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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 known as a high importance nosocomial microorganism isolated specially from intensive care units (ICUs), that causes severe infections. *A. baumannii* is usually multi drug resistant (MDR), showing resistance to the third generation cephalosporins, aminoglycosides, and fluoroquinolone (1, 2). Newly, *A. baumannii* isolates have proved to be extensively drug-resistant (XDR) which is determined as resistance to all antibacterial agents except tigecycline and colistin. The treatment of XDR *A. baumannii* infection physicians unable to treat well and limited management experience. Unfortunately, the extend 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, because of chromosomally encoded cephalosporinase production and low level of membrane impenetrability, b) acquired, transfering of foreign genetic information or mutation in endogenous structural or regulatory genes. Efflux systems are extensively available in microorganisms and admit resistance to various compounds including antibiotics, by deporting of the drug. As an important clinical issue are mutational events in the regulators of efflux systems, which simply can admit multidrug resistance (MDR) to the host by over expression of the pump. The pumps of the resistance-nodulation-cell division (RND) superfamily are common in Gram-negative bacteria and have the broadest substrate ranges (4).

Up to now, overexpression of three RND systems, AdeABC (5), AdeFGH (6), and AdeIJK (7), has been related with MDR in A. baumannii (8). The first pump explicated, AdeABC, admits resistance to aminoglycosides, tetracyclines, fluoroquinolones, chloramphenicol, and trimethoprim, and decreased susceptibility to tigecycline (9-11). AdeABC, initially, and AdeFGH play a main role in acquired resistance (8), while AdeIJK is responsible for intrinsic resistance (7). Production of AdeABC is controlled by a two-component regulatory system, AdeRS (12).

The another efflux pump, AbeM, which be a part of 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 help of this system to antimicrobial resistance in clinical isolates is unknown.

2. Objectives

This study aims to determine the prevalence of *adeA*, *adeB*, *adeC*, and *abeM* genes among *A. baumannii* strains isolated from patients admitted to Imam Reza medical center, Tabriz, IR Iran.

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3. Materials and Methods

This study was perform in the Imam Reza medical center laboratory in Tabriz in East Azerbaijan, northwest of IR Iran from June 2013 to march 2014.

3.1. Bacterial isolates

Sixty-one strains of *A. baumannii* were isolated from blood, wound, abscesses, urine, sputum, respiratory tract, and fluid bodies of patients from Imam Reza medical center in Tabriz, IR Iran. The isolates were assigned by conventional biochemical methods (14) and confirmed for bla_{OXA-51} to the species.

3.2. Susceptibility testing

In this study, antibiotic susceptibility pattern was performed on Mueller-Hinton Agar (Merck, Germany) by Kirby Bauer's disk diffusion technique. This method was done according to CLSI recommendations 2012 (15). Antimicrobial agents tested were the antibiotic disks which were comprised 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 enterpreted 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 (AB Biodisk, Solna, Sweden) were determined using E- test method according 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 microtubes 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 at 14000 rpm for 5 min. Supernatant containing DNA was extracted and used as template for PCR techniques.

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

Efflux pump genes, *adeA*, *adeB*, *adeC*, *and abeM*, were detected by PCR with designed specific primers, which are presented in Table 1. The PCR assay was done 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 5 min at 95°C for initial denaturation step, 30 cycles of 30s at 95°C, 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 done according to a final extension step at 72°C for 5 min. PCR products were analyzed by 1% agarose gel and stained with ethidium bromide. Statistical analyses were calculated by using SPSS16 software, X_2 statistical significance was defined as P-value <0.05.

3.6. Nucleotide Sequencing

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.

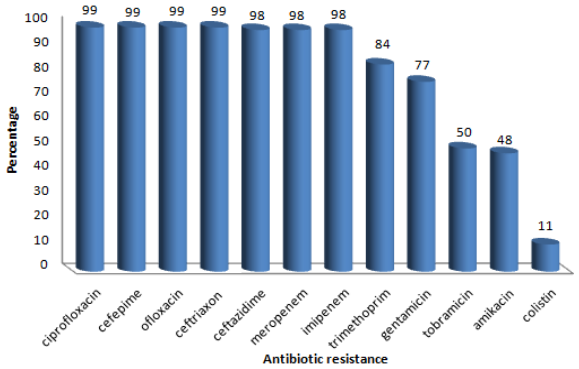
4. Results

In this study, sixty-one strains were isolated from Imam Reza medical center. 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 cases. 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: trimethoprim-sulfamethoxazole 51 (84%), ceftazidime 59 (98%), ciprofloxacin 60 (99%), amikacin 29 (48%), gentamicin 46 (77%), tobramicin 30 (50%), imipenem 60 (99%), meropenem 60 (99%), ceftriaxon 60 (99%), cefepime 60 (99%), ofloxacin 60 (99%), colistin 6 (11%) (Table 2).

Table 1. The targeted genes and related design primers used for amplification.

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. Antibiotic resistance of *A.baumannii* strains.



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 strains. Sequencing of PCR products revealed conserved regions for the restricted

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sequence of *adeA, adeB, adeC, and abeM* genes, which was confirmed by BLAST in NCBI.

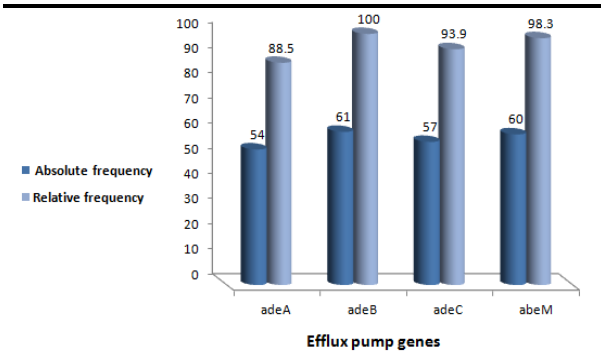

Figure 1. Absolute and relative frequency of efflux pumps genes in sample isolates of *A .baumannii* by PCR method.

