ORIGINAL ARTICLE

Distribution of Plasmid-Mediated Quinolone Resistance, Integrons and AdeABC Efflux Pump Genes in Nosocomial Isolates of *Acinetobacter baumannii*

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Abstract:

Background: Acinetobacter baumannii is an opportunistic pathogen associated with nosocomial infections. Extensive use of quinolones has resulted in an increase of resistance in this organism worldwide. *Aim and Objectives:* To study the association between PMQR genes, integron carriage as well as the possible role of AdeABC efflux pump in ciprofloxacin-resistance as well as multidrug resistance in clinical isolates of *A. baumannii*. We studied the presence of Plasmid-Mediated-Quinolone Resistance (PMQR); AdeABC efflux pump genes and integron carriage in Intensive Care Unit (ICU) isolates of *A. baumannii*. *Material and Methods:* Fifty six non-duplicate clinical isolates of *A. baumannii* were obtained from two hospital ICUs in Tehran from March 5th 2014 to July 20th 2015. Susceptibility to 10 antibiotics was determined by disc diffusion. Presence of PMQR (*aac(6')-Ib-cr*, *qnrA*, *qnrB*, *qnrC*, *qnrD* and *qnrS*), a*deABC* efflux and class I and II integron genes were detected by Polymerase Chain Reaction (PCR). *Results:* All isolates were Multidrug-Resistant (MDR) among which, *qnrB* and *aac(6')-Ib-cr* were detected in 7.1% and 26.8% of the isolates, respectively. However, *qnrA*, *qnrC*, *qnrD* and *qnrS* were not observed. Presence of *adeA* and *adeB* was observed in 100% and *adeC* in 73.2% of the isolates. Overall, integron carriage was observed in (94.6%) of the isolates including *qnrB* positive and 73.3% of the *aac(6')-Ib-cr* carrying isolates. *Conclusion:* Our results show that quinolone-resistance is not associated with PMQR genes. On the other hand, the AdeABC efflux pump is clearly responsible for MDR in our *A. baumannii* isolates including resistance to quinolones. No association was found between PMQR and integron carriage.

Keywords: *Acinetobacter baumannii*, Quinolone, Nosocomial Infections, Integron

Introduction:

Acinetobacter baumannii is an opportunistic pathogen associated with nosocomial infections, especially among patients admitted to Intensive Care Unit (ICU). The bacterium is capable of causing various infections including; urinary tract, wound, skin and soft tissue infections, pneumonia and meningitis [1, 2]. Over the past few years, the extensive use of quinolones and fluoroquinolones in humans has resulted in an increase of resistance to these agents worldwide [3]. Quinolone resistance mechanisms in *A. baumannii* include chromosomal mutations in DNA gyrase (*gyrA*/*gyrB*), topoisomerase IV (*parC*/*parE*), and Plasmid-Mediated Quinolone Resistance (PMQR) [3-5]. PMQR determinants include *qnr* genes which encode pentapeptide repeat proteins QnrA, QnrB, QnrS, QnrC, and QnrD which protect DNA gyrase and topoisomarase IV from fluoroquinolones [4-6]. In addition, other fluoroquinolone resistance mechanisms involve enzymatic modification of fluoroquinolones by the aminoglycoside acetyltransferase, Aac(6')-Ib-cr (capable of reducing the activity of Norfloxacin

and Ciprofloxacin), and efflux pump-mediated resistance by QepA and OqxAB [7, 8]. Furthermore, the AdeABC efflux pump from the RND family of efflux pumps is reported to be a major factor in Multidrug Resistance (MDR) in *A. baumannii* [9]. The AdeABC consists of an *adeA* gene encoded membrane fusion protein, an *adeB*-encoding membrane protein and an *adeC*-encoding outer membrane protein [10]. The *adeABC* operon is found in 81% of *A. baumannii* strains and its overexpression is thought to be responsible for MDR in this organism [11]. AdeABC efflux pump was shown to be responsible for decreased susceptibility to a broad spectrum of antibiotics including some β-lactams, aminoglycosides, tetracyclines, erythromycin, chloramphenicol, trimethoprim and fluoro-quinolones [10]. In addition, problems arise when antibiotic resistance genes are located on genetic elements such as integrons. Presence of integrons on plasmids often facilitates their horizontal transmission [12]. Among the antibiotic resistance integrons, classes I and II are most frequently found in Gram-negative pathogens including MDR isolates of *A. baumannii* [13-16]. The aim of this research was to study the association between PMQR genes, integron carriage as well as the possible role of AdeABC efflux pump in Ciprofloxacin-resistance as well as multidrug resistance in clinical isolates of *A. baumannii*.

