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gyrA Mutations in Ciprofloxacin-resistant Clinical Isolates of *Pseudomonas aeruginosa* in a Silesian Hospital in Poland

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A b s t r a c t

Among 73 clinical isolates of *Pseudomonas aeruginosa* 48 strains were ciprofloxacine (CIP) susceptible and 25 CIP resistant (Minimal inhibitory concentration – MIC>32 μg/ml – 14 strains) or of intermediate susceptibility to CIP (MIC≥1,5 – 32 μg/ml – 11 isolates). Mutations in the quinolone-resistance-determining region (QRDR) of *gyrA* gene were searched in groups of CIP resistant and of intermediate susceptibility to CIP isolates. Two methods: restriction fragment length polymorphism (RFLP) analysis and DNA sequencing analysis allowed to detect three different mutations. The nucleotide substitutions observed led to the following amino acid replacements: Thr-83 → Ile, Asp-87→Asn, Asp-87→Gly. One mutated strain among the group of mutants analyzed showed double mutation (Thr-83→Ile, Asp-87→Gly) and additional silent mutation (Val-103→Val); whilst the rest of the isolates showed different single missense mutations. The most frequently detected mutation in the *gyrA* gene (16 out of 25 mutants) was the Thr-83→Ile substitution.

K e y w o r d s: *Pseudomonas aeruginosa*, ciprofloxacin, resistance, mutation, *gyrA*

Introduction

Pseudomonas aeruginosa possesses intrinsic mechanisms of resistance to a wide variety of antibiotic. Fluoroquinolones (FQ_s) are broad-spectrum antibiotics, which are known to be effective in the treatment of a wide range of infections. Ciprofloxacin has emerged as one of the most effective quinolones against *P. aeruginosa* (Mouneimńe *et al.*, 1999). However, the extensive use of fluoroquinolones has resulted in an increasing incidence of FQ_s resistance (Yonezawa *et al.*, 1995). Opportunistic infections caused by *Pseudomonas aeruginosa*, especially quinolone-resistant isolates, pose a serious medical problem. The antibacterial effect of the FQ_s depends on the inhibition of bacterial topoisomerases: DNA gyrase and topoisomerase IV (Bearden and Danziger, 2001; Dricla and Zhao, 1997). DNA gyrase and topoisomerase IV are heterotetrameric proteins composed of two subunits designated A and B. The genes encoding the A and B subunits are referred to as *gyrA* and *gyrB* (DNA gyrase) or *parC* and *parE* (DNA topoisomerase IV). Both subunits constitute the active form of the enzyme: A_2B_2 (Dricla and Zhao, 1997). DNA gyrase activity is strongly inhibited by quinolones. Alterations in DNA gyrase or topoisomerase IV caused by mutations in the QRDR appear to play a major role in fluoroquinolone resistance in clinical isolates of *P.* aeruginosa (Yoshida *et al*., 1990). Recently, several species of bacteria have been studied in order to determine the influence of *gyrA* mutation on quinolone resistance (Weigel *et al.*, 1998).

The authors have not found any Polish publications concerning mutations in *gyrA* gene in fluoroquinolone-resistant clinical isolates of *P. aeruginosa.*

The aim of our study was to find mutations responsible for ciprofloxacin-resistance in *gyrA* gene of *P. aeruginosa* clinical strains, isolated in Silesia region.

Experimental

Materials and Methods

Bacterial isolates. 73 clinical isolates of *Pseudomonas aeruginosa* were isolated at Microbiological Laboratory, Saint Barbara Hospital No 5 in Sosnowiec, Poland, in 2003 from various clinical materials, collected from infirmary and hospitalized patients, showing this species infections.

Antimicrobial susceptibility testing. Initial MIC profiles were screened using the disc diffusion method in accordance with the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS, 2000). The MICs of ciprofloxacin were determined by the E-test (AB Biodisk, Solna, Sweden). The range of CIP concentration in susceptibility tests was between 0.002 and 32 μg/ml. The results were evaluated after 24 hours of incubation at 35°C. The *Pseudomonas aeruginosa* strain ATCC 27853 was included as a control.

