

Original article

Association between mutations in GyrA/B and ParC/E genes of *Acinetobacter baumannii* clinical isolates and fluoroquinolones resistance

Mohammed Shees Ahmed Shahhat ¹, Essam Abo El Yazed Gaballa ¹, Raafat Zaher Abd El Rohman ², Mohammed Nabil Mohammed ¹, Ayman Sabry Yassin ^{*1}

1- Department of Microbiology and Immunology, Faculty of Medicine, Al-Azhar University.

2- Department of Microbiology and Immunology, Military Medical Academy.

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ABSTRACT

Background: *Acinetobacter baumannii* (*A. baumannii*) is an important organism in hospital acquired infections. *Acinetobacter baumannii* has emerged as quinolones resistant organism. **Objectives:** Determination of mutations of gyrA/B and parC/E genes using Restriction Fragment Length Polymorphism PCR (RFLP - PCR) among fluoroquinolones resistant clinical isolates of *A. baumannii* showed in ICU patients at Al-Azhar University hospitals. **Methods:** The study was conducted on 100 isolates of *A.baumannii* which were subjected to molecular study of mutations of gyrA, gyrB, parC and parE genes beside determination of minimal inhibitory concentration (MIC). **Results:** Most *A. baumannii* isolates had MIC >128 µg/ml (61.1%). The resistant isolates showed combined mutations in both gyrA& parC genes most frequently (79.2%) then gyrA (9.7%), parC (8.3%) and gyrB (2.8%). **Conclusions:** Mutations in gyrA and parC are the most common in fluoroquinolones resistant *A. baumannii* isolated from Al-Azhar University hospitals.

Introduction

Acinetobacter species are distributed in nature, and have been determined in soil, water, animals, and humans. Some of them can exist for weeks in environment & cause hospital acquired infections [1]. They inhabit in human pores and skin and are often isolated from the throat and respiratory tract of hospitalized patients [2].

More than thirty known and unknown *Acinetobacter* species had been described [3]. Some *Acinetobacter* spp have medical importance, which includes *Acinetobacter baumannii* (*A. Baumannii*), *Acinetobacter* gen3, and *Acinetobacter Gen. Sp. 13TU*, at the same time a different species, like *Acinetobacter johnsonii*, *Acinetobacter*

schindleri and *A. Ursingii*, additionally can incidentally associated to cause infections [4].

Acinetobacter baumannii has emerged in the ultimate years as a completely important infectious agent inflicting fitness care related infections. It's one among what is called "ESKAPE" microorganism (*Staphylococcus aureus*, *Enterococcus faecium*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Enterobacter* species and *Pseudomonas aeruginosa*) [5].

In addition, *A. baumannii* resistant to carbapenem is one of the critical-precedence pathogens on the global Health Organization precedence listing of antibiotic-resistant microorganism for powerful drug development [6].

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* Corresponding author: Ayman Sabry Yassin

E-mail address: asabry_2008@yahoo.com

Ciprofloxacin has wide spectrum bactericidal activity, good oral bioavailability, high tissue penetration, more safe and tolerable and used to treat a wide types of infections [7], ciprofloxacin will be taken as a model of fluoroquinolones in current study according to **Ardebili A et al.** [8].

The maximum crucial mechanism of fluoroquinolones resistance is the mutation in genes that encode the subunits of DNA gyrase (gyrA and gyrB) and topoisomerase IV (parC and parE). GyrA gene and its homologous region of the parC gene are most significant mutations incorporate mainly the quinolone resistance figuring out regions (QRDRs). On the opportunity hand, the mutations in gyrB and parE genes regions are of less significance [9,10]

Aim of study

This study aimed to detect the presence of gyrA/B and parC/E genes mutations by restriction fragment length polymorphism PCR (RFLP PCR) among fluoroquinolones resistant *A. baumannii* isolates in patients with infections in intensive care units at Al-Azhar university hospitals in Cairo.

Subjects and Methods

The study was done between February 2019 to July 2020. The study was approved by Al Azhar faculty of Medicine ethical committee. Samples collected from inpatients (each isolate from individual patient), including sputum, endotracheal tubes, urine, infected wounds and bed sores. The collected samples were subjected to: Direct microscopic examination of a Gram-stained smear, culture on MacConkey and blood agar media and biochemical tests [11]. Further identification to species level was done by VITEK 2 automated system [12].

