Iran Red Crescent Med J. 2017 September; 19(9):e58798. doi: 10.5812/ircmj.58798.

Published online 2017 July 26. **Brief Report**

Determination of Different Fluoroquinolone Mechanisms Among Clinical Isolates of *Acinetobacter baumannii* in Tehran, Iran

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Received 2016 November 23; **Revised** 2017 February 06; **Accepted** 2017 March 04.

Abstract

Background: *Acinetobacter baumannii* isolates resistant to fluoroquinolones, such as levofloxacin and ciprofloxacin are being increasingly developed every day.

Objectives: In this study, ciprofloxacin resistance in *A. baumannii* isolates was determined by the presence or absence of efflux pump inhibitors, as the efflux pumps play an important role in the creation of ciprofloxacin resistance.

Methods: One hundred and three *Acinetobacter* isolates were collected from ventilator-associated pneumonia (VAP) and burn patients of Tehran hospitals, Iran, during six months of 2014. Susceptibility rates of the isolates to levofloxacin and ciprofloxacin antibiotics were assessed using the agar disk diffusion and broth microdilution. The effects of the efflux pump inhibitors including phenylalanine-arginine β-naphthylamide (PAβN) and 1-(1-naphtylmethyl)-piperazine (NMP) on ciprofloxacin resistance were investigated. Further, the quinolone resistance *qnrA*, *qnrB*, *qnrS*, and *adeABC* genes were evaluated using the polymerase chain reaction (PCR) test. Finally, to examine the mutation in quinolone resistance-determining regions, the PCR products of the *gyrA* and *parC* genes were sequenced.

Results: According to the results of the antibiogram test, 74.7% and 33% of the studied isolates were resistant to ciprofloxacin and levofloxacin, respectively. Also, there was a significant relationship between the type of the specimen and resistance to ciprofloxacin (P = 0.02) and resistance to levofloxacin (P = 0.04). As for the synergistic study of the inhibitors with ciprofloxacin, the reduction of minimum inhibitory concentration (MIC) was observed in 40% and 56.6% of the isolates in the presence of PAβN and NMP, respectively. The prevalence rates of *qnrA*, *qnrB*, *qnrS*, *AdeA*, *AdeB*, and *AdeC* genes were 0%, 0%, 3.9%, 100%, 100% and 100%, respectively. In all the resistant isolates, mutation of in the *gyrA* gene was observed, but no mutation was seen in the *parC* gene.

Conclusions: The presence of the efflux pumps and the *gyrA* gene mutation are still considered as the most important factors causing fluoroquinolone resistance; however, identification of the *qnr* genes for the first time in Tehran hospitals, Iran, can lead to further concerns in the future.

Keywords: A. baumannii, Resistance Patterns, Fluoroquinolones

1. Background

Acinetobacter baumannii is a Gram-negative, nonfermentative and oxidase-negative coccobacillus which, in nature and especially in hospital environments, causes opportunistic infections such as meningitis, pneumonia, respiratory and urinary tract infections, infections of patients hospitalized in intensive care unit (ICU), and burn infections. (1-4). Bacteremia is caused by toxic shock in 25% to 30% of the cases and usually occurs due to disseminated intravascular coagulation. Colonization might occur after invasive infection and often among burn patients (5, 6). The mortality rate in the hospitals is high, about 23% and 43% in the hospitalized patients and patients in ICU, respectively (7). Today, *Acinetobacter* isolates have become resistant to many antibiotics. According to the reports, more than 80% of the isolates are aminoglycoside-resistant; further, resistance to quinolones is also expanding, which leads to an increase in therapeutic problems and concerns (8). Both inherent and acquired mechanisms could result in such resistances (9). Resistance to quinolones is developed through different methods, one of which is the changes occurred in the expression of the efflux pumps. The efflux pump in *A. baumannii* is the AdeABC pump and is of great importance in terms of resistance creation (10). One of the mechanisms to deal with the antibiotic resistance is to block or disrupt the mechanisms leading to

