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Novel Gyrase Mutations and Characterization of Ciprofloxacin-resistant Clinical Strains of *Enterococcus faecalis* Isolated in Poland

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A b s t r a c t

The activity of ciprofloxacin, sparfloxacin and moxifloxacin was determined for 205 *Enterococcus faecalis* isolates from patients of five hospitals (Warsaw, Poland; collected from 2000 to 2002). Ciprofloxacin resistant and intermediate isolates were numerous (53.7%). Among them, highly resistant (MIC ≥16 mg/l) isolates predominated (98%). Isolates resistant to ciprofloxacin were also resistant to sparfloxacin and moxifloxacin. The *parC* and *gyrA* QRDRs (quinolone-resistance-determining region) of 11 isolates with ciprofloxacin MICs from 1 to 256 mg/l were analysed by DNA sequencing. In ParC one kind of amino acid substitution (of Ser-85 to Ile) in 9 *E. faecalis* strains with MICs from 16 to 256 mg/l was observed. In GyrA Ser-84 was changed to one of four different amino acids: Arg, Ile, Cys or Tyr, however no association between the amino acid type and MIC value was found. The last two substitutions have not been reported to date for *E. faecalis*. Moreover, our results may suggest that mutations within *parC* and *gyrA* are associated with development of a high-level of ciprofloxacin resistance.

K e y w o r d s: *Enterococcus faecalis*, fluoroquinolone resistance, *gyrA*, *parC*, QRDR

Introduction

Enterococcus faecalis is the most commonly isolated among enterococci. Recently, an increasing number of enterococcal infections including live-threatening nosocomial infections have been observed. It may be a result of a growing multi-resistance to many antimicrobial agents commonly used to treat enterococcal infection, that limits therapeutic choices (Eliopoulos, 1993; Hunt, 1998). The widespread use of fluoroquinolones has led to an increasing number of fluoroquinolone-resistant clinical isolates of *E. faecalis.* This problem was observed in many countries (Schaberg *et al.*, 1992; Barišić and Punda-Polić*,* 2000), also in Poland. Zaręba and Hryniewicz (1995) reported that 14% of clinical *E. faecalis* isolates collected between 1991 and 1993 were resistant to ciprofloxacin. However, among strains collected from patients between 1996 and 2005, Kawalec *et al.* (2007) found that 72% were resistant to ciprofloxacin.

The main mechanism of antibacterial action of fluoroquinolones depends on the inhibition of the target enzymes: DNA gyrase and topoisomerase IV. Mutations associated with resistance to fluoroquinolones in *E. faecalis* have been documented in conserved regions of the *gyrA* and *parC* genes (Korten *et al.*, 1994; Tankovic *et al.*, 1996; Kanematsu *et al.*, 1998). These regions are referred to as the quinolone-resistance-determining region (QRDR). Despite the rapidly growing resistance of *E. faecalis* to quinolones in Poland, little is known about the type and heterogeneity of mutations within the *gyrA* and *parC* QRDRs in Polish isolates. Thus, we have analysed the *parC* and *gyrA* QRDRs and their adjacent regions in selected fluoroquinolone resistant strains of *E. faecalis*.

Experimental

Materials and Methods

Bacterial strains. The 205 clinical isolates of *E. faecalis* originated from patients of five hospitals in Warsaw, Poland (collected from 2000 to 2002) were tested for resistance to ciprofloxacin, sparfloxacin and moxifloxacin. Identification was performed by

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a standard biochemical tests according to the scheme of Faclam *et al.* (1999). The resistance to ciprofloxacin was determined by using a twofold agar dilution method (Oxoid, England) according to guidelines recommended by the Clinical and Laboratory Standards Institute – CLSI (Clinical Laboratory Standards Institute, 2006), while MICs of the two remaining agents were examined by application of E-test (AB Biodisk, Sweden). MIC interpretation criteria for ciprofloxacin followed those of the CLSI whilst no criteria were available for sparfloxacin and moxifloxacin (CLSI, 2006). For molecular analysis of the *gyrA* and *parC* QRDRs 11 isolates with ciprofloxacin MICs from 1 to 256 mg/l were randomly selected from the all 205 tested strains (Table II).

