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Quinolone-Resistant Isolates of *Acinetobacter baumannii* in a Teaching Hospital in Iran

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ABSTRACT

Background: *Acinetobacter baumannii* could develop resistance through different mechanisms, leading to the emergence of strains resistant to all commercially accessible antibiotics. This research aimed to evaluate the antimicrobial resistance pattern and the prevalence of genes encoding quinolone resistance in quinolone-resistant isolates.

Materials & Methods: In this study, 114 *A. baumannii* strains were isolated from patients admitted to a teaching hospital in Kashan, during 2013-2014. Antimicrobial susceptibility testing was performed using the Kirby-Bauer disk-diffusion breakpoint assay. Polymerase chain reaction (PCR) was employed to identify quinolone resistance encoding genes (*gyrA* and *parC*).

Findings: All *A. baumannii* strains showed resistance to piperacillin, ceftriaxone, ceftazidime, and cefotaxime, and all of them were susceptible to colistin and polymyxin B. In addition, 100% of *A. baumannii* strains were MDR (Multi-drug resistance), and 68.4% (78 isolates) of them were XDR (Extensively-drug resistant), while none of them were PDR (Pan-drug resistant). All *A. baumannii* strains isolated in this study were positive for the presence of *parC* and *gyrA* genes.

Conclusion: MDR *A. baumannii* strains were highly prevalent among hospitalized patients in this study. Based on these comes about, novel prevention and treatment procedures against *A. baumannii* infections are justified. Moreover, these information may help in reexamining treatment rules and territorial arrangements in care units to moderate the rise of antimicrobial resistance.

Keywords: *Acinetobacter baumannii*, Antibiotic resistance, Fluoroquinolone resistance, *gyrA*, *parC*, MDR.

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Introduction

Acinetobacter baumannii could develop antibiotic resistance via different mechanisms, leading to the emergence of resistant strains to most commercially accessible antimicrobials, especially in ICU (intensive care unit) patients [1, 2]. Cephalosporins, fluoroquinolones, and carbapenems are used to treat *Acinetobacter* infections [3]. Empirical antibiotic treatment used to treat *Acinetobacter* infections ought to contain a carbapenem, a broad-spectrum cephalosporin, or a beta-lactam/beta-lactamase inhibitor combination (e.g., a sulbactam-containing combination). Recently, resistant strains of *A. baumannii* have been frequently identified as major pathogens of hospital-acquired infections worldwide [4]. For the treatment of patients with *Acinetobacter* infections in an environment where resistance to the selected antibiotic is high, a fluoroquinolone, an aminoglycoside, or colistin is used as the second drug of choice. Fluoroquinolones inhibit by targeting of enzymes involved in DNA replication and transcription. These antibiotics hinder topoisomerase IV and DNA gyrase as necessary factors in DNA replication. Resistance to fluoroquinolones in *A. baumannii* strains is increasing, which is associated with overuse of these antibiotics by people in the community and hospitals [5].

The emergence of quinolone-resistant strains is related with the amount of antibiotic use and the duration of antibiotic treatment. Quinolone resistance also occurs in ICUs, which is usually accompanied by the development of resistance to other antibiotics. Quinolone resistance could be induced by chromosomal gene mutations or acquisition of antibiotic resistance genes (on plasmids) [6].

As quinolones are critical for treating *A. baumannii* infections, investigating the sensitivity to these antibiotics is crucial for proper antibiotic treatment of MDR *A. baumannii* infections. The progress of resistance to quinolones in *A. baumannii* is mostly attributed to specific point mutations within the quinolone resistance-determining regions (QRDRs) of the *parC* and *gyrA* genes. Notably, mutations occurring at codons Ser80, Glu84, and Asp87 of *parC*, as well as codon Ser83 of *gyrA*, have been identified as the most significant changes associated with quinolone resistance. These mutations directly impact the target structure of the topoisomerase IV and gyrase proteins, which are essential for the activity of quinolones [7-10].

