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Mutations in *gyrA* and *parC* genes in nalidixic acid-resistant *Escherichia coli* strains from food products, humans and animals

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Mutations in quinolone targets were analysed in 80 unrelated nalidixic acid-resistant (NAL^R) *Escherichia coli* strains whose nalidixic acid and ciprofloxacin MICs ranged from 32 to >256 mg/L and 0.03–64 mg/L, respectively. These strains were isolated from food products (23) and faecal samples from humans (15) and healthy animals (42). Thirteen nalidixic acid-susceptible (NAL^S) *E. coli* strains were also analysed. Mutations in *gyrA* and *parC* genes were studied by PCR and sequencing. No amino acid changes were detected in GyrA or ParC proteins of the 13 NAL^S strains. A single change in the GyrA protein was detected in all 61 NAL^R strains with ciprofloxacin MICs ≤ 2 mg/L with the following substitutions (number of strains): Ser-83→Leu (54), Ser-83→Ala (one), Ser-83→Val (one), Asp-87→Asn (two), Asp-87→Tyr (two) and Asp-87→Gly (one). A double change in GyrA was found in 18 of 19 NAL^R strains with ciprofloxacin MICs ≥ 4 mg/L. Amino acid substitutions were Ser-83→Leu, with an additional change [Asp-87→Asn (15), Asp-87→Tyr (two) or Asp-87→His (one)]. The remaining strain (ciprofloxacin MIC 4 mg/L) showed a single Ser-83→Leu substitution. In respect of the ParC protein, a single change at Ser-80 or Glu-84 was found in 25 of 42 strains, with ciprofloxacin MICs ranging from 0.5 to 32 mg/L. A double substitution (Ser-80→Ile and Glu-84→Gly) was found in one strain (ciprofloxacin MIC 64 mg/L). No amino acid changes were detected in the GyrB protein of 18 NAL^R strains.

Keywords: *Escherichia coli*, quinolone resistance

Introduction

Quinolones are used in medicine and veterinary practice for treatment of infectious diseases caused by enteric bacteria such as *Escherichia coli.* In recent years, a substantive increase in quinolone resistance in human and animal *E. coli* isolates has been observed.[1,2]

The most frequent mechanism of resistance to quinolones in *E. coli* includes alterations in genes that encode subunits of the quinolone targets DNA gyrase (in *gyrA* and *gyrB* genes) and topoisomerase IV (in *parC* and *parE*).[2–4] These alterations involve mainly mutations located in the quinolone resistance-determining region (QRDR) of the *gyrA* gene and its homologous region of the *parC* gene.[3,5,6] In contrast, mutations in *gyrB* and *parE* genes are of minor importance and are rare contributors to quinolone resistance.[4,7] Other mechanisms of resistance, such as efflux pump systems or modifications of porins, can decrease susceptibility to quinolones.[2,5,7,8]

Most of the studies on mechanisms of quinolone resistance in *E. coli* have been performed in human clinical strains and very few studies have focused on strains from animals or foods.[2,7] In this study, mutations in *gyrA*, *parC* and *gyrB* have been analysed in nalidixic acid-resistant (NAL^R) *E. coli* strains from food products, humans and animals.

Materials and methods

Eighty unrelated NAL^R *E. coli* strains (MIC ≥ 32 mg/L) recovered from different sources[1] were included. All strains showed different pulsed-field gel electrophoresis patterns following digestion of chromosomal DNA with the restriction

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enzymes *Xba*I and *Sfi*I (data not shown). The origins of these strains were: food products of animal origin (23), faecal samples from healthy animals (broilers, 27; pigs, nine; dogs, six) and human faecal samples (in- and outpatients, 13; healthy volunteers, two). Thirteen nalidixic acid-susceptible (NAL^S) *E. coli* (animals, seven; foods, two; humans, four) were also included.[1]

The MICs of nalidixic acid and ciprofloxacin were determined by an agar dilution method in Mueller–Hinton agar, according to NCCLS guidelines.

