Preliminary investigations into fluoroquinolone resistance in *Escherichia coli* strains resistant to nalidixic acid isolated from animal faeces

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Summary

Resistance to fluoroquinolones was evaluated in 17 nalidixic acid-resistant *Escherichia coli* strains isolated from animal faeces. Polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), hybridisation and sequencing were used to reveal point mutations in DNA gyrase subunits A and B and the presence of the *qnr* gene as evidence of plasmid resistance. Chromosomal resistance and class 1 integrons were found in 10 of the 17 *E. coli* strains examined, while the *qnr* gene was not found in any of these. Mutation Ser83-Leu was found in six strains and in one strain mutation Ser83-Ala was found in *gyrA*. Mutations in *gyrA* Asp87 codons and in *gyrB* Asp426 or Lys 447 codons were not identified. Sequencing of the *E. coli* strain ATCC 25922 subjected to resistance induction revealed a *gyrB* mutation of the Lys 447 residue which had been replaced by arginine.

Keywords

Antimicrobial resistance, *Escherichia coli*, Hybridisation, Nalidixic acid, Polymerase chain reaction, Restriction fragment length polymorphism, Sequencing, Resistance.

Introduction

Increased resistance to antibiotics is considered to be one of the major public health problems worldwide, in both human and veterinary medicine. In animals, not only are antibiotics used to treat or prevent infectious diseases, but in the past they have also been widely used as growth promoters (9).

Exposure to antibiotics has exerted a selective pressure on bacteria which has resulted in increased resistance to these drugs. The transmission of resistance between pathogenic bacteria has been amply demonstrated in hospitals, with the horizontal transmission of resistance genes between commensal and pathogenic bacteria considered more likely than their transfer from one pathogen to another (1). The importance of commensal bacteria as mediators in the transmission and spread of resistance genes has been widely reported in the literature (6, 19) and both the correlation between the use of antibiotics in animals and increased numbers of resistance genes in the human microbiota (14) and the function of these bacteria as a reservoir of resistance genes that spread through the various ecosystems (15) have been demonstrated. Animals and animal products thus constitute a 'stockpile' from which resistant bacteria can be transferred to

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humans, or from which resistance determinants can be transferred to zoonotic bacteria (15).

The *Enterobacteriaceae*, and specifically *Escherichia coli*, are among the bacteria that have been studied in greatest depth in an attempt to clarify the phenomena underlying antibiotic resistance, especially in human medicine. *E. coli* has been identified as an indicator micro-organism ('alert organism surveillance'), as it is ubiquitous and highly prone to horizontal resistance gene transfer (13).

Quinolones and fluoroquinolones are broad-spectrum antibiotics that are used in both human and veterinary medicine and act on a wide range of bacteria. Resistance to nalidixic acid is considered an indicator of increased resistance to fluoroquinolones (11).

In Europe, *E. coli* strains isolated from animals in 2007 showed resistance above all to tetracycline, nalidixic acid and ampicillin. Strains isolated from chicken droppings were found to have the greatest resistance of all to both nalidixic acid (17.4%) and ciprofloxacin (37.8%) (3). Resistance to this drug class can be both chromosomal and plasmid (4) and is related to mutations of the target enzymes of these compounds, DNA gyrase and topoisomerase IV. DNA gyrase is a tetrameric enzyme which catalyses negative DNA supercoiling and consists of two subunits A and two subunits B. Topoisomerase IV is also a tetrameric enzyme, consisting of two subunits C and two subunits E, and is involved in the separation of daughter chromosomes during DNA replication (5). Resistance caused by point mutations arises spontaneously and results in the substitution of amino acids within the gyrase and topoisomerase genes (*gyrA*, *gyrB*, *parC* and *parE*), often in combination with reduced membrane porin expression and over-expression of the efflux pump system (5).