Objectives: This research aims to evaluate the prevalence of *parC* and *gyrA* mutations in quinolone-resistant *A. baumannii* strains from patients in Kashan, Iran.

Material and Methods

Bacterial isolates: This cross-sectional study was performed on 114 *A. baumannii* isolates collected from patients admitted to Beheshti hospital in Kashan, Iran, during 2013-2014. The protocol of this research was approved by the Ethics Committee of Kashan University of Medical Sciences. The specifications of the study participants and their clinical samples are summarized in Table 1. Species identification was done using Microwell Strip API (Microgen Bioproducts Co., UK) (Figure 1).

Detection of bla_{OXA-51} gene to identify *A. baumannii*: DNAs of isolates were extracted through boiling technique. Before DNA extraction the isolates were cultured in LB broth at 37°C for 18 hours. Bacteria were pelleted from 1.5 mL LB broth and suspended in 200 µL of sterile deionized water and incubated at 100°C for 10 minutes. The supernatants were used as a template DNA after centrifugation of the lysate. The collected strains were subjected to PCR (polymerase chain reaction) amplification of the *blaoxa-*$_{51}$ gene (specific to *A. baumannii* species in the database) and confirmed by blast analysis

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(http://www.ncbi.nlm.nih.gov/GenBank). The following primer pairs were used for PCR amplification of the bla_{OXA-51} gene with a 353 bp chain: Forward: 5'-TAATGCTTTGATC-GGCCTTG-3' and reverse: 5'-TGGATTG-CACTTCATCTTGG-3' [11]. In this study, the standard strain *A. baumannii* ATCC 19606 was employed as the reference isolate [12].

Antimicrobial susceptibility testing: Antibiotic sensitivity of the isolates was determined by employing Kirby-Bauer disk-diffusion technique on Mueller-Hinton agar culture medium (Merck, Germany) according to Clinical and Laboratory Standards Institute (CLSI) guidelines the version CLSI 2021 guidelines. Accordingly, *A. baumannii* strains were categorized as resistant, intermediate, or susceptible to antibiotics.

Figure 1) Image of the final result of *Acinetobacter baumannii* isolates identified using Microwell Strip API (Microgen Bioproducts Co., UK) in this study (Fig.1).

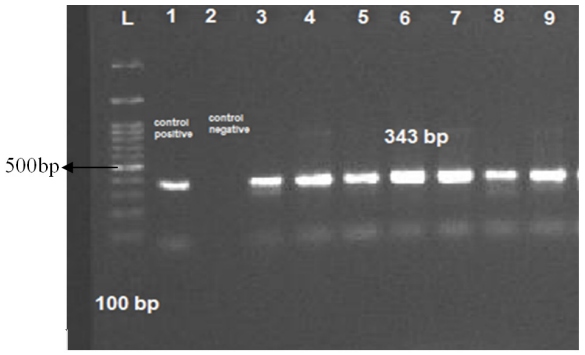

Figure 2) Detection of genes encoding *gyrA* by PCR. M: 100 bp DNA ladder, Lane 1: positive control, Lane 2: negative control, Lanes 3 to 9: isolates of *A. baumannii* containing *gyrA* genes in this study

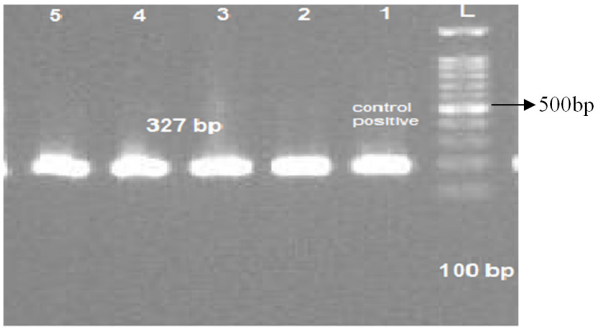

Figure 3) Detection of genes encoding *parC* by PCR. M: 100 bp DNA ladder, Lane 1: positive control, Lanes 2 to 5: isolates of *A. baumannii* containing *parC* genes in this study

