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PCR-RFLP-Based Detection of Mutations in the Chromosomal Fluoroquinolone Targets *gyrA* and *parC* Genes of *Acinetobacter baumanii* Clinical Isolates from a Tertiary Hospital in Cairo, Egypt

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Abstract **Background and Aim:** *Acinetobacter baumannii* is one of the most antimicrobial resistant nosocomial pathogens encountered clinically worldwide. The emergence of fluoroquinolone resistance among *A. baumannii* isolates is currently of concern. This study aimed to investigate the antimicrobial susceptibility patterns among 49 clinical isolates of *A baumannii* collected from a tertiary care hospital in Egypt. These isolates were analysed for the mechanism of fluoroquinolone resistance based on the presence of mutations in the quinolone resistance-determining regions (QRDRs) of the chromosomal quinolone resistance determinants *gyrA* and *parC* genes. **Methods:** *A. baumannii* isolates were identified using conventional biochemical testing, VITEK 2 automated system and polymerase chain reaction (PCR) assay targeting the intrinsic $bla_{\text{OXA-51-like}}$ gene of *A. baumannii* species. Antimicrobial susceptibility to different antimicrobial agents was tested using agar disk diffusion method and the minimum inhibitory concentration (MIC) of ciprofloxacin was determined using E-test (bioMérieux, France) on Mueller-Hinton agar medium following the Clinical and Laboratory Standards Institute guidelines. The QRDRs of the *gyrA* and *parC* genes in *A. baumannii* isolates were amplified by PCR using specific primers. Mutations in theses QRDRs were detected by *Hinf*I restriction fragment length polymorphism (RFLP) of PCR products and sequencing. **Results:** The $bla_{\text{OXA-51-like}}$ gene was detected in all isolates confirming identification as *A. baumannii*. Antimicrobial susceptibility study showed that all isolates (100 %) were MDR. They were 100 % resistant to the tested fluoroquinolones, ciprofloxacin and levofloxacin. The MIC of ciprofloxacin ranged from 4 to ≥ 32 μg/mL. All *A. baumannii* isolates (100 %) were found to harbour *gyrA* and *parC* genes. *Hinf*I restriction analysis showed a detectable mutation in QRDRs at position Ser-83 of *gyrA* gene and Ser-80 of *parC* gene. There was single mutation in either *gyrA* or *parC* in 11 isolates showed ciprofloxacin MIC of < 32 μg/mL, while 38 isolates with MIC of ≥ 32 μg/mL had double mutations in QRDRs of both genes. **Conclusions:** Resistance of *A. baumannii* isolates to fluoroquinolones in Egypt is alarming as all MDR *A. baumannii* isolates in the current study were mostly highly resistant to ciprofloxacin with MIC ≥ 32 μg/mL, limiting the remaining therapeutic options and a public health policy on appropriate prescribing and thus the rational use of antimicrobial agents is required. Double mutation with substitutions at positions Ser-83 and Ser-80 of *gyrA* and *parC* genes, respectively, could lead to high - level ciprofloxacin resistant phenotype than a single mutation in one of them. Further extensive studies including a larger number of isolates from different geographic areas in Egypt and investigating other fluoroquinolone resistance mechanisms are warranted.

Keywords: *PCR-RFLP, A. baumannii, gyrA, parC, fluoroquinolone*

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1. Introduction

Multi-drug resistant (MDR) *A. baumannii* is a rapidly emerging pathogen in the health care settings where the risk factors such as long stay in intensive care units, recent mechanical ventilation or invasive procedures. In addition, the selective pressure exerted by the extensive use of broad-spectrum antimicrobials is a critical risk factor. Accordingly, *A. baumannii* has become one of the most difficult nosocomial Gram-negative bacilli to control and treat [1,2]. *A. baumannii* can cause a variety of serious infections including bacteraemia, pneumonia, meningitis,

urinary tract infection and wound infection. These infections that caused by MDR strains are associated with remarkable mortality rate and hospital costs [3].

