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High level fluoroquinolone resistance in *Escherichia coli* isolated from animals in Turkey is due to multiple mechanisms

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Abstract: The aim of this study was to determine the molecular mechanisms of fluoroquinolone resistance in *E. coli* isolated from cattle, goats, sheep, cats, and dogs in Turkey. Twenty nonreplicate *E. coli* isolates (chosen on the basis of RAPD pattern) from food-producing animals were selected for the study. To identify phenotypic differences between isolates, the sum of the MIC values of 14 antimicrobials was calculated; values ranged from 565 to 2520 µg/mL, indicating the diversity in antimicrobial resistance present in the panel of isolates. PCR and qRT-PCR were used to characterize the presence and expression levels of known molecular mechanisms of fluoroquinolone resistance. The number of *E. coli* isolates having single, double, and triple topoisomerase mutations was 2, 10, and 5, respectively. Moreover, the number of *qnrA* - , *qnrS* - , *oqxB* -, and *aac(6')Ib-cr*-containing *E. coli* isolates was 1, 4, 1, and 17, respectively. Increased expression of *acrB* and *soxS* was detected in 2 and 9 isolates, respectively. The results of this study show a wide range of different mechanisms of fluoroquinolone resistance in *E. coli* isolates in Turkey.

Key words: Fluoroquinolone resistance, *E. coli*, animals, PCR, qRT-PCR

1. Introduction

The worldwide dissemination of fluoroquinolone (FQ) resistant *Escherichia coli* isolates is a significant challenge to antibiotic treatment and infection control policies (1). In *E. coli*, a gram-negative bacterium, resistance to FQs primarily occurs from mutations in the quinolone resistance determining region (QRDR) of the genes encoding the drug target enzymes (DNA gyrase and topoisomerase IV) (2). In addition, plasmid-mediated quinolone resistance (PMQR) genes, such as the target protection PMQR gene *qnr*, the enzymatic modification gene *aac(6')Ib-cr*, and efflux pump genes *qepA* and *oqxB*, have also been shown to increase the MICs of FQs (3–5). Overexpression of the multidrug efflux pump AcrAB–TolC has been shown to directly contribute to multidrug resistance (MDR) in *E. coli* and controls the susceptibility of *E. coli* strains to many structurally unrelated antibiotics, including β-lactams, FQs, and aminoglycosides (6). AcrAB is regulated primarily by the local repressor AcrR and global regulators such as MarA and its homologs SoxS and Rob in *E. coli* (7).

Although FQs have been widely used in human and veterinary medicine in Turkey, most studies of FQ resistance focused on human isolates (8,9). Therefore, the aims of the present study were to identify QRDR (*gyrA*, *gyrB*, *parC*, and *parE*) mutations, to detect PMQR genes (*qnrA*, *qnrB*, *qnrS*, *qepA*, *oqxB*, and *aac(6')Ib-cr*), and to evaluate the expression of genes encoding MarA, AcrB, and SoxS in *E. coli* isolates of animal origin.

2. Materials and methods

2.1. Strains and susceptibility testing

Random amplified polymorphic DNA (RAPD) analysis was used to determine the genetic relatedness of 58 *E. coli* isolates from cattle, goats, sheep, cats, and dogs. Twenty different RAPD patterns were observed among these isolates and one representative isolate was chosen from each pattern based on resistance genotype.

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Broth microdilution testing was carried out to determine the MICs of nalidixic acid (NAL), enrofloxacin (ENR), danofloxacin (DAN), ciprofloxacin (CIP), orbifloxacin (ORB), moxifloxacin (MFX), ampicillin (AMP), cefotaxime (CTX), tetracycline (TET), erythromycin (ERY), gentamicin (GEN), chloramphenicol (CHL), sulfamethoxazole (SMX), and trimethoprim (TMP) according to the guidelines of the Clinical Laboratory Standards Institute (10). The specific breakpoints, as set in previous studies (10–12), used in this study to determine susceptibility were as follows: CIP > 1 µg/mL, MFX > 1 µg/mL, AMP > 8 µg/mL, CTX > 2 µg/mL, GEN > 4 µg/mL, CHL > 8 µg/mL, SMX > 4 µg/mL, TMP > 4 µg/mL, NAL > 16 µg/mL, ENR > 4 µg/mL, ORB > 8 µg/mL, and TET > 4 µg/mL. To identify differences in the degree of resistance between the isolates, the sum of MIC values was calculated by the addition of the MIC values for 14 antimicrobial agents.