Studies of strains isolated from clinical sources demonstrate that most mutations that induce quinolone resistance are found in gyrase subunit A (*gyrA*) (10). These are mainly found in a region called the quinolone resistance-determining region (QRDR). A single mutation in this region results in a high resistance to nalidixic acid but not to fluoroquinolones, for which additional mutations are necessary. For this reason, the minimum inhibitory concentration (MIC) of nalidixic acid can be used as a genetic marker of Gram-negative bacterial resistance to the quinolone family (10).

In plasmid-mediated resistance, the *qnr* gene (4, 5, 16) is passed from one bacteria to another by horizontal transfer, increasing mutation frequency and thus the likelihood of increased resistance (18). Plasmid-mediated quinolone resistance is related to the presence of structures called integrons, which are mobile DNA elements made up of two conserved segments which flank a central region containing a fragment ('cassette') that codes for antibiotic resistance and may play an important role in the acquisition and spread of antibiotic resistance genes. The *qnr* gene is also found within an integron (16). In Gram-negative bacteria, class 1 integrons are predominant (4).

Given the fact that the chromosomal location of *qnr* genes was recently demonstrated by different authors, it was insufficient to analyse samples only for the plasmid.

The molecular screening was the first step since bacterial fluoroquinolone resistance can be supported by both plasmid and chromosomal genetic elements. We attempted to determine the molecular basis of resistance phenotypes of fluoroquinolone resistant strains. After finding a mutation on chromosomal genes *gyrA* and *gyrB*, the research focused not only on the presence of plasmids, but also on fluoroquinolone resistance genes (*qnr*) carried by the plasmid.

This study evaluated, in nalidixic acid-resistant *E. coli* isolated from faeces of cattle and chicken, possible quinolone and fluoro-quinolone resistance through integron detection via hybridisation and subsequent polymerase chain reaction (PCR) to reveal the *qnr* gene. Strains were also subjected to PCR-restriction fragment length polymorphism (RFLP) and sequencing to reveal any *gyrA* and *gyrB* point mutations. *In vitro* resistance was

induced in *E. coli* American Type culture collection (ATCC) 25922 with increasing levels of antibiotic (enrofloxacin) to evaluate the onset of resistance.

Materials and methods

A total of 85 samples of chicken faeces and 55 samples of cattle faeces collected at the abattoir upon slaughter were examined. Samples were sown on MacConkey agar and incubated aerobically for 24 h to 48 h at 37°C. After incubation, lactose-positive samples were identified biochemically using the Vitek system (Biomérieux, Florence). The biochemical strains identified as *E. coli* underwent antibiogram testing by agar disc diffusion or Kirby-Bauer (2) using the procedures and inhibition zone interpretation methods described by the National Committee for Clinical Laboratory Standards (NCCLS) (7). The strains were tested on a panel of some of the most representative antibiotics (Beckton-Dickinson, St Louis, Missouri), including nalidixic acid (30 µg), ciprofloxacin (5 µg) and enrofloxacin (5 µg).

A total of 17 nalidixic acid-resistant *E. coli* strains were selected and all strains were from chicken faeces sampled on poultry farms in the Abruzzo region, while *E. coli* ATCC 25922 was used as the reference strain; this strain does not harbour plasmid that confers resistance.

Hybridisation was performed using a commercially available kit (GE Healthcare, Milan) in accordance with the instructions of the manufacturer. The PCR primers were designed on Blast (8) (Table I). DNA extraction and purification were performed using a commercial kit and 2× Master mix (Mo Bio Laboratories, Inc., Carlsbad, California). Amplification was performed on the thermal cycler GeneAmp PCR system 9700 (Applied Biosystems, Carlsbad, California) in the following conditions:

- 94°C for 5 sec as initial denaturation
- 33 cycles of: 94°C for 30 sec, 53°C for 30 sec, 72°C for 30 sec and final extension at 72°C for 7 sec.

The amplification product was separated by 1% agarose gel electrophoresis (Eppendorf, Milan) and stained with Sybr Safe DNA gel stain (Promega, Madison, Wisconsin). UV transillumination was used to observe the image. Hinf I (Promega, Madison, Wisconsin) was used for RFLP. The restriction product was analysed with 3% agarose gel electrophoresis.