Fluoroquinolone drugs such as ciprofloxacin, with a wider spectrum of antibacterial activity particularly against Gram-negative bacteria [4], have established as a necessary member of the antimicrobial arsenal [5]. However, Resistance of Gram-negative bacteria, including *A. baumannii*, to fluoroquinolones has become common in the last decades leading to the emergence of resistance to these antibacterial agents [6]. The main targets of fluoroquinolones are the bacterial DNA topoisomerase II (DNA gyrase) and DNA topoisomerase IV, which are essential for DNA replication. Fluoroquinolones inhibit the control of supercoiling within the cell exerted by these two enzymes resulting in impaired DNA replication and consequently inhibiting cell division [6,7]. DNA gyrase enzyme is composed of two GyrA and two GyrB subunits, and topoisomerase IV enzyme is composed of two ParC and two ParE subunits. When either DNA gyrase or topoisomerase IV induces transient double-strand DNA breaks, they first bind to the DNA to form enzyme-DNA complexes before breaking the bound DNA, passing another segment of DNA through this break, and re-joining the original DNA segment. When a fluoroquinolone drug is present, it binds to DNA gyrase or topoisomerase IV, thus, the complex is altered into a drug-enzyme-DNA complex which is unable to re-ligate the DNA substrate. The binding of fluoroquinolones to these enzymes occurs at the helix-4 in either GyrA or ParC subunits [6,7,8,9].

The most frequent mechanism of resistance to fluoroquinolones is the alterations in the genes that encode the subunits of quinolone targets DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*). These alterations involve mainly the mutations located in the quinolone resistance-determining regions (QRDRs) of *gyrA* gene and its homologous region of the *parC* gene. In contrast, mutations in *gyrB* and *parE* genes are of minor significance and are rarely contribute to fluoroquinolone resistance [10,11,12]. There are few data from Egypt on the molecular basis of fluoroquinolone resistance based on the mutations in the genes encodes for DNA gyrase and topoisomerase IV in *A. baumanii*. Thus, in this study, we aimed to screen *A. baumanii* clinical isolates from a tertiary hospital in Cairo for the chromosomal quinolone resistance determinants *gyrA* and *parC* genes, and specifically assess the presence of mutations in the QRDRs of both genes.

2. Materials and Methods

2.1. *A. baumannii* Clinical Isolates Included in This Study

From November 2015 to May 2016, a total of 49 non-duplicate *A. baumannii* clinical isolates were collected from International Medical Centre (IMC), Cairo, Egypt. These 49 *A. baumannii* isolates were recovered from diverse clinical samples, collected from inpatients (each isolate from individual patient), including endotracheal tube (16 isolates), blood (9 isolates), wound swabs (11 isolates), central venous pressure (CVP) catheter tips (6 isolates), urine (5 isolates) and sputum (2 isolates).

2.2. Identification of *A. baumannii* Isolates

A. baumannii isolates were identified using conventional biochemical testing and cultural characteristics and VITEK 2 automated system (bioMe´rieux, Marcy l'E´toile, France). Identification was confirmed by polymerase chain reaction (PCR) assay targeting the intrinsic $bla_{\text{OXA-51-like}}$ gene of *A. baumannii* species.

2.3. Antimicrobial Susceptibility Testing of *A. baumannii* Isolates

The agar disk diffusion method was used to investigate the antimicrobial susceptibility patterns of *A. baumannii* isolates to 15 different antimicrobial agents, representing different antimicrobial classes, following Kirby-Bauer method on Mueller-Hinton agar medium [13]. The antimicrobial disks were the product of Oxoid, UK. They included piperacillin (100 µg), piperacillin/tazobactam (100/100 µg), ampicillin/sulbactam (10/10 µg), ceftazidime (30 µg), cefotaxime (30 µg), cefepime (30 µg), ceftriaxone (30 µg), imipenem (10 µg), meropenem (10 µg) gentamicin (10 µg), amikacin (30 µg), tetracycline (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg) and trimethoprim/sulfamethoxazole (1.25/23.75 µg). The interpretation of the results of antimicrobial susceptibility testing as susceptible, intermediate or resistant to examined antimicrobial agents was performed following Clinical and Laboratory Standards Institute guidelines [14].