Mechanisms of resistance to quinolones

Detection of mutations in the QRDR of the *gyrA* gene, as well as in the analogous region of the *parC* gene, was performed in the 80 NAL^R and 13 NAL^S strains by PCR.[4,6] Amplified fragments were purified (Qiagen), and both strands were automatically sequenced (ABI 310, Applied Biosystems, Madrid, Spain) using the same set of primers as for the PCR. Mutations in the *gyrB* gene were also analysed by sequencing specific amplicons in 18 NAL^R strains.[9] Sequences obtained were compared with those previously reported for *gyrA* (GenBank accession no. X06373), *gyrB* (X04341) and *parC* genes (M58408 with the modification included in L22025).

Results and discussion

Table 1 shows the nalidixic acid and ciprofloxacin MICs for the 93 *E. coli* strains (80 NAL^R, 13 NAL^S) and the deduced amino acid changes in the QRDR of GyrA and ParC proteins.

No amino acid changes were detected in GyrA or ParC proteins in the 13 NAL^S isolates, whereas at least one amino acid substitution in the GyrA protein (position 83 and/or 87) was detected in all NAL^R strains. A single amino acid substitution in the QRDR of GyrA protein was detected in 62 NAL^R strains (ciprofloxacin MIC range 0.03–4 mg/L). The substitutions were as follows (number of strains): Ser-83→Leu (55), Ser-83→Val (one), Ser-83→Ala (one), Asp-87→Asn (two), Asp-87→Tyr (two) and Asp-87→Gly (one). Double changes in the GyrA protein were detected in the remaining 18 NAL^R strains (ciprofloxacin MIC range 4–64 mg/L). All exhibited the Ser-83→Leu substitution, and an additional substitution of Asp-87 for Asn (15 strains), Tyr (two) or His (one). The change Ser-83→Leu was the substitution most frequently identified, as previously reported.[2,3,6,9] To our knowledge, this is the first report of the Ser-83→Val substitution in the GyrA protein of *E. coli* strains as a spontaneous mutation, and the Ser-83→Ala substitution in a strain of food origin. Tavio *et al.*[10] reported the Ser-83→Ala change in a human *E. coli* clinical isolate and also described the Ser-83→Val substitution in an *in vitro* mutant selected under antibiotic pressure. In respect of changes in position 87, our results revealed that the Asp-87→Tyr and Asp-87→Gly single substitutions in the GyrA protein were associated with a lower ciprofloxacin MIC (0.06 mg/L) than the Asp-87→Asn change (MICs 0.25–0.5 mg/L) (Table 1). The substitutions of Ser-83 for Leu, Ala or Val have resulted not only in the loss of the hydroxyl group of serine and thus the ability to form hydrogen bonds, but also in the replacement by an aliphatic chain. All the changes in Asp-87 for Asn, Tyr, Gly or His involve the loss of a negatively charged amino acid.[6] These changes suggest that the ability to form hydrogen bonds and the negative charge at these positions seem to be important for quinolone interactions with the DNA gyrase–DNA complex.

Analysis inside the QRDR of the *gyrA* sequences of all 93 strains (NAL^R and NAL^S), in comparison with the *gyrA* sequence previously reported (GenBank accession no. X06373), revealed five different patterns (A–E), according to silent mutations identified at positions 255, 261, 267, 273, 282 and 300 (Table 2). All strains exhibited a T→C transition at nucleotide 267. Mutations at positions 261 and 282 have not been reported previously. Table 2 shows the relationship between silent mutation patterns and amino acid substitutions in the GyrA protein.

A single amino acid change in the QRDR of the ParC protein was found in 26 NAL^R strains, which also showed changes in the GyrA protein (ciprofloxacin MIC range 0.5–64 mg/L) (Table 1). The amino acid substitutions were as follows: Ser-80→Ile (16), Ser-80→Arg (seven), Glu-84→Lys (two) and Glu-84→Val (one). To our knowledge this is the first time Glu-84→Val has been described as a single substitution in the ParC protein. A double amino acid change in the ParC protein (Ser-80→Ile and Glu-84→Gly) reported previously[5] was detected in one strain. In respect of amino acid substitutions in the ParC protein, the ability to form hydrogen bonds is reduced when Ser-80 is substituted for Ile or Arg, whereas the change of Glu for Lys, Val or Gly at position 84 involves the loss of a negative charge. These observations are similar to those for the GyrA protein.

