Molecular basis of quinolone resistance in *Escherichia coli* from wild birds

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Abstract

Nine quinolone resistant (minimal inhibitory concentration [MIC] was > 32 μg/mL for nalidixic acid, > 1 μg/mL for ciprofloxacin) isolates of *Escherichia coli* have been found in wild birds with septicemia. All of the isolates were aerobactin positive. The mechanisms of resistance were characterised by sequencing the quinolone resistance-determining region (QRDR) of the *gyrA, gyrB, parC,* and *parE* genes. Sequence analysis of the *gyrA* gene in all isolates identified only 1 nucleotide substitution at codon Serine-83 for Leucine-83. Sequence analysis of the *gyrB, parC,* and *parE* QRDR genes revealed no mutations in any of the isolates. This study was conducted to determine the importance of these genes in the susceptibility of *E. coli* strains isolated from wild birds to quinolones.

Résumé

*Neuf isolats d'*Escherichia coli *résistants aux quinolones (concentration minimale inhibitrice de > 32 μg/mL pour l'acide nalidixique et > 1 μg/mL pour le ciprofloxacin) ont été trouvés chez des oiseaux sauvages septicémiques. Tous les isolats étaient positifs pour l'aérobactine. Les mécanismes de résistance ont été caractérisés par le séquençage de la région déterminant la résistance aux quinolones (QRDR) des gènes* gyrA, gyrB, parC *et* parE. *L'analyse de la séquence du gène* gyrA *de tous les isolats a permis d'identifier une substitution d'une seule base au codon sérine-83 pour leucine-83. L'analyse de la séquence des gènes* gyrB, parC *et* parE *n'a pas permis de démontrer de mutation chez aucun des isolats. Cette étude a été effectuée afin de déterminer l'importance de ces gènes dans la sensibilité aux quinolones des isolats de* E. coli *provenant d'oiseaux sauvages.*

(Traduit par Docteur Serge Messier)

Problems attributed to coliform infections in birds and poultry are often caused by strains of *Escherichia coli.* There is a marked variation in severity that ranges from severe acute infections with sudden and high mortality to mild infections of a chronic nature with low morbidity and mortality. Infections may result in a respiratory disease from an air sac infection, a septicemic disease called colibacillosis. One of the most important substances involved in this infection is aerobactin, an iron sequestering system (1).

Fluoroquinolones are potent broad-spectrum antimicrobial agents that are increasingly used to treat *E. coli* infection. Despite initial optimism, resistance to these antibiotics has increased significantly since their introduction into medicine and agriculture in the late 1980's and early 1990's. Consequently, the mechanism of resistance in fluoroquinolone-resistant bacteria has been the subject of intense research and, in recent years, dramatic advances have been made in understanding of these mechanisms (2,3).

The primary target of fluoroquinolone action is DNA gyrase and topoisomerase IV. The 2 enzymes are tetramers, composed of 2 A subunits and 2 B subunits, encoded by the *gyrA* and *gyrB* genes for DNA gyrase (topoisomerase II) and *parC* and *parE* genes for topoisomerase IV. The mechanism of resistance to fluoroquinolones has been mainly linked to mutations in the quinolone-resistance-determining region (QRDR) of *gyrA* and *parC* genes and, less frequently, in the *gyrB* and *parE* genes.

Most of the information about the mechanisms of resistance to fluoroquinolones in isolates is from commercially produced poultry because of the economic losses. Little is known about the incidence of resistance in wild birds and what mechanisms of resistance these strains posess.

The aim of this study was to determine the importance of the 4 gene mutations in the acquisition of quinolone-resistance in pathogenic *E. coli* strains isolated from wild birds.

For this purpose, 9 *E. coli* strains isolated from wild birds with septicemia were studied (Table I). The 9 birds involved in this study had just arrived at Grupo para la Recuperación de la Fauna Autóctona (GREFA) Wildlife Hospital. The necropsies and the bacterial cultures from spleen, kidney, lungs, and heart were carried out on McConkey agar and Columbia agar (5% sheep blood) (bioMérieux, France).

To determine the aerobactin virulence gene, a polymerase chain reaction (PCR) was carried out as described previously (4).

Antimicrobial susceptibility was evaluated for each isolate using a diffusion technique. The antibiotics used were nalidixic acid (NA), 30 μg (bioMérieux, France), and ciprofloxacin (CIP), 5 μg (bioMérieux,

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Table I. Susceptibility of 9 *Escherichia coli* isolates from wild birds to quinolones and amino acid substitutions within Gyr A, Gyr B, Par C, and Par E

