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ORIGINAL ARTICLE

Quinolone-resistant clinical strains of *Pseudomonas aeruginosa* isolated from University Hospital in Tunisia

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Abstract In this study, we examined mutations in the quinolone resistance-determining regions (QRDRs) of the *gyrA* and *parC* genes of *Pseudomonas aeruginosa* (*P. aeruginosa*) clinical isolates collected from patients hospitalized in University Hospital of Monastir, Tunisia. A total of 81 *P. aeruginosa* strains, obtained from clinical specimens, were included in the present study. Isolates were tested against 11 different antibiotics by a disk diffusion method. Minimum inhibitory concentrations (MICs) of ciprofloxacin were evaluated by E test method. The *gyrA* and *parC* sequences genes amplified by polymerase chain reaction (PCR) were sequenced. The highest resistance rates were found for ciprofloxacin (100%), gentamicin (96%) and ticarcillin (93%). The lower resistance rates were obtained for imipenem (74%) and ceftazidime (70%). Notably, 54% of isolates resistant to ciprofloxacin were determined to be multi-drug resistant. The investigation of mutations in the nucleotide sequences of the *gyrA* and *parC* genes showed that 77% of isolates have a single mutation in both *gyrA* (Thr-83 → Ile) and *parC* (Ser-87 → Leu). The emergence of ciprofloxacin resistance in clinical *P. aeruginosa* requires the establishment of appropriate antibiotherapy strategies in order to prescribe the most effective antibiotic treatment for preventing the emergence of multi-drug-resistant (MDR) *P. aeruginosa* strains.

Keywords Ciprofloxacin · *gyrA* · Mutations · *Pseudomonas aeruginosa* · *parC* · Resistance

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Introduction

P. aeruginosa is an important opportunistic human pathogen responsible for a wide spectrum of serious nosocomial infections (Obritsch et al. 2005; Alhazmi 2015). Antipseudomonal agents including some beta-lactams, aminoglycosides, and fluoroquinolones have been the most important antibiotics used for treatments of *P. aeruginosa* infections. Surveillance of antimicrobial resistance of *P. aeruginosa* has revealed the emergence of multi-drug-resistant (MDR) strains worldwide. The emergence of such strains is due to the excessive use of broad-spectrum antibiotics as well as the ability of *P. aeruginosa* to acquire resistance genes (Sievert et al. 2013; Breidenstein 2011; Livermore 2002). Fluoroquinolones (FQs) and carbapenems have been widely used in the treatment of MDR *P. aeruginosa* infections (Pfaller and Jones 2000; Thomson 1999). However, the resistance to carbapenems has been reported in countries worldwide, namely European countries (Italy, France, Greece, Spain, Turkey), African countries (Tunisia, Algeria, Kenya, and South Africa), and Asian countries (China, Korea) (Fang et al. 2014; Lee and Ko 2012; Pfaller and Jones 2000). Moreover, FQs resistance among *P. aeruginosa* isolates has increased at an alarming rate leading to treatment failure in infections and limiting their usefulness (Amudhan et al. 2012; Hancock 1998, Jaffe et al. 2001; Jalal et al. 2000). The main mechanisms of resistance to FQs are mutations in the quinolone resistance-determining regions (QRDRs) within DNA gyrase (GyrA and GyrB subunits) and topoisomerase IV (ParC and ParE subunits) and in regulatory genes for drug efflux pumps (Aeschlimann 2003; Akasaka et al. 2001; Le Thomas et al. 2001; Ziha-Zarifi et al. 1999). Mutations in the GyrA and ParC subunits have a major role in conferring high-level FQs resistance

in *P. aeruginosa* (Belland et al. 1994). Clinical isolates of *P. aeruginosa* resistant to FQs have been reported worldwide (De Francesco et al. 2013; Falagas and Bliziotis 2007; Pasca et al. 2012; Rossolini and Mantengoli 2005; Salma et al. 2013). Therefore, it is important to determine the antibiotic susceptibility pattern of bacteria so that clinicians can prescribe the appropriate antibiotic. In our study, we have evaluated the antibiotic resistance of *P. aeruginosa* clinical strains isolated from University Hospital of Monastir and determined the amino acid mutations in the QRDRs (*gyrA* and *parC* genes) within ciprofloxacin-resistant isolates.