Two substitutions outside the QRDR of the ParC protein (Ala-56→Thr and Ser-57→Thr) were identified in two strains. The substitution Ala-56→Thr was detected in one NAL^R *E. coli* from a pig (ciprofloxacin MIC 0.5 mg/L) and the Ser-57→Thr change in one strain from food (*E. coli* Co7). This strain also presented a double amino acid substitution in the GyrA protein (Ser-83→Leu + Asp-87→Tyr) and a single amino acid change in the ParC protein (Glu-84→Lys) (Table 1). In addition, *E. coli* Co7 also showed a total of 14 different silent mutations inside the QRDR of the *parC* gene.

Analysis inside the QRDR of the *parC* sequences of all 93 strains, in comparison with the *parC* sequence previously reported (GenBank accession nos M58408 and L22025) revealed six different patterns (from I to VI) according to the silent mutations identified at positions 240, 243, 273 and 321 (Table 2). No silent mutations were found in the 22 strains

Table 1. Amino acid changes in the QRDR of GyrA and ParC proteins in 93 *E. coli* strains (80 NAL^R and 13 NAL^S) of different origins and the corresponding nalidixic acid and ciprofloxacin MICs

E. coli strains and origin		MIC (mg/L)		Amino acid changes in the QRDR of	
origin (no.)	total no.	NAL	CIP	GyrA[a]	ParC[b]
F (2), B (1), H (4), P (4), D (2)	13	≤1–16	0.007–0.06	wild	wild
F (1), H (1)	2	32, 64	0.06	Asp-87→Tyr	wild
H (1)	1	64	0.06	Asp-87→Gly	wild
B (1)	1	64	0.25	Asp-87→Asn	wild
F (1)	1	128	0.125	Ser-83→Ala	wild
F (7), B (7), H (9), P (5), D (3)	31	64–>256	0.03–0.25	Ser-83→Leu	wild
F (4), B (3), H (1), P (2)[c], D (2)	12	128–>256	0.5	Ser-83→Leu	wild
H (1)	1	256	0.5	Ser-83→Val	wild
F (1)	1	256	0.5	Asp-87→Asn	wild
H (1)	1	>256	0.5	Ser-83→Leu	Ser-80→Ile
B (3)	3	>256	0.5	Ser-83→Leu	Ser-80→Arg
F (2), B (1)	3	>256	1	Ser-83→Leu	wild
F (1), B (1)	2	>256	1	Ser-83→Leu	Ser-80→Arg
B (1)	1	>256	2	Ser-83→Leu	Glu-84→Val
D (1)	1	>256	2	Ser-83→Leu	Ser-80→Ile
B (1)	1	>256	4	Ser-83→Leu	Ser-80→Ile
B (1)	1	>256	4	Ser-83→Leu + Asp-87→Asn	Ser-80→Ile
B (2)	2	>256	8	Ser-83→Leu + Asp-87→Asn	Ser-80→Arg
F (1), B (6), P (1)	8	>256	8–16	Ser-83→Leu + Asp-87→Asn	Ser-80→Ile
P (1)	1	>256	8	Ser-83→Leu + Asp-87→Asn	Glu-84→Lys
F (1)[d]	1	>256	16	Ser-83→Leu + Asp-87→Tyr	Glu-84→Lys
F (1)	1	>256	16	Ser-83→Leu + Asp-87→Tyr	Ser-80→Ile
F (1)	1	>256	16	Ser-83→Leu + Asp-87→His	Ser-80→Ile
F (1)	1	>256	32	Ser-83→Leu + Asp-87→Asn	Ser-80→Ile
F (1)	1	>256	64	Ser-83→Leu + Asp-87→Asn	Ser-80→Ile
H (1)	1	>256	64	Ser-83→Leu + Asp-87→Asn	Ser-80→Ile + Glu-84→Gly

