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Original Article

Detection of Efflux Pump Genes (*adeA, adeB, adeC and abeM)* in *Acinetobacter baumannii* Isolated from Hospitalize Patients, North-west of Iran

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Background: The importance of this research was to determine the prevalence of efflux pump genes among *Acinetobacter baumannii* isolates from hospitalized patients in Imam Reza hospital in Tabriz, Iran.
Materials and Methods: This descriptive study was conducted in the Imam Reza hospital, Tabriz, IR Iran during June 2013 to March 2014. Twenty-six strains were isolated from female patients (42.6%) and thirty-five from male patients (57.4%). Clinical specimens were cultured for isolation of the microbial agents of *A. baumannii*. The isolated bacteria were identified using biochemical tests. Disk diffusion susceptibility test was used to determine the antimicrobial susceptibility, and E-test methods were also used. The prevalence of efflux pump genes was detected by PCR and sequencing methods.
Results: The resistance of *A. baumannii* isolates against tested antibiotics was analyzed as follows: 51 (84%) to trimethoprim-sulfamethoxazole, 59 (98%) to ceftazidime, 60 (99%) to ciprofloxacin, 29 (48%) to amikacin, 46 (77%) to gentamicin, 30 (50%) to tobramicin, , 60 (99%) to imipenem,, 60 (99%) to meropenem,, 60 (99%) to ceftriaxon,, 60 (99%) to cefepime,, 60 (99%) to ofloxacin, 6 (11%) to colistin. By using E-test, 45 (73.3%) to imipenem, 57 (93.3%) to ciprofloxacin, 23 (38%) to amikacin were also analyzed. The prevalence of *adeA, adeB, adeC,* and *abeM* genes was 54 (88.5%), 61 (100%), 57 (93.9%), and 60 (98.3%), respectively.
Conclusion: The result of this study showed high incidence of AdeABC efflux pump in MDR *A. baumannii* isolates and the growing number of nosocomial infections associated with XDR *A. baumannii* complex, leading to difficulties in antibiotic therapy.

Keywords: A. baumannii, Efflux pumps, PCR

1. Background

Acinetobacter baumannii is known to be an important nosocomial pathogen isolated predominantly from intensive care units (ICUs), and responsible for severe infections. *A. baumannii* is usually multidrug resistant (MDR), showing resistance to the third generation cephalosporins, aminoglycosides, and fluoroquinolone (1, 2). Recently, *A. baumannii* isolates have almost always proved to be extensively drug-resistant (XDR) which is defined as resistance to all antibacterial agents except tigecycline and colistin. The treatment of XDR *A. baumannii* infections is a major challenge which faces physicians with the lack of effective treatment options and limited management experience. Unfortunately, the increased use of tigecycline and colistin as salvage therapy has been associated with the emergence of pan-drug resistance (PDR), against which no known antibacterial agents retain activity (3).

The most common mechanisms for resistance can be a) intrinsic, due to production of a chromosomally encoded cephalosporinase and low level of membrane permeability, b) acquired, following transfer of foreign genetic information or mutation in endogenous structural or regulatory genes. Efflux systems are widely found in microorganisms and confer resistance to various compounds including antibiotics, by extrusion of the drug. Of particular clinical importance are mutational events in the regulators of efflux systems, which in a single step can confer multidrug resistance (MDR) to the host by over expression of the pump. The pumps of the resistance-nodulation-cell division (RND) superfamily are ubiquitous in Gram-negative bacteria and have the broadest substrate ranges (4).

To date, overexpression of three RND systems, AdeABC (5), AdeFGH (6), and AdeIJK (7), has been associated with MDR in A. baumannii (8). The first pump described, AdeABC, confers resistance to aminoglycosides, tetracyclines, fluoroquinolones, chloramphenicol, and trimethoprim, and reduced susceptibility to tigecycline (9-11). AdeABC, primarily, and AdeFGH play a major role in acquired resistance (8), whereas AdeIJK is responsible for intrinsic resistance (7). Production of AdeABC is controlled by a two-component regulatory system, AdeRS (12).