Material and Methods:

Bacterial Isolates:

Fifty six MDR isolates of *A. baumannii* were employed. The test isolates were collected from Imam Hossein (n=38) and Ebnesina (n=18) hospitals in Tehran from March 5th 2014 to July 20th 2015 based on their resistance to multiple antibiotic classes. Among these, 46 were from sputum specimens, three were from urine, three from wound, two from catheters and two from blood. Bacteria were identified using standard biochemical tests as well as the presence of the bla_{OXA-51} gene intrinsic in *A. baumannii.* The isolates were stored at -20°C in brain heart infusion broth containing 8% dimethyl sulfoxide (v/v) until use.

Antibiotic Susceptibility Testing:

Antibiotic susceptibility profiles of the test isolates were confirmed against 10 antibiotics, performed by disc diffusion according to the 2017 CLSI Guidelines using commercially available discs (Mast, UK) including: cefepime (30 μg), cefotaxime (10 μg), amikacin (10 μg), gentamicin (30 μg) ciprofloxacin (5 μg), piperacillin (100 μg), piperacillin-tazobactam (110 μg), imipenem (10 μg), meropenem (10 μg) and aztreonam (30 μg) [17].

DNA extraction and Polymerase Chain Reaction (PCR) amplification of bla_{oxa-51} gene:

DNA extraction was carried out using the phenol:chloroform method [18]. The DNA was then stored at -20 °C before use. Detection of bla_{oxa-51} gene was carried out using primer: 5'-TAATGCTTTGATCGGCCTTG-3' (forward), and 5'-TGGATTGCACTTCATCTTGG-3' (reverse), resulting in an amplification product of 353 bps [13]. PCR reaction mixtures (25 μL) contained 1 μL of DNA template, 1.4 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 μM primer, and 0.6 U of Taq DNA polymerase in the buffer provided by the manufacturer (CinnaGen, Tehran, Iran). The amplifications were performed in a Peltier thermocycler (MG25^{+}, Long Gene Scientific

Instruments, China) using the following program: initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 25 sec, 52°C for 40 sec and 72°C for 50 sec, followed by a final elongation step at 72°C for 6 minutes. PCR products were separated on 1% agarose gels and visualized after staining with RedSafe (iNtRON Biotechnology, Korea) using an image analysis system (UVLtec; St John's Innovation Centre, UK). Detection of *adeA*, *adeB, adeC, intI* and *intII* genes was carried out using the primers listed in Table 1 [19-25]. PCR reaction mixtures employed were the same as those used for PMQR genes except that 1 μL of template DNA was used. Gene amplification was carried out using conditions presented in Table 2.

Table 1: Primers Used for Amplification of Quinolone-Resistance Genes

Gene	Primer	Sequence (5' → 3')	Product Length, bp	Reference
qnrA	Forward Reverse	TTCTCACGCCAGGATTTGAG TGCCAGGCACAGATCTTGAC	571	19
qnrB	Forward Reverse	GATCGTGAAAGCCAGAAAGG ACGATGCCTGGTAGTTGTCC	469	20
qnrS	Forward Reverse	GACGTGCTAACTTGCGTGAT AACACCTCGACTTAAGTCTGA	388	19
qnrC	Forward Reverse	GGGTTGTACATTTATTGAATCG CACCTACCCATTTATTTTCA	307	21
qnrD	Forward Reverse	CGAGATCAATTTACGGGGAATA AACAAGCTGAAGCGCCTG	540	22
aac(6')-Ib-cr	Forward Reverse	TTGCGATGCTCTATGAGTGGCTA CTCGAATGCCTGGCGTGTTT	482	23
adeA	Forward Reverse	ATCTTCCTGCACGTGTACAT GGCGTTCATACTCACTAACC	513	24
adeB	Forward Reverse	TTAACGATAGCGTTGTAACC TGAGCAGACAATGGAATAGT	541	24
adeC	Forward Reverse	TACGGACTGCTACGCTTAAT AACAGGATGACCTGCTAACA	527	24
intI	Forward Reverse	ACGAGCGCAAGGTTTCGGT GAAAGGTCTGGTCATACATG	565	25
intII	Forward Reverse	GTGCAACGCATTTTGCAGG CAACGGAGTCATGCAGATG	403	25