Antibiotic discs used in this study included: polymyxin B (300 unit), colistin (10 µg), trimethoprim-sulfamethoxazole (1.25/23.75 mg), tetracycline (30 µg), levofloxacin (5 µg), ciprofloxacin (5 µg), gentamicin (10 µg), amikacin (30 µg), meropenem (10 µg), imipenem (10 µg), cefepime (30 µg), ceftriaxone (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), piperacillin-tazobactam (100/10 µg), ampicillin-sulbactam (10/10 µg), and piperacillin (100 µg), which were prepared from MAST (MAST Group, Merseyside, U.K). In this study, the standard strain *Escherichia coli* ATCC 25922 was employed as a quality control in each susceptibility experiment [13].

PCR amplification of quinolone resistance-encoding genes: To detect QRDR-related mutations in *gyrA* and *parC* genes, PCR amplification as well as the following oligonucleotide primers (Bioneer, Korea), which were obtained from well-known sequences of *gyrA* and *parC* with 343 and 272 bp lengths, were employed: for *gyrA*: forward: 5'-AAATCTGCCCGTGTCGTTGGT-3' and reverse: 5'-GCCATACCTACGGCGATACC-3' [8], and for *parC*: forward: 5'-AAAAATCAGCGC-GTACAGTG-3' and reverse: 5'-CGAGAGTTTG-GCTTCGGTAT-3' [14]. PCR was performed as follows. One to five colonies grown on nutrient agar (Merck) were re-suspended in 50 mL of sterile distilled water, followed by boiling for 10 minutes. After a short 5-min-

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Table 1) Demographic Characteristics of the Study Population in this study

Parameters	No. of Cases (%)	XDR (%)
Age, y ≤ 40	33(28.9)	22(28.2)
Age, y > 40	81(71.1)	56(71.8)
Sex (male/female)	71/43(62.3/37.7)	44/34(56.5/43.5)
Hospital sampling ward		
ICU	66(57.9)	46(59)
Internal medicine	23(20.2)	17(21.7)
Emergency room	21(18.4)	13(16.8)
Pediatrics	4(3.5)	2(2.5)
Length of stay in hospital (days)		
≤ 30	73(64)	46(59)
> 30	41(36)	32(41)
Death ratio	12/114(10.5)	6/78(7.6)

ute centrifugation step at 20,000 g, the DNA-containing supernatant was gathered. PCR amplification was performed using 20 mL reaction mixtures, consisting of 100 ng of *A. baumannii* genomic DNA, 1.5 U of Taq polymerase, 1x PCR buffer (20 mM Tris-HCl, pH 8.4), 200 mM dNTP each, 1.5 mM $MgCl_2$, 50 mM KCl, and 0.5 mM primer each. PCR assay was conducted using a thermal cycler (Mastercycler gradient; Eppendorf, Hamburg, Germany). The reaction mixtures were amplified under the following thermal cycling conditions: 36 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 2 min for *gyrA* as well as 36 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 2 min for *parC*. Amplification was performed in a DNA thermal cycler. Then PCR amplification products (10 µL) were subjected to 1% agarose gel electrophoresis in TAE (tris-acetate-EDTA) buffer to separate DNA fragments. Ethidium bromide (0.5 mg/mL) (Ethidium bromide for gel staining is a very toxic substance) was used to stain the gel. The produced bands in agarose gel electrophoresis were visualized and photographed under ultraviolet (UV) light. A 100-bp ladder was employed to separate amplicons of various sizes. The standard strain *A. baumannii* ATCC 19606 was employed as the reference isolate [12].

Sequencing method: PCR was performed to amplify the *parC* and *gyrA* genes using specific primer pairs as previously explained [14]. Sanger sequencing method was used to sequence the purified PCR amplification products (Applied Biosystems 3730/3730xl DNA Analyzers; Bioneer). The sequences obtained from Sanger sequencing were compared with those in the GenBank database (www.ncbi.nlm.nih.gov/GenBank).

