ANTIMICROBIAL RESISTANCE

Analysis of mutations to *gyrA* in quinolone-resistant clinical isolates of *Enterobacter cloacae*

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The *gyrA* subgenes of a quinolone-resistant *Enterobacter cloacae* clinical isolate (ofloxacin MIC, 16 mg/L) and of a control, *E. cloacae* NCTC 10005 (ofloxacin MIC, 0.03 mg/L), were amplified by polymerase chain reaction (PCR) and sequenced. The resistant isolate had mutations at the codons for amino acids 83, 89 and 90. The first of these mutations led to replacement of serine-83 by tyrosine, whereas the other mutations were silent. Digestion of PCR-amplified DNA fragments with the restriction enzyme *HinfI* detected mutations at the same site in *gyrA* in six further quinolone resistant *E. cloacae* isolates.

Introduction

An increase in the incidence of serious diseases caused by *Enterobacter cloacae* has been observed recently [1, 2]. Nosocomial infections caused by *E. cloacae* are often treated with fluoroquinolones, because of resistance to many other agents [3]. Quinolones act primarily by inhibiting DNA gyrase (topoisomerase II) [4–6], which is essential for bacterial growth [7]. DNA gyrase transiently breaks the DNA strands and introduces negative superhelical turns in an ATP-dependent process [8]. Apart from being involved in DNA replication, this enzyme regulates the initiation of transcription of several genes. The enzyme is a tetramer with two A and two B subunits encoded by the genes *gyrA* and *gyrB*, respectively.

Mechanisms of resistance to fluoroquinolones have been studied in several gram-negative organisms including *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Salmonella typhimurium* [9–12] and were found to be chromosomally determined. In most cases, high-level fluoroquinolone resistance in these organisms maps to mutations in *gyrA* [13, 14]. Such mutations result in a simultaneous increase in resistance towards all quinolones. Most mutations in *Esch. coli* lie in the 5' end of *gyrA*, between nucleotides 199 (corresponding to Ala-67 in the protein product) and 318 (Gln-106). This region

contains the regulatory centre, tyrosine 122 [15, 16] and has been termed the 'quinolone-resistance-determining region' (QRDR). The present study determined the nucleotide sequence of the QRDRs of quinolone-sensitive and -resistant strains of *E. cloacae*.

Materials and methods

Bacterial strains

E. cloacae strains were collected from different clinical specimens at the University Hospital of Vienna and were identified by the API 20E system (bioMérieux, Marcy l'Etoile, France). *E. cloacae* NCTC 10005 and *Esch. coli* ATCC 25922 were used as reference strains for antimicrobial susceptibility testing. *Esch. coli* S17-1, carrying the plasmid pLA2917, and *Esch. coli* S17-1 carrying the plasmid pNJR3-2, were used as the donor strains for conjugation [7].

Antimicrobial susceptibility testing

MICs were determined by a microdilution method in Mueller Hinton Broth (Merck, Darmstadt, Germany) with final inocula of 10^5 cfu. The quinolones used were ciprofloxacin (Bayer, Vienna, Austria), ofloxacin (Hoechst, Vienna, Austria) and nalidixic acid (Sterling Winthrop, Dijon, France).

Conjugation

Plasmid pLA2917 (tet⁺), carrying a tetracycline resistance gene and pNJR3-2 (tet⁺, *gyrA*⁺), carrying