2.4. Determination of the Minimum Inhibitory Concentration (MIC)

The MIC of ciprofloxacin was determined against *A. baumannii* isolates by agar diffusion method using E-test (bioMérieux, France) on Mueller-Hinton agar medium following the manufacturer's instructions. Concentrations of ciprofloxacin tested ranged from 0.002 - 32 µg/mL. Mueller-Hinton agar plates were inoculated with *A. baumannii* bacterial suspension equivalent to 0.5 McFarland standard, then ciprofloxacin (CIP) E-test strips were placed on each. Plates were incubated at 37ºC for overnight and the MIC was read at the intersect where the ellipse of growth inhibition intersects the strip. The results were interpreted using the interpretive MIC susceptibility breakpoints of ciprofloxacin against *A. baumannii* of CLSI (2016): MIC $\leq$ 1 µg/mL for susceptible, MIC 2 µg/mL for intermediate and MIC $\geq$ 4 µg/mL for resistant.

2.5. PCR Amplification of $bla_{\text{OXA-51-like}}$, *gyrA* and *parC* Genes

The $bla_{\text{OXA-51-like}}$ gene and the QRDRs of the *gyrA* and *parC* genes in *A. baumannii* isolates were amplified by PCR. PCR oligonucleotide primers used in this study, synthesized by Invitrogen, UK, are listed in Table 1. The concentration of each primer was adjusted to 10 pmol/µl using nuclease free water (Promega, USA). Total DNA was extracted from all tested isolates using boiling method

by heating bacterial cells suspension in sterile distilled water at 95°C for 10 min, followed by removal of cellular debris by centrifugation at 14,000 rpm for 1 min. The supernatant was collected and used as template DNA for PCR amplification.

PCR reaction mixtures were prepared in total volumes of 20 µl. Each reaction contained 2 µl of template DNA, 1 µl (equivalent to 10 pmol concentration) of each primer and 10 µl of GoTaq® Green Master 2× Ready Mix (Promega, USA), then the volume was completed to 20 µl by adding 6 µl of nuclease free water. The PCR amplification programmes were as follows: initial denaturation for 5 min at 95°C, then 30 cycles of denaturing at 95°C for 30 seconds, annealing for 30 seconds at 52°C for $bla_{\text{OXA-51-like}}$, 47°C for *parC* gene, 53°C for *gyrA* gene, and extension at 72°C for 45 seconds, followed by a final extension at 72°C for 7 min. the PCR amplicons were sequenced at Clinilab, Cairo using an ABI 3730XL DNA Analyzer (Applied Biosystem Inc., Forster City, CA, USA).

2.6. Detection of *gyrA* and *parC* Mutations by PCR-restriction Fragment Length Polymorphism (RFLP)

Detection of mutations in the QRDRs of both *gyrA* and *parC* genes in ciprofloxacin resistant isolates was performed by *Hinf*I restriction digestion. The *gyrA* and *parC* PCR amplified products were subjected to restriction analysis with *Hinf*I restriction enzyme (NEB, UK) following the manufacturer's recommended conditions to yield the restriction digestion products indicating mutations on TAE-agarose gel electrophoresis according to Vila et al. [10] for *gyrA* and Vila et al. [11] for *parC*.

2.7. TAE (Tris-acetate-EDTA)-agarose Gel Electrophoresis

DNA fragments of PCR products and restriction digestion analysis were detected through TAE agarose gel (1 %) (Bioline, UK) electrophoresis in 1× TAE buffer containing ethidium bromide for DNA visualisation. DNA fragments were visualized by placing the gel on a UV light source and the fragments' sizes were estimated and photographed directly. GeneRuler 1 kb DNA molecular weight marker (Thermo Scientific, USA) was used for sizing the PCR products and separated DNA fragments of restriction analysis.

