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Plasmid-mediated quinolone resistance in *Escherichia coli* strains isolated from animals in Turkey*

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Summary: Chromosomal mutations and resistance genes transferred by plasmids are the main factors of quinolone resistance particularly in *Escherichia coli* strains isolated from both animals and humans. In this study a total of 259 *E. coli* strains were examined for the resistance to nalidixic acid and ciprofloxacin by agar dilution method and for the presence of *qnr*A, *qnr*B and *qnr*S genes that are known as plasmid-mediated quinolone resistance determinants by PCR. According to antimicrobial susceptibility tests 47 (50.0%) of 94 chicken, 3 (4.5%) of 66 sheep, 5 (9.6%) of 52 cattle and 3 (6.4%) of 47 dog *E. coli* strains were found to be resistant to ciprofloxacin. Among all plasmid-mediated quinolone resistance determinants that were investigated in this study, only *qnr*A gene was found in 5 (5.3%) of 94 *E. coli* chicken isolates. This is the sole report from Turkey that evaluated the plasmid-mediated quinolone resistance in animal isolates of *E. coli* strains.

Key words: Animal, *Escherichia coli*, plasmid-mediated, quinolone-resistance

Türkiye'de hayvanlardan izole edilen *Escherichia coli* suşlarında plasmid ilişkili kinolon direnci

Özet: Hayvanlarda ve insanlarda çok çeşitli infeksiyonlara neden olan *Escherichia coli*'lerde kinolon direnç gelişimine neden olan başlıca faktörler kromozomal mutasyonlar ve plazmidler ile aktarılan direnç genleridir. Bu çalışmada toplam 259 *E. coli* suşu, nalidiksik asit ve siprofloksasin direnci, agar dilusyon yöntemi kullanılarak ve plazmid ilişkili kinolon direnç belirleyicilerinden olan *qnr*A, *qnr*B ve *qnr*S genlerinin varlığı PCR yöntemi kullanılarak araştırıldı. Antibiyotik duyarlılık test sonuçlarına göre 94 tavuk *E. coli* suşundan 47 (%50)'si, 66 koyun *E. coli* suşundan 3 (%4.5)'ü, 52 sığır *E. coli* suşundan 5 (%9.6)'i, 47 köpek *E. coli* suşundan 3 (%6.4)'ü siprofloksasin'e dirençli bulundu. Bu çalışmada araştırılan bütün plazmid ilişkili kinolon direnç belirleyicileri içerisinden, izole edilen 94 *E. coli* tavuk suşu arasında *qnr*A geni, sadece 5 (%5.3) tavuk izolatında bulundu. Bu çalışma, Türkiye'de *E. coli* hayvan izolatlarında plazmid ilişkili kinolon direncinin değerlendirildiği tek rapordur.

Anahtar sözcükler**:** *Escherichia coli*, hayvan, kinolon direnci, plazmid ilişkili

Introduction

Escherichia coli is one of the most common microorganism, which affects both animals and humans worldwide by a wide spectrum of diseases ranging from opportunistic wound infection to severe systemic infections. The zoonotic potential, complicated antigenic structure and toxins give importance to *E. coli* in prophylaxis and treatment regimes (5).

Quinolones are broad-spectrum antibiotics widely used against Gram negative bacterial infections in both human and veterinary medicine nevertheless intensive and misuse of quinolones led to bacterial resistance. Chromosomal mutations in the quinolone resistance-determining regions of DNA gyrase and topoisomerase IV and genes transferred by plasmids to susceptible bacterial strains are the main factors in acquiring rapid resistance to quinolones (22).

In 1998, plasmid-mediated quinolone resistance (PMQR) was first reported in a pMG252 plasmid of *Klebsiella pneumoniae* isolated from urine of a patient at the University of Alabama at Birmingham Medical Center (12). After this first record, *qnr* gene which encodes a 218 amino-acid protein (Qnr) of pentapeptide repeat family and produces low-level quinolone resistance, was cloned and sequenced from pMG252 plasmid (19). It was also reported that the Qnr protein protects DNA gyrase from ciprofloxacin (1) and increases resistance to fluoroquinolones and nalidixic acid (12, 15, 16).