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resistance. Inhibiting the efflux pumps is a strategy of acting through disrupting the energy system required for drug disposal, blocking the efflux pump, or preventing the formation and assembly of the pump. The PAβN inhibits the efflux pump by blocking, and exhibits a state of competition with antibiotics, while the mechanism of 1-(1-naphtylmethyl)-piperazine (NMP) inhibitor has not been known yet. Determining ciprofloxacin resistance in *A. baumannii* isolates was performed both in the presence and in the absence of efflux pump inhibitors (11-14). Another mechanism that causes resistance to quinolones is the presence of quinolone resistance (*qnr*) genes located on the plasmid, which leads to a low-level resistance to quinolones. These proteins are of low frequency, and most of the studies have been conducted on the bacteria of the Enterobacteriaceae family. Studies performed to identify the *qnr* genes in *A. baumannii* isolates have not been so successful (15). Another important mechanism in the creation of quinolones resistance is mutation in quinolone resistance-determining regions (QRDR), where the target enzymes of DNA gyrase (*gyrA*) and Topoisomerase IV (*parC*) are affected. The major impact of quinolones is on the target enzymes such as DNA gyrase, which inhibit the transcription process by binding to and causing mutation in the gene of this enzyme (16).

Based on the above-mentioned requirements of increased *Acinetobacter* resistance to most of the antibiotics and the importance of fluoroquinolones in the treatment of infections caused by these isolates, the present study investigated various mechanisms of resistance to fluoroquinolones, efflux pump, *qnr* genes or mutation, as well as reduction of resistance through inhibition of efflux pumps by two efflux pump inhibitor compounds of PAβN and NMP.

2. Methods

2.1. Bacterial Isolates and Identification

In this cross sectional study, 103 *Acinetobacter* isolates were collected from two general hospitals in Tehran, Iran, during six months of 2014. Seventy isolates were collected from the ventilator-associated pneumonia (VAP) patients from the ICU of Rasool Akram Hospital and 33 isolates from burn patients of Shahid Motahhari Hospital. To confirm phenotypic and genotypic of the specimens of *A. baumannii*, biochemical tests and polymerase chain reaction (PCR) test were used. After culturing the bacteria on the nutrient agar medium, routine tests including growth at temperatures of 45°C and 37°C, and acid production in oxidative fermentative glucose (Merck, Germany) were conducted to identify the species of *A. baumannii* (7). For final confirmation of the *Acinetobacter* isolates, inherent genes, including bla$_{OXA\text{-}51\text{-}like}$ and *gyrB*, which are the main characteristics of *A. baumannii* species, were tested using the PCR test) Ependorf, Mastercycler Gradient). After confirmation, specimens were transmitted to a medium consisting of 15% glycerol and 85% brain heart infusion (BHI) liquid medium) Merck, Germany) for storage and maintenance in a freezer with the temperature of -70°C (7).

2.2. Antibiotic Susceptibility Testing

Determination of susceptibility to levofloxacin and ciprofloxacin antibiotics (MAST, England) was conducted using a disk agar diffusion method. To analyze the susceptibility rates, the diameters of the zones of inhibition were measured and categories of susceptible, intermediate or resistant were determined. After examination of the antibiogram results, ciprofloxacin resistant and intermediate isolates were used to conduct minimum inhibitory concentration (MIC) for ciprofloxacin (sigma, Aldrich Belgium) using broth microdilution. The CLSI 2015 criteria were used in both methods (17).