Table 1. Sequences of PCR primers used in this study

Target gene	Primer sequence (5' – 3')	Amplicon size	Source
*bla*OXA-51	Forward TAATGCTTTGATCGGCCTTG	353 bp	15
	Reverse TGGATTGCACTTCATCTTGG		
gyrA	Forward AAATCTGCCCGTGTCGTTGGT	343 bp	16
	Reverse GCCATACCTACGGCGATACC		
parC	Forward AAACCTGTTCAGCGCCGCATT	327 bp	
	Reverse AAAGTTGTCTTGCCATTCACT		

3. Results

3.1. Identification of *A. baumannii* Isolates

A. baumannii isolates were identified using Vitek 2 automated system with 99 % probability. Isolates were catalase positive and oxidase negative, the observations which differentiated them from *Pseudomonas* spp., and grew at 42°C which differentiated them from *A. calcoaceticus* according to UK Standards for Microbiology Investigations of Public Health England [17,25]. Identification was confirmed by detection of the intrinsic $bla_{\text{OXA-51-like}}$ specific gene in all 49 *A. baumannii* isolates as it was amplified from all isolates using $bla_{\text{OXA-51-like}}$ specific primers (Figure 1).

3.2. Antimicrobial susceptibility Pattern of *A. baumannii* Isolates

All 49 *A. baumannii* isolates included in the present study were MDR as they were resistant to at least three different classes of the tested antimicrobial agents. All isolates (100 %) were resistant to piperacillin, piperacillin/tazobactam, ampicillin/sulbactam, cefepime, ceftriaxone, cefotaxime, ceftazidime, imipenem, meropenem, ciprofloxacin and levofloxacin. The resistance rates to gentamicin, amikacin, tetracycline and trimethoprim/sulfamethoxazole were 39/49 (79.6 %), 38/49 (77.5 %), 36/49 (73.5 %) and 44/49 (89.8 %), respectively (Table 2).

Table 2. Antimicrobial susceptibilities of *A. baumannii* isolates included in this study using agar disk diffusion method

Antimicrobial agent	Sensitive No. (%)*	Intermediate No. (%)*	Resistant No. (%)*
Piperacillin	0 (0)	0 (0)	49 (100)
Piperacillin/Tazobactam	0 (0)	0 (0)	49 (100)
Ampicillin/Sulbactam	0 (0)	0 (0)	49 (100)
Cefepime	0 (0)	0 (0)	49 (100)
Cefotaxime	0 (0)	0 (0)	49 (100)
Ceftazidime	0 (0)	0 (0)	49 (100)
Ceftriaxone	0 (0)	0 (0)	49 (100)
Ciprofloxacin	0 (0)	0 (0)	49 (100)
Levofloxacin	0 (0)	0 (0)	49 (100)
Gentamicin	6 (12.2)	4 (8.2)	39 (79.6)
Amikacin	7 (14.3)	4 (8.2)	38 (77.5)
Imipenem	0 (0)	0 (0)	49 (100)
Meropenem	0 (0)	0 (0)	49 (100)
Tetracycline	3 (6.1)	10 (20.4)	36 (73.5)
Trimethoprim/ sulfamethoxazole	5 (10.2)	0 (0)	44 (89.8)

*Percentages correlated to the total number of isolates (49).

3.3. MIC of Ciprofloxacin to *A. baumannii* Isolates

The MIC of ciprofloxacin against *A. baumannii* isolates, determined by E-test, ranged from 4 to ≥ 32 µg/mL (Table 3 and Table 4, Figure 1).