DNA amplification and sequencing. Total genomic DNA template for PCR from 11 tested strains and the type strain was obtained as described previously (Gierczyński *et al.*, 2004). The *gyrA* and *parC* QRDRs were amplified by PCR using primers designed in this study. A 575-bp fragment from the *gyrA* gene (Gen Bank Accession No AB059405) was amplified with oligonucleotide primers 5'-GCAATGAG TGTTATCGT AGCC-3' and 5'-TCTGGTCCAGGTA ACACTTCC-3' while a 549-bp *parC* (GenBank Accession No AB 059406) fragment was amplified with oligonucleotide primers 5'-CCTACCAGATATTC GAGATGG-3' and 5'-TCTGGTCCTGGAATGTAT TCC-3'. PCRs were carried out in 100 µl reaction volumes in a Mastercycler (Eppendorf), with 5 U of Taq DNA Polymerase (Fermentas), Mg-free PCR buffer with $(NH_4)_2SO_4$, each deoxynucleoside triphospate at a concentration of 0.2 mM, 1.5 mM $MgCl_2$, each primer at a concentration 0.4 µM and 10 µl of the template DNA solution. A program consisting of 35 cycles for 60 seconds of each denaturation at 94°C, annealing at 58°C and elongation at 72°C was used for amplification. Finally, DNA synthesis was completed at 72°C for 3 min. Prior to cycling, a 3 min denaturation step at 94°C was included. PCR-products were sequenced using Big Dye®Terminator v3.1 cycle sequencing kit (Applied Biosystem) in both the forward and reverse directions by using ABI PRIZM 377 automatic sequencer.

Results and Discussion

Ciprofloxacin MICs of tested strains and their breakdown for the all tested strains (n = 205) are shown in Table I. Among them, 53.7% were intermediate or resistant to ciprofloxacin. Notably, the majority (98%) of the resistant isolates were highly resistant to ciprofloxacin, with MICs raging from 16 to 256 mg/l. These results are in agreement with those reported by Tankovic *et al*. (1996), who tested a large panel of *E. faecalis* clinical strains isolated in France from 1986 to 1999. The *E. faecalis* strains that were resistant to ciprofloxacin, revealed sparfloxacin MICs above 32 mg/l. In this group, moxifloxacin MICs ranged from 1 to 32 mg/l, however strains with MICs from 16 to 32 mg/l predominated (72%). These findings demonstrate that clinical *E. faecalis* strains from Poland which were highly resistant to ciprofloxacin were also highly cross-resistant to the newer fluoroquinolones and are in agreement with the cross-resistance reported by Tancovic *et al*. (1996). For strains with intermediate resistance to ciprofloxacin MICs of sparfloxacin and moxifloxacin were lower and varied from 0.38 to 1 mg/l and from 0.094 to 1 mg/l, respectively. Among the ciprofloxacin sensitive isolates the range of MICs sparfloxacin and moxifloxacin were 0.25–0.75 mg/l and 0.064–1 mg/l, respectively. The type strain *E. faecalis* ATCC 29212 was used as a quinolone-sensitive reference.

The amino acid alterations in QRDRs of GyrA and ParC proteins together with respective codon sequences detected in tested strains are shown in Table II, in relevance to ciprofloxacin, sparfloxacin and moxifloxacin MICs. Strains ES-7 and EI-1 with ciprofloxacin MIC ≤ 2 mg/l revealed no amino acid alterations when compared with the reference fluoroquinolone sensitive strain *E. faecalis* ATCC 29212 whereas, the remaining nine highly resistant strains (MICs 16–256 mg/l) had a single amino acid change in both GyrA and ParC QRDRs. However, no alterations in the adjacent regions were detected that may suggest a crucial role of the QRDRs in resistance of *E. faecalis* to fluoroquinolones.

In ParC only one kind of a single amino acid change has been observed that was related with substitution of

Table I
Susceptibility to ciprofloxacin of clinical *E. faecalis* strains

	MICs of ciprofloxacin (mg/l):									
	Sensitive		Intermediate	Resistant						
	0.5	1	2	4	8	16	32	64	128	256
No. of strains	21	74 [1]*	50 [1]*	1	–	1 [1]*	2 [1]*	12 [2]*	41 [3]*	3 [2]*
Total (%)	95 (46.3%)		50 (24.4%)	60 (29.3%)						

[ ]* – number of strains analyzed by DNA sequencing.