PCR analysis. Primers were designed to amplify the DNA fragment including the putative quinolone resistance-determining region (Yoshida *et al.*, 1990). For the QRDR of *gyrA* (GenBank access number L29417), a pair of primers, PaGA 1 (5'-TGACGGCCTGAAGCCGGTGCAC-3') and PaGA 4 (5'-TATCGCATGGCTGCGGCGTTG-3') (Takenouchi *et al.*, 1999), was used. These primers allowed amplification of the *gyrA* gene region, including codons 38 to 122, to encompass the region containing codons 67 to 106. The amplification procedure comprised of initial denaturation at 94°C for 3 min followed by 38 cycles of denaturation (30 s at 94°C), annealing (30 s at 55°C), and polymerization (60 s at 72°C), and then a final extension cycle: 10 min at 72°C. The reactions were conducted in a final volume of 50 μl with 2.5 U of *Taq* DNA polymerase (Fermentas).

PCR-RFLP analysis. PCR products were treated with *Cfr* 42 *I* enzyme (*an isoschizomer of Sac*II), (MBI Fermentas) at 37°C for 2 hours, and the restriction fragments were separated in 3% low-melting-point agarose gel and visualized by means of ethidium bromide staining (Takenouchi *et al.*, 1999). The *Cfr* 42 *I* site (CCGCŻGG) is present in the wild-type *gyrA* gene between nucleotides 512 and 517, whereas it is absent in the mutant *gyrA* gene.

Nucleotide sequencing. Prior to the sequencing reaction, the PCR products were purified by isopropanol precipitation with 2M $NaClO_4$ and glycogen in low-TE buffer. Amplicons were analyzed by direct sequencing using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). DNA sequencing analysis was performed with the same primers as those used for PCR. Sequence reactions products were purified with the use of SigmaSpin columns (Sigma-Aldrich) and air-dried. Samples were heated at 96°C for 4 min in 5 μl of loading buffer (25 mM EDTA, pH 8,0 with 50 mg/ml blue dextran and deionized formamid with a ratio of 1:5) and chilled rapidly with ice before loading with 5% denaturing polyacrylamide gel in an Applied Biosystem 377 DNA Sequencer according to the manufacture's instructions (Kureishi *et al.*, 1994; Yonezava *et al.*, 1995; Nakano *et al.*, 1997; Takenouchi *et al.*, 1999). The nucleotide sequences obtained both for sense and antisense primers were compared with the GeneBank Database (http://www.ncbi.nlm.nih.gov/blast) in order to check their homology.

Results and Discussion

The first step of our study was to perform a drug susceptibility test. Using the E-test method, we evaluated the lowest ciprofloxacin concentration visibly preventing growth of *P. aeruginosa,* (MIC) (Fig. 1). The strains analyzed showed a various susceptibility to ciprofloxacin. Figure 1 presents ciprofloxacin MIC values for analyzed strains of *P. aeruginosa*. 48 isolates (65.7%) were CIP susceptible (MIC ≤1 μg/ml), whilst 14 strains (19.2%) were CIP resistant with MIC >32 μg/ml, and 11 strains (15.1%) were of inter-