The study was done on 100 clinical isolates of *A. baumannii* (68 males, 32 females) who developed clinical evidence of infection. Full history was taken including duration of hospital stay, underlying diseases, risk factors (e.g. Diabetes) & previous operation.

Consent was taken from the patient's family to be enrolled in the study.

***Acinetobacter* was identified by:**

- 1) Gram stain: short, Gram-negative rods, arranged in pairs or clusters.
- 2) Colony appearance: on MacConkey agar media colonies appear smooth, convex, glistening, sometimes pale yellow & mucoid.

3) Biochemical reaction: oxidase test negative and catalase test positive.

4) VITEK 2 automated microbiology system.

Antibiotic sensitivity test was done by modified Kirby Bauer disc diffusion method, according to CLSI guidelines [13].

Minimal inhibitory concentration for ciprofloxacin

Detection of MIC for ciprofloxacin among isolates of *Acinetobacter baumannii* was done by broth macrodilution approach consistent with the Clinical Laboratory Standards Institute instructions. Ciprofloxacin serial dilutions have been done using Muller Hinton broth and bacterial isolates with attention same to half McFarland have been brought to every tube then incubated for 18 hours at 37° C. Minimum inhibitory concentration is described as the least concentration without a seen growth. *Acinetobacter baumannii* isolates considered as intermediate resistant at MIC 2 µg/ml and full resistant at ≥4 µg/ml [14].

DNA Extraction

Acinetobacter baumannii isolates had been subjected to extraction of DNA using boiling technique. At first colonies were suspended in 1ml of sterile distilled water then boiled for 10 minutes & then centrifuged for another 10 minutes. Then preserve supernatant in sterile eppendorf at -20 °C till amplification [15].

Amplification technique

Amplification technique achieved by adding 2µl of the extracted DNA with 20 µl of amplification mixture (Qiagen) [16]. The used primers were summarized in **table (1)**.

The amplified products were purified by the use of QIA quick PCR purification kit according to the manufacturer's protocol. Then the eluant incubated at 37°C for 2.5 hours with 10 U of *HinfI* enzyme which used for the digestion. Then digests were separated by electrophoresis in 1.5% agarose gel stained with ethidium bromide and photographed with UV illumination.

Table 1. Primers sequences of the target genes.

Target Gene	Primer sequence (5'-3')	Amplicon size
gyr A	Forward AATCTGCCCGTGTCGTTGGT	343 bp
	Reverse GCCATACCTACGGCGATACC	
gyrB	Forward GAAATGACCCGCCGTAAA	490 bp
	Reverse ACGACCGATACCACAGCC	
parC	Forward AATCTGCCCGTGTCGTTGGT	327 bp
	Reverse GCCATACCTACGGCGATACC	
parE	Forward CTGACCGAAAGCTACGTCAACC	417bp
	Reverse CGTTCGGCTTGCTTTCTTG	

Results

Among the *Acinetobacter* group, *A.baumannii* was the main isolated_spp. (77.5%) while *A.lwoffii* (16.3%)and *A. Junii* were (6.2%) (Figure 1).

Different types of samples were included in this current study, 36 wound, 38 sputum, 11 blood and 2 urine samples. While 13 isolates were recovered from miscellaneous samples (endotracheal tube (ETT), bronchial lavage and urinary catheter) (Table2).

Table 2. Distribution of *A. baumannii* isolates in clinical samples.

Samples	No.
Blood Culture	11
Sputum	38
Wounds Culture	36
Urine Culture	2
Others	13
Total	100

Most effective antibiotics against *A. baumannii* were imipenem (88%), ciprofloxacin (33%), ceftriaxone (11%), ofloxacin (8 %), cefepime (7%) and amikacin (2%). While *A. baumannii* were 100% resistant to ampicillin/sulbactam, amoxicillin/clavulinate, trimethoprim/sulfamethozole, cefazolin and cefuroxime (Table 3).