2.3. Examining the Effects of PAβN and NMP Compounds on Minimum Inhibitory Concentration of Ciprofloxacin

The PAβN and NMP (sigma, Aldrich Belgium) compounds were prepared and added to ciprofloxacin. Based on the protocol, the concentration of PAβN should be 5 mg/mL; thus, 5 mg of this material was solved in 1 mL of distilled water; further, the amount that should be added to each well was 2 μL; therefore, the inhibitor was mixed with the culture medium and then added to each well. The size of the culture medium was reduced and an inhibitor of the same amount was added, so that in each microplate, there were 48 μL of the adjusted Mueller-Hinton medium (Merck, Germany) and 2 μL of PAβN. After dilution of the antibiotic, the bacterial suspension was added by the same amount, ie, 50 μL (final volume of 100 μL), and all the remaining steps were conducted as MIC (18). As for the NMP inhibitor, based on the protocol, first 100 mg of the inhibitor was solved in 2 mL of DMSO, and then 2 mL of 0.25 molar HCL was added to it. Next, the volume reached 10 mL using distilled water. Then, after preparation, it was added to the culture medium, the same way as PAβN, and the MIC steps were performed (19).

2.4. Determination of Frequency of Antibiotic-Resistant Genes

The PCR test was used to examine the presence of genes including *qnrA*, *qnrB*, *qnrS*, and *adeABC* among *Acinetobacter* isolates. DNA of the specimens was extracted using the phenol-chloroform-isoamyl alcohol method. Then, the

used primers were diluted, in accordance with the protocol, by adding a certain amount of sterile distilled water and, thereby, the solutions were provided (Table 1). The final volume for each reaction was 25 μL. For preparation of the reactions, the following steps were performed: The master mix was provided based on the number of specimens supposed to undergo PCR. Twenty μL of master mix and 5 μL of the specimen were added to the vial; thus, the total volume for the test was 25 μL. The vials were placed inside the thermocycler. The temperature conditions of the PCR reaction were: the initial denaturation at 95°C for 1 minutes, 35 cycles at 94°C for 54 seconds, annealing at 53°C for 43 seconds, extension at 72°C for 1 minutes, and final extension at 72°C for 10 minutes. After the PCR reaction on the DNA resulted from the specimens, the products reproduced in PCR were electrophoresed on 1.5% agarose to observe DNA pieces. The stain safe DNA (SYBR) was used for staining DNA pieces. The voltage required for electrophoresis was 100 V and its duration was 50 minutes. Five microliters of the PCR product was mixed with 4 μL of Loading Buffer 6X (Thermo Scientific) and then placed in the gel wells for electrophoresis (BIO-RAD power pac basic electrophoresis power supply) (20, 21).

2.5. Sequencing the Quinolone Resistance-Determining Regions to Examine Mutation

For sequencing the QRDR region, first the PCR with the volume of 50 μL was conducted for *gyrA* and *parC* genes; then, the reaction products were confirmed through observing the size band in the agarose gel (Gel Doc XR+, Bio-Rad). Afterwards, the PCR products of both genes were sequenced through double-stranded sequencing using the primer pairs. The sequencing was performed by the Korean Macrogen Inc, and the subsequent steps, including the mutagenic sequence analysis and nucleotide conversion to amino acid, were performed using ClustalW (Lasergene MegAlign software package v.6.1).

2.6. Statistical Analysis

Chi-square analysis using the SPSS software version 21 was used for statistical analyses. A P value $\leq$ 0.05 was considered as statistically significant.

3. Results

3.1. Phenotypic and Genotypic Verification of Specimens

After the complementary biochemical tests, all of the one hundred and three isolates were identified as *A. baumannii*. All the specimens contained *gyrB* and bla$_{OXA-51}$ inherent genes identified as *A. baumannii*.