The PCR product was purified for sequencing with the QIAquick purification kit (Quiagen, Milan), Big Dye Terminator kit v3.1 (Applied Biosystems, Foster City, California) was used for the sequencing reaction, following the instructions reported for use with the

Table I
Primer sequences used

Target	Primers	Sequence
qnrS1 <121-538>	*Qnr*S1 for	ACG ACA TTC GTC AAC TGC AA
	*Qnr*S1 rev	TTA ATT GGC ACC CTG TAG GC
qnrS2 <1-657>	*Qnr* S2 for	ATG GAA ACC TAC CGT CAC
	Qnr S2 rev	CTA GTC AGG AAA AAC AAC
qnrA <1-632>	*Qnr* A for	ATG GAT ATT ATT GAT AAA GTT TTT CAG
	Qnr A rev	AAG GGT TCC AGC AGT TGC
qnrB1 <1-681>	*Qnr* B1 for	ATG ACG CCA TTA CTG TAT AA
	Qnr B1 rev	CTA ACC AAT CAC CGC GAT
QRDR gyrA <25-613>	ENTGYRA_for	ACA CCG GTC AAC ATT GAG GA
	ENTGYRA_rev	TGC TGA TGT CTT CAT CAT CG
QRDR gyrB <795-1485>	ENTGYRB_for	CAT CTA CTG (CT)AC CAA C
	ENTGYRB_rev	GAT GAT GAT GCT GTG (AG)TA

QRDR quinolone resistance-determining region

The sequencing reaction product was purified with Agencurt CleanSEQ and Dye Terminator Removal (Agencourt, Bioscience Corporation, Madison, Wisconsin). Sequencing was performed with the Avant Genetic Analyzer 3100 (Applied Biosystems, Foster City, California).

For resistance induction, an initial bacterial suspension of *E. coli* ATCC 25922 was prepared at a concentration of 0.5 McFarland. This was sown onto 50 brain infusion broth agar (Becton Dickinson, St Louis, Missouri) dishes using a calibrated 10 µl loop and aerobically incubated at 37°C for 18 h to 24 h. The resulting colonies were then diluted in brain infusion broth (Becton Dickinson, St Louis, Missouri) and sown on brain infusion agar (Becton Dickinson, St Louis, Missouri) with an initial enrofloxacin concentration of 0.008 µg/ml, which was doubled up at each step to 655.36 µg/ml (11th step, T11) in which resistant strains were obtained.

At each concentration, the resistant strains underwent sequencing, using for DNA target the GyrA QRDR fragment, and were compared against the reference strain.

Results

Of the strains that underwent the antibiogram, 17 were resistant to nalidixic acid and were sensitive to enrofloxacin and ciprofloxacin.

The integron-specific hybridisation and subsequent PCR revealed that only 10 of the 17 *E. coli* strains had class 1 integrons and the *qnr* gene was not found in any of these, demonstrating that the resistance was chromosomal only.

For both *gyrA* and *gyrB*, a 600 bp product was revealed with PCR (Figs 1 and 2), which was then digested with Hinf I and subjected to RFLP. The Hinf I restriction site is found at the *gyrA* Ser83 codon (11).

Analysis of the *gyrA*, digestion products revealed two different restriction patterns (Fig. 3) , in which 4 strains (strains 3, 7, 8 and 9) showed no mutation while the remainder revealed a mutation of the Ser83 codon, 7 of these resulted in replacement while others were silent mutations that have not produced substantial changes of gyrase.

For *gyrB*, no profile other than that of the original ATCC was seen for any sample (Fig. 4).

PCR product sequencing revealed a mutation of Se83-Leu in six strains: one strain revealed a mutation of Ser83-Ala, numerous strains showed silent mutations for *gyrA* and none for *gyrB*.

The position of mutation in DNA gyrase on the QRDR region can create different resistance patterns, especially towards fluoroquinolones.