The sensitivity and specificity of disc diffusion method as compared to the MIC in detection of ciprofloxacin resistant were found to be 90% and 100%, respectively (Table 4).

All resistant strains of *A. baumannii* to ciprofloxacin had mutations in *gyrA* ,*gyr B* and *parC*. The most frequent mutations were combined mutations in both genes (79.2%) then mutation *gyrA* (9.7%), *parC*(8.3%), *gyrB* (2.8%) and *par E* (0%) (Table 5) (Figures 2,3&4).

Acinetobacter baumannii with resistance to ciprofloxacin had MIC ≥ 4 $\mu\text{g/ml}$. Most isolates had MIC >128 $\mu\text{g/ml}$ (61.1%) (Table 6).

The isolates of *A. baumannii* with MIC more than 128 $\mu\text{g/ml}$; 44 (61.1%) had combined mutations in the *gyrA* and *parC*. These findings indicate that combined mutations are associated with high ciprofloxacin MIC (Table 7). In relation to type of specimens it was found that *A. baumannii* with resistance to ciprofloxacin had MIC >128 $\mu\text{g/ml}$ more in sputum and wounds culture (Table 7).

Table 3. Antibiotic resistance among isolates of *A. baumannii*.

Antimicrobial agent	Disc content	Zone diameter (mm)		Sensitive No.	Intermediate No.	Resistant No.
		Sensitive	resistant			
Ampicillin-sulbactam	(20 µg)	≥15	≤11	0	0	100
Amoxicillin-Clavulante	(30 µg)	≥18	≤13	0	0	100
Trimethoprim-sulfamethoxazole	(1.25/23.75µg)	≥15	≤12	0	0	100
Amikacin	(10 µg)	≥17	≤14	2	0	98
Ofloxacin	(5 µg)	≥16	≤12	8	15	77
Cefazolin	(30 µg)	≥18	≤14	0	0	100
Cefuroxime	(30µg)	≥18	≤14	0	0	100
Ceftriaxone	(30µg)	≥21	≤13	11	17	72
Cefepime	(30µg)	≥18	≤14	7	60	33
Ciprofloxacin	(5 µg)	≥21	≤15	33	19	48
Imipenem	(10µg)	≥22	≤18	88	3	9

Table 4. Comparison between disc diffusion test and MIC for detection of ciprofloxacin resistance.

Procedure used	CIP sensitive isolates		CIP resistant isolates	
	No.	%	No.	%
Disc diffusion test	33	33%	67	67%
MIC tube	28	28%	72	72%

Table 5. Genetic mutation among *A. baumannii* isolates.

Characteristics	No.	%
gyrA	7	9.7%
gyrB	2	2.8%
ParC	6	8.3%
ParE	0	0%
Combined gyrA and parC	57	79.2%
Total	72	100%

Table 6. *Acinetobacter baumannii* resistance by MIC.

	<i>A. baumannii</i> resistance by MIC (µg/ml)													
	4		8		16		32		64		128		> 128	
	No	%	no	%	No	%	no	%	no	%	no	%	no	%
<i>A.baumannii isolates</i>	11	15.2	6	8.3	2	2.8	2	2.8	3	4.2	4	5.6	44	61.1

Table 7. Genetic mutation among resistant *A. baumannii* isolate in relation to MIC (µg/ml).

	4		8		16		32		64		128		> 128	
	no	%	no	%	no	%	no	%	no	%	no	%	no	%
<i>gyrA</i>	3	4.2	0	0	0	0	2	2.8	2	2.8	0	0	0	0
<i>gyrB</i>	0	0	2	2.8	0	0	0	0	0	0	0	0	0	0
<i>parC</i>	0	0	0	0	2	2.8	0	0	0	0	4	5.6	0	0
<i>parE</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Combined gyrA and parC</i>	8	11.2	4	5.6	0	0	0	0	1	1.4	0	0	44	61.1

Figure 1. *Acinetobacter baumannii* distribution among different species of *Acinetobacter*.

