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Ciprofloxacin-resistant *Escherichia coli* isolated from the intestinal microbiota of goats in Greece in the absence of selective pressure ☆

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ABSTRACT

The presence of ciprofloxacin resistance commensal *Escherichia coli* (C-R-Ec) was determined for goats in the absence of selective pressure in Northern and Central Greece. The C-R-Ec was categorized in 3 groups with respect to their phenotypic resistance to other antibiotics as well as the carriage of antibiotic resistance genes. The first group consisted of 7 C-R-Ec that were found also resistant to tetracycline. Among them *tet*(B) (n = 7), *qnr*(S) (n = 7), and *qnr*(B) (n = 3) producers were identified by polymerase chain reaction. The second group consisted of 10 C-R-Ec that were found sensitive to all other antibiotics, and their phenotypic resistance to ciprofloxacin was not attributed to the presence of resistance genes. Finally, the third group consisted of 2 C-R-Ec also resistant to sulfamethoxazole. These strains were not carrying any transferable elements that contribute to resistance either to ciprofloxacin or to sulfamethoxazole. This is the first report of ciprofloxacin-resistant *E. coli* isolated from goats in Greece.

1. Introduction

Ciprofloxacin is a synthetic second-generation fluoroquinolone with activity against gram-negative pathogens, especially those resistant to cephalosporins and aminoglycosides (Acar and Goldstein, 1997). By inhibiting the activity of gyrase and topoisomerase IV, ciprofloxacin inhibits bacterial DNA transcription and replication. Soon after ciprofloxacin was introduced as therapeutic compound, its extensive use and misuse in human, as well as in veterinary medicine, have led to the emergence and spread of resistant bacterial strains (Vatopoulos et al., 1999).

Initially, quinolone resistance is believed to arise either from chromosomal mutations in genes encoding target enzymes or a decreased accumulation of the drug inside the bacteria (Piddock, 2002). Later, plasmid-like transmissible elements were described having the potential to horizontally transfer quinolone resistance genes (Tran and Jacoby, 2002). The locus responsible for this plasmid-mediated quinolone resistance, named *qnr*(A), *qnr* (B), and *qnr*(S), has been identified in species of Enterobacteriaceae (Kehrenberg et al., 2006; Robicsek et al., 2006a, 2006b). A number of studies reported that the genes encoding quinolone resistance to Enterobacteriaceae are located on plasmids, along with other resistance elements like CTX-M-15β-lactamase (Giamarellou and Poulakou, 2009; Jacoby et al., 2006). In order to find ways of limiting the spreading of these life-threatening resistant plasmids, their prevalence among livestock is monitored worldwide (Endimiani et al., 2012).

During the past decade, plasmid-mediated quinolone resistance has been increasingly reported from around the world among *Escherichia coli* strains isolated from the colonic microbiota of food animals (European Food Safety Authority Panel on Biological Hazards (BIOHAZ), 2010). In Greece, plasmid-mediated ciprofloxacin resistance in *E. coli* isolates of human origin has already been reported (Chaniotaki et al., 2004; Galani et al., 2010; Mavroidi et al., 2012; Vasilaki et al., 2008). Although the antibiotic resistance of Enterobacteriaceae isolated from food animals of Greek herds has been studied (Filioussis et al., 2008; Solomakos et al., 2009), the molecular mechanisms responsible for ciprofloxacin resistance in the *E. coli* of small ruminants remain unknown. We herein report on the experience with the community of goats in Greece where ciprofloxacin resistance in commensal *E. coli* (C-R-Ec) was found to be present notwithstanding that the community has retained an absence of ciprofloxacin exposure.

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2. Materials and methods

2.1. Sample collection

Ten goatherds were enrolled in the study with the inclusion criteria that none of the animals was given fluorquinolones. Six the herds were medium-sized (120 animals/farm on average) and were located in Macedonia region (Northern Greece). Additionally, the rest 4 were larger (250 animals/farm on average) and were located in Thessaly region (Central Greece). During the initial visit, a questionnaire was administered to gather information on basic flock-level antimicrobial use (antimicrobials used, route of administration, diseases treated).

Twenty healthy female goats per farm, aged 4 to 8 years old, were sampled. Only 1 sample was taken per animal. The faecal samples were collected with rectal swabs, placed in separate sterile plastic containers, and transferred to the laboratory within less than 4 h of collection, maintained at 4–8° C using insulated boxes. The specimens were initialized, as $Spec_{x.y}$ where x was the number of the sampled herd, ranged from 1 to 10, and y was the identification number of the sampled goat, ranged from 1 to 20. All specimens delivered at the laboratory were processed immediately for *E. coli*.

2.2. Isolation, biochemical identification, and polymerase chain reaction (PCR) confirmation of E. coli

Faecal carriage of ciprofloxacin-resistant *E. coli* was investigated by a direct plating method. Each faecal swab was spread onto a macConkey agar plate (1.10748 EMD Merck KGaA, Darmstadt, Germany), supplemented with 2 μg/mL of ciprofloxacin (17850 FLUKA). After incubation at 37° C for 24–48 h, plates were inspected for colonies growth. One lactose fermented colony per plate was purified on nutrient agar (70148 FLUKA) and further identified as *E. coli* by API 20E identification strips (bioMerieux, Marcyl'Etoile, France). Species identification of the isolates was performed by PCR that detects *usp*(A) gene, which is characteristic for *E. coli* strains (Chen and Griffiths, 1998).