2.2. PCR amplification

DNA was extracted from bacterial cultures using the Genomic DNA Purification Kit (Fermentas) according to the manufacturer's instructions. QRDR and PMQR genes were amplified using specific primers as described in Table 1 and PCR products of *gyrA*, *gyrB*, *parC*, and *parE*

Table 1. Primers and reaction conditions used in the study.

Target	Primers	Annealing temp. (ng)	Size (bp)	Reference
RAPD	GTCATCGCAG ATGTAAGCTCCTGGGGATTCAC TGGTGGCCTCGAGCAAGAGAACAAAG			
QRDR				
gyrA	F - ACGTACTAGGCAATGACTGG R - AGAAGTCGCCGTCGATAGAAC	55	189	(24)
gyrB	F - CAGACTGCCAGGAACGCGAT R - AGCCAAGTGCGGTGATAAGA	55	203	(24)
parC	F - TGTATGCGATGTCTGAACTG R - CTCAATAGCAGCTCGGAATA	55	264	(24)
ParE	F - TACCGAGCTGTTCCTTGTGG R - GGCAATGTGCAGACCATCAG	55	266	(24)
PMQR				
qnrA	F - ATTTCTCACGCCAGGATTTG R - GATCGGCAAAGGTTAGGTCA	53	516	(5)
qnrB	F - GATCGTGAAAGCCAGAAAGG R - ACGATGCCTGGTAGTTGTCC	53	469	(5)
qnrS	F - ACGACATTCGTCAACTGCAA R - TAAATTGGCACCCTGTAGGC	53	417	(5)
qepA	F - CTTCTCTGGATCCTGGACAT R - TGAAGATGTAGACGCCGAAC	53	720	This study
oqxB	F - ATCGGTATCTTCCAGTCACC R - ACTGTTTGTAGAACTGGCCG	56	541	This study
aac(6')Ib - cr	F - TTGCGATGCTCTATGAGTGGCTA R - CTCGAATGCCTGGCGTGTTT	59	482	(15)
MDR				
16S rRNA	F - ATGCCGCGTGTATGAAGAA R - GGTAACGTCAATGAGCAAAGG	60	93	UPL*, Roche
marA	F - CCGTAAGATGACGGAAATCG R - CCAGATAGAGTATCGGCTCGTT	60	62	UPL, Roche
acrB	F - GCGGGAACCATCCTGATT R - CGCGTCTGACCTCTACTGAA	60	65	UPL, Roche
soxS	F - GCAATGGACCTGGGTTATGT R - CAAACTGCCGACGGAAAA	60	61	UPL, Roche

*Universal Probe Library.

were sequenced by Macrogen, Inc. The presence of *qnrA*, *qnrB*, *qnrS*, *qepA*, and *oqxB* genes was determined by PCR amplification as described previously (3,13,14). The *aac(6') Ib* gene was amplified as described previously by Park et al. (15); PCR amplimers representing the *aac(6')Ib* gene were sequenced by Macrogen, Inc. in order to identify *aac(6') Ib-cr*.

2.3. Quantitative real-time-PCR (qRT-PCR)

RNA was extracted using the RNA Purification Kit (Fermentas) according to the manufacturer's instructions. Genomic DNA was removed by DNase I according to the manufacturer's instructions (Fermentas). The synthesis of cDNA used the 'RevertAid' First Strand cDNA Synthesis Kit (Fermentas). Template RNA (1 μg/mL) and oligo $(dT)_{18}$ primers in a final volume of 12 μL were incubated at 65 °C for 5 min and then the following components were added: 4 μL of 5X reaction buffer, 1 U/μL RiboLock RNase inhibitor, 1 mM dNTP mix, and 10 U/μL RevertAid M-MuLV reverse transcriptase. The 20-μL mixture was incubated at 42 °C for 60 min. An additional incubation at 70 °C for 5 min terminated the reactions. The cDNA was diluted 1:10 with nuclease-free water prior to the qRT-PCRs. 16S rRNA was used as a housekeeping gene. *E. coli* AG100 was used as a control strain. Overexpression was defined as a 1.5-fold increase in the expression of *marA*, *soxS*, and *acrB* as described previously by Karczmarczyk et al. (16). Amplification reactions were carried out in duplicate.

2.4. Accumulation of Hoechst 33342 by *E. coli* isolates

Efflux activity of the 20 *E. coli* isolates was determined by measuring accumulation of the fluorescent dye Hoechst 33342 (bisbenzimide: 2.5 μM). Measurements were taken at excitation and emission wavelengths of 350 nm and 460 nm, respectively, over 120 min using a FLUOstar OPTIMA as previously described (17).