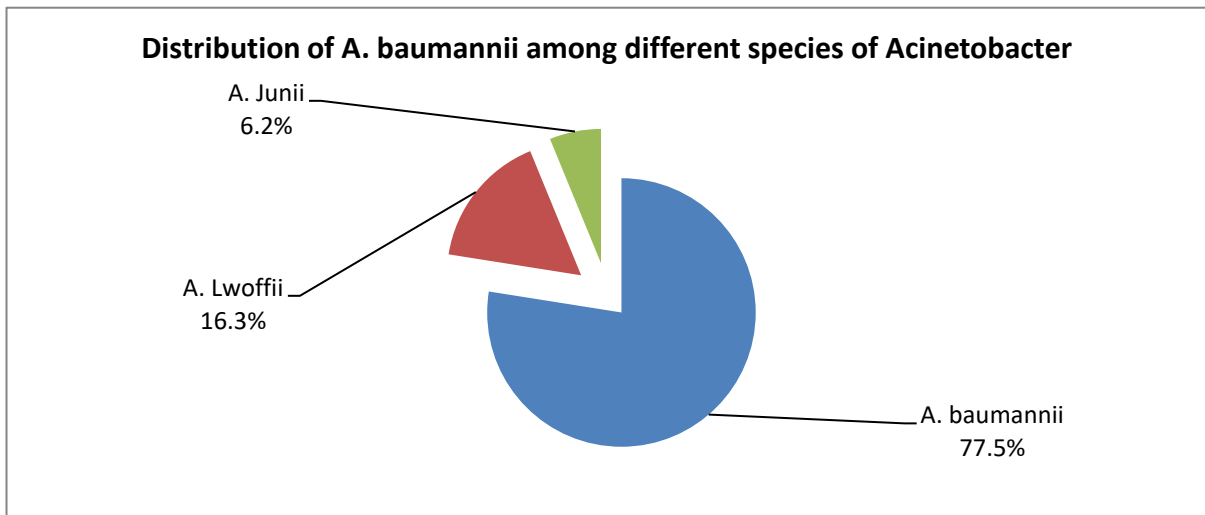




Fig. 2: Gel electrophoresis of PCR amplification for *gyrA* gene in *A. baumannii* isolates (343 bp): Lane 2,3,5, 6,8,9,10 positive cases - Lane 1,4,7,11 negative cases.

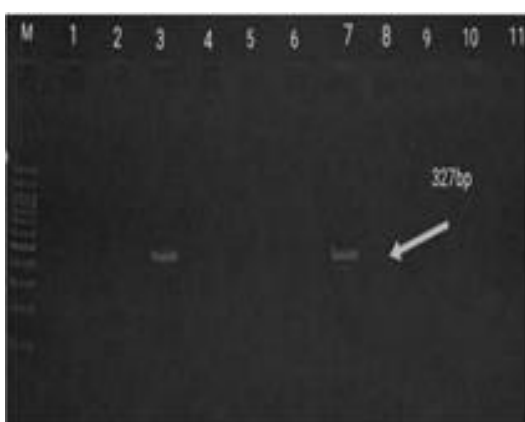


Fig.3: Gel electrophoresis of PCR amplification for *par C* gene in *A. baumannii* isolates (327 bp): lane 3,7 positive cases - Lane 1,2,4,5,8,9,10,11 negative cases.

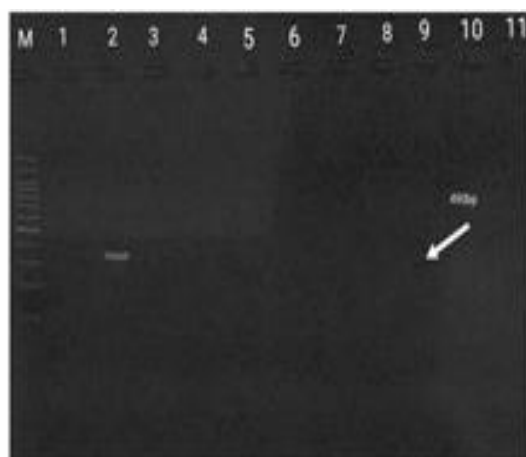
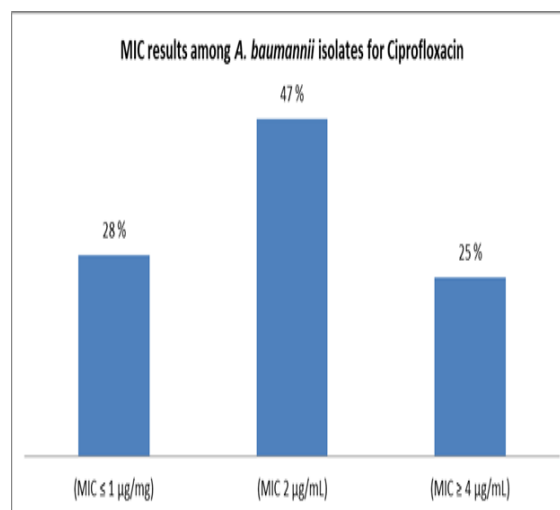