2.3. Phenotypic resistance to antibiotics

The chosen antibiotics were ciprofloxacin (17850-FLUKA), amoxicillin (A8523- SIGMA), gentamicin (G1264-SIGMA), sulfamethoxazole (S7507-FLUKA), ceftazidime (C3809-SIGMA), cefuroxime (C4417-FLUKA), and tetracycline (T7660-SIGMA). The minimal inhibitory concentrations (MICs) were determined on Mueller Hinton broth (70192 FLUKA) by broth microdilution according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2011). Susceptibility results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing breakpoints (European Committee on Antimicrobial Susceptibility Testing, 2012).

2.4. PCR detection of antibiotic resistance genes

Molecular detection of genes that encode resistance to ciprofloxacin, tetracycline, and sulfamethoxazole was performed on the phenotypically resistant *E. coli* strains. The antibiotic resistance genes were amplified by PCR using *Taq* DNA polymerase in accordance with the supplier's directions (Roche Diagnostics, Basel, Switzerland) The oligonucleotides used for PCRs, the annealing temperatures, and the size of the amplicons are listed in Table 1. The PCR amplicons of the resistance genes were sequenced on an ABI Prism 3100 genetic analyser (Applied Biosystems, Foster City, CA, USA) using dRodamine-labeled terminators. Nucleotide sequences were aligned and compared to GenBank sequences using the BLAST program of the National Center for Biotechnology Information, Bethesda, MD, USA. The sequence comparisons with sequences in the EMBL/GenBank database were made using the BLAST program.

3. Results and discussion

A total number of 200 faecal samples from equal healthy adult goats representing 10 herds from 2 Greek regions were collected. The samples were obtained during the coldest 4 months of the year because the lambing period in Northern and Central Greece spans from late winter to early spring. A recent study reported that the prevalence of *E. coli* carriage among food animals does not account for seasonal variation (Endimiani et al., 2012). Therefore, we suggest that our results are representative of the variation across a whole year. Overall, 19 of the samples collected from equal number of goats yielded ciprofloxacin-resistant *E. coli* isolates. The positive animals were stabled in 3 different herds of Macedonia region. To our knowledge, this is the first report of the ecological impact of antibiotic resistance in goats in Greece. Consequently, our results are not comparable to other reports from previous studies in the country.

Following the characterization of the specimens, the isolates were initialized as C-R-$Ec_{x.y}$ where x and y stands for the number of the positive herd and the number of the positive goat, respectively. The MICs of the isolates against ciprofloxacin and 6 other antibiotics broadly used in veterinary medicine were determined. Overall, the isolates were characterised with respect to their phenotypic resistance to all tested antibiotics, as well as the carriage of antibiotic resistance genes. As a result, 3 different groups could be distinguished (Table 2).

The first group consisted of 7 isolates that phenotypically exhibited a combined resistance to ciprofloxacin and tetracycline. The MICs against ciprofloxacin ranged from 8 mg/L to 16 mg/L (media 8 mg/L) and against tetracycline from 32 mg/L to 64 mg/L (media

Table 1
Oligonucleotides and PCR conditions used for the detection of resistance genes.

Primer	Sequence	Amplicon size (bp)	Annealing temperature (°C)	Detected gene	Primer reference
*tet*A-L	5′- GGCGGTCTTCTTCATCATGC −3′	502	64	*tet*(A)	21
*tet*A-R	5′-CGGCAGGCAGAGCAAGTAGA-3′				
*tet*B-L	5′- CATTAATAGGCGCATCGCTG-3′	930	64	*tet*(B)	21
*tet*B-R	5′- TGAAGGTCATCGATAGCAGG −3′				
*sul*I-L	5′- GTGACGGTGTTCGGCATTCT-3′	799	68	*sul*(I)	21
*sul*I-R	5′-TCCGAGAAGGTGATTGCGCT-3′				
*sul*II-L	5′-CGGCATCGTCAACATAACCT-3′	721	66	*sul*(II)	21
*sul*II-R	5′- TGTGCGGATGAAGTCAGCTC-3′				
grnA-fw	5′- TCAGCAAGAGGATTTCTCA-3′	627	50	*qnr*(A)	20
grnA-rv	5′-GGCAGCACTATGACTCCCA-3				
grnB-fw	5′-TCGGCTGTCAGTTCTATGATCG-3′	640	54	*qnr*(B)	20
grnB-rv	5′- TCCATGAGCAACGATGCCT-3′				
grnS-fw	5′-TGATCT CACCTTCACCGCTTG-3′	566	58	*qnr*(S)	20
grnS-rv	5′-GAATCAGTTCTTGCTGCCAGG-3′				