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Association between mutations in *gyrA* and *parC* genes of *Acinetobacter baumannii* clinical isolates and ciprofloxacin resistance

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ABSTRACT

Objective(s): We investigated the contribution of *gyrA* and *parC* mutational mechanism in decreased ciprofloxacin susceptibility of *Acinetobacter baumannii* isolated from burn wound infections.
Materials and Methods: Ciprofloxacin susceptibility of 50 *A. baumannii* isolates was evaluated by disk diffusion and agar dilution methods. PCR and sequencing were performed for detection of mutation in *gyrA* and *parC* genes.
Results: The 44 and 4 isolates of *A. baumannii* exhibited full and intermediate-resistant to ciprofloxacin, respectively. Overall, the 42 isolates with double mutations of *gyrA* and *parC* genes showed a higher level of ciprofloxacin resistance than the 3 isolates with single mutations of *gyrA* or *parC*.
Conclusion: Simultaneous mutations in *gyrA* and *parC* genes are expected to play a major role in high-level fluoroquinolone resistance in *A. baumannii*; albeit a single mutation in DNA topoisomerase IV could occasionally be associated with intermediate-resistance to these antimicrobials.

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Introduction

A list of organisms has been identified by the Infectious Disease Society of America as being responsible for the majority of healthcare associated drug resistant infections. These organisms are the members of the notorious "ESKAPE" (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) (1). Among these problematic organisms, *A. baumannii* has nowadays emerged as a particular concern worldwide; because it is responsible for a variety of infections, such as blood infection, meningitis, ventilator associated pneumoniae (VAP), and wound infections, especially among patients admitted in burn and intensive care units (2, 3). In addition, this organism is a successful pathogen to escape the effects of several classes of antimicrobials, including fluoroquinolones and represents a significant challenge to infectious disease specialists (4).

The antimicrobial activity of fluoroquinolones is to form ternary complexes, including enzymes, such as DNA gyrase or topoisomerase IV, drug, and DNA which can block DNA replication and transcription, probably before DNA cleavage occurs (5). There is remarkable conservation of protein sequences between the DNA gyrase subunit A (GyrA) and topoisomerase IV subunit C (ParC) in the quinolone resistance determining region (QRDR). Resistance to fluoroquinolone in bacteria is mediated mainly by spontaneous mutations in the QRDR of *gyrA* and *parC* genes (8). In *Escherichia coli*, the most important mutations leading to a quinolone-resistant phenotype are Ser83Leu and Asp87Asn in the *gyrA* gene, and Ser80Arg and Glu84Val in the *parC* gene (6, 7).

Many fluoroquinolone-resistant *A. baumannii* clinical isolates have been reported in Iran (8-9). There are, however, few studies about the prevalence of mutations in the genes for DNA gyrase and topoisomerase IV in this organism. So, the present study aimed to specifically assess the presence of mutations in the *gyrA* and *parC* genes and their effects on resistance to fluoroquinolones in *A. baumannii* isolates from patients at a teaching hospital in Tehran.

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Materials and Methods

Bacterial isolates

Motahari Hospital is a 120-bed university affiliated Burn and Reconstruction Center located in Tehran, Iran. A total of 50 non-repetitive *A. baumannii* isolates were recovered between April 2012 and March 2013 from burn wounds of patients admitted in this hospital. All isolates were assigned to *A. baumannii* by bacteriologic and traditional phenotypic methods, including gram's staining, oxidase and catalase tests, motility, oxidative-fermentative (OF) test, and growth at 37 °C and 44 °C. The PCR of the intrinsic $bla_{Oxa\text{-}51\text{-}like}$ gene was done to confirm *A. baumannii* species (10).