the tetracycline resistance gene in addition to the cloned wild-type *gyrA* gene of *Esch. coli* were used. Each of these plasmids was transferred into 10 randomly chosen isolates, with ofloxacin MICs ≥ 1 mg/L. To achieve transfer, the isolates were cultured in 10 ml volumes of Mueller Luria Broth [7], whilst the donor strains were cultured overnight in 10-ml amounts of Mueller Luria broth containing tetracycline (Wyeth-Lederle, Vienna, Austria) 5 mg/L. Equal volumes (1 ml of donor and recipient were then mixed and the mixtures were centrifuged (13 200 g) for 5 min at 4°C. The pellets were suspended in 50- μ l amounts of 0.15 M NaCl, spread on Mueller Luria agar and incubated for 5 h at 37°C to allow mating, then harvested into 3-ml volumes of sterile 0.15 M NaCl. Dilutions containing c. 10^2 and 10^4 cfu/ml were made in 10-ml volumes of sterile water, and 100- μ l volumes were spread on MacConkey agar (Oxoid, Basingstoke, Hants) containing tetracycline at 0, 20, 100 and 200 mg/L. Presumptive transconjugates recovered on this medium after incubation overnight at 37°C were subcultured on MacConkey agar plates containing tetracycline 100 mg/L. They were then confirmed as *E. cloacae* and MICs were determined as above, but with tetracycline (5 mg/L) added to the Mueller Hinton broth to conserve the plasmids.

Polymerase chain reaction (PCR)

Two oligonucleotide primers (Dr Müller, Oligo-Ocom, Vienna Biocenter, Austria), Pri 1 (5'-TACACCGGT-CAACATTGAGG-3') and Pri2 (5'-TTAATGATTG-CCGCCGTCGG-3') were designed, on the basis of the known *gyrA* sequences of *Esch. coli* K-12. Pri1 was identical in sequence to nucleotide positions 24–43, and Pri2 was complementary to positions 652–671. These were used to amplify *gyrA* by PCR [17]. Template DNA was prepared from each strain by heat (37°C) and cold shock (–196°C) treatment of single colonies suspended in water, followed by centrifugation. The template DNA was taken from the resulting supernate and amplification was performed in a final volume of 50 μ l containing 5 μ l of DNA template (30–50 ng), 0.8 μ l of each primer (Pri1 and Pri2 each at 20 μ M), 5 μ l of deoxynucleoside triphosphates (dATP, dCTP, dGTP and dTTP; 2 mM each; Perkin-Elmer, Branchburg, NJ, USA), and 5 μ l of *Taq* buffer containing 1.0–2.0 U of *Taq* polymerase (Perkin-Elmer), which was added after the sample had been heated. Thirty amplification cycles were used, with the following temperature profiles: 92°C for 35 s; 64°C for 1 min and 74°C for 2 min.

Sequence analysis

PCR fragments were subjected to phenol-chloroform extraction and then precipitated with ethanol [18]. Double-stranded PCR fragments were sequenced by an automated DNA sequencing system, with a fluores-

cence sequencer (LI-COR; Lincoln, Nebraska, USA) [19].

Restriction fragment length polymorphism (RFLP) of PCR-amplified DNA

The 648-bp fragment amplified by PCR was digested with *Hinf*I [17] (Amersham, Bucks) at 37°C for 1 h. The cleavage products were profiled by electrophoresis in gels containing agarose 1.5% in TAE buffer and ethidium bromide staining [17].

Results

Antimicrobial susceptibility

The susceptibilities of clinical *E. cloacae* isolates are shown in Table 1. The most resistant isolates were nos. 89 and 132; isolates 5, 15, 19, 32, 67, 68, 105, 110 had diminished susceptibility to fluoroquinolones as compared to isolate 136 and *E. cloacae* NCTC 10005. All the isolates except no. 136 were resistant to nalidixic acid.

Dominance test

The susceptibilities of donor strains, *E. cloacae* isolates and transconjugants are shown in Table 1. All transconjugants with plasmids pLA2917 and pNJR3-2 became resistant to tetracycline. Four isolates (nos. 5, 15, 32 and 105) of those acquiring plasmid pNJR3-2 retained diminished susceptibility to ofloxacin, but six (isolate nos. 19, 67, 68, 89, 110 and 132) became more sensitive to this quinolone. Notably, the isolates in which acquisition of pNJR3-2 reduced or eliminated resistance were those that began with the highest levels of resistance to the quinolones. In contrast to its *gyrA* coding derivative, pLA2917 had no effect on quinolone MICs.