Table 3. MIC distribution of ciprofloxacin, PCR detection of *gyrA* and *parC* and detection of *gyrA* and *parC* mutations

Susceptibility pattern to ciprofloxacin No. (%)			Distribution of MIC of Ciprofloxacin (µg/mL)								
S	I	R	≤ 1	2	4	6	8	12	16	24	≥ 32
0	0	46 (100)	0	0	4	0	0	0	3	4	38
gyrA PCR detection			-	-	+ve (4)	-	-	-	+ve (3)	+ve (4)	+ve (38)
RFLP analysis-based *gyrA* mutation			-	-	+ve (3) -ve (1)	-	-	-	+ve (2) -ve (1)	+ve (2) -ve (2)	+ve (38)
parC PCR detection			-	-	+ve (4)	-	-	-	+ve (3)	+ve (4)	+ve (38)
RFLP analysis-based *parC* mutation			-	-	+ve (1) -ve (3)	-	-	-	+ve (1) -ve (2)	+ve (2) -ve (2)	+ve (38)

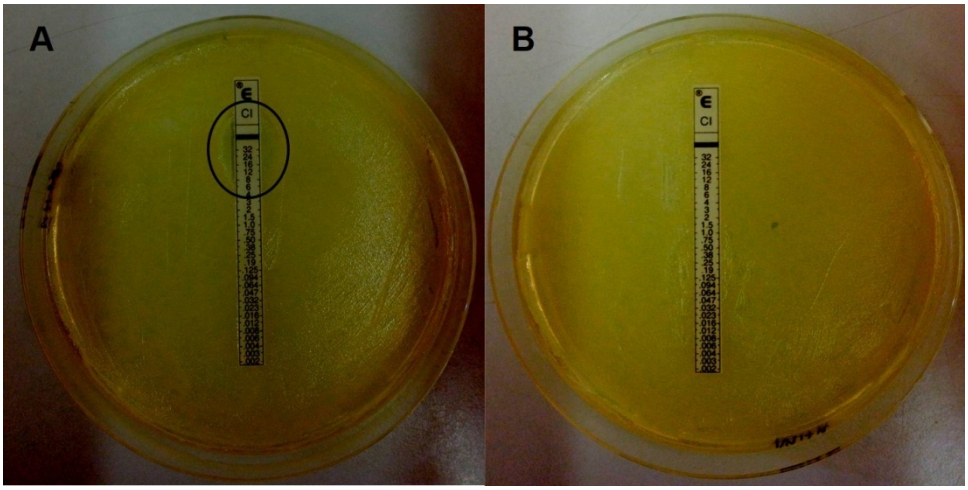

Figure 1. Representative results of ciprofloxacin (CIP) E-test for isolates of *A. baumannii*. A, isolate showing MIC 4 µg/mL; B, isolate showing MIC ⩾ 32 µg/mL

Table 4. Distribution of MIC of *A. baumannii* isolates to ciprofloxacin by E-test based on type of specimens

Specimen (No. of isolates)	Distribution of MIC of Ciprofloxacin (µg/mL)								
	≤ 1	2	4	6	8	12	16	24	≥ 32
Endotracheal tube (16)	0	0	3	0	0	0	0	1	12
Blood (9)	0	0	1	0	0	0	0	0	8
Wound swabs (11)	0	0	0	0	0	0	3	0	8
Urine (5)	0	0	0	0	0	0	0	0	5
Sputum (2)	0	0	0	0	0	0	0	0	2
Central venous pressure catheter tips (6)	0	0	0	0	0	0	0	0	6

3.4. PCR Detection *gyrA* and *parC* Genes

All *A. baumannii* isolates (100 %) were found to harbour the genes *gyrA* and *parC* indicated by PCR amplification results of both genes from the 49 isolates (Figure 2).

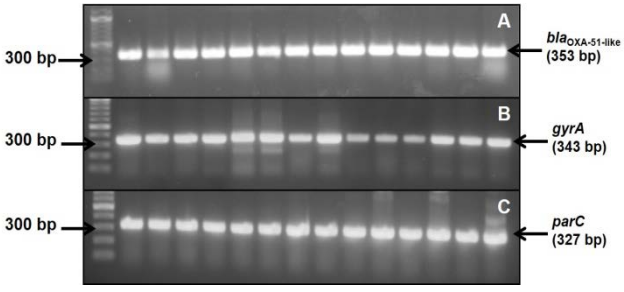

Figure 2. Agarose gel electrophoresis (1 % agarose) of PCR amplicons of A, $bla_{\text{OXA-51-like}}$ gene; B, *gyrA* gene; C, *parC* gene. First lane in each panel is GeneRuler DNA M.W. marker