The second efflux pump, AbeM, which belongs to the multidrug and toxic compound extrusion (MATE) family, has also been characterized in isolates of A. baumannii (13). Substrates for the abeM efflux pump include gentamicin, ciprofloxacin, erythromycin, and trimethoprim (13). The contribution of this system to antimicrobial resistance in clinical isolates is unknown.

2. Objectives

The aim of our study was to determine the prevalence of *adeA, adeB, adeC,* and *abeM* genes among *A. baumannii* strains isolated from patients admitted to Imam Reza hospital, Tabriz, IR Iran.

3. Materials and Methods

This study was carried out in the Imam Reza hospital laboratory in Tabriz in East Azerbaijan, northwest of IR Iran from June 2013 to march 2014.

3.1. Bacterial isolates

Sixty-one isolates of *A. baumannii* were recovered from blood, wound, abscesses, urine, sputum, respiratory tract, and fluid bodies of patients from Imam Reza hospital in Tabriz, IR Iran. The isolates were identified by conventional biochemical methods (14) and also confirmed for bla_{OXA-51} gene by PCR.

3.2. Antimicrobial susceptibility testing

In the present study, antimicrobial susceptibility testing was carried out on Mueller-Hinton Agar (Merck, Germany) using disk diffusion (Kirby Bauer's) technique. This method was employed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines 2012 (15). Antimicrobial agents tested were the antibiotic disks which werecomprised of: imipenem (IPM)(10μg), meropenem (MER)(10μg), ceftazidime (CAZ)(30μg), cefotaxime (CTX)(30μg), amikacin (AN)(30μg), colistin(CL)(10μg), ciprofloxacin (CP)(5μg), cefepime (FEP)(30 μg), trimethoprim-sulfamethoxazole (TS) (2.5μg), tobramicin (TOB)(10μg), ceftriaxon (CRO)(30μg), and gentamicin (GM)(10μg) (Hi Media, India; padtanteb, Iran).The results were interpreted based on inhibition zone. *E. coli* ATCC 25922 was used as the quality control strain for antimicrobial susceptibility test.

3.3. E-test

The MICs of imipenem, ciprofloxacin, and amikacin were determined by E- test method (AB Biodisk, Solna, Sweden) according to the guidelines of the CLSI 2012 (15).

3.4. Genomic DNA extraction

Genomic DNAs for PCR were prepared by boiling method briefly; the bacteria were cultured on nutrient agar. After one overnight, a colony was inoculated in 3 mL of luria Bertoni broth. After that, it was inoculated at 37°C on incubator for 20-24 hr. After incubation, 1.5 mL of the culture medium was poured into micro tubes and centrifuged for 5 min 14000 rpm. Then Supernatant was removed and 500 mL distilled water added to the pellet. The suspension was heated for 10 min at 95°C. The heated suspension was centrifuged again for 5 min in 14000 rpm. Supernatant containing DNA was extracted and used as template for PCR techniques.

3.5. Detection of adeA, adeB, adeC, and abeM genes by PCR

Efflux pump genes, *adeA, adeB, adeC, and abeM,* were determined by PCR with designed specific primers, which are shown in Table 1. The PCR assay was performed in 20µL of reaction mixture containing: 1.5U Taq DNA polymerase (Fermentas), 0.5µL dNTPs (Fermentas), 5mM $MgCl_2$, 2.5µL of 10X PCR buffer, 1 µL of the purified nucleic acid solutions, and a 1µM of each primer. The thermal profile involved an initial denaturation step at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30s, primer annealing temperature was set up for adeA at 44°C for 30s, for *adeB* at 46°C for 30s, for *adeC* and *abeM* at 55°C for 30s. The extension was also set up at 72°C for 30s.The cycling was followed by a final extension step at 72°C for 5 min. PCR product was electrophoresed in a 1% agarose gel and stained with ethidium bromide. Statistical analyses were calculated by using SPSS software (version 16), X_2 statistical test and P-value <0.05 were considered as significant.

3.6. Sequencing method

PCR purification kit (Bioneer Co., Korea) was used to purify PCR products, and sequencing was performed by the Bioneer Company (Korea). The nucleotide sequences were analyzed with the Chromas 1.45 software and the BLAST program from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST). The nucleotide sequences of *adeA, adeB,* and *adeC* were submitted to the GenBank database and assigned accession numbers from AB982118.1 to AB982122, LC016875.1 to LC016884.1 and LC016625.1 to LC016633.1.