Table II
Alterations in GyrA and ParC in clinical strains of *E. faecalis*

Strain	MICs (mg/l)[a]			Amino acid (codon)[b]	
	CIP	SPX	MXF	GyrA position 84	ParC position 85
E. faecalis[c] ATCC 29212	1	0.38	0.125	Ser (AGT)	Ser (AGC)
ES-7[*]	1	0.25	0.125	Ser (AGT)	Ser (AGT)
EI-1	2	1	0.38	Ser (AGT)	Ser (AGT)
E-28	16	>32	1	Cys (TGT)	Ile (ATC)
E-34	32	>32	4	Tyr (TAT)	Ile (ATT)
E-39	64	>32	8	Ile (ATT)	Ile (ATT)
E-30	64	>32	16	Ile (ATT)	Ile (ATC)
E-45	128	>32	8	Tyr (TAT)	Ile (ATT)
E-06	128	>32	32	Ile (ATT)	Ile (ATC)
E-48	128	>32	16	Ile (ATT)	Ile (ATC)
E-17	256	>32	8	Arg (AGA)	Ile (ATC)
E-18	256	>32	32	Ile (ATT)	Ile (ATT)

[a] CIP, ciprofloxacin; SPX, sparfloxacin; MXF, moxifloxacin;
[b] Positions correspond to Ser-83 (GyrA) and Ser-80 (ParC) of *E. coli*;
[c] Reference ciprofloxacin sensitive strain
[*] – ciprofloxacin sensitive wild-type strain

the serine at position 85 (that corresponds to Ser-80 in *E. coli*) to isoleucine. Interestingly, two different codons ATC and ATT were found to be responsible for this substitution in tested isolates. The same phenomenon was observed by Kanematsu *et al.* (1998) who suggested that the codon variety for the same amino acid reflects diversity of parental strains and independent selection of ParC mutants. Consequently, it may imply that not only clonal dissemination but an emergence of independent genetic events in previously quinolone-susceptible strains are responsible for increase and spread of resistant strains in Poland.

In GyrA, the serine at position 84 (Ser-83 in *E. coli*) was changed to one of four amino acids: arginine, isoleucine, cysteine and tyrosine. Similar alterations (Ser to Arg or Ile) of *E. faecalis* have been already reported (Kanematsu *et al.*, 1998). On the other hand, in *E. faecalis* the substitution of the serine at position 84 to cysteine and tyrosine were not reported to date. Therefore, we considered them novel GyrA mutations in *E. faecalis*. Notably, El Amin *et al.* (1999) described substitution of the serine at position 84 to tyrosine for *E. faecium*.

The obtained results are in agreement with observations by Kanematsu *et al.* (1998) who suggested that the high-level resistance (MIC of ciprofloxacin ≥16 mg/l) in *E. faecalis* is associated with simultaneous mutations in ParC and GyrA. Previous reports by Korten *et al.* (1994) and Tankovic *et al.* (1996) who tested GyrA QRDR only attributed the high-level ciprofloxacin resistance in *E. faecalis* to alterations in GyrA. In the present study, we did not find any strain with ciprofloxacin MICs in the 16 to 256 mg/l range with unaltered ParC. Strains without any alterations in the ParC were sensitive or showed intermediate susceptibility to ciprofloxacin (MIC≤2 mg/l). In this light, our results are in agreement with Onodera *et al.* (2002), who found that mutation in ParC is indispensable for the high level resistance to ciprofloxacin. Nevertheless, our results show that ciprofloxacin MICs in *E. faecalis* strains with the altered ParC may range from 16 to 256 mg/l (Table II). Noteworthy, all the highly resistant strains in this study had also alterations in GyrA. Taken together, our findings may suggest that factors other than sole mutations in QRDRs of *gyrA* and *parC* genes are responsible for highly elevated ciprofloxacin MIC (256 mg/l) in *E. faecalis* strains. Onodera *et al.* (2002) and Oyamada *et al.* (2006) suggested that a contribution of mutations in genes *gyrB* and *parE*, or activity of some efflux pumps may be such agents. However, to date no reports regarding the influence of alterations in GyrB and ParE on fluoroquinolone resistance have been published for *E. faecalis* isolated from clinical specimens. Data from Gram-positive bacteria other than *E. faecalis* indicate that mutations in the aforementioned subunits occur rarely and probably play a minor role in quinolone resistance. In addition, little is known about the efflux pumps in clinical isolates of *E. faecalis* (Jonas *et al.*, 2001).