3. Results

In the present study, *E. coli* isolated from cattle (n = 17), sheep (n = 2), and a cat (n = 1) demonstrated a high level of resistance to 14 different antimicrobial agents. The susceptibility profiles of the 20 isolates were determined and the sum of MIC values ranged from 565 to 2520 μg/mL. The MICs of NAL, ENR, DAN, ORB, MFX, AMP, CTX, ERY, CHL, SMX, and TMP against the 20 *E. coli* isolates were higher than the breakpoint concentrations of these antibiotics. Fifteen of the *E. coli* isolates (75%) were resistant to all antibiotics tested in the study. *E. coli* E94 was susceptible to CHL; *E. coli* E95 and *E. coli* E247 were susceptible to CTX and GEN; *E. coli* E248 was susceptible to CIP, CTX, GEN, CHL, and TMP; and *E. coli* E308 was susceptible to CTX, TET, and CHL.

The DNA sequences of *gyrA*, *gyrB*, *parC*, and *parE* were amplified, sequenced, and analyzed using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). *E. coli* isolates carried a variety of alterations in *gyrA* (Ser – 83 → Leu, Asp – 87 → Asn), *parC* (Ser – 80 → Ile), and *parE* (Ser – 458 → Ala). The number of *E. coli* isolates having single, double, and triple topoisomerase mutations was 2, 10, and 5, respectively. The number of *E. coli* isolates with mutations in *gyrA*, *parC*, and *parE* was 16, 16, and 5, respectively; mutation in the *gyrB* gene was not observed (Table 2). The number of *qnrA1* -, *qnrS1* -, *oqxB* -, and *aac(6')Ib-cr*- containing *E. coli* isolates was 1, 4, 1, and 17, respectively; *qnrB* and *qepA* were not detected in any of the *E. coli* isolates. PMQR genes and the number of *gyrA*, *parC*, *gyrA*/*parC*, and *gyrA*/*parC*/*parE* mutations in *aac(6')Ib-cr*-containing *E. coli* isolates was 1, 1, 9, and 4, respectively. QRDR mutations were not detected in *qnrA1*-containing *E. coli* E101 and *qnrS1*-containing *E. coli* E103 and E248 isolates. However, *qnrS1*- and *oqxB*-containing *E. coli* E247 showed mutations at codons 83 and 87 of *gyrA* and codon 80 of *parC*. In addition to amino acid substitutions in *gyrA* and *parC*, *qnrS1*-containing *E. coli* E308 showed point mutations in *parE*.

The relative changes in the expression of *marA*, *acrB*, and *soxS* are shown in Table 2. Increased expression of *acrB* and *soxS* was detected in 2 and 9 isolates, respectively; increased expression of *marA* was not detected in any of the *E. coli* isolates. The most resistant isolate according to the sum of MIC values, E97, and *E. coli* E100 had mutations in the QRDR genes, and *aac(6')Ib-cr* plus increased *acrB* expression. *E. coli* isolates in the study were grouped as isolates with decreased efflux activity (E78, E81, E84, E85, and E95) and those with increased a efflux ctivity (E73, E75, E92, E93, E94, E96, E97, E100, E101, E102, E103, E104, E247, E248, and E308) (Table 2).

4. Discussion

Twenty *E. coli* isolates with an MDR phenotype and distinct RAPD banding patterns were identified. Antimicrobial resistance profiles of 128 isolates from environmental and clinical sources were established on the basis of total MIC values of 14 different antimicrobial agents by Afifi et al. (18); their results showed that environmental and clinical sources were potential reservoirs for MDR. The majority of the MDR *E. coli* were isolated from cattle in the present study. This result indicates that cattle may be an important vector for the dissemination of MDR *E. coli* among animals as well as from animals to humans.

In the present study, amino acid substitution in the QRDRs was also at the most frequently identified sites (2); codons 83 and 87 of *gyrA*, 447 of *gyrB*, 80 of *parC*, and 458 of *parE*. *E. coli* E81, E85, E94, E97, and E308 have *parE* mutations plus *gyrA* and *parC*; these isolates were more resistant than the others having *gyrA* and *parC* mutants in QRDR. The *oqxB* and *gnrS* genes were detected in

Table 2. Phenotypic and molecular characterizations of *E. coli* isolates.