Materials and methods

Bacterial strains

A total of 81 *P. aeruginosa* strains were collected from pathological specimens of patients hospitalized in University Hospital of Monastir, between November 2014 and May 2015. The bacterial isolates were recovered from different clinical specimens (Table 1). The isolated bacteria were characterized based on isolation on Pseudomonas Selective Agar, Base (Cetrimide Agar), gram staining, and biochemical tests using the API 20NE system (BioMérieux, Marcy l'Etoile, France).

Confirmation of species identification was performed by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (AutoflexTM; Bruker Daltonics, Bremen, Germany) with the Flex control software (Bruker Daltonics) MALDI-TOF. A score value > 1.9 was considered for confirmation *P. aeruginosa* identification as reported previously (Seng et al. 2009). *P. aeruginosa* ATCC 27853 and PAO1 (Accession: NC 002516.2) strain were used as control strains.

Antimicrobial susceptibility testing

Susceptibility to antipseudomonal antimicrobials: beta-lactams (extended-spectrum penicillins: piperacillin + tazobactam/ticarcillin + clavulanic acid), cephalosporins (ceftazidime) carbapenems (imipenem), aminoglycosides (gentamicin, tobramicin, amikacin), FQs (ciprofloxacin, levofloxacin), colistin, and fosfomycin, was assessed by an agar disk diffusion method. Antimicrobial breakpoints were interpreted according to the recommendations of the Comité de l'antibiogramme de la Société Française de Microbiologie (CA-SFM) (2014) (www.sfm-microbiologie.org). Minimum inhibitory concentration (MIC) of CIP was determined by *E* test method (Biomerieux, Marcy l'Etoile, France) according to the manufacturer's instructions. *P. aeruginosa* ATCC 27853 was used as the control strain for susceptibility testing.

Antibiotic resistance was categorized into three groups: (1) resistance to extended-spectrum penicillins, and/or resistance to cephalosporins, and/or resistance to carbapenems (imipenem), (2) resistance to aminoglycosides, and (3) resistance to quinolones (ciprofloxacin).

DNA extraction and polymerase chain reaction (PCR)

The chromosomal DNA of all *P. aeruginosa* isolates and PAO-1 strain was extracted by the heat shock method and used as templates for PCR. To detect resistance-mediating mutations in the QRDRs of the target genes *gyrA* and *parC*, amplification was performed with specific primers previously described (Pasca et al. 2012). The cycling conditions were as follows: initiation at 94 °C for 10 min; 30 cycles of denaturation at 94 °C for 1 min, annealing at 69 °C for 1 min for *gyrA* and 60 °C for *parC*, elongation at 72 °C for 1 min; and final elongation for 10 min at 72 °C.

Table 1 Distribution of *P. aeruginosa* isolates by source of isolation

Samples	Unit Origin					
	ICU	ICUN	IDU	PNU	ORLU	ORPU
Pus (20)	2	4	3		6	5
Urine (4)	4					
Tracheal aspirate (28)	28					
Distal Bronchial (12)	12					
Drainage liquid (8)				8		
Septum (9)	3			6		
Total 81						

ICU intensive care unit, *IDU* Infectious disease Unit, *ICUN* Intensive Care Unit of Nephrology, *PNU* Pneumology Unit, *ORPU* Orthopedic Unit, *ORL* Oto-Rhino-Laryngology

DNA sequencing

Amplicons were sent for DNA sequencing to Biotools platform (http://www.biotools.eu). The aligned sequences were then analyzed with the BioEdit sequence program, and similarity searches for the nucleotide sequences were performed with the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