Table 1. Oligonucleotide Primers Used in the Study

Gene	Nucleotide Sequence (5'-3')	Amplicon Size (bp)	References
bla$_{OXA-51-like}$	TAATGCTTTGATCGGCCTTG TGGATTGCACTTCATCTTGG	353	(22)
gyrB	TGG ATT GCA CTT CAT CTT GG	294	(23)
	AACGGAGCTT-GTCAGGGTTA		
	TGG CAC TTC ACT ATC AAT AC	490	
qnrA	AGAGGATTTCTCACGCCAGG TGCCAGGCACAGATCTTGAC	580	(20)
qnrB	GGMATHGAAATTCGCCACTG TTTGCYGYYCGCCAGTCGAA	264	(20)
qnrS	GCAAGTTCATTGAACAGGGT TCTAAACCGTCGAGTTCGGCG	428	(20)
Ade A	CTCTAGCCGATGTCGCTCAA ATACCTGAGGCTCGCCACTG	510	(24)
Ade B	AAGTATGAATTGATGCTGC AACGATTATATTGTTGTGG	862	(24)
Ade C	TGCGGCAGGTTAGCCCATCG GCGGAACAGGATGACCTGCT	435	(24)
parC	AAACCTGTTCAGCGCCGCATT AAAGTTGTCTTGCCATTCACT	327	(25)
gyrA	AAATCTGCCCGTGTCGTTGG GCCATACCTACGGCGATACC	285	(26, 27)

3.2. Determination of Susceptibility to Antibiotics

According to the results of the antibiogram test, 74.7% and 33% of the studied isolates were resistant to ciprofloxacin and levofloxacin, respectively. The antibiotic susceptibility pattern of the studied *A. baumannii* strains based on the type of the specimen showed that levofloxacin had a greater effect on *A. baumannii* species compared to ciprofloxacin (Table 2). Also, statistical analyses showed that there was a significant relationship between the resistance to ciprofloxacin (P = 0.02) and levofloxacin and the type of the specimen (P = 0.04). Also, the results of the broth microdilution method showed that 34.5% of the isolates had a MIC of 256 for ciprofloxacin.

3.3. Examining the Effects of PAβN and NMP Compounds on the Minimum Inhibitory Concentration of Ciprofloxacin

The results obtained from combining ciprofloxacin with efflux pump inhibitors showed that 56.6% of the

Table 2. Antibiotic Susceptibility Pattern of *A. baumannii* Strains According to the Types of the Specimen

Antimicrobial	Specimen				Total (N = 103) (%)
	VAP (%)		Burn (%)		
Ciprofloxacin	Resistant	67	Resistant	90	74.7
	Intermediate	9	Intermediate	4	6.8
	Susceptible	24	Susceptible	6	18.4
Levofloxacin	Resistant	40	Resistant	18	33
	Intermediate	11	Intermediate	6	9.7
	Susceptible	49	Susceptible	76	56.3

Abbreviation: VAP, ventilator-associated pneumonia.

isolates demonstrated MIC reduction in the presence of NMP, while the same value for phenylalanine-arginine β-naphthylamide (PAβN) was about 40%. It seems that NMP had a better effect on reduction of MIC and, consequently, on reduction of resistance compared to PAβN. However, 2.7% and 6.10% of the isolates became susceptible to ciprofloxacin in the presence of NMP and PAβN, respectively. Phenotypic and genotypic characteristics of highly ciprofloxacin resistant *A. baumannii* isolated from burns and VAP samples have been shown in Tables 3 and 4, respectively.

3.4. Determination of Frequency of Antibiotic-Resistant Genes

All isolates were positive for *AdeA*, *AdeB* and *AdeC* genes (100%). For the first time, 3.9% of the strains contained the *qnrS* gene. No strains carried *qnrA* and *qnrB* genes (0%).

3.5. Sequencing the Quinolone Resistance-Determining Regions to Examine Mutation

The *gyrA* gene was observed to have a length of 344 and, after purification, the PCR product was sequenced. Converting this sequence into the amino acid sequence and its comparison with the *gyrA* protein sequence of the wild-type strain of (ATCC 19606) accession no. AF100557 revealed that in all the ciprofloxacin-resistant isolates, the mutation has occurred in the *gyrA* gene, which was predictable as this mutation is considered the main mechanism of inherent resistance to ciprofloxacin. However, no mutation was seen in *parC*, and this protein is less important in terms of inherent resistance.