Data analysis was performed by DNA sequencing analysis (ABI) system (CLC Genomics Workbench). Analyzes were performed using blastn and ExPASy-Translate tool online and CLC Genomics Workbench software (Ver. 3.6.5) offline. DNA sequences

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Table 2) The frequency percent of antibiotic resistance patterns of 114 *Acinetobacter baumannii* isolates of hospitalized patients in kashan in this study

Antibiotics	Resistant No. (%)	Intermediate No. (%)	Susceptible No. (%)
Piperacillin	114(100)	0(0)	0(0)
Ampicillin-sulbactam	101(88.6)	6(5.3)	7(6.1)
Piperacillin-tazobactam	112(98.2)	0(0)	2(1.8)
Ceftazidime	114(100)	0(0)	0(0)
Cefepime	113(99.1)	1(0.9)	0(0)
Cefotaxime	113(99.1)	0(0)	1(0.9)
Meropenem	104(91.2)	0(0)	10(8.8)
Imipenem	101(88.6)	1(0.9)	12(10.5)
Amikacine	92(80.7)	8(7)	14(12.3)
Gentamicine	96(84.2)	4(3.5)	14(12.3)
Levofloxacin	113(99.1)	0(0)	1(0.9)
Ciprofloxacin	113(99.1)	1(0.9)	0(0)
Tetracycline	88(77.2)	17(14.9)	9(7.9)
Sulfamethoxazole	111(97.4)	2(8.1)	1(0.9)
Polymyxin B	0(0)	0(0)	114(100)
Colistin	0(0)	0(0)	114(100)

of *A. baumannii* strains were compared with those of *A. baumannii* OCU-Ac20.

Nucleotide sequence accession numbers: The following GenBank accession numbers were assigned to the *parC* and *gyrA* genes detected in the current re search: *parC* (OQ513837; OQ513838), *gyrA* (OQ513836;OQ513835; OQ513839).

Statistical analysis: The statistical data was showed using (SPSS Inc. Version 26.0, Chicago, IL, USA). Descriptive statistics were used and the chi-square test was used to compare antibiotic resistance rates. A p value of $< .05$ was considered as statistically significant. The statistical data was conducted using (SPSS Inc. Version 26.0, Chicago, IL, USA).

Findings

MDR, XDR, and PDR definitions: Multi-drug resistance (MDR) is described as non-susceptibility to at least one antibiotic in three or more antibiotic categories. Extensively-drug resistance (XDR) is described as showing resistance to at least one antibiotic in all but two or fewer antibiotic categories (the strain is sensitive to only one or two antibiotic categories). Pan-drug resistance (PDR) is described as non-susceptibility to all antibiotics in all antibiotic categories (the organism is not sensitive to any antibiotic) [6].

A total of 114 patients in the age range of 23 to 95 years were enrolled in this research. The average age of the study participants was 54.62 ± 18.12 years. Of whom, 71 (62.3%) cases were male, and 43 (37.7%) cases were female. Table 1 summarizes the specifications of the study participants. The strains were isolated from tracheal tube (n= 63, 55.3%), blood (n=22, 19.3%), sputum (n=8, 7%), pleural fluid (n=7, 6.1%), urine (n=6, 5.3%), cerebrospinal fluid (n=3, 2.6%), catheter (n=3, 2.6%), and wound (n=2, 1.8%) samples. All *A. baumannii* isolates were MDR, and

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Table 3) Mutations in genes *gyrA* and *parC*

***Acinetobacter baumannii* clone**	***parC* mutation**		
	Nucleotide position	Nucleotide exchange	Amino acid exchange
PC.2	10-11-14	--- (Nucleotide deletion)	I3KY
***Acinetobacter baumannii* clone**	***gyrA* mutation**		
	Nucleotide position	Nucleotide exchange	Amino acid exchange
GA.1	299 302	GCT→ GCC ATT→ATC	I96I A97A
GA.2	299 302	GCT→ GCC ATT→ ATC	I96I A97A