Table 2: Programs Used for Amplification of PMQR, *adeABC, intI* and *intII* Genes in Clinical Isolates of *A. baumannii*

Gene	PCR conditions					
	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension	No. cycles
qnrA	94°C, 5 m	94°C, 1 m	57°C, 1 m	72°C, 1m	72°C, 10 m	30
qnrB	94°C, 5 m	94°C, 1 m	53°C, 45s	72°C, 1m	72°C, 10 m	35
qnrS	94°C, 5 m	94°C, 1 m	57°C, 1m	72°C, 1m	72°C, 10 m	30
qnrC	94°C, 5 m	94°C, 1 m	55°C, 1m	72°C, 1m	72°C, 10 m	35
qnrD	94°C, 5 m	94°C, 1 m	48°C, 1m	72°C, 1m	72°C, 10m	35
aac(6')-Ib-cr	94°C, 5 m	94°C, 1 m	54°C, 1m	72°C, 1m	72°C, 10m	30
adeA	94°C, 5m	94°C, 1 m	56°C, 1 m	72°C, 1m	72°C, 7 m	30
adeB	94°C, 5m	94°C, 1 m	56°C, 1 m	72°C, 1m	72°C, 7 m	30
adeC	94°C, 5m	94°C, 1 m	56°C, 1 m	72°C, 1m	72°C, 7 m	30
intI	95°C, 5m	94°C, 1 m	54°C, 1m	72°C, 1m	72°C, 10 m	30
intII	95°C, 5m	94°C, 30 s	52°C, 30 s	72°C, 2m	72°C, 7 m	30

Results:

Among the 56 ICU clinical isolates of *A. baumannii,* 46 (82.2%) were from sputum specimens, three (5.3%) from wound, three (5.3%) from urine, two (3.6%) from blood and two (3.6%) were recovered from catheters. The identification of all isolates was confirmed by both biochemical tests as well as the presence of bla_{OXA51} gene. Antibiotic resistance results showed that 100% of the isolates were resistant to cefepime, cefotaxime, piperacillin, piperacillin-tazobactam, imipenem, meropenem, aztreonam, amikacin and ciprofloxacin. Resistance to gentamicin was detected in 51 isolates (91.1%). These results show that regardless of the source, all isolates were multidrug-resistant (MDR).

Fig. 1 is representative image of PCR amplification results for PMQR genes, as well as *adeABC, intI* and *intII* genes. Among PMQR genes, 4/56 isolates (7.1%) had the *qnrB* and 15/56 (26.8%) carried the *aac(6')-Ib-cr* gene. One isolate carried both *aac(6')-Ib-cr* and *qnrB* gene. The majority of the *aac(6')-Ib-cr* harboring isolates (12/15, 80%) were from Imam Hossein ICU patients and the three remaining isolates were from Ebnesina Hospital. Other PMQR genes (*qnrA*, *qnrC*, *qnrD* and *qnrS*) were not observed. On the other hand, efflux pump genes *adeA*, *adeB* were present in all isolates (100%) and the *adeC* was detected in 41/56 isolates (73.2%).

