Association between mutations in \textit{gyrA} and \textit{parC} genes of \textit{Acinetobacter baumannii} clinical isolates and ciprofloxacin resistance

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\textbf{ABSTRACT}

\textbf{Objective(s):} We investigated the contribution of \textit{gyrA} and \textit{parC} mutational mechanism in decreased ciprofloxacin susceptibility of \textit{Acinetobacter baumannii} isolated from burn wound infections.

\textbf{Materials and Methods:} Ciprofloxacin susceptibility of 50 \textit{A. baumannii} isolates was evaluated by disk diffusion and agar dilution methods. PCR and sequencing were performed for detection of mutation in \textit{gyrA} and \textit{parC} genes.

\textbf{Results:} The 44 and 4 isolates of \textit{A. baumannii} exhibited full and intermediate-resistant to ciprofloxacin, respectively. Overall, the 42 isolates with double mutations of \textit{gyrA} and \textit{parC} genes showed a higher level of ciprofloxacin resistance than the 3 isolates with single mutations of \textit{gyrA} or \textit{parC}.

\textbf{Conclusion:} Simultaneous mutations in \textit{gyrA} and \textit{parC} genes are expected to play a major role in high-level fluoroquinolone resistance in \textit{A. baumannii}; albeit a single mutation in DNA topoisomerase IV could occasionally be associated with intermediate-resistance to these antimicrobials.

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\textbf{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" (\textit{Enterococcus faecium}, \textit{Staphylococcus aureus}, \textit{Klebsiella pneumoniae}, \textit{Acinetobacter baumannii}, \textit{Pseudomonas aeruginosa}, and \textit{Enterobacter} species) (1). Among these problematic organisms, \textit{A. baumannii} has nowadays emerged as a particular concern worldwide; because it is responsible for a variety of infections, such as blood infection, meningitis, ventillator 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 (\textit{GyrA}) and topoisomerase IV subunit C (\textit{ParC}) in the quinolone resistance determining region (QRDR). Resistance to fluoroquinolone in bacteria is mediated mainly by spontaneous mutations in the QRDR of \textit{gyrA} and \textit{parC} genes (8). In \textit{Escherichia coli}, the most important mutations leading to a quinolone-resistant phenotype are Ser83Leu and Asp87Asn in the \textit{gyrA} gene, and Ser80Arg and Glu84Val in the \textit{parC} gene (6, 7).

Many fluoroquinolone-resistant \textit{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 \textit{gyrA} and \textit{parC} genes and their effects on resistance to fluoroquinolones in \textit{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 blaOxa-51-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, UK) 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'-AAATCTGCTGTTGTTGTTGG-3', reverse, 5'-GCCATACCTACAGCAATACC-3', and parC primer: forward, 5'-AAGCCGTACAGCGCCGTATT-3', reverse, 5'-AAAGTTATCTTGCCATTGCCT-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/lMgCl2, 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 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,
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 QRDRs 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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