78 (68.4%) isolates were XDR, while none of the isolates were PDR. Six out of 78 patients (7.6%) harboring XDR *A. baumannii* isolates died (Table 1). All the strains showed resistance to piperacillin, ceftriaxone, ceftazidime, and cefotaxime. All of them were susceptible to polymyxin B and colistin. Antibiotic susceptibility testing results are presented in Table 2. Bla_{oxa-51} unique to *A. baumannii* species was detected in 100% of the isolates. All *A. baumannii* strains isolated in this study were positive for the presence of *parC* and *gyrA* genes. Figures 2 and 3 show PCR amplification of the *parC* and *gyrA* genes detected in this study, respectively. DNA sequences of some *A. baumannii* isolates were compared with those of *A. baumannii* OCU-Ac20 in the GenBank database.

Alignment of DNA sequences of QRDRs from some *A. baumannii* isolates with those of *A. baumannii* OCU-Ac20 showed few nucleotide substitutions in the former (Table 3). The results showed that many nucleotides comprising QRDRs were conserved.

Discussion

Resistance of *A. baumannii* strains to many antibiotics is increasing. Variation in resistance rate to different antibiotics in different bacteria is a serious challenge in the treatment of nosocomial (hospital-acquired) infections [15].

This study showed that patients with MDR and XDR *A. baumannii* infections are at higher risk of mortality. The present study findings showed that the prevalence of XDR and MDR *A. baumannii* strains in Kashan is high, which are the major causes of clinically important nosocomial infections.

A. baumannii is considered as one of the main bacterial causes of hospital-acquired infections worldwide; however, the management of this bacterium has recently become a major challenge due to the increasing prevalence of its MDR strains. MDR strains of *A. baumannii* have a high potential for dissemination among ICU patients [16, 17]. The emergence of MDR and XDR strains may occur during the treatment of *A. baumannii* infections, leading to increased morbidity and mortality rates [18, 19].

Salehi et al. reported that 97% of *Acinetobacter* species were MDR and 41% as XDR. They exhibited that more than 90% of *Acine-*

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tobacter species showed resistance to carbapenem and all the isolates were found to be susceptible to colistin and most of them (77%) were non-susceptible to tigecycline [20]. In another study by Dizbay et al. (2008), 80.5% of *A. baumannii* isolates were MDR, all of them were susceptible to colistin, and resistance rates to ciprofloxacin, cefepime, imipenem, meropenem, and cefoperazone/sulbactam were 95.5, 72.7, 80.3, 71.2, and 68.2%, respectively [21]. In the study of Mirzaei et al. (2020), the prevalence of XDR and MDR strains was 73.13 and 74.75%, respectively [22]. The frequency of XDR and MDR *A. baumannii* isolates in the current research was extensively high. The high rate of XDR and MDR isolates in this study may be due to the high probability of spreading resistance factors in hospital environments. Also, this high prevalence is alarming, and it is recommended to conduct more research and apply more preventive procedures to decrease the prevalence of this infection among hospitalized patients [23]. In all isolates, fluoroquinolone resistance phenotypes were related to detected *parC* and *gyrA* gene bands. Resistance to carbapenems, extended-spectrum cephalosporins, ampicillin-sulbactam, quinolones, and aminoglycosides among MDR *A. baumannii* strains is expanding [24]. Quinolones are among the most broadly prescribed antimicrobial agents in the world. The increase in antibacterial resistance, as observed in *A. baumannii* isolates, is due to the increasing and excessive use of antibiotics, including quinolones [25]. Fluoroquinolone resistance in *A. baumannii* is often caused by point mutations within DNA gyrase QRDRs [26]. These mutations are often point changes that occur at Ser80 in *parC* or Ser83 in *gyrA*, which cause changes within the amino acid group of QRDRs and quinolone resistance. Sequencing of the QRDRs of *parC* and *gyrA* revealed an ILe3→Lys or Tyr change in *gyrA* and a silent mutation in *parC*, a mutation that does not cause a major amino acid change.