4. Discussion

Due to the widespread prevalence of multidrug-resistant *Acinetobacter*, the treatment of *A. baumannii* infections is considered as a clinical problem in many European countries (28). The present study investigated the resistance of these isolates to fluoroquinolone antibiotics (ciprofloxacin and levofloxacin) and the mechanisms that cause resistance. In this study, the antibiotic resistance rates of 103 *Acinetobacter* isolates to ciprofloxacin and levofloxacin were 74.7% and 33%, respectively. In contrast to ciprofloxacin, most of the isolates were susceptible to levofloxacin; this was also confirmed by the results of the broth microdilution method, so that 34.5% of the isolates had a MIC of 256 for ciprofloxacin. It seems that levofloxacin has a higher efficiency than ciprofloxacin. The study by Adams et al. on 65 *A. baumannii* isolates led to similar results, that is, 95.9% of the isolates were ciprofloxacin-resistant (29). In another study (2015) in Iran by Gholami et al., 100% (n = 65) of the *A. baumannii* isolates were resistant to ciprofloxacin (24). All of these results indicated the high level of ciprofloxacin resistance in *A. baumannii* isolates. Results of the present study showed the MIC reduction of ciprofloxacin among *A. baumannii* isolates in the presence of the efflux pump inhibitors including NMP and PAβN. Moreover, examining the MIC status of ciprofloxacin in the presence of the two efflux pump inhibitors and comparing them with each other revealed that NMP has a more effective role in reduction of MIC compared to PABN. By investigating the effect of NMP and PAβN on ciprofloxacin resistance, Golanbar et al. showed that the MIC of the *A. baumannii* isolates is reduced in the presence of both compounds (30). Further, Waltin et al. demonstrated the considerable effect of both NMP and PAβN compounds on MIC of ciprofloxacin; the inhibitory effect of NMP was higher than that of PAβN (18). In contrast to the above-mentioned studies, Ribera et al. observed that PAβN had no effect on MIC of ciprofloxacin (31); however, in general, in many studies such as the present one, the inhibitory effect of both compounds on the performance of efflux pumps has been observed (32). In the current study, the efflux pump genes, *adeABC*, also were evaluated using the PCR test. All isolates harbored

Table 3. Phenotypic and Genotypic Characteristics of Highly Ciprofloxacin Resistant *A. baumannii* Isolated From Burn Samples

NO	MIC CIP μg/mL	MIC (CIP+NMP μg/mL)	MIC (CIP+PAβN μg/mL)	*Gyr A* Ser 83 to Leu	*Par C*
1	64	64	32	+	-
2	32	8	16	+	-
3	64	8	32	+	-
4	32	8	16	+	-
5	32	32	8	+	-
6	128	128	128	+	-
7	32	32	16	+	-
8	128	64	128	+	-
9	32	32	16	+	-
10	256	256	256	+	-
11	128	128	64	+	-
12	128	128	64	+	-
13	256	256	64	+	-
14	256	128	64	+	-
15	256	128	64	+	-
16	128	128	16	+	-
17	256	256	128	+	-
18	256	256	128	+	-
19	256	256	128	+	-
20	256	256	128	+	-
21	256	64	128	+	-
22	256	32	256	+	-
23	256	256	128	+	-

Abbreviations: CIP, ciprofloxacin; NMP, 1-(1-naphtylmethyl)-piperazine; PaβN, phenylalanine-arginine β-naphthylamide.