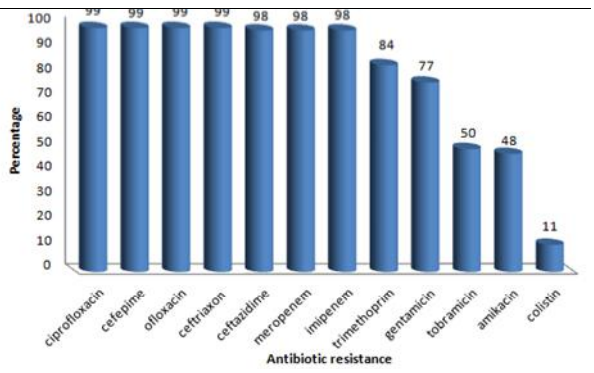
4. Results

In this study, sixty-one strains were isolated from Imam Reza hospital. Twenty-six strains were isolated from female patients (42.6%) and thirty-five from male patients (57.4%). Of the 61 isolates, 30 (49.3%) from tracheal tube were isolated, 7 (11.6%) from wound, 10 (16.7%) from blood, 4 (6.5%) from urine, 5 (8.4%) from sputum, and 5 (7.5%) from CSF, asites, and other samples. The age range of the patients was from 1 to 90 years. The isolates were obtained from patients in different age groups: 1–29 years (n = 7), 30–39 years (n = 6), 40–49 years (n = 8), 50–59 years (n = 14), 60–69 years (n = 12), and 70–79 years (n = 10), and four isolates were isolated from patients more than eighty years old. The resistance of *A. baumannii* isolates against tested antibiotics was analyzed as follows: 51 (84%) to trimethoprim-sulfamethoxazole, 59 (98%) to ceftazidime, 60 (99%) to ciprofloxacin, 29 (48%) to amikacin, 46 (77%) to gentamicin, 30 (50%) to tobramicin, , 60 (99%) to imipenem,, 60 (99%) to meropenem,, 60 (99%) to ceftriaxon,, 60 (99%) to cefepime, 60 (99%) to ofloxacin, 6 (11%) to colistin (Table 2).

Table 1. The targeted genes and related design primers used in this study.

Gene	Primers	Product size
*ade*A- F	5'- CTGATATTACAGGGGTGTG -3'	408 bp
*ade*A- R	5'-GCTTCTCTCAATAAAGCTGAAG -3'	
*ade*B- F	5'- ATTTGGATTGCTGAGCATTC - 3'	340 bp
*ade*B- R	5'- GTAAACCTTGCTGACGTACA - 3'	
*ade*C- F	5'- ATGCATCATCTGAACTGAAAG -3'	222 bp
*ade*C- R	5'- GTGCATGTGTAGCAAGTGCA - 3'	
*abe*M- F	5'- TATTACTTACCTTGCAACGCAG - 3'	283 bp
*abe*M- R	5'- GTGGTTGCAATCATGATGCCA - 3'	

Table 2. Antimicrobial resistance of clinical isolates of *A.baumannii*.



The prevalence of *adeA, adeB, adeC,* and *abeM* genes among 61 *A. baumannii* isolates was 54 (88.5%), 61 (100%), 57 (93.9%), and 60 (98.3%), respectively (Figure1- 5). bla_{OXA-51} was investigated and detected in all isolates. Sequencing of PCR products showed conserved regions for the restricted sequence of *adeA, adeB, adeC, and abeM* genes, which was confirmed by BLAST in NCBI.

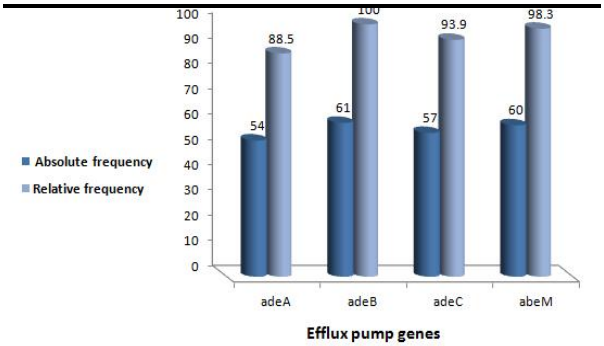


Figure 1. Absolute and relative frequency of efflux pumps genes in clinical isolates of *A .baumannii* by PCR method.