The studied *P. aeruginosa* isolates were collected from specimens of patients hospitalized in different units of Monastir Hospitals, mainly, from intensive care unit (65%); 34% of strains were recovered from tracheal aspirate, 24% from pus, 9% from drainage liquid, and the remaining isolates from other different clinical specimens. The resistance profiles of all strains were determined by antibiogram. The term "multi-drug resistance" was used to describe the resistance to three or more classes of antimicrobial primarily, beta-lactams (antipseudomonal penicillins, carbapenems, cephalosporins), aminoglycosides, and FQ agents, according to previous reports (Falagas et al. 2005; McCracken et al. 2009).

The determination of antimicrobial susceptibility was performed by agar disk diffusion method, and the results were analyzed in accordance with the recommendations established by CA-SFM. The interpretation of the antibiograms was carried out by measuring the diameters of the inhibition zones and comparing them with the critical diameters defined for each antibiotic. The results shown in Table 2 revealed that all strains were resistant to ciprofloxacin. Gentamicin (96%) and ticarcillin (93%) were the low active antibiotics among antimicrobial agents tested. The resistance rates for carbapenems and cephalosporins were 74% for imipenem and 70% for ceftazidime, respectively. The colistin was the most active antibiotic as no colistin-resistant strain was recorded. Table 3 summarizes the resistance patterns identified according to isolates resistance frequencies among different antibiotic classes. Our results showed that 54% were MDR *P. aeruginosa*.

The ciprofloxacin resistance of all *P. aeruginosa* strains was confirmed by *E* test method. Different values of ciprofloxacin MICs were found ranging from 2 to $\geq$ 32 mg/ml (Table 4).

The amplification of *gyrA* and *parC* genes gave two fragments of size 416 and 363 bp, respectively. DNA sequences of all isolates were compared with the corresponding sequences of *P. aeruginosa* PAO1 (Accession: NC 002516.2). Results analysis showed that all isolates presented one mutation Thr-83 → Ile in *gyrA* and 63 isolates showed one mutation Ser-87 → Leu in *ParC*. Mutations in nucleotide sequence and protein sequence of *gyrA* and *parC* are shown in Figs. 1 and 2.

Discussion

P. aeruginosa is an important opportunistic pathogen causing nosocomial infections (Wolska and Szweda. 2009). In fact, over the past decade the widespread use of FQ class is an alarming increase in the prevalence of FQ resistance among *P. aeruginosa* strains (Linder et al. 2005; Neuhauser et al. 2003). More importantly, resistance to ciprofloxacin was significant associated with cross-resistance to other antimicrobial agents (Flamm et al. 2004; Hsu et al. 2005). Several studies have described *P. aeruginosa* strains with reduced susceptibility or resistant to FQs and imipenem

Table 3 Resistance groups of *P. aeruginosa* isolates

No. (% resistance)	Antibiotic classes
58:81 (71)	R1
56:81 (69)	R2
74:81 (91)	R3
54:81 (66)	R1 + R2
55:81 (67)	R1 + R3
71:81 (87)	R2 + R3
44:81 (54)[a]	R1 + R2 + R3

R1 beta-lactam resistant, *R2* aminoglycoside resistant, *R3* fluoroquinolone resistant

[a] Multi-drug resistant

Table 2 Antimicrobial susceptibility patterns of *Pseudomonas aeruginosa* isolates

Antimicrobial agents	Ticarcillin–tazobactam		Piperacillin–clavulanate		Imipenem		Ceftazidime		Gentamicin		Tobramicin		Amikacin		Ciprofloxacin	
	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R
Critical diameters (mm)	$\geq$ 18	< 18	$\geq$ 18	18 <	$\geq$ 20	< 17	$\geq$ 16	< 16	$\geq$ 15	< 15	$\geq$ 16	< 16	$\geq$ 18	< 15	$\geq$ 25	< 22
% Strains	7	93	8	82	19	74	30	70	4	96	7	93	29	67	0	100