			Resistance pattern		MIC (μg/mL)		Substitutions	
E. coli isolates	Hosts	Diseases	NA	CIP	NA	CIP	Gyr A	Gyr B, Par C, Par E
Case 1 1Q	Stone curlew	Septicemia	R	R	> 32	2	83 (Ser→Leu)	—
Case 2 3Q	Long-eared owl	Septicemia	R	R	> 32	1	83 (Ser→Leu)	—
Case 3 4Q	Little owl	Septicemia	R	R	> 32	2	83 (Ser→Leu)	—
Case 4 5Q	White stork	Septicemia	R	R	> 32	1	83 (Ser→Leu)	—
Case 5 6Q	Lesser kestrel	Septicemia	R	R	> 32	1	83 (Ser→Leu)	—
Case 6 8Q	Booted eagle	Septicemia	R	R	> 32	1	83 (Ser→Leu)	—
Case 7 9Q	Osprey	Septicemia	R	R	> 32	2	83 (Ser→Leu)	—
Case 8 10Q	Lesser kestrel	Septicemia	R	R	> 32	2	83 (Ser→Leu)	—
Case 9 11Q	Scops owl	Septicemia	R	R	> 32	1	83 (Ser→Leu)	—

NA — Nalidixic acid; CIP — Ciprofloxacin; R — Resistant; MIC — Minimum inhibitory concentration

Table II. GeneBank accession numbers to complete sequence to the quinolone resistance-determining region (QRDR) in all genes

Escherichia coli	GENBANK accession number			
isolates	QRDR *gyrA*	QRDR *gyrB*	QRDR *parC*	QRDR *parE*
1Q	AY065791	AY065800	AY065809	AY065818
3Q	AY065792	AY065801	AY065810	AY065819
4Q	AY065793	AY065802	AY065811	AY065820
5Q	AY065794	AY065803	AY065812	AY065821
6Q	AY065795	AY065804	AY065813	AY065822
8Q	AY065796	AY065805	AY065814	AY065823
9Q	AY065797	AY065806	AY065815	AY065824
10Q	AY065798	AY065807	AY065816	AY065825
11Q	AY065799	AY065708	AY065817	AY065726

France). The minimal inhibitory concentrations (MIC) of NA (Bayer, Germany) and ciprofloxacin (Bayer, Germany) were determined by the broth macrodilution test tube method. Both of these assays were carried out according to the guidelines of the National Committee for Clinical Laboratory Standard guidelines (5,6).

To identify *gyrA, gyrB, parC,* and *parE* mutations in resistance isolates, PCR and direct DNA sequencing were applied. A DNA fragment of 648 base pairs (bp), from nucleotides 24 to 671 of the QRDR of *gyrA* gene, was obtained according to a previously described procedure (7). To amplify a 448 bp fragment of the QRDR of *gyrB,* from nucleotides 995 to 1442, 2 20-mer oligonucleotide primers, 5′-GCGCGTGAGATGACCCGCCGT-3′ and 5′-CTGGCGGTAGAAGAAGGTCAG-3′ were used. These specific primers were constructed based on the *E. coli* gene sequence data (GeneBank accession number D87842). To amplify the 359 bp fragment of the QRDR of *parC* and to amplify the 483 bp fragment of the QRDR of the *parE* gene the previously described procedures, respectively (8,9), were used.

In all of the cases, *E. coli* strains were isolated in pure culture. All of the 9 strains were aerobactin positive. Aerobactin seems to be an important virulence factor, particularly in invasive diseases in birds.

The strains showed high-level nalidixic acid resistance and low-level resistance to ciprofloxacin (Table I). These patterns of resistance, obtained by in vitro Antimicrobial Susceptibility Testing (AST), are similar to those described in previous reports (10).

The mutations in the *gyrA* gene leading to the amino acid substitutions and MICs are shown in Table I. All the isolates showed a change in Ser-83 (polar amino acid) to Leu (nonpolar amino acid) in the Gyr A subunit. Gyr A alterations of Ser-83 are responsible for the low-level resistance to fluoroquinolones (7). In this study, we have found that a change in Ser-83 in the Gyr A protein generated MICs for ciprofloxacin of 1 μg/mL (strains 3Q, 5Q, 6Q, 8Q, 11Q) and 2 μg/mL (4Q, 9Q, 10Q). However, it has been previously stated that a single mutation in *gyrA,* that encodes the change Ser-83 to Leu-83, can give a MIC of 16 μg/mL ciprofloxacin (11).

The majority of veterinary *E. coli* isolates resistant to fluoroquinolones, isolated in the United Kingdom, had mutations only in the QRDR of the *gyrA* gene (3). Additional mutations in the *gyrB* gene have also been implicated in resistance to quinolones in amino acids Asp-426 and Lys-447 of the Gyr B protein (7). Possible amino acid substitutions in both Par C and Gyr A led to an increased level of resistance (8).

The *parE* gene plays a role in the development of quinolone resistance. Overall, this region is homologous to the QRDR of the *gyrB* gene (8), particularly residues Asp-420 and Lys-441 of Par E, which are homologous to Asp-426 and Lys-447 in the B subunit of DNA gyrase.

We did not find concomitant mutations in *gyrA* in our sequences and no single or double mutation that conferred detectable amino acid substitutions in the QRDR of Gyr B, Par C, or Par E was found either.

In conclusion, our results clearly show that the high frequency of mutation at codon 83 of the *gyrA* gene is the most important point in the acquisition of resistance in pathogenic *E. coli* strains from wild birds.

The complete sequence of QRDR of the 4 genes has been deposited in the GeneBank. The accession number of these sequences is available in Table II.

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