However, it is difficult to define which of the resistance mechanisms contributed, DNA gyrase mutation or a different accumulation of the antibiotic within the cell (due to changes in the efflux pump system) or other mechanisms. Consequently, it is difficult to identify the causes of these differences because the MIC is unknown.

In the resistance induction procedure, there was a constant reduction in the sensitivity zone over each doubling of enrofloxacin concentration. The colonies found to be resistant at each concentration were sequenced. A comparison of the sequences of the colonies obtained at each step revealed that with respect to the initial *E. coli* ATCC 25922 strain, resistance induction had resulted in a *gyrB* mutation at the Lys 447 residue, which had been substituted for arginine.

Sequencing of the *E. coli* strains isolated from animals showed that no mutation was observed in *gyrB*.

Discussion

The correlation between *gyrA* mutations and resistance to quinolones and fluoroquinolones has been widely described for various Gram-negative bacteria. Within the *gyrA* QRDR, the greatest quinolone resistance is conferred by mutations to the Ser83 and Asp87 codons (5, 13, 20).

In this study, all the strains tested were resistant to nalidixic acid and sensitive to ciprofloxacin and enrofloxacin. These results are in line with other studies that have found

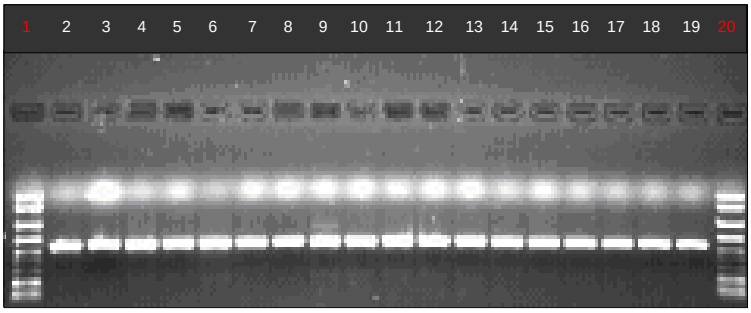

Figure 1
Agarose gel of *gyrA* polymerase chain reaction products
Lanes 1 and 20: markers
Lanes 2 to 18: *Escherichia coli* strains isolated from animals
Lane 19: *E. coli* ATCC 25922

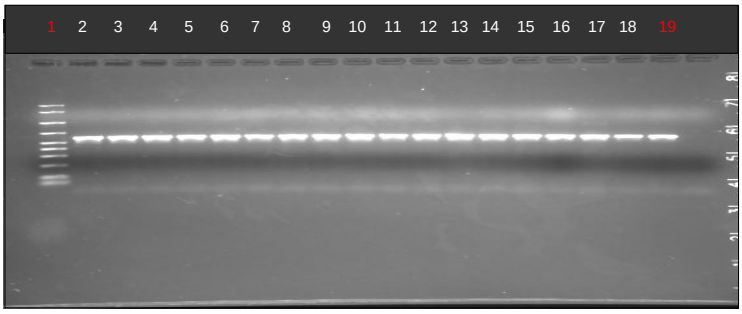

Figure 2
Agarose gel of *gyrB* polymerase chain reaction products
Lane 1: marker
Llanes 2 to 18: *Escherichia coli* strains isolated from animals
Lane 19: *E. coli* ATCC 25922

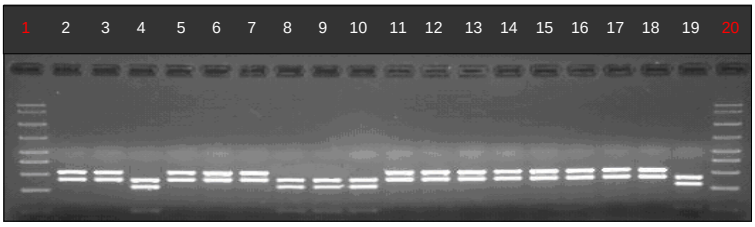

Figure 3
GyrA restriction Hinf I pattern of 17 *Escherichia coli* strains
Lanes 1 and 20: markers
Lanes 2-18: *E. coli* isolated from animals
Lane 19: *E. coli* ATCC 25922