R resistant, *S* susceptible, % percentage of strains

Table 4 Distribution of ciprofloxacin-resistant *P. aeruginosa* isolates by *gyrA* and *parC* mutations

Mutation	No. of isolates	No. of isolates with MIC µg/ml											
		< 1	1	2	3	4	6	8	12	16	24	32	> 32
gyrA T83I	18			2		4	3		2	1			6
gyrA T83I +*parC* S87L	63			12				4	6	12		8	21

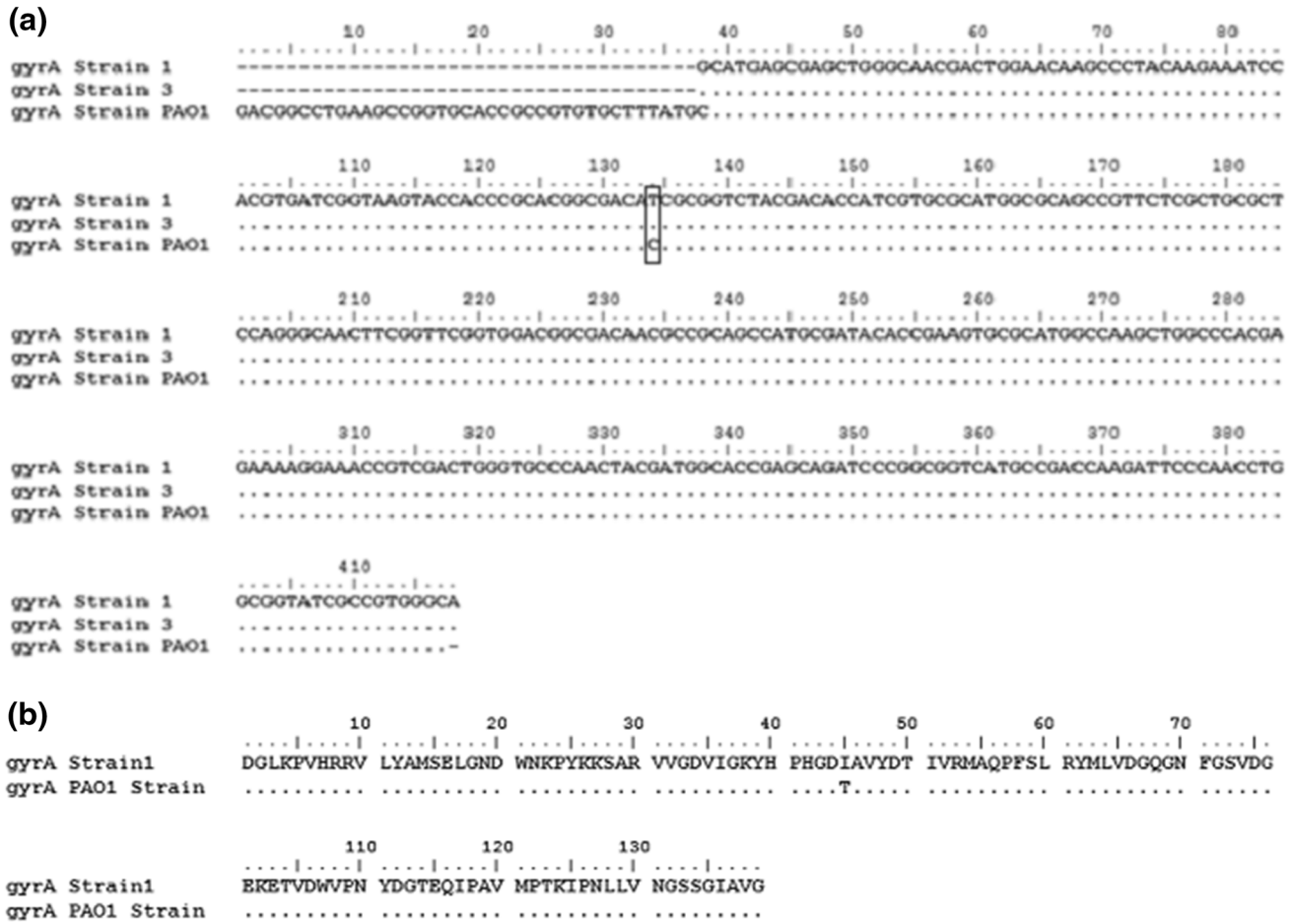

Fig. 1 Nucleotide sequences (**a**) and amino acid sequences (**b**) comparison of *gyrA*