Isolate	Animal	Sum of MICs (µg/mL)	QRDR mutations				Presence of PMQR						Gene expression*			Efflux activity[a]
			gyrA	*gyrB*	*parC*	*parE*	*qnrA*	*qnrB*	*qnrS*	*qepA*	*oqxB*	*aac(6') Ib - cr*	*marA*	*acrB*	*soxS*	
E248	Sheep	565	-	-	-	-	-	-	+	-	-	-	↓↓	↓	↓	↑
E103	Cattle	1444	-	-	-	-	-	-	+	-	-	+	↓↓	↓	↑	↑
E104	Cattle	1592	-	-	+	-	-	-	-	-	-	+	↓	↓	↓	↑
E78	Cattle	1612	+	-	+	-	-	-	-	-	-	+	↓	↓↓↓	↓	↓
E73	Cattle	1644	+	-	+	-	-	-	-	-	-	+	↓	↓	↓↓	↑
E100	Cattle	1644	+	-	+	-	-	-	-	-	-	+	↓	↑	↓↓	↑
E102	Cattle	1724	+	-	-	-	-	-	-	-	-	+	↓↓	↓	↓	↑
E101	Cattle	1740	-	-	-	-	+	-	-	-	-	+	↓↓↓	↓↓	↑	↑
E247	Sheep	1748	+	-	+	-	-	-	+	-	+	-	↓↓↓	↓↓↓	↑↑	↑
E92	Cattle	1772	+	-	+	-	-	-	-	-	-	+	↓↓↓	↓	↑	↑
E95	Cattle	1788	+	-	+	-	-	-	-	-	-	+	↓↓↓	↓↓↓	↑↑	↓
E84	Cattle	1832	+	-	+	-	-	-	-	-	-	+	↓↓↓	↓	↓	↓
E96	Cattle	1836	+	-	+	-	-	-	-	-	-	+	↓	↓	↓	↑
E94	Cattle	1921	+	-	+	+	-	-	-	-	-	+	↓↓	↓↓↓	↑	↑
E75	Cattle	1980	+	-	+	-	-	-	-	-	-	+	↓↓	↓	↓	↑
E81	Cattle	2104	+	-	+	+	-	-	-	-	-	+	↓↓	↓↓	↑	↓
E308	Dog	2256	+	-	+	+	-	-	+	-	-	-	↓	↓	↓	↑
E93	Cattle	2308	+	-	+	-	-	-	-	-	-	+	↓↓↓	↓	↑	↑
E85	Cattle	2512	+	-	+	+	-	-	-	-	-	+	↓↓	↓↓↓	↑	↓
E97	Cattle	2520	+	-	+	+	-	-	-	-	-	+	↓↓↓	↑	↓	↑

↑: 1–5 fold increased; ↑ ↑: 5–10 fold increased; ↓: 1–5 fold decreased; ↓↓: 5–10 fold decreased; ↓↓↓: ≥ 10 fold decreased;
*: compared to AG100; [a]: as measured in Hoechst assay compared to AG100.

one *E. coli* isolate having 2 QRDR mutations; this isolate exhibited resistance against 11 of the 14 antimicrobials. Recently, Wong et al. (19) also reported that *Salmonella typhimurium* isolates were positive for both *oqx* and *aac(6')Ib-cr* and had a single mutation in *gyrA*. The *aac(6') Ib-cr* was the most widely (85%) detected transmissible resistance gene and only two of the six PMQR genes were found in *aac(6')Ib-cr*-containing *E. coli* isolates. Overall, the *E. coli* isolates were very resistant against the selected antimicrobials, including PMQR positive isolates, whereas PMQR determinants conferred low-level quinolone resistance (20). Reduced susceptibility by PMQR determinants is likely the most important factor that facilitates the selection of mutants with higher-level resistance (21). When both *qnr* and *aac(6')-Ib-cr* genes are present in the same cell, resistance increases more than that conferred by *qnr* genes alone (15).

The highly resistant isolates (E97, E100) carried a mix of different genetic determinants (QRDR mutations, *aac(6')Ib-cr*, and overexpressed *acrB*). However, there were more isolates that demonstrated derepression of *soxS* than those that carried PMQR genes. Oregan et al. (22) indicated that deletion of *marA* or *soxS* decreased ciprofloxacin MICs by 8–16-fold, and MDR isolates in which both *marA* and *soxS* were inactivated lost their MDR phenotype. The MIC changes are small and not clinically significant in the absence of the AcrAB–TolC system even if mutations in QRDR or PMQR genes are present (23). However, it has also been demonstrated that alternative regulatory pathways exist that can result in overexpression of AcrAB–TolC independently of *marA* and *soxS* (16). In the present study, a mix of resistance genes was found to be responsible for the high level of resistance in *E. coli*, but the role of the genes could not be estimated individually.

This study is the first study to investigate MDR at the molecular level and to identify *oqxB* and *aac(6')Ib-cr* in *E. coli* isolates of animal origin in Turkey. The 20 distinct *E. coli* isolates, all with extensive antibiotic resistance, showed that accumulation of resistance genes in a reservoir creates a high risk for public health from direct contact with animals or consumption of their products.

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