Antimicrobial susceptibility testing

The susceptibility of *A. baumannii* isolates to ciprofloxacin disk (5 µg) (Mast, Merseyside, U.K) was assessed by the standard disk agar diffusion method on Mueller Hinton agar plates. The MIC for ciprofloxacin was determined by the broth macrodilution method in accordance to the Clinical and Laboratory Standards Institute (CLSI) criteria (11). Briefly, a serial dilutions of ciprofloxacin was prepared in Mueller Hinton broth tubes with the bacteria at a density of 5×10^6 CFU/ml. Microbial tubes were incubated at 37 °C for 18 hr and finally, the lowest concentration of antibiotic with no visible bacterial growth was defined as the MIC. Quality control was done using *Pseudomonas aeruginosa* ATCC 27853 strain, and results were compared to MIC ranges of CLSI. *A. baumannii* isolates were considered as intermediate-resistant and full-resistant to ciprofloxacin when the MIC was 2 µg/ml and ≥4 µg/ml, respectively.

PCR amplification and sequencing

The QRDRs of the *gyrA* and *parC* genes in *A. baumannii* clinical isolates were amplified by PCR assay. Two pairs of oligonucleotide primers used for the PCR reactions were *gyrA* primer: forward, 5'-AAATCTGCTCGTGTCGTTGG-3'; reverse, 5'-GCCATACCTACAGCAATACC-3', and *parC* primer: forward, 5'-AAGCCCGTACAGCGCCGTATT-3'; reverse, 5'-AAAGTTATCTTGCCATTCGCT-3'. Extraction of genomic DNA from *A. baumannii* colonies was performed using genomic DNA purification kit (Fermentas, Germany), based on the manufacturer's instructions. For all amplification reactions, a PCR mixture was used that contained 12.5 µl of 2× Master Mix (Ampliqon, Denmark), including 1× PCR buffer, 1.5 mmol/l $MgCl_2$, dNTPs at a concentration of 0.15 mmol/l each dNTP, 1.25 U of Taq DNA polymerase, 0.5 µl of 0.8 µM of each primer, 1 µl of template DNA (0.5 µg), and sterile distilled water up to 25 µl. DNA amplification was performed in the Mastercycler gradient instrument (Eppendorf, Germany). For *gyrA*, PCR conditions consisted of an initial denaturation at 95 °C for 1 min; 35 cycles of denaturation at 95 °C for of 30 sec, 30 sec of annealing at 52 °C, and 2 min of extension at 72 °C; ending with a final extension at 72 °C for 10 min. The temperature profile for *parC* gene was comprised of 95 °C for 2 min, followed by 35 cycles of 1 min at 95 °C, 1 min at 60 °C and 2 min at 72 °C, with 10 min at 72 °C for final extension step. DNA fragments were analyzed by electrophoresis on agarose gel (2%, wt/vol) containing 0.5 mg of ethidium bromide per liter and photographed with ultraviolet illumination. Direct sequencing of the PCR products in both directions was performed by using an ABI3730XL DNA analyzer (Applied Biosystems, Forster, USA). Nucleotide sequences data were analyzed at the National Center for Biotechnology Information (NCBI), available at the website (http:/www.ncbi.nlm.nih.gov/BLAST/).

Statistical data analysis

SPSS version 11.5 (SPSS, Inc., Chicago, IL, USA) was employed for statistical analysis. Descriptive statistics and Pearson's chi-square tests were used to evaluate correlation between mutation and ciprofloxacin resistance. Statistical significance was defined as *P*-value less than 0.05.

Results

Ciprofloxacin susceptibility and amino acid substitutions

The susceptibility testing by disk diffusion method determined that 4%, 8%, and 88% of *A. baumannii* clinical isolates were susceptible,