(Henwood et al. 2001; Karlowsky et al. 2003; Thomson 1999). The resistance of *P. aeruginosa* to FQ was widely studied, but this work represents to our knowledge the first study in Tunisia.

In this work, we reported that among all characterized isolates 54% of strains were MDR, but all were susceptible to colistin. However, strains resistant to colistin, beta-lactams, aminoglycosides, and FQs have been previously detected but fortunately remain rare (Johansen et al. 2008; Rossolini and Mantengoli 2005). It has been documented that colistin could be a useful alternative treatment, while having a considerable toxicity (Falagas et al. 2005).

P. aeruginosa strains are a major threat to patients, especially, in the Intensive Care Unit (ICU). Higher rates of antimicrobial resistance are recorded in ICUs due to multiple factors, including increased use of broad-spectrum antimicrobials, high occurrence of invasive procedures, and increased chance of resistant bacteria transmission among patients (Hanberger et al. 2009; Rosenthal et al. 2012; Sader et al. 2014, Sievert et al. 2013). In our study,

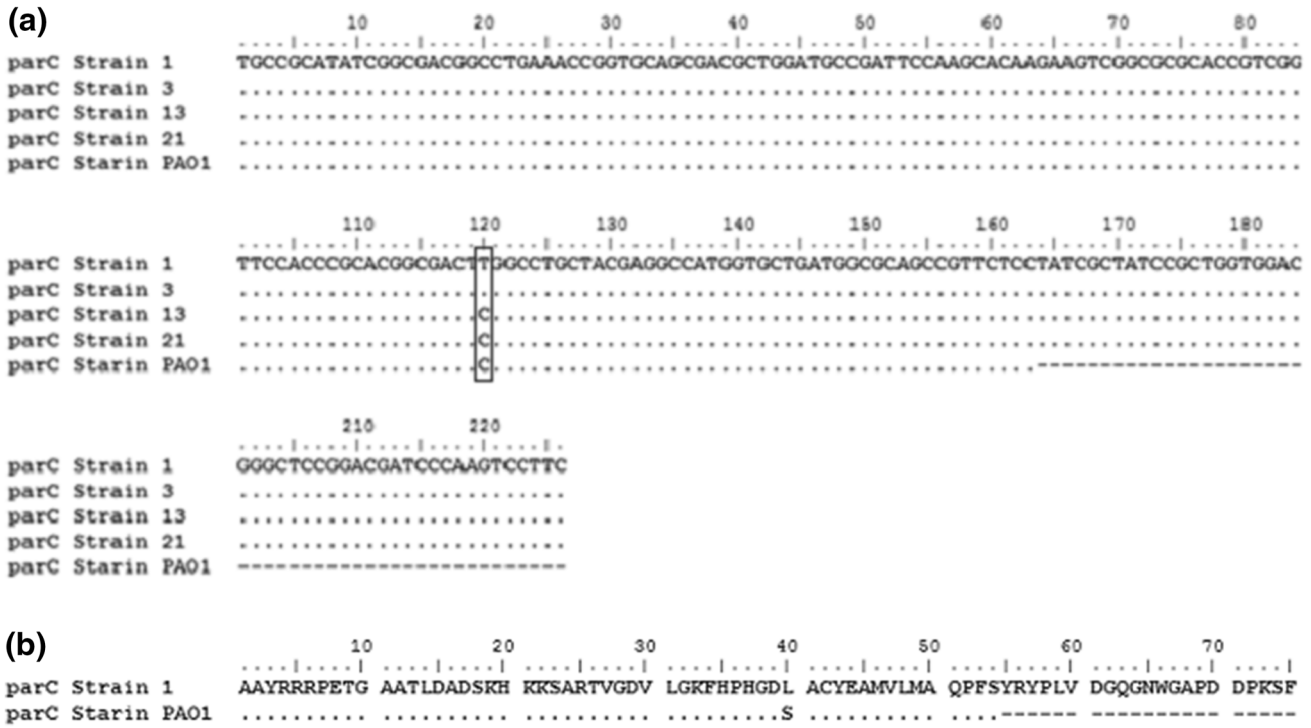

Fig. 2 Nucleotide sequences (**a**) and amino acid sequences (**b**) comparison of *parC*

62% of MDR strains identified were isolated from patients hospitalized in ICU. *P. aeruginosa* seems to be emerged in ICU worldwide (Rosenthal et al. 2012; Sader et al. 2014). Surveillance studies of the occurrence frequency and antimicrobial susceptibility of *P. aeruginosa* isolated from ICU patients are available (Rosenthal et al. 2012; Sader et al. 2014; Sievert et al. 2013). Recent report of Sader et al. (2014) showed that ICU isolates from USA and Europe were MDR, and they were susceptible to only colistin (99.4% susceptible) and amikacin (97.3% in USA and 84.9% in EU). The surveillance study conducted by the International Nosocomial Infection Control Consortium (INICC) between January 2004 and December 2009 focused on data from 36 countries reported high levels of ciprofloxacin-resistant strains isolated from patients in ICUs (Rosenthal et al. 2012).

FQs resistance in *P. aeruginosa* is mediated primarily through amino acid mutations in the QRDRs of *gyrA*, *gyrB*, *parC* and *parE* genes (Jalal and Wretlind 1998; Mouneimné et al. 1999).