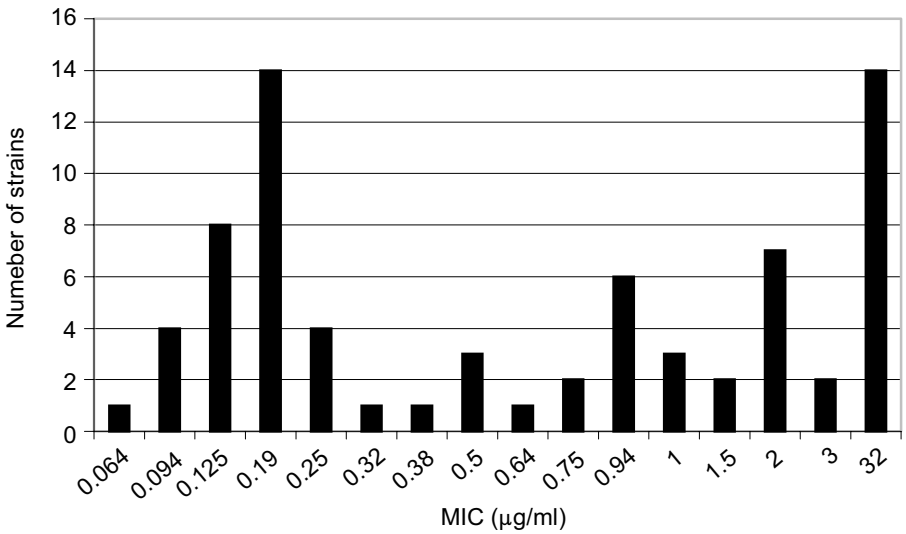

Fig. 1. Ciprofloxacin MIC profile for different strains of *P. aeruginosa*

Table I
Types of point mutations in *gyrA* and ciprofloxacin MIC range for the resistant or of intermediate susceptibility *P. aeruginosa* strains

PCR-RFLP method	DNA sequencing method			Number and (%) of strains with mutation	Number and (%) of strains without mutation
	Type of mutation	Amino acid replacement	Nucleotide replacement		
*Cfr*42 I site present	Wild type	–	–	–	2 (8)
	Single mutation	**Asp-87 → Asn**	GAC→AAC	7 (28)	–
*Cfr*42 I site not detected	Single mutation	**Thr-83 → Ile**	ACC→ATC	15 (60)	–
	Double mutation	**Thr-83 → Ile** **Asp-87 → Gly**	ACC→ATC GAC→GGC	1 (4)	–
	Silent mutation	**Val-103 → Val**	GTA→GTC		
Total number of strains with and without mutation in the *gyrA* gene				23 (92)	2 (8)
Total number of analyzed strains				25 (100)	

mediate susceptibility to CIP with MIC ≥1.5–32 μg/ml. Only the resistant strains and the isolates of intermediate susceptibility were subjects of our further studies (25 strains). The PCR reaction confirmed the presence of the *gyrA* gene in all of the strains. In order to detect mutations two methods were used for each examined analyzed strain: PCR-RFLP with *Cfr 42 I* (*Sac*II) and DNA sequencing analysis. PCR-RFLP analysis with *Cfr 42 I* was performed as a screening method. The restrictase *Sac*II recognizes the CCGC↓GG site, present only in a wild type *gyrA* gene between 512 and 517 nucleotides at codon 83, therefore this method allows mutation at the Cfr 42 *I* site to be detected, however it does not exclude the possibility of mutation elsewhere. In our study, using the PCR-RFLP method, we found that 9 out of 25 isolates (36%) have *Cfr* 42 *I* restriction site and 16 strains (64%) did not have the site recognized by this enzyme (Table I). Of the 16 isolates without *Cfr* 42 *I* restriction site, 14 showed resistance to CIP; furthermore, the MIC_s values were above the maximum E-test concentration (>32 μg/ml) (Table II).

In order to perform a more accurate analysis of the entire fragment of the *gyrA* gene, a DNA sequencing analysis was conducted for each examined strain of *P. aeruginosa*. Using this method we discovered *gyrA* mutation in 23 strains (92%). Gene alterations were located at codon 83 and 87. We found 15 strains with a single mutation Thr-83→Ile (13 strains resistant to CIP, 2 of intermediate susceptibility to CIP); 7 strains with a single mutation Asp-87→Asn (all of them of intermediate susceptibility to CIP), and 1 strain (#47) with a double mutation and additional silent mutation Thr-83→Ile, Asp-87→Gl, Val-103→Val (resistant to CIP) (Table I, Fig. 2).

Table II
Correlation between MIC values and presence of mutations in the *gyrA* gene of *P. aeruginosa*

Strain number	MIC μg/ml	Amino acid alteration in *gyrA* gene at codon		
		83	87	103
ATCC 27853	0.25	Thr (ACC)	Asp (GAC)	Val (GTA)
2	3		Asn (AAC)	
3	3		Asn (AAC)	
4	2		Asn (AAC)	
5	1.5			
6	2		Asn (AAC)	
8	2			
10	2	Ile (ATC)		
26	2		Asn (AAC)	
27	> 32	Ile (ATC)		
36	2		Asn (AAC)	
40	> 32	Ile (ATC)		
43	1,5		Asn (AAC)	