PCR products and point mutations in *gyrA*

PCR products consistently had the mobility expected for a 648-bp DNA fragment (Fig. 1). Sequencing of the 338-bp fragment corresponding to the N-terminal region of GyrA from the most quinolone-resistant isolate (no. 89) showed three alterations in comparison with that of the quinolone-susceptible control *E. cloacae* NCTC 10005 (Table 2). Silent mutations were present in the codons determining amino acids 89 (ATC to ATT) and 90 (GTT to GTC); in addition, the codon corresponding to amino acid 83 was altered from TCC in the control to TAC in the resistant isolate, indicating a serine to tyrosine substitution.

PCR-RFLP

PCR products of QRDR region were 648 bp long and were expected to have two *Hinf*I recognition sites. Thus, *Hinf*I digestion was predicted to yield three

Table 1. MICs for ofloxacin, ciprofloxacin and tetracycline for donors, *E. cloacae* isolates and their transconjugants

Strains and plasmids	MIC mg/L			
	Nalidixic acid	Ciprofloxacin	Ofloxacin	Tetracycline
<i>Esch. coli</i> S17-1 (pLA2917)	...	≤ 0.01	≤ 0.01	128
<i>Esch. coli</i> S17-1 (pNJR3-2)	...	≤ 0.01	≤ 0.01	128
<i>E. cloacae</i> NCTC10005	2	≤ 0.01	0.03	—
<i>E. cloacae</i> 136	4	0.01	0.06	—
<i>E. cloacae</i> 5 (no plasmid)	32	0.12	1	32
5 (pLA2917)	...	0.12	1	≥ 256
5 (pNJR3-2)	...	0.12	1	≥ 256
<i>E. cloacae</i> 15 (no plasmid)	128	0.12	0.5	32
15 (pLA2917)	...	0.12	0.5	≥ 256
15 (pNJR3-2)	...	0.12	0.5	≥ 256
<i>E. cloacae</i> 19 (no plasmid)	≥ 256	0.5	2	8
19 (pLA2917)	...	0.5	2	≥ 256
19 (pNJR3-2)	...	0.03	0.25	≥ 256
<i>E. cloacae</i> 32 (no plasmid)	32	0.12	1	32
32 (pLA2917)	...	0.12	1	≥ 256
32 (pNJR3-2)	...	0.12	1	≥ 256
<i>E. cloacae</i> 67 (no plasmid)	≥ 256	0.25	2	2
67 (pLA2917)	...	0.25	2	256
67 (pNJR3-2)	...	≤ 0.01	0.06	256
<i>E. cloacae</i> 68 (no plasmid)	≥ 256	0.25	2	2
68 (pLA2917)	...	0.25	2	256
68 (pNJR3-2)	...	≤ 0.01	0.06	256
<i>E. cloacae</i> 89 (no plasmid)	≥ 256	4	16	16
89 (pLA2917)	...	4	16	≥ 256
89 (pNJR3-2)	...	0.25	1	≥ 256
<i>E. cloacae</i> 105 (no plasmid)	≥ 256	1	4	32
105 (pLA2917)	...	1	4	≥ 256
105 (pNJR3-2)	...	1	4	≥ 256
<i>E. cloacae</i> 110 (no plasmid)	≥ 256	0.25	1	2
110 (pLA2917)	...	0.25	1	256
110 (pNJR3-2)	...	≤ 0.01	0.06	256
<i>E. cloacae</i> 132 (no plasmid)	≥ 256	1	4	16
132 (pLA2917)	...	1	4	≥ 256
132 (pNJR3-2)	...	0.06	0.25	≥ 256

Fig. 1. Agarose gel electrophoresis of PCR products of *gyrA* from: (a) *E. cloacae* strains sensitive to or with low-level resistance to quinolones: lane 1, DNA marker λ DNA-*Hind* III digest; 2–7, 648-bp amplification products of *E. cloacae* isolates 5, 15, 32, 105, 136 and NCTC 10005, respectively; 8, negative control (–); (b) *E. cloacae* strains with resistance to quinolones: 1, DNA marker λ DNA-*Hind* III digest; 2–7, 648-bp amplification products of *E. cloacae* isolates 19, 67, 68, 89, 110 and 132, respectively; 8, negative control (–).

