by Jia et al. (17) showed that isolates carrying blaOXA-23 and AdeABC efflux pump genes were the most prevalent in MDR *A. baumannii* and the co-expression of carbapenemase and efflux pump proteins are considered the main reason for the resistance of these bacteria in the intensive care unit (ICU).

**Table 3.** Antimicrobial Susceptibility in the Presence and Absence of Efflux Pump Inhibitor (Phe-Arg-$\beta$-naphthylamide) of *Acinetobacter baumannii* Clinical Isolates

| Isolate No. | | AK | GM | IPM | MEM | CAZ | CTX | CIP | LVX | TE | TG | PI | CT |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| K1 | | | | | | | | | | | | | |
| | Alone | 128 | 32 | 64 | 128 | 16 | 128 | 32 | 4 | 16 | 2 | 128 | 0.5 |
| | +EPI | 16 | 2 | 16 | 32 | 16 | 128 | 4 | 0.5 | 2 | 0.25 | 32 | 0.5 |
| K2 | | | | | | | | | | | | | |
| | Alone | 256 | 64 | 256 | > 256 | 128 | 256 | 64 | 16 | 64 | 1 | 128 | 0.5 |
| | +EPI | 32 | 4 | 64 | 128 | 128 | 128 | 4 | 2 | 4 | 0 | 16 | 0.5 |
| K3 | | | | | | | | | | | | | |
| | Alone | 256 | 64 | 256 | > 256 | 128 | 256 | 32 | 16 | 128 | 4 | 256 | 0.5 |
| | +EPI | 32 | 8 | 64 | 64 | 64 | 256 | 2 | 2 | 16 | 0.5 | 32 | 0.5 |
| K4 | | | | | | | | | | | | | |
| | Alone | 64 | 16 | 128 | 64 | 16 | 16 | 16 | 8 | 2 | 0.5 | 256 | 2 |
| | +EPI | 8 | 1 | 32 | 8 | 16 | 16 | 2 | 2 | 0.25 | 0 | 64 | 2 |
| K5 | | | | | | | | | | | | | |
| | Alone | 256 | 64 | > 256 | 128 | 64 | > 256 | 64 | 16 | 4 | 0.5 | 256 | 8 |
| | +EPI | 32 | 4 | 128 | 16 | 32 | 256 | 8 | 1 | 1 | 0 | 64 | 8 |
| K14 | | | | | | | | | | | | | |
| | Alone | 256 | 64 | > 256 | > 256 | 128 | 128 | 64 | 16 | 128 | 32 | 256 | 2 |
| | +EPI | 64 | 2 | 64 | 128 | 128 | 64 | 16 | 2 | 16 | 2 | 32 | 2 |
| Fold of reduction In MIC + EPI | | 4 - 8 | 8 - 32 | 2 - 8 | 2 - 8 | 0 - 2 | 0 - 2 | 4 - 16 | 4 - 16 | 4 - 16 | 1 -16 | 2 - 8 | 0 |

Abbreviations: MIC, minimum inhibitory concentration; EPI, efflux pump inhibitor (Phe-Arg-$\beta$-naphthylamide); amikacin (AK), gentamicin (GM), imipenem (IPM), meropenem (MEM), ceftazidime (CZ), cefotaxime (CTX), ciprofloxacin (CIP), levofloxacin (LVX), Tetracycline (TE), tigecycline (TGC), piperacillin (PI), and colistin (CT).

**Table 4.** Prevalence of Efflux Pump Genes Among Carbapenem-Susceptible *Acinetobacter baumannii* Isolates

| Is. No. | IPM | MEM | adeA | adeB | adeC | adeRS |
|---|---|---|---|---|---|---|
| K6 | S | S | - | - | - | + |
| K13 | S | S | - | - | + | - |
| K22 | S | S | - | - | + | - |
| K23 | I | S | + | - | - | - |
| K25 | S | S | + | - | + | - |
| K26 | S | S | + | - | + | - |
| K28 | S | S | + | - | + | - |
| K39 | S | S | + | - | + | + |
| K55 | S | S | + | - | + | - |
| K89 | S | S | + | - | + | + |
| K90 | S | S | + | - | + | - |
| K93 | S | S | + | - | - | + |
| K96 | S | S | + | - | + | + |

Abbreviations: S, sensitive; I, intermediate; IPM, imipenem; MEM, meropenem.

Our results are concordant with the findings of Marchand et al. (4), who claimed that adeC gene is not essential for the MDR *A. baumannii* phenotype resulted from the efflux pump, since an inactivation of adeC gene will not change the resistance of bacteria to different antibiotics. Another study suggested that OXA-23 carbapenemase encoded by the blaOXA-23 gene on the chromosome and overexpression of the AdeABC efflux pump may contribute to carbapenem resistance in clinical isolates of *A. baumannii* (24). It was shown that the present results disagree with the data of some previous studies, which indicated that AdeABC efflux pump genes existed in both carbapenem-resistant and sensitive strains, therefore they may not have contributed to the carbapenems resistance of *A. baumannii* (25, 26).

The current study demonstrated the main contribution of the gene adeB and its regulatory system in multidrug and carbapenems resistance in clinical isolates of *A. baumannii*. The high levels of carbapenem-resistance in *A. baumannii* might be due to the cooperation among blaOXA and AdeABC genes.

## Acknowledgments

This study was supported by the biotechnology department, institute of genetic engineering and biotechnology for postgraduate studies, University of Baghdad, Baghdad, Iraq. The authors are thankful to the head of the department of biotechnology.

## Footnotes

**Authors' Contribution:** Kais Kassim Ghaima performed the laboratory work. Shurook Mohammad K. Saadedin designed the study, and Kifah Ahmed Jassim provided advice for the study.

**Funding/Support:** This study was supported by University of Baghdad, Baghdad, Iraq

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