Strain number	MIC μg/ml	Amino acid alteration in *gyrA* gene at codon		
		83	87	103
47	> 32	Ile (ATC)	Gly (GGC)	Val (GTC)
54	> 32	Ile (ATC)		
67	> 32	Ile (ATC)		
69	> 32	Ile (ATC)		
70	> 32	Ile (ATC)		
71	> 32	Ile (ATC)		
76	> 32	Ile (ATC)		
91	> 32	Ile (ATC)		
93	> 32	Ile (ATC)		
94	> 32	Ile (ATC)		
95	> 32	Ile (ATC)		
102	> 32	Ile (ATC)		
119	2	Ile (ATC)		

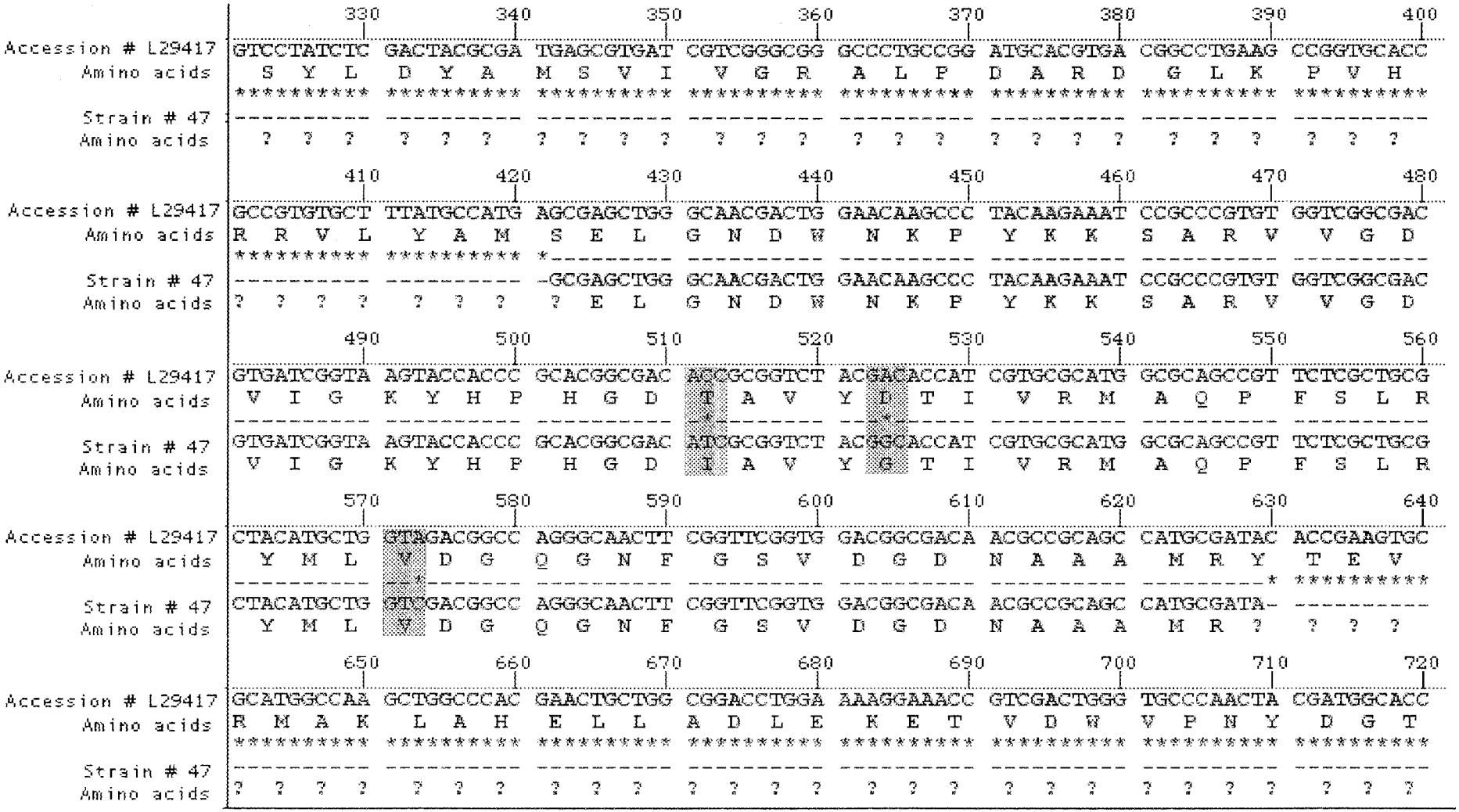

Fig. 2. Nucleotide and amino acid sequence matching between isolate #47 with double mutation and silent mutation of *gyrA* gene and wild type of *gyrA* gene (GB ACC L29417, Kureishi *et al.*, 1994)

Akasaka and co-workers (2001) collected clinical isolates of *P. aeruginosa*, and sequenced bacterial DNA to search for type II topoisomerase mutations. They found mutations in *gyrA* gene in 119 out of 150 isolates with reduced susceptibilities to levofloxacine (79.3%); the replacement(s) of amino acid(s) referred to: Thr-83→Ile or Ala, Asp-87→Asn, Gly or Tyr. The principal replacement observed by the researchers was Thr-83→Ile (74.7%). In our study we found similar types of mutation in *gyrA* gene at codon 83 and 87. As a result of nucleotide substitutions we detected the following mutations: Thr-83→Ile, Asp-87→Asn, Asp-87→Gly. In addition to point mutations in the *gyrA* gene followed by a single amino acid change in 22 strains: 7 Asp-87→Asn and 15 Thr-83→Ile, we found one strain with one double mutation and additional silent mutation: Thr-83→Ile, Asp-87→Gly, Val-103→Val. Moreover, the replacement Thr-83→Ile was the most frequent (65.2%).

The point mutation followed by Thr-83→Ile (ACC?ATC) amino acid replacement was also reported to be the most frequent by Mouneimné *et al.* (1999), Yonezawa and co-authors (1995), and Nakano's group (1997). Contrary to our study, mutation at codon 87: Asp-87→Asn has been reported only sporadically by other authors (Yonezawa *et al.*, 1995; Nakano *et al.*, 1997).