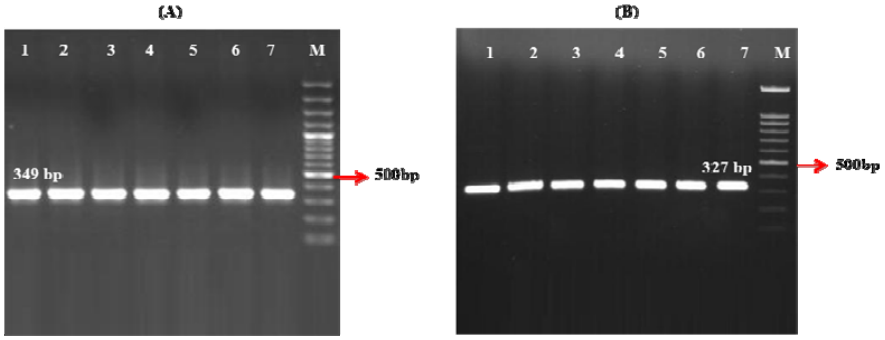

Figure 1. PCR amplification of the QRDRs of the *gyrA* (A) and *parC* (B) genes in *Acinetobacter baumannii* isolates. Lanes: 1- 7, PCR products of the corresponding genes; M, 100 bp Plus DNA Ladder

Table 1. Point mutations in the QRDRs of the *gyrA* and *parC* genes of *Acinetobacter baumannii* isolates, and the ciprofloxacin MIC in the isolates of mutation groups

Mutation group	No. (%) of isolates	No. (%) of isolates with MIC (µg/ml) to ciprofloxacin								***gyrA*** mutations		***parC*** mutations		
		2	4	8	16	32	64	128	>128	S83 (tca)	G81(ggt)	S80(tcg)	E84(gaa)	G78(ggt)
I	1 (2.2)	1 (2.2)								L (tta)				
II	2 (4.4)		2 (4.4)											C (tgt)
III	17 (37.8)				2 (4.4)	4 (8.9)	7 (15.6)	2 (4.4)	2 (4.4)	L (tta)		L (ttg)		
IV	6 (13.3)			1 (2.2)		1 (2.2)	1 (2.2)	2 (4.4)	1 (2.2)		D (gat)	L (ttg)		
V	19 (42.2)					3 (6.7)	10 (22.1)	3 (6.7)	3 (6.7)	L (tta)			K (aaa)	

intermediate-resistant and full-resistant to ciprofloxacin, respectively. The MIC range of ciprofloxacin in 44 full-resistant isolates was 4 to ≥128 µg/ml. To determine the changes in the structure of DNA gyrase and topoisomerase IV enzymes, the QRDRs of corresponding genes, *gyrA* and *parC*, were analyzed by PCR sequencing technique in all 50 clinical isolates with intermediate-resistant and full-resistant to ciprofloxacin. Amplification of *gyrA* and *parC* genes yielded PCR products of 349 and 327 bp, respectively (Figure 1). The nucleotide sequencing results revealed that 45 (90%) of the 50 isolates had amino acid alteration in *gyrA* and *parC*, as follow: 1 (2.2%) isolate in *gyrA* only, 2 (4.4%) isolates in *parC* only, and 42 (93.3%) isolates in *gyrA* and *parC*, concurrently. The *A. baumannii* isolates were divided into five groups based on the amino acid substitutions associated with resistance to fluoroquinolone (Table 1). Single mutations encoding Ser83Leu and Gly81Asp were found in the QRDR of *gyrA* in 37 (82.2%) and 6 (13.3%) of the 45 mutated isolates, respectively. Forty four of the 45 *Acinetobacter* isolates (95.5%) had a single mutation in *parC* encoding Ser80Leu (23; 51.1%), Glu84Lys (19; 42.2%), and Gly78Cys (2; 4.4%).

MIC for ciprofloxacin in the QRDR mutants

Groups I and II mutants had a single mutation in *gyrA* and *parC*, respectively; but their ciprofloxacin MIC ranged from 2 to 4 µg/ml. *Acinetobacter* isolates present in the mutation groups III, IV, and V showed double mutations in *gyrA* and *parC* and were resistant to ciprofloxacin with a MIC range of 8 to ≥128 µg/ml. Overall, isolates with double mutations of *gyrA* and *parC* genes showed a higher level of ciprofloxacin resistance than isolates with single mutations of *gyrA* or *parC* ($P< 0.05$).