B, broiler; F, food product; P, pig; D, dog; H, human; NAL, nalidixic acid; CIP, ciprofloxacin; wild, no amino acid changes.
[a]The deduced GyrA amino acid sequence was compared with that previously reported (GenBank accession no. X06373).
[b]The deduced ParC amino acid sequence was compared with that previously reported (GenBank accession no. M58408 with the modifications included in L22025).
[c]One of these strains showed a change outside the QRDR of ParC (Ala-56→Thr).
[d]This strain (*E. coli* Co7) showed a change outside the QRDR of ParC (Ser-57→Thr).

included in pattern I, whereas the remaining 71 strains presented a transition (a guanine instead of an adenine) at nucleotide 273. Table 2 shows the relationship between silent mutation patterns and amino acid substitutions in ParC.

No changes were observed in the GyrB protein of the 18 NAL^R strains.

A correlation between the number of changes in GyrA and ParC proteins and the level of quinolone resistance in *E. coli* strains was observed. This is in accordance with previous observations.[4,5,7] None of the NAL^S *E. coli* showed amino acid changes either in GyrA or in ParC. A single substitution in the GyrA protein was associated with a decrease in susceptibility to ciprofloxacin (MIC 0.03–1 mg/L) and two amino acid changes (one in GyrA and the other in ParC) with a low level of resistance to ciprofloxacin (2–4 mg/L). Three amino acid substitutions (two in GyrA and one in ParC) were associated with a moderate or high level of ciprofloxacin resistance (8–32 mg/L), and four substitutions (two in GyrA and two in ParC) with the highest ciprofloxacin MIC observed (64 mg/L). Several strains did not follow the above schedule (Table 1): six strains with ciprofloxacin MICs ranging from 0.5 to 1 mg/L showed two amino acid changes (one in GyrA and other in ParC), and one strain with a ciprofloxacin MIC of 4 mg/L had three amino acid changes (two in GyrA and one in ParC proteins). MICs of all seven strains could be explained by increasing drug permeability.[4] However, in contrast to these

Table 2. Silent mutations detected in *gyrA* and *parC* genes from 80 NAL^R *E coli* strains with different amino acid changes in GyrA and ParC proteins, and from 13 NAL^S *E. coli* strains

	Nucleotide mutations in *gyrA* at the indicated positions								
					282				
Pattern of silent mutations in *gyrA*	255 C→T	261 C→T	267 T→C	273 C→T	G→A	G→C	300 T→C	No. of strains	Amino acid changes in GyrA protein detected in strains belonging to each pattern (no. of strains)
A			+					13	wild (5 NAL^S), S83L (7), S83L+D87N (1)
B	+		+	+			+	76	wild (7 NAL^S), S83L (45), S83V (1), S83A (1), D87N (2), D87Y (2), D87G (1), S83L+D87N (14), S83L+D87Y (2), S83L+D87H (1)
C	+		+	+	+		+	1	wild (1 NAL^S)
D	+		+	+		+	+	2	S83L (2)
E	+	+	+	+			+	1	S83L (1)

	Nucleotide mutations in *parC* at the indicated positions					
Pattern of silent mutations in *parC*	240 C→T	243 C→T	273 A→G	321 C→G	No. of strains	Amino acid changes in ParC protein[a] detected in strains belonging to each pattern (no. of strains)
I					22	wild (5 NAL^S, 12 NAL^R), A56T (1), S80R (2), E84V (1), S80I+E84G (1)
II			+		48	wild (5 NAL^S, 26 NAL^R), S80I (12), S80R (3), E84K (1), S57T+E84K (1)[a]
III	+		+		14	wild (11 NAL^R), S80R (2), S80I (1)
IV	+		+	+	7	wild (3 NAL^S, 3 NAL^R), S80I (1)
V			+	+	1	S80I (1)
VI		+	+	+	1	S80I (1)

Wild, no amino acid changes. When wild sequence is indicated, the number of strains NAL^S and NAL^R are included in parentheses. A, alanine; D, aspartic acid; E, glutamic acid; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; N, asparagine; R, arginine; S, serine; T, threonine; V, valine; Y, tyrosine.