3.5. Detection of Mutation in QRDRs of *gyrA* and *parC* genes by *Hinf*I Restriction Analysis

*Hinf*I restriction analysis showed a detectable mutation in QRDR of *gyrA* gene and/or *parC* gene in *A. baumannii* isolates in this study indicted by no digestion of PCR products of *gyrA* and *parC* by *Hinf*I enzyme as there was only one DNA band detected on agarose gel at 343 bp and 327 bp, respectively (Figure 2). There was single mutation in QRDR of either *gyrA* or *parC* in isolates showed ciprofloxacin MIC of < 32 µg/mL, while isolates with MIC of ≥ 32 µg/mL had double mutations in QRDRs of both genes. There was no mutation in QRDR of *gyrA* gene indicted by digestion of *gyrA* PCR product in one isolate of MIC 4 µg/mL, one isolate of MIC 16 µg/mL and two isolates of MIC 24 µg/mL, giving two bands of sizes 291 bp and 52 bp. There was no mutation in QRDR of *parC* gene indicted by digestion of PCR in one isolate of MIC 4

µg/mL, one isolate of MIC 16 µg/mL and two isolates of MIC 24 µg/mL, giving two bands of sizes 206 bp and 121 bp (Table 3, Figure 3).

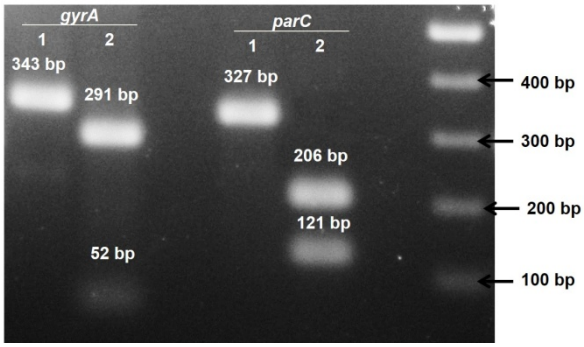

Figure 3. Agarose gel (1 %) detection of the mutation in the *gyrA* gene and *parC* gene by *Hinf*I restriction fragment length polymorphism of PCR products. Lane 1 for each gene: non-digested PCR product indicating mutation in the QRDR of this gene. Lane 2 for each gene: digested PCR product indicating no mutation in the QRDR of this gene. The rightest lane: DNA molecular weight marker

4. Discussion

In developing countries, the frequency of nosocomial infections caused by MDR bacteria is very high owing to inadequate infection control practices, overcrowding of hospitals and inappropriate use of antimicrobials [18]. *A. baumannii* is one of the most important bacterial pathogen causing nosocomial infections worldwide. Unfortunately, it has been noting that the infections caused by *A. baumannii* are difficult to control because of multiple resistance to available antimicrobial agents, accordingly the therapeutic options are very limited, particularly in critically ill and debilitating patients [2]. Antimicrobial resistance patterns among this important pathogen varies considerably from country to country or among hospitals within the same country and even within the same country and/or hospital over time [19,20]. Thus, the frequent surveillance of nosocomial pathogens for their antimicrobial susceptibility patterns, especially in a developing country like Egypt, to guide appropriate selection for empirical therapy is necessary [20]. Accordingly, in this study, a total of 49 nosocomial *A. baumannii* clinical isolates, recovered from various clinical specimens, were screened for their antimicrobial susceptibility patterns. In addition, these isolates were investigated for the quinolone resistance mechanism through mutations in QRDRs of the chromosomal fluoroquinolone targets *gyrA* and *parC* genes.