The silent mutation Val-103→Val observed in the present study was one of 7 different types of silent mutation in the *gyrA* gene reported by Takenouchi and co-authors (1999). Takenouchi's group found two double mutations, three of which were previously unknown: Ala-67→Ser, Asp-87→Gly, Ala-84→Pro and Gln-106→Leu. One of them, Asp-87→Gly, was also described by us in strain #47 carrying a double *gyrA* mutation, and showed resistance to CIP ≥32 μg/ml. The resistance of mutant Thr-83→Ile to FQ, as mentioned in other articles, was also observed in our study (87.5%) (Mouneimné *et al.*, 1999; Weigel *et al.*, 1998; Akasaka *et al.*, 2001; Nakano *et al.*, 1997; Takenouchi *et al*., 1999).

According to Ball (1994), suitable drug dosing and medical procedure supervision can minimize or even prevent bacterial resistance to quinolones. He emphasizes that bacteria causing intrahospital infections through steady contact with a wide range of chemotherapeutics easily develop resistance to drugs and, as a consequence, the drug resistance phenomenon occurs mostly among hospitalized patients. In our study, 10 of the 14 isolates (71.4%) recognized as CIP resistant (MIC ≥32 μg/ml) were obtained from hospitalized patients, which confirms Ball's (1994) observation.

Our results and the data of other authors show that the analyzed sequence of the *gyrA* gene plays a crucial role in counteracting ciprofloxacin and DNA gyrase, however, the participation of a mutation localized in different regions of *P. aeruginosa* genome cannot be excluded. A wide spectrum of genes has recently been analyzed with regard to FQ resistance, *e.g.* mutations in *parC*, *mexR* or *nfxB* genes have been the subject of intense discussion (Nakajima *et al.*, 2002; Nakano *et al.*, 1997). Further studies on the mechanism of *P. aeruginosa* resistance to FQ_s are necessary. Such studies may not only improve the efficiency of FQ therapy and help to prevent the new bacterial resistance phenomenon, but also can help to synthesize new drugs that enable initiation of the more effective therapy of *P. aeruginosa* infections.

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