the adeABC genes. This reflects the role of efflux pumps to the resistance of ciprofloxacin antibiotic. Ardebili et al. investigated the presence of *adeABC* genes among the 68 *A. baumannii* isolates in Iran and showed that all isolates were resistant to ciprofloxacin, and positive for *adeABC* genes (33). Srinivasan et al. detected the *adeB* efflux gene among *A. baumannii* isolates (n = 83) originated from two hospital settings in central Ohio, the *adeB* efflux gene was found in 53% (44/83) of the isolates only (26). The results of the present study were consistent with previous studies that demonstrate the important role of efflux pumps in the resistance to ciprofloxacin. However, the expressions of genes coding for these pumps were measured by real-time PCR and this is a limitation of our study. Another mechanism leading to the quinolone resistance is the presence of QNR proteins that cause low-level resistance to the quinolones (34). Due to the limited number of studies on these proteins among *A. baumannii* isolates, the present study investigated the isolates in terms of genes, which encode this protein. The *qnrA* and *qnrB* genes were observed in none of the isolates, but unexpectedly, 4 isolates had the *qnrS* gene. Based on this report, of the 4 isolates containing this gene, 2 isolates were ciprofloxacin-resistant and 2 were intermediate. Hujer et al. investigated the antibiotic resistance genes among 75 isolates of *A. baumannii* and the QNR genes were not observed in any of the isolates (35). As for sequencing, it was shown that all the resistant strains had the mutation of leucine83 $\rightarrow$ arginine in the *gyrA* gene, but no mutation leading to resistance was observed in this *parC*. Wisplinghoff et al. sequenced the QRDR regions of 147 *A. baumannii* isolates. In some of the isolates resistant to ciprofloxacin, any mutations leading to resistance have been observed that appear to be other mechanisms involved in resistance (36). Most of the previous studies have focused on the mutation in these two genes (22) and their results are quite similar to

the results of the present study. In a study by Hamonda et al., of the 9 ciprofloxacin-resistant isolates, 2 isolates exhibited mutation in the *parC* gene (8). Moreover, in most of the studies, the mutation in the *gyrA* gene was significantly higher than that in the *parC*, and the *parC* gene was considered as the second objective of the fluoroquinolone antibiotics. In the present study, it seems that the efflux pumps and mutation in the *gyrA* gene plays an important role in inherent resistance to ciprofloxacin.

Acknowledgments

This study was supported by Ilam University of Medical Sciences (grant code number 931025/50). We gratefully thank all the staff of the clinical microbiology research center of Ilam University of Medical Sciences, Ilam, Iran, for their technical support. Preparation and editing of this manuscript was totally performed in the Razi herbal medicines research center of Lorestan University of Medical Sciences, Khorramabad, Iran.

Footnote

Financial Disclosure: The authors declare that they have no conflicts of interests regarding the publication of this paper.

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Table 4. Phenotypic and Genotypic Characteristics of Highly Ciprofloxacin Resistant *A. baumannii* Isolated From Ventilator-Associated Pneumonia Samples

NO	MIC (CIP μg/mL)	MIC (CIP+NMP μg/mL)	MIC (CIP+PAβN μg/mL)	*Gyr A* (Ser 83 to Leu)	*Par C*
1	256	256	256	+	-
2	32	16	2	+	-
3	128	128	128	+	-
4	64	16	88	+	-
5	256	256	256	+	-
6	256	128	256	+	-
7	128	32	64	+	-
8	128	32	32	+	-
9	128	128	64	+	-
10	32	8	8	+	-
11	32	4	32	+	-
12	128	128	128	+	-
13	256	256	64	+	-
14	256	256	256	+	-
15	256	256	256	+	-
16	128	64	64	+	-
17	128	128	64	+	-
18	32	32	32	+	-
19	256	256	128	+	-
20	128	32	64	+	-
21	256	128	32	+	-
22	128	128	64	+	-
23	64	16	64	+	-
24	256	256	256	+	-
25	64	32	64	+	-
26	32	16	32	+	-
27	256	256	256	+	-
28	128	64	128	+	-
29	256	256	64	+	-
30	256	256	256	+	-
31	32	8	4	+	-
32	256	256	256	+	-
33	256	256	128	+	-
34	128	128	128	+	-
35	64	64	8	+	-
36	64	64	32	+	-
37	256	256	128	+	-
38	64	32	32	+	-
39	256	128	128	+	-
40	32	16	32	+	-
41	256	256	64	+	-
42	128	32	64	+	-
43	128	128	32	+	-
44	256	256	128	+	-

Abbreviations: CIP, ciprofloxacin; MIC, minimum inhibitory concentration; NMP, 1-(1-naphtylmethyl)-piperazine; PaβN, phenylalanine-arginine β-naphthylamide.