All isolates in this study were identified using VITEK 2 automated system (bioMe´rieux, Marcy l'E´toile, France) which indicated *A. baumanii* complex in all isolates with 99 % probability. Identification of *A. baumannii* from other *Acinetobacter* species requires a complex battery of phenotypic tests, otherwise genotypic methods are perfect although they are beyond the capabilities of routine microbiology laboratories [21]. The VITEK 2 system detects metabolic changes by fluorescence-based methods which facilitate the rapid identification of Gram-negative bacteria, based on the kinetics of bacterial growth. The VITEK 2 system correctly identified *A. baumannii* isolates in the present study, providing fast and accurate method for identification of this bacterium. However, for testing antimicrobial activities, it would be better for clinical microbiologists to evaluate their own MICs rather than trust the limitations imposed by the VITEK 2 system [21]. Accordingly, antimicrobial susceptibly testing in the current study was performed using agar diffusion disk and E-test susceptibility methods. Moreover, the identification of isolates as *A. baumanii* was confirmed by detection of the $bla_{\text{OXA-51-like}}$ gene which is specific to *A. baumanii* species. It has been evidenced that $bla_{\text{OXA-51}}$ gene is an *A. baumannii* naturally occurring carbapenemase encoding gene intrinsic to this species. Thus, PCR detection of this gene is more reliable than biochemical identification of *A. baumannii*, and the ability to distinguish it properly from other members of the genus is highly valuable [15,22,23].

A. baumannii is highly resistant to the most of available antimicrobial agents, which has been partly attributed to the overwhelming use of antimicrobial agents, including fluoroquinolones (e.g., ciprofloxacin), that resulted in the emergence of more resistant forms of this bacterium [24,25]. Based on the antimicrobial susceptibility testing, all *A. baumanii* isolates (100 %) in this study were significantly MDR isolates based on Magiorakos et al. [2] definition of multidrug resistance in bacteria; the resistance to at least one antimicrobial agent in different three or more classes of antimicrobial agents. This result is consistent with Nowroozi et al. (2014) as 100 % of *Acinetobacter* isolates were MDR. In the current study, all *A. baumannii* isolates (100 %) were resistant to piperacillin, ampicillin/sulbactam, piperacillin/tazobactam, cefepime, ceftriaxone, cefotaxime, ceftazidime, imipenem, meropenem, ciprofloxacin and levofloxacin. In addition, *A. baumanii* showed high frequencies of resistance to other classes of antimicrobials including gentamicin (79.6 %), amikacin (77.5 %), tetracycline (73.5 %) and trimethoprim/sulfamethoxazole (89.8 %). This high resistance could be explained by that our isolates were mainly of nosocomial infections origin. Unfortunately, these observations revealed that these antimicrobial agents are not suitable for initiation of empirical therapy of *A. baumannii* caused infection in a developing country like Egypt that make the treatment of these infections is challenging. This could be due to the overuse and/or misuse as well as the hospitals' indiscriminate routine use of these antimicrobial agents in developing countries which resulted in increasing the resistance rates to them. Although previous studies revealed the effectiveness of several antimicrobial agents, such as imipenem, ceftazidime and quinolone, as a potential empirical therapy for *A. baumannii* infections [20,26]. This resistance can be inherited or acquired through resistance genetic factors [25].

The fluoroquinolone drugs such as ciprofloxacin have shown good activities against *Acinetobacter* spp. However, the widespread use of fluoroquinolones caused these drugs to face resistance from infectious bacteria which represents a rapidly increasing major clinical issue worldwide [27]. Using E-test, all *A. baumanii* isolates in this study were resistant to ciprofloxacin with MIC ≥ 4. Of 49 *A. baumanii* isolates, 38 isolates showed ciprofloxacin MICs $\geq$ 32 µg/mL, indicating that these isolates were

highly resistant to ciprofloxacin, in addition to 11 isolates had ciprofloxacin MICs less than 32 µg/mL. In an Egyptian study conducted in 2014, the resistance rate of ciprofloxacin among *A. baumanii* was 75 % [28], indicating increasing the resistance to this drug in the last years in Egypt. The resistance to ciprofloxacin in this study was comparable to other studies. The resistance percentages were 95.6 % in a study from Iran [29], 96.2 % in a study from Saudi Arabia [30], 97.3 % in a study from Pakistan [31], 97.7 % in a study from Nepale [32], however the percentage was 19.4 % in a study from Tawian [33].