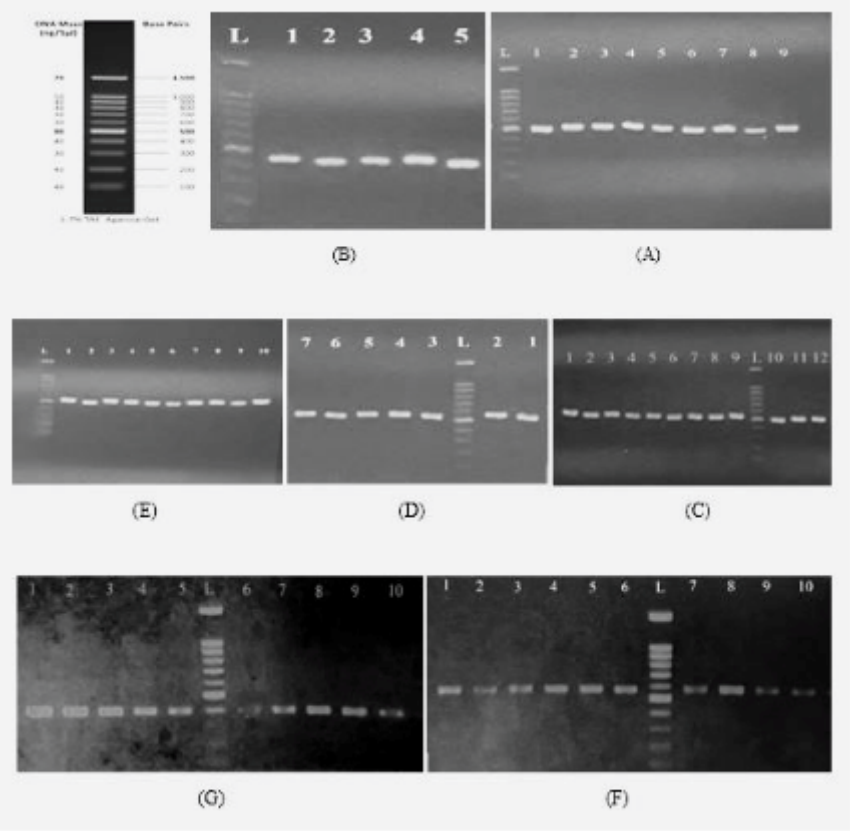

Fig. 1: PCR Amplification Products of [A] *aac(6')-Ib-cr* (482 bp); [B] *qnrB* (469 bp); [C] *adeA* (513 bp); [D] *adeB* (541 bp); [E] *adeC* (527 bp); [F] *int1* (565 bp); [G] *int2* (403 bp) Genes in a Number of *Acinetobacter baumannii* isolates. [L] 100bp DNA Ladder, Positive Controls are shown in Lane 1 of all Gels

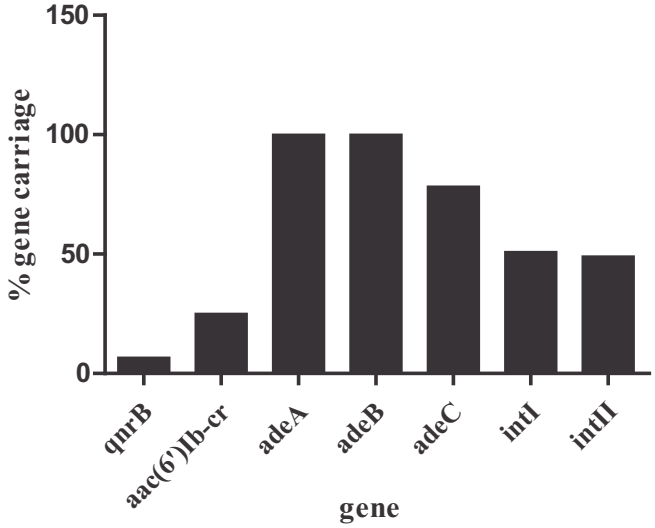

Fig. 2: Distribution of PMQR, a*deABC* and Integron Genes in Clinical Isolates of *A. baumannii*

Integron carriage was observed in 53 isolates (94.6%) of which 27 (50.9%) had class I and 26 (49.1%) harbored class II integron. Among these, 11 (20.7%) carried both integron classes. All *qnrB* positive isolates and the majority of the *aac(6')-Ib-cr* harboring isolates (11/15, 73.3%) carried integrons. There was no association between PMQR gene carriage with ciprofloxacin resistance or integron class type. However, integron carrige was significantly associated with the presence of the AdeABC efflux pump in our MDR *A. baumannii* isolates. Overall, the results of the present research show that along with the other tested antibiotics, ciprofloxacin resistance in our 56 MDR *A. baumannii* isolates was due to the presence of the AdeABC efflux pump and not the *qnr* genes Fig. 2 represents the overall distribution of quinolone-resistance, a*deABC* and integron genes in our MDR clinical isolates of *A. baumannii.*

Discussion:

Quinolones are among the antibiotic agents which are frequently employed for treatment of Gram-negative related bacterial infections. In this study, we investigated the presence of PMQR genes, the AdeABC efflux pump and integron carriage in 56 clinical isolates of *A. baumannii*. Of the six PMQR genes studied, *qnrB* and *aac(6')-Ib-cr* genes were found in 7.1% and 26.8% of isolates, respectively. The other *qnr* genes were not observed in any of the isolates. Two studies from China have reported the presence of *qnrB* in 8.1% and 7.7% of *A. baumannii* clinical isolates in 2014 and 2016, respectively [26, 27]. Similar to our results, they also showed that the other PMQR genes (*qnrA*, *qnrC*, *qnrD*, *qepA* and *oqxAB*) were not found in their test isolates. The frequency of *aac(6')-Ib-cr* gene carriage in our clinical isolates of *A. baumannii* was 26.8%. Yang *et al.* reported that the *aac(6')-Ib* gene was found in 56.4% of *A. baumannii* clinical isolates in China in 2015 [27] Khorsi *et al.*, showed that the rate of the *aac(6')-Ib* gene carriage was 31.5% in Algeria [28]. Overall, the low frequency of PMQR gene carriage in the present study as well as the other reports, suggest that PMQR genes may not play a key role in quinolone resistance in *A. baumannii*.

Another important mechanism of antibiotic resistance in Gram-negative pathogens is the AdeABC efflux pump which has been shown to have a role in resistance to multiple antibiotics including quinolones [13]. In this study, the AdeABC efflux pump genes, *adeA*, *adeB* were found in all isolates whereas *adeC* was present in 73.2% of the isolates. Modersi *et al*. (2008) showed the presence of *adeA* (92%), *adeB* (61.5%) and *adeC* (84.6%) in 65 MDR *A. baumannii* ICU isolates [29]. Gholami *et al*. (2013) showed that the frequency of *adeA*, *adeB* and *adeC* genes was 60%, 100%, and 85% in Iranian isolates of *A. baumannii*, respectively [30]. Japoni-Nejad *et al*. (2014) also showed that 100% of their MDR *A. baumannii* isolates carried *adeA*, *adeB* genes and 96.5% harbored *adeC* [31]. Wong *et al.* (2009) showed that 92.3% of carbapenem-resistant *A. baumannii* carried *adeA*; *adeB* and 18.9% had *adeC* [32]. In a study from Iraq, Jassim *et al.* (2015) reported that 77.4%, 100% and 83.3% of MDR *A. baumannii* isolates carried *adeA*, *adeB* and *adeC* genes, respectively [33]. Majority of the studies report a lower frequency of the *adeC* gene in clinical isolates of *A. baumannii* compared to *adeA* and *adeB*. In a comprehensive study, Marchand *et al.* reported that the *AdeB* and *AdeA* are essential for antibiotic resistance in *A.*

baumannii. However, presence of the *AdeC* protein in the outer membrane is not essential and could be replaced by other outer membrane proteins [11].

In this research, integron carriage was detected in 53 isolates (94.6%) which had similar rates of class I and class II integron carriege (n=27, 50.9% and n=26, 49.1%, respectively. In addition, 11 (20.7%) carried both integron classes. A number of Iranian studies have shown the presence of class I and II integrons in clinical isolates of *A. baumannii.* Mirnejad *et al*. (2013) showed a higher frequency of class II integron carriage clinical isolates of *A. baumannii* [16]. However, the majority of the studies show a higher frequency of class 1 integron in MDR clinical isolates of *A. baumannii* [14, 30, 31, 34, 35]. Nourbakhsh *et al*. (2005) showed that the incidence of class I, II and III integrons as 100%, 44% and 3%, in clinical isolates of *A. baumannii,* respectively [36]. Lin *et al*. showed an association between class 1 integron carriage and *adeABC* in MDR isolates of *A. baumannii* [24]. Similarly, we also found a strong association between integron carriage and *adeABC* efflux genes in our MDR *A. baumannii* isolates.

Conclusion:

Our results showed that quinolone-resistance is not associated with PMQR genes. On the other hand, the AdeABC efflux pump was clearly responsible for MDR in our *A. baumannii* isolates including resistance to quinolones. No association was found between PMQR and integron carriage.

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