Fig. 4: Gel electrophoresis of PCR amplification for *gyrB* gene in *A. baumannii* isolates (490 bp): Lane 2 positive case - Lane 1,3, 4,5,6,7,8,9,10,11 negative cases.

Figure 5. Minimum inhibitory concentration results among *A. baumannii* isolates for ciprofloxacin.



Discussion

Acinetobacter baumannii has emerged as an essential organism in hospital acquired infections in last few years. The importance of *A. baumannii* came from its extensive antimicrobial resistance that make little antibiotic alternatives for treatments of its infections [17].

In the current study the most effective antibiotics against *A. baumannii* were imipenem (88%), ciprofloxacin (33%), ceftriaxone (11%), ofloxacin (8%), cefepime (7%) and amikacin (2%). While *A. baumannii* were 100% resistant to ampicillin/sulbactam, amoxicillin/clavulanate, trimethoprim/sulfamethozole, cefazolin and cefuroxime. This results is consistent with **Enas et al.** and **Nowroozi et al.** [18,19]. In the current study all *A. baumannii* isolates (100%) were significantly multidrug resistant isolates based on definition of multidrug resistance [15]. This result is also matching with **Nowroozi et al.** [19].

In our study from a total 72 *A. baumannii* isolates resistant to ciprofloxacin it was found that 44 (61.1%) isolates showed ciprofloxacin MICs > 128 µg/mL, indicating that these isolates were highly resistant to ciprofloxacin, in addition to 28(38.9%) isolates had MICs <128 µg/mL. Ciprofloxacin resistance among *A. baumannii* was 75% in a study conducted in Egypt in 2014 [20]. This points to that there is increase in ciprofloxacin resistance in the last years in Egypt among *A. baumannii* isolates. Meanwhile resistance were 95.6% in a study from Iran [8], 97.3% in a study from Pakistan [21], and 96.2% in a study from Saudi Arabia [22].

In the current study the sensitivity and specificity of disc diffusion method as compared to

the MIC in detection of ciprofloxacin resistance showed that 90% and 100%, respectively, this results concomitant with **Karami et al.** [23]. These results differed from **Stone et al.** [24], who reported that disc diffusion technique turned into a great fast screening technique to MIC for detection of antimicrobial sensitivity. Our results were matching with **Maysaa et al.** and **Valentine et al.** [25, 26].

A baumannii isolates with MIC >128 µg/mL had mixed mutations within the *gyrA* and *parC*. These findings suggest that mixed mutations are related to excessive MIC resistance to ciprofloxacin. The mixed mutations in those genes are related to remarkable decrease binding affinity for quinolones in the resistant organisms. These outcomes had been in settlement with **Maysaa et al.** and **Redgrave et al.** [25, 27].

Mixed mutations of *gyrA* & *parC* are mentioned to be related to a high-degree of resistance to quinolones, Six isolates in this study show mutations in *parC* without mutations in *gyrA*, suggesting that *parC* may have a role in ciprofloxacin resistance without mutation in *gyrA*. This result is consistent with **Ardebili et al.** [8]. While there had been two isolates in this result had mutations in *gyrB* gene alone those suggest that mutations in *gyrB* had little role in ciprofloxacin resistance, that is consistent with **Vila et al.** [28].

Conclusion

From this study we can conclude that resistance to ciprofloxacin was common in clinical isolates of *A. baumannii*. The most frequent mutations were present in *gyrA* and *parC*. However, mutations in *gyrB* or *parC* alone were not uncommon.

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