[a]This strain (Co7) had other 13 silent nucleotide mutations.

observations, three amino acid changes were detected in one *E. coli* strain with a ciprofloxacin MIC of 64 mg/L. The presence of active efflux pumps or mutations that affect regulatory loci (e.g. *mar* and *sox*) might be associated with this high ciprofloxacin MIC.[2,5,8] These other mechanisms might also be responsible for the wide range of ciprofloxacin MICs (4–64 mg/L) detected in strains that show the same amino acid substitutions in GyrA (Ser-83→Leu + Asp-87→Asn) and in ParC proteins (Ser-80→Ile).

In the present study, a large variety of amino acid changes in GyrA and ParC proteins were detected in the NAL^R *E. coli* strains of different origins. A correlation was found between the number of changes in the QRDR of GyrA and ParC proteins and the level of quinolone resistance of the *E. coli* strains.

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References

1. Sáenz, Y., Zarazaga, M., Briñas, L., Lantero, M., Ruiz-Larrea, F. & Torres, C. (2001). Antibiotic resistance in *Escherichia coli* isolates obtained from animals, foods, and humans in Spain. *International Journal of Antimicrobial Agents* **18**, 353–8.

2. Everett, M. J., Fang Jin, Y., Ricci, V. & Piddock, L. J. V. (1996). Contributions of individual mechanisms to fluoroquinolone resistance in 36 *Escherichia coli* strains isolated from humans and animals. *Antimicrobial Agents and Chemotherapy* **40**, 2380–6.

3. Piddock, L. J. V. (1999). Mechanisms of fluoroquinolone resistance: an update 1994–1998. *Drugs* **58**, 11–8.

4. Vila, J., Ruiz, J., Goñi, P. & Jiménez de Anta, T. (1996). Detection of mutations in *parC* in quinolone-resistant clinical isolates of *Escherichia coli*. *Antimicrobial Agents and Chemotherapy* **40**, 491–3.

5. Heisig, P. (1996). Genetic evidence for a role of *parC* mutations in development of high-level fluoroquinolone resistance in *Escherichia coli*. *Antimicrobial Agents and Chemotherapy* **40**, 879–85.

6. Oram, M. & Fisher, L. M. (1991). 4-Quinolone resistance mutations in the DNA gyrase of *Escherichia coli* clinical isolates identified by using the polymerase chain reaction. *Antimicrobial Agents and Chemotherapy* **35**, 387–9.

7. Giraud, E., Leroy-Sétrin, S., Flaujac, G., Cloeckaert, A., Dho-Moulin, M. & Chaslus-Dancla, E. (2001). Characterization of high-level fluoroquinolone resistance in *Escherichia coli* O78:K80 isolated from turkeys. *Journal of Antimicrobial Chemotherapy* **47**, 341–3.

8. Kern, W. V., Oethinger, M., Jellen-Ritter, A. S. & Levy, S. B. (2000). Non-target gene mutations in the development of fluoroquinolone resistance in *Escherichia coli*. *Antimicrobial Agents and Chemotherapy* **44**, 814–20.

9. Vila, J., Ruiz, J., Marco, F., Barcelo, A., Goñi, P., Giralt, E. *et al.* (1994). Association between double mutation in *gyrA* gene of ciprofloxacin-resistant clinical isolates of *Escherichia coli* and MICs. *Antimicrobial Agents and Chemotherapy* **38**, 2477–9.

10. Tavio, M. D., Vila, J., Ruiz, J., Martín-Sánchez, A. M. & de Anta, M. T. J. (1999). Mechanisms involved in the development of resistance to fluoroquinolones in *Escherichia coli* isolates. *Journal of Antimicrobial Chemotherapy* **44**, 735–42.