Table 2. Nucleotide sequence and amino-acid composition of QRDR of *gyrA* from the quinolone-sensitive *E. cloacae* strain NCTC 10005 and the most quinolone-resistant isolate *E. cloacae* no. 89

NCTC 1005	GGT	<u>GAT</u>	<u>TCC</u>	GCG	GTG	TAC	GAC	ACC	ATC	GTT
Amino acid	Gly 81	Asp	Ser 83	Ala	Val 85	Tyr	Asp 87	Thr	Ileu 89	Val
no. 89	GGT	<u>GAT</u>	<u>TAC</u>	GCG	GTG	TAC	GAC	ACC	ATT	GTC
Amino acid	Gly 81	Asp	Tyr 83	Ala	Val 85	Tyr	Asp 87	Thr	Ileu 89	Val

Amino acid changes and point mutations are marked with bold letters. *HinfI* restriction site is underlined.

Fig. 2. Agarose gel electrophoresis of *HinfI* digests of *gyrA* amplification products from: (a) quinolone-sensitive *E. cloacae* clinical isolates and those with low level resistance: lane 1, DNA marker λ DNA-*Hind*III digest; 2–7, digests from isolates 5, 15, 32, 105, 136 and NCTC 10005, respectively; 8, negative control (–); (b) digests from quinolone-resistant *E. cloacae* isolates: 1, DNA marker λ DNA-*Hind*III digest; 2–7, digests from isolates 19, 67, 68, 89, 110 and 132, respectively; 8, negative control (–).

DNA fragments with sizes of 99, 221 and 328 bp. This was seen with DNA from isolates 5, 15, 32, 105, 136 and *E. cloacae* NCTC 10005 (Fig. 2, panel a). However, mutation at the sequence corresponding to amino acid position 83 – as found in isolate 89 – removes one *HinfI* site, so that digestion generated only two fragments, with sizes of 320 and 328 bp (Fig. 2, panel b). All the clinical isolates (19, 67, 68, 89, 110, 132) for which the MICs of ofloxacin were ≥ 1 mg/L, and that gave reversion of resistance in the dominance test, showed these two-band patterns after *HinfI* digestion and electrophoresis.

Discussion

Quinolone resistance is often associated with point mutations in *gyrA*. This can be tested by a complementation method in which plasmids encoding quinolone-sensitive GyrA are transferred into resistant isolates. Sensitive *gyrA* is dominant to resistant so, if the original resistance is caused by mutation to *gyrA*, it is lost [7]. This method detects all mutations to *gyrA*, but gives no data on their nature. To address this aspect, *gyrA* genes from the most quinolone-resistant isolate (no. 89) and the control *E. cloacae* NCTC

10005 were amplified by PCR, and sequenced [20]. Analysis showed that *gyrA* from the resistant isolate had a mutation leading to replacement of serine 83 by tyrosine, together with silent mutations in the codons determining amino acids 89 and 90. Mutation at serine 83 has also been reported as the first mutation step in quinolone resistance in *Esch. coli* [2]. *HinfI* digestion of PCR-amplified DNA implied mutation at serine 83 in the *gyrA* genes of six further isolates with ofloxacin MICs ≥ 1 mg/L. PCR-RFLP was a useful method to detect *gyrA* mutants at amino position 83, serine, and, in contrast to the dominance tests, was simple and rapid [21]. This method could provide a rapid primary characterization of highly fluoroquinolone-resistant isolates.

Quinolone resistance can be associated with reduced impermeability of the outer membrane, active efflux, and mutations in *gyrB* [14, 21, 23, 24]. Four strains in this study retained diminished susceptibility after conjugation, implying the presence of one of these mechanisms, rather than *gyrA* mutation.

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