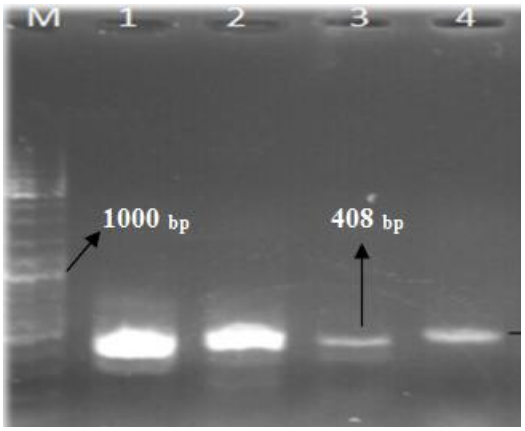

Figure 2. PCR results of *adeA*(408_{bp}) gene *A. baumannii* isolated in Tabriz, Northwest of Iran. M: 100 bp DNA size marker, line 1: positive control, line 2 through 4 respectively represents samples.

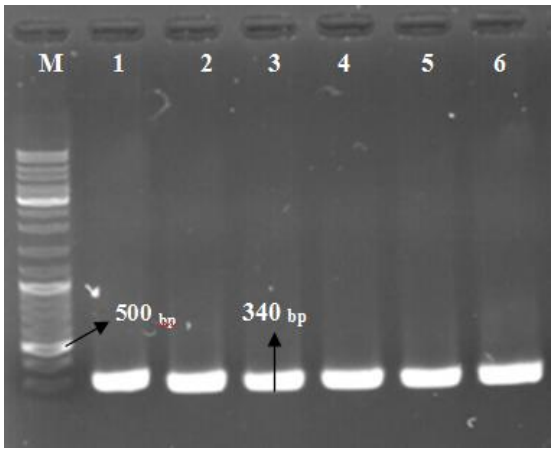

Figure 3. PCR product of *adeB* (340_{bp}) gene *A. baumannii* isolated in Tabriz, Northwest of Iran. M: 100 bp DNA size marker, line 1 through 6 respectively represent samples.

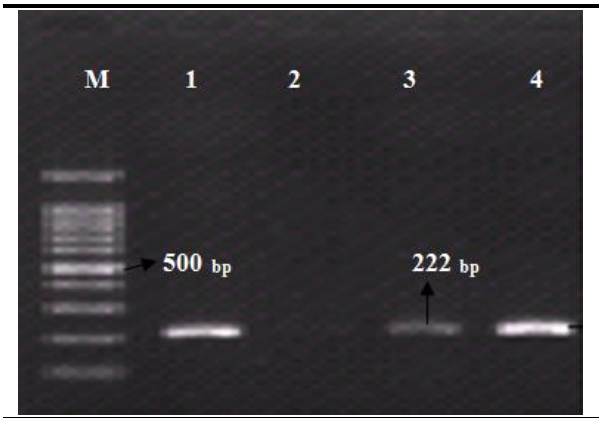

Figure 4. Results of PCR product of *adeC* (222_{bp}) gene *A. baumannii* isolated in Tabriz, Northwest of Iran. M: 100 bp DNA size marker, line 1: Positive control, line 2: Negative control, line 3 through 4 respectively represent samples.

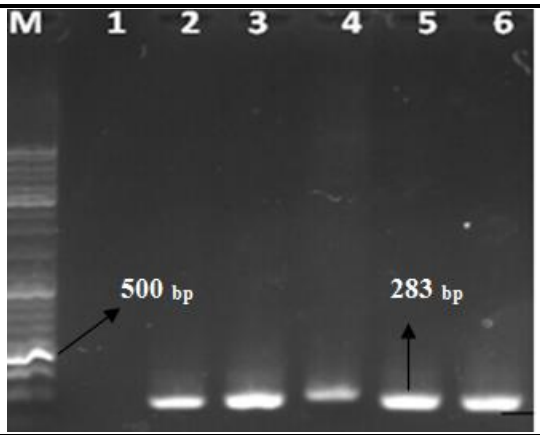

Figure 5. PCR product of *abe*M (283_{bp}) gene *A. baumannii* isolated in Tabriz, Northwest of Iran. M: 100 bp DNA size marker, line 1: Negative control, line 2: Positive control, line 3 through 6 respectively represent samples.

5. Discussion

Nosocomial outbreaks of MDR *A. baumannii* have been demonstrated in many studies (16, 17, 18). Many studies has been shown 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 point in the current study was concerned problem is related to their multidrug resistance which restricts the treatment procedure (14). Several studies have clearly indicated 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 pump for the transmembrane protein of AdeABC. All isolates in the current study were found to carry the *adeB* gene. As illustrated 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

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the *adeA, adeB, adeC,* and *abeM* genes among *A. baumannii* strains isolated from patients admitted to Imam Reza medical center, 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 our 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. described that the majority of the isolates (75%), generally acquiring high level of multidrug resistance, were positive for AdeABC and AdeIJK , implying a potential correlation between these genes and multidrug resistance (26).

6. Conclusion

A. baumannii is a 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. Hence, using standard assays for susceptibility test and MIC breakpoint could be achieved to accurately monitor the resistant strains. Furthermore , a new approach would be the effort to develop efflux inhibitors as a possible way for the proceeding of new agents to control antimicrobial resistance in nosocomial pathogens, developed hygiene procedures and optimal drug use are vital 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 instructor in the project and assessment to the analysis of the data.

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