In conclusion, our results supported findings and provide evidence that mutations within *parC* and *gyrA* QRDRs are associated with development of the high-level ciprofloxacin resistance. The absence of mutations in the same regions in ciprofloxacin intermediate *E. faecalis* strain suggests that other mechanisms (like mutations at the remaining gyrase and topoisomerase IV loci and/or decreased drug accumulation connected with efflux systems) may be responsible for this resistance trait.

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Literature

Barišić Z. and V. Punda-Polić. 2000. Antibiotic resistance among enterococcal strains isolated from clinical specimens. *Internat. J. Antimicrob. Agents* 16: 65–68.

Clinical Laboratory Standards Institute. 2006. Performance standards for antimicrobial susceptibility testing; sixteenth informational supplement M100-S16, vol.26. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania, USA.

El Amin N., S. Jalal and B. Wretlind. 1999. Alterations in GyrA and ParC associated with fluoroquinolone resistance in *Enterococcus faecium*. *Antimicrob. Agents Chemother.* 43: 947–949.

Eliopoulos G.M. 1993. Increasing problems in the therapy of enterococcal infections. *Eur. J. Clin. Microbiol. Infect. Dis.* 6: 409–412.
Facklam R. R, D. F. Sahm and L. M. Teixeira. 1999. *Manual of Clinical Microbiology*, 7th ed. ASM Press, Washington D.C.
Gierczyński R., S. Kałużewski, A. Rakin, M. Jagielski, A. Zasada, A. Jakubczak, B. Borkowska-Opacka and W. Rastawiecki. 2004. Intriguing diversity of *Bacillus anthracis* in eastern Poland – the molecular echoes of the past outbreaks. *FEMS Microbiol. Lett.* 239: 235–240.
Hunt C.P. 1998. The emergence of enterococci as a cause of nosocomial infection. *Br. J. Biomed. Sci.* 55: 149–156.
Jonas B. M., B. E. Murray and G. M. Weinstock. 2001. Characterization of *emeA*, a *norA* homolog and multidrug resistance efflux pump in *Enterococcus faecalis. Antimicrob. Agents Chemother.* 45: 3574–3579.
Kanematsu E., T. Deguchi, M. Yasuda, T. Kawamura, Y. Nishino and Y. Kawada. 1998. Alterations in the GyrA subunit of DNA gyrase and the ParC subunit of DNA topoisomerase IV associated with quinolone resistance in *Enterococcus faecalis. Antimicrob. Agents Chemother.* 42: 433–435.
Kawalec M., Z. Pietras, E. Daniłowicz, A. Jakubczak, M. Gniadkowski, W. Hryniewicz and R.J.L. Willems. 2007. Clonal structure of *Enterococcus faecalis* isolated from Polish hospitals: the characterization of epidemic clones. *J. Clin. Microbiol.* 45: 147–153.
Korten V., W.M. Huang and B.E. Murray. 1994. Analysis by PCR and direct DNA sequencing of *gyrA* mutations associated with fluoroquinolone resistance in *Enterococcus faecalis. Antimicrob. Agents Chemother.* 38: 2091–2094.
Onodera Y., J. Okuda, M. Tanaka and K. Sato. 2002. Inhibitory activities of quinolones against DNA gyrase and topoisomerase IV of *Enterococcus faecalis. Antimicrob. Agents Chemother.* 46: 1800–1804.
Oyamada Y., H. Ito, M. Inoue and J. Yamagishi. 2006. Topoisomerase mutations and efflux are associated with fluoroquinolone resistance in *Enterococcus faecalis. J. Med. Microbiol.* 55: 1395–1401.
Schaberg D.R., W.I. Dillon, M.S. Terpenning, K.A. Robinson, S.F. Bradley and C.A. Kauffman. 1992. Increasing resistance of enterococci to ciprofloxacin. *Antimicrob. Agents Chemother.* 36: 2533–2535.
Tankovic J., F. Mahjoubi, P. Courvalin, J. Duval and R. Leclercq. 1996. Development of fluoroquinolone resistance in *Enterococcus faecalis* and role of mutations in the DNA gyrase *gyrA* gene. *Antimicrob. Agents Chemother.* 40: 2558–2561.
Zaręba T. and W. Hryniewicz. 1995. Patters of antibiotic resistance among enterococcal strains isolated from clinical specimens and food in Poland. *Eur. J. Clin. Microbiol. Infect. Dis.* 14: 69–71.