Sequencing the target genes *gyrA* and *parC* showed that 77% isolates have mutations in both *gyrA* and *parC*. In g*yrA*, the substitution of isoleucine for threonine at position 83 (Thr-83 → Ile) was detected in all tested strains (Fig. 1). In *parC*, the substitution of leucine for serine in position 87 was found in 63 isolates (Fig. 2). The alteration in *gyrA* (Thr-83 → Ile) is the most frequent substitution identified in several studies on clinical isolates of *P. aeruginosa*. The substitution in *parC* (Ser-87 → Leu) was also reported previously (Akasaka et al. 2001; Mouneimné et al. 1999; Oh et al. 2003). These findings are consistent with previously published mutations (Akasaka et al. 2001; Higgins et al. 2003; Jalal and Wretlind 1998; Nakano et al. 1997).

According to our results, strains with a double *gyrA*-*parC* mutation have high-level resistance (MIC ≥ 32 mg/L). However, four resistant isolates having single mutations in *gyrA* showed also MICs ≥ 32 mg/L. We suggested that *gyrA* is the main target of FQs; a mutation in *gyrA* seems to be the main factor leading to FQs resistance. Indeed, previous studies reported that the alteration in *parC* occurs after the *gyrA* alteration and is associated with the development of higher-level FQs resistance (Akasaka et al. 2001; Salma et al. 2013).

Conclusion

Our results are in agreement with other studies as they showed that a mutation in *gyrA* (Thr-83 → Ile) gene plays a crucial role in occurrence resistance to FQs. The limited activity of ciprofloxacin could be due to the overuse of this antimicrobial agent in clinical settings. The ongoing surveillance of this bacterium is important to help in

directing antimicrobial therapy and monitoring the emergence of potentially drug-resistant strains.

Author contribution statement Mouna Ben Nejma proposed the idea of the project and contributed substantially in writing the manuscript. Olfa Sioud collected and identified the strains. Maha Mastouri is the director of laboratory and supervised the work.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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