Significantly, in this study, the E-test results completely agreed with the results of disk diffusion method. This finding was consistent with the record of Howard et al. [34] when compared the activities of ciprofloxacin and other fluroquinolones against *Acinetobacter* spp. using both methods. The authors also confirmed that E-test to be an excellent and practical alternative to the use of the broth microdilution method for quinolones against *Acinetobacter* spp.; a finding supported by similar success with this method for gatifloxacin by Biedenbach et al. [35]. E-test was also used successfully by number of studies for determining MIC of ciprofloxacin against *Acinetobacter* spp. [19,24].

Most studies emphasized that spontaneous mutations in QRDRs of both *gyrA* and *parC* genes are essential for resistance of *A. baumannii* to ciprofloxacin [25,36,37,38]. In this study, the 49-ciprofloxacin resistant *A. baumanii* isolates showed a mutation of *gyrA* gene and/or *parC* in PCR-RFLP analysis and sequencing. Based on Vila et al. [10] and Vila et al. [11], the presence or absence of *gyrA* and *parC* mutations at codon 83 and codon 80, respectively, can be determined by the digestion of the PCR products with *Hinf*I restriction enzyme. The *Hinf*I restriction analysis in the current study was consistent with the records of Vila et al. [10] and Vila et al. [11] as *Hinf*I digestion of the PCR product from *A. baumannii* isolates with no mutation in *gyrA* gene generated two fragments of 291 bp and 52 bp, and in *parC* gene generated two fragments of 206 and 121 bp while the *Hinf*I restriction site in isolates carrying a mutation at both codons in both genes was abolished, resulting in no digestion of the fragment containing the full-length PCR product.

A. baumanii isolates showed MIC to ciprofloxacin less than 32 µg/mL had only one mutation in the QRDRs of *gyrA* or *parC*, that resulted in the substitution of Ser-83 to Leu in GyrA protein or Ser-80 to Leu in ParC protein. While there were double mutations in the QRDRs of both *gyrA* and *parC* genes (Ser → Leu in both proteins) in isolates with MIC ≥ 32 µg/mL. These results may indicate that mutations in both *gyrA* and *parC* genes resulted in increasing the MIC and accordingly a high level of resistance to ciprofloxacin. From these observations, it looks like the two mutations with Ser-83→Leu in GyrA and Ser-80→Leu ParC are common among ciprofloxacin-resistant clinical isolates of *A. baumannii* in Egypt. This finding revealed that the presence of mutation in the *parC* gene alongside the mutation in the *gyrA* gene contributed to a higher-level of ciprofloxacin resistance than a single mutation in one of them due to ten times less binding affinity for quinolones [16,27,39,40,41]. In addition, the finding of substitution of Ser-83 to Leu in GyrA protein and Ser-80 to Leu in ParC protein was consistent with the records of many previous studies revealed that theses mutations are the principal mutation associated with ciprofloxacin resistance in *A. baumannii* [10,16,23,37,38,42].

In conclusion, antimicrobial resistance in *A. baumannii* has increased significantly in the past decade. In the current study, we found that most of *A. baumannii* isolates were highly resistant to fluoroquinolones (i.e ciprofloxacin) with MIC ≥ 32 µg/mL. Resistance of *A. baumannii* isolates to ciprofloxacin in Egypt is alarming; since these isolates were MDR, thus, the remaining therapeutic options are limited. Therefore, a public health policy on appropriate prescribing and the rational use of antimicrobial agents is a warranted. There is a need to explore new synthetic quinolone analogues. Double mutation with substitution at position Ser-83 of both *gyrA* gene and *parC* genes, was frequently identified in ciprofloxacin-resistant *A. baumannii*, which could lead to high-level ciprofloxacin resistant phenotype than a single mutation in one of them by modulating the MIC level. The present work could serve as the basis for more extensive studies including a larger number of isolates from different areas in Egypt and investigating other fluoroquinolone resistance mechanisms other than mutations in *gyrA* and *parC* leading to resistance to fluoroquinolones in *A. baumannii* isolates.

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