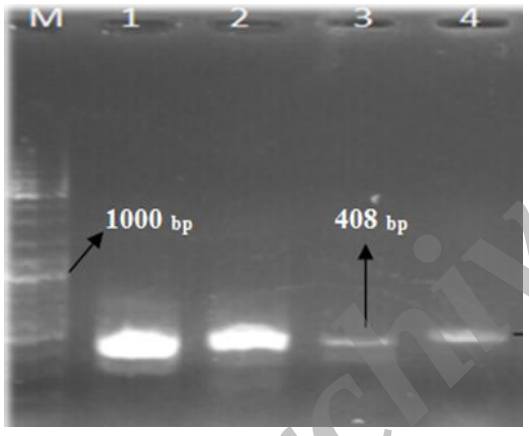


Figure 2. PCR product of *adeA*(408_{bp}) gene *A. baumannii* isolated in Tabriz, Northwest of Iran. M: 100 bp DNA size marker, line 1: positive control, line 2 through 4 respectively represents samples.

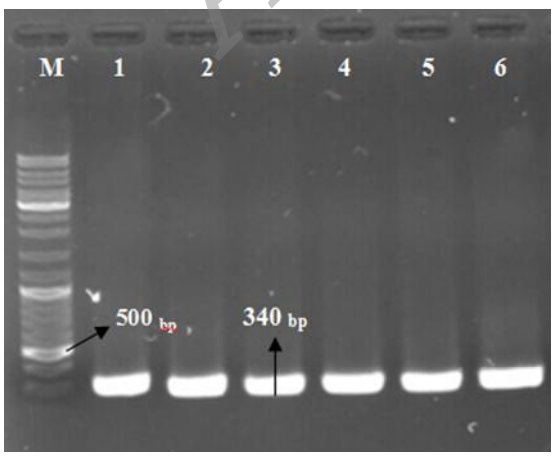


Figure 3. PCR product of *adeB* (340_{bp}) gene *A. baumannii* isolated in Tabriz, Northwest of Iran. M: 100 bp DNA size marker, line 1 through 6 respectively represent samples.

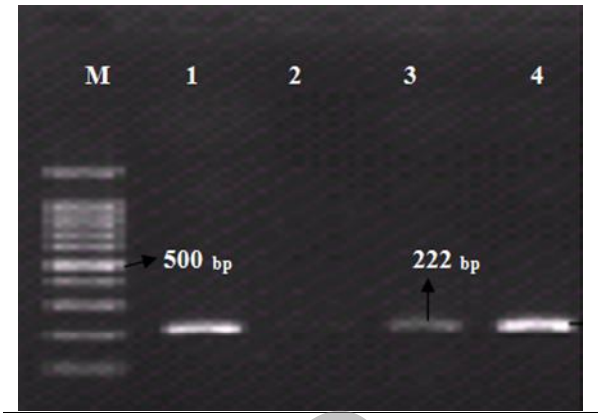


Figure 4. PCR product of *adeC* (222_{bp}) gene *A. baumannii* isolated in Tabriz, Northwest of Iran. M: 100 bp DNA size marker, line 1: Positive control, line 2: Negative control, line 3 through 4 respectively represent samples.

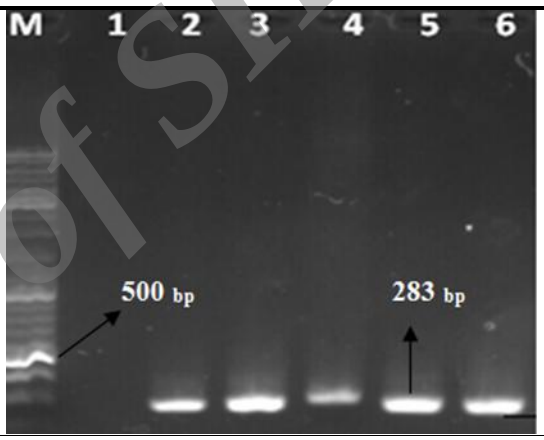


Figure 5. PCR product of *abe*M (283_{bp}) gene *A. baumannii* isolated in Tabriz, Northwest of Iran. M: 100 bp DNA size marker, line 1: Negative control, line 2: Positive control, line 3 through 6 respectively represent samples.