Nucleotide sequence accession number

The nucleotide sequences data reported in the present study have been deposited in the Pubmed/NCBI/GenBank nucleotide sequence database under accession numbers for *gyrA* (KJ195830.1 and KJ195831.1) and *parC* (KJ756512, KJ756513 and KJ756514) genes.

Discussion

Previous studies have shown that fluoroquinolones are one of the first line therapies for *A. baumannii* infections (12, 13). However, our results in agreement with other studies revealed a considerable increase in ciprofloxacin resistance in Iran (8, 9). Resistance to ciprofloxacin in *Acinetobacter* isolates is alarming; since many of such strains are usually multi-drug resistant (MDR), and at these circumstances, the remaining therapeutic options are colistin (a relatively toxic drug), tigecycline (a bacteriostatic agent, i.e. a disadvantage when using in immunocompromised patients), and sulbactam (with a limited antibacterial spectrum only) (14).

It is accepted that changes in the structure of the antibiotic targets DNA gyrase and DNA topoisomerase IV are one of the most significant mechanisms in conferring a resistance to fluoroquinolone in gram negative bacilli (7). In *E. coli*, three or four mutations in both *gyrA* and *parC* genes are necessary to obtain high-level resistance to ciprofloxacin, even as double mutations at positions 83 (Ser83) of *gyrA* and 80 (Ser80) of *parC* cause only moderate-level resistance (6, 7). Nevertheless, the situation in *A. baumannii* is rather different from that in *E. coli*, since we found in the present study that only a double mutation could lead to high-level resistant phenotype. So that, among 44 ciprofloxacin-resistant isolates, 11 (25%) had MIC range of 8-32 µg/ml and the majority (31; 70.4%) of isolates had MICs of 128 µg/ml or higher, that indicate these isolates are highly resistant to fluoroquinolones.

Our sequencing results revealed serine to leucine mutation at position 83 of *gyrA* subunit in 38 of 44 ciprofloxacin-resistant *Acinetobacter* isolates. Similar to study by Park *et al* (15), this is indicative of the fact that Ser83Leu substitution is the principal mutation in *A. baumannii* for resistance to fluoroquinolones. Other mutations in the *gyrA* gene resulting in Ala84Pro or Gly81Val reported in ciprofloxacin resistant isolates of previous study (16) were not observed in our included isolates. Instead, we found a novel mutation in the *gyrA* gene, leading to Gly81Asp substitution DNA gyrase, in six resistant isolates with a MIC range at 8 to ≥128 µg/ml.

In *A. baumannii*, topoisomerase IV is a target of quinolones and mutations at residues Ser80 and Glu84 of *parC* contribute to decreased fluoroquinolone susceptibility (16). Although *parC* mutations always along with mutations in *gyrA* are needed to acquire a high-level resistance to quinolones (4), two

clinical isolates in our study had mutations in *parC* without *gyrA*, suggesting that *parC* might not only be a secondary target for quinolones but is really as important as *gyrA* to cause a decreased susceptibility to fluoroquinolones in *A. baumannii*. On the other hand, QRDRs of the three isolates with intermediate-resistance to ciprofloxacin in the current study did not possess alterations associated with fluoroquinolones resistance in the sequence of either genes, indicating other resistance mechanisms, such as efflux systems should be considered in these isolates (17).

Conclusion

Although a single point mutation in DNA gyrase is enough for resistance to fluoroquinolone in *A. baumannii*, the concurrent mutations within QRDR regions of the *gyrA* and *parC* genes are expected to significantly contribute to high-level fluoroquinolone resistance. Further studies are required to elucidate mechanisms, other than alterations in *gyrA* and *parC*, leading to decreased susceptibility to quinolones in *A. baumannii* isolates.

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Conflicts of interest

No declarations were made by the authors of this paper.

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