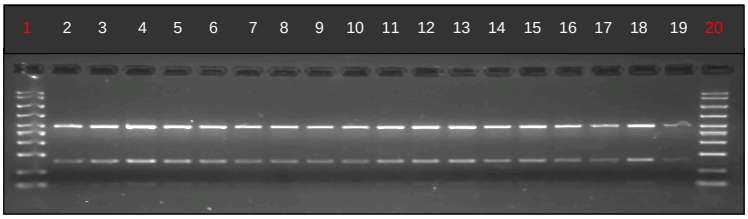

Figure 4
GyrB restriction Hinf I pattern of 17 *Escherichia coli* strains
Lanes 1 and 20: markers
Lanes 2-18 to 18: *E. coli* strains isolated from animals
Lane 19: *E. coli* ATCC 25922

that not all *gyrA* or *gyrB* mutations confer the same level of quinolone resistance (4, 5).

Finally, no mutation to the *gyrA* Asp87 codon was identified in the 17 samples resistant to nalidixic acid, while at *gyrA* Ser83 codon, 6 strains showed type Ser83-Leu mutations and one strain showed a Ser83-Ala mutations, while there was no mutation identified in *gyrB* Asp426 and Lys 447 codons.

In *gyrB*, mutations were observed in the Asp426 and Lys 447 codons (21); Asp426 mutations conferred resistance to all the quinolones tested, while the Lys 447 mutation conferred resistance to nalidixic acid but the strains were hypersensitive to amphoteric quinolones, such as ciprofloxacin (4).

In general, amino acid substitutions can impair the capacity to form hydrogen bonds and the negative charge of amino acids in these positions appears to be important for the interaction between quinolones and the DNA gyrase complex (12).

Moreover, some amino acids in the QRDR may be more important for the bonds between the GyrA and GyrB subunits than for the activity of the holoenzyme itself. Together, these factors may help to explain why some substitutions have a greater effect on fluoroquinolone resistance than others (17).

From the sequencing of *E. coli*, ATCC 25922 subjected to induction of resistance showed a mutation in the remaining Lys 447 in *gyrB*, which was replaced by arginine, thereby confirming that antibiotic pressure alone can produce mutations.

Given the low number of strains tested and the presence of silent mutation, the study needs to be extended to include quinolone- and fluoroquinolone-resistant strains isolated from veterinary sources, including both animals and food, in order to study the mechanism that creates resistance and its correlation with the resistance mechanism in strains isolated from human clinical sources, which has been widely described and studied in the literature.

Conclusions

Exposure to antibiotics has exerted a selective pressure on bacteria which has resulted in increased resistance to these drugs among bacteria from both animals and humans. This ability evolves through different mechanisms and once one or more resistance genes are generated, bacteria can transfer the genetic information by horizontal transmission of resistance genes between commensal and pathogenic bacteria. Antibiotic resistance is becoming a common problem in many parts of the world due to the fact that antibiotics are used frequently and, sometimes, incorrectly. Other factors that contribute to the spread of the phenomenon include incorrect diagnosis, unnecessary prescriptions, improper use of antibiotics by patients and the use of antibiotics as livestock food additives for growth promotion.

Only 10 of the 17 *E. coli* strains had class 1 integrons and the *qnr* gene was not found in any of these, demonstrating that the resistance was chromosomal only.

Mutations were revealed in seven strains in Ser83 codon in *gyrA* that has already been identified in human strains. Similarly, induction of resistance by a mutation in the remaining *gyrB* Lys 447, which was replaced by arginine, was also found in human strains.

Since strains isolated from animals showed the same mutations found in human isolates and the appearance of mutations in response to induction of resistance in susceptible strains it is suggested that the study be extended to include a larger number of isolates, in order to explain the real effect that the use of antibiotics has on the resistance of the strains isolated from animals and the likelihood of a possible transfer of this resistance to humans through food.

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