5. Discussion

Nosocomial outbreaks of MDR *A. baumannii* have been demonstrated in many studies (16, 17, 18). Many studies have reported the outbreak of PDR *A. baumannii*. In this study *A. baumannii* was highly sensitive to colistin (89%) and moderately sensitive to amikacin (52%) and tobramycin (50%). Despite the high sensitivity of *Acinetobacter* to colistin, its use should be limited to life threatening conditions because of serious neurological and renal side effects (19, 20). Another concerned problem is related to their multidrug resistance which restricts the treatment procedure (14). Various studies have clearly demonstrated that the incidence of resistant *A. baumannii* strains is increaseing worldwide (21). In general, susceptibility rates of *A. baumannii* isolates to third- and fourth-generation cephalosporins, carbapenems, fluoroquinolones, and trimethoprim/sulfamethoxazole (SXT) were very low.

In this study, the resistance to imipenem, ciprofloxacin, and amikacin by using E-test were 73.3, 93.3, and 38%, respectively, while resistance rates were reported by Boroumand et al. 53.4 and 24.6% to ciprofloxacin and imipenem, respectively (22). The gene *adeB* codes multidrug efflux pupm for the transmembrane protein of AdeABC. All isolates in the present study were found to carry the *adeB* gene. As described by Magnet et al., disruption of this gene leads to the loss of multidrug resistance (5). Because of its

necessity for AdeABC function, we investigated the prevalence of the *adeA, adeB, adeC,* and *abeM* genes among *A. baumannii* strains isolated from patients admitted to Imam Reza hospital, Tabriz, IR Iran by using PCR. Our study, showed high incidence of *adeA, adeB, adeC,* and *abeM* genes (88.5, 100, 93.9, and 98.3%, respectively) among 61 *A. baumannii* isolates. The results suggest that multidrug efflux pumps play a role in the mechanism of the resistance in *A. baumannii* strains. It has recently been reported that resistance to antibiotics is due to the overexpression of the AdeABC pump (12). In the Gholami study, the *adeA* and *adeB* were detected in 60 (100%) isolates, while *adeC* was detected in 51 (85%) isolates (23). The *adeA*, *adeB,* and *adeC* genes were found in 100, 100, and 96.5% of the isolates, respectively (24). The AdeABC operon was present in 80% (from 53 to 97%) of the A. baumannii strains (8). Similarly, Yan et al. reported the high distribution of *ade*B (100%) and *abeM* (100%) in genotypically, which emphasizes the multidrug resistance of genes that may possess along with the potential of horizontal gene transfer between polyclonal MDR *A. baumannii* strains (25). Also, Li Lin et al. reported that the majority of the isolates (75%), generally displaying high level of multidrug resistance, were positive for AdeABC and AdeIJK , suggesting a potential linkage between these genes and multidrug resistance (26).

6. Conclusion

A. baumannii is a typical opportunistic pathogen in hospital settings. The acquisition of resistance and overexpression of efflux pumps provide a successful strategy to survive, adapt, and be selected in this environment. Thus, using standard assays for susceptibility test and MIC breakpoint could be carried out to accurately monitor the resistant strains. Moreover, a novel approach would be the attempt to develop efflux inhibitors as a possible way for the development of new agents to control antimicrobial resistance in nosocomial pathogens, improved hygiene procedures and optimal drug use are necessary to limit the selection and reduction of such microorganisms.

Conflict of Interests

The authors declare they have no conflict of interests.

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Authors' Contribution

Goli Angoti and Maryam Hajizadeh collected the samples; Goli Angoti and Amaneh Kouchaki performed the experiments and wrote the manuscript; Maryam zarringhalam moghaddam analyzed data; Hossein Goudarzi and Mojgan Bandehpour were an advisor in the project and contributed to the analysis of the data.

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