In topoisomerase IV, transformations happen more regularly in *parC* than in *parE*. Topoisomerase IV is the auxiliary target of quinolones in most Gram negative organisms. *GyrA-parC* twofold mutants show the next level of resistance to quinolones compared to *gyrA* single mutants, and the highest resistance levels are detected in mutants with double changes in *parC* and *gyrA* each [7, 25]. Changes in codon 80 of *parC* and codon 83 of *gyrA* are the foremost basic changes in *A. baumannii*. Changes in *gyrA* codons at amino acids Glu87, Ala84, and Gly81 have been reported in several studies [27]. Upregulated efflux pumps may also confer fluoroquinolone resistance in *A. baumannii* [28]. Studies have reported changes in codon 83 of *gyrA* in *E. coli* (Ser→Leu), *Klebsiella pneumonia* (Ser→Tyr), and *Pseudomonas aeruginosa* (Thr→Ile) [24]. Other studies have also reported changes in codon 80 of *parC* in *E. coli* (Ser80 Ile or Arg) and *Staphylococcus aureus* (Ser80 Tyr) [29, 30].

In a research by Tantawy et al. (2020), all *A. baumannii* isolates showed resistance to levofloxacin and ciprofloxacin, and all of them harbored both *parC* and *gyrA* genes [31]. In another study by Nowroozi et al. (2014), all *A. baumannii* strains were MDR, and all of them showed resistance to ciprofloxacin; in addition, *parC* and *gyrA* gene bands were detected in all the isolates [14].

Most specimens in the present research were collected from tracheal secretion samples, especially in the ICU. Thus, disinfection of equipment related to the respiratory system could significantly help prevent the spread of this infection in hospital environments. In this study, 100% of *A. baumannii* strains isolated were MDR, and 99.1% of them showed resistance to fluoroquinolones. Also, 100% of *A. baumannii* isolates were positive for the presence of *parC* and *gyrA*. This suggests

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that *A. baumannii* may be highly resistant to quinolone antimicrobial agents because *parC* and *gyrA* coexist in this species. In addition to mutations in *parC* and *gyrA,* other mechanisms (e.g., intrinsic membrane impermeability, alterations in *gyrB* and *parE,* or active efflux pumps) might be involved in the growth of resistance in *A. baumannii* isolates [32].

A. baumannii isolates were mainly sensitive to polymyxin B and colistin, but these antibiotics are toxic. MDR and XDR strains may emerge during the treatment of *A. baumannii* infections, causing increased morbidity and mortality [24, 25]. Since *A. baumannii* has recently developed resistance to some antimicrobials, these antimicrobials are preferably not recommended to treat related infections. With the emergence of XDR and MDR *A. baumannii* strains, antibiotics options for the treatment of related infections have become very limited. Irrational use of antiseptics and broad-spectrum antibiotics has led to an increase in antimicrobial resistance. Antibiotic stewardship, application of infection control procedures, including standard and contact precautions, staff education, and surveillance should be done with effective management to solve this problem [31].

limitation

Ethidium bromide used for gel staining is a very toxic substance whose use is obsolete.

Conclusion

A. baumannii is considered as one of the main bacterial causes of hospital-acquired infections. Several investigations have reported the presence of this organism in many parts of the world, including Iran. This study results indicate that *A. baumannii* has become resistant to many antibiotics including ceftazidime, ceftriaxone, cefotaxime and piperacillin. All of the isolates shown susceptibility to polymyxin B and colistin. Results of this study display that a MDR a system of prevention and control of *Acinetobacter spp*. infections is necessary. Given the magnitude and costs associated with hospital acquired infections, and the increase in multidrug-resistant organisms, it is value re-evaluating our present methods. Thus prevention and control system for MDR *Acinetobacter* infections is required.

Conflicts of interests: The authors declare that have no personal or financial conflicts of interest.

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