Alteration of drug target (DNA gyrase, topoisomerase IV), decreasing membrane permeability by changing porin protein structures, eliminating the effect of drug by using active efflux-pumps and plasmid-mediated bacterial resistance are the known mechanisms

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involved in quinolone resistance (15,16). Plasmid-mediated bacterial resistance is determined by *qnr*A, formerly named *qnr* (*qnr*B, *qnr*S, *qnr*C, *qnr*D are variants of *qnr* gene, and encodes QnrB, QnrS, QnrC and QnrD proteins respectively), *aac(6')-Ib-cr* (encodes an aminoglycoside acetyltransferase for enzymatic inactivation of quinolones) and *qep*A (encodes a new quinolone efflux pump protein, QepA) genes located on plasmids (15, 16).

There are several reports in other countries of the world that have investigated PMQR in veterinary clinical isolates of *E. coli* (3, 7, 10, 23). Although, quinolones are widely used in veterinary medicine in Turkey, there is no report of PMQR in *E. coli* strains of animal origin. This study aimed to investigate the prevalence of resistance to nalidixic acid and ciprofloxacin and the presence of *qnr*A, *qnr*B and *qnr*S genes as which are the PMQR determinants in *E. coli* strains isolated from animals of different origins.

Materials and Methods

*Samples and bacteriological culture***:** Rectal swab, cloacal swab and stool samples collected from animals raised in commercial farms of Ankara province were investigated in Ankara University, Faculty of Veterinary Medicine, Department of Microbiology. Samples were inoculated onto MacConkey Agar (Oxoid, CM0007B), Eosin Methylene Blue Agar (Oxoid, CM0069B) and 5-7% sheep Blood Agar (Oxoid, CM0055B) for bacteriological culture. After incubation at 37°C for 24-36 h, suspected colonies were Gram stained and evaluated according to Bergey's Manual of Determinative Bacteriology (6). A total of 259 *E. coli* strains; 94 from broiler chickens, 66 from sheep, 52 from cattle and 47 from dogs were stored in Brain Heart Infusion Broth (Oxoid, CM1135B) with 15% glycerol at -20°C for further investigation.

*Antimicrobial susceptibility test***:** A modified agar dilution technique was used to determine minimal inhibitory concentration (MIC) values of nalidixic acid and ciprofloxacin according to the guidelines of Clinical and Laboratory Standards Institute (CLSI) (2). Briefly, from 512 µg/ml to 0.125 µg/ml serial two-fold dilutions of nalidixic acid and ciprofloxacin was prepared in Mueller-Hinton Agar (Oxoid, CM0337B). Bacterial suspensions were adjusted to McFarland 0.5 turbidity standard with sterile physiological saline and 2 µl of this suspension was inoculated onto Mueller-Hinton Agar plates. After aerobic incubation at 37°C for 16-20 h, agar plates were evaluated for MIC values. MICs of ciprofloxacin ≥4 µg/ml and nalidixic acid ≥32 µg/ml for resistance, MICs of ciprofloxacin ≤1 µg/ml and nalidixic acid ≤8 µg/ml were accepted as susceptible breakpoints as defined by CLSI (2). *E. coli* ATCC 25922 was used as quality control in all tests.

*Detection of qnr genes***:** In order to determine PMQR, all *E. coli* isolates were subjected to polymerase chain reaction (PCR). DNA was extracted by boiling method (17) and *E. coli* J53Azir containing the plasmid pMG254 was used as *qnr*A positive quality control strain. Primers used for amplification of *qnr*A, *qnr*B and *qnr*S genes and sizes of amplified products are shown in Table 1. Thermal cycling conditions for *qnr*A-specific PCR were as follows: 30 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 30 sec and extension at 72°C for 1 min (8). Same cycling conditions were applied for *qnr*B *and qnr*S except with a lower annealing temperature of 53°C. PCR products were resolved by 1.5% agarose gel electrophoresis and visualized with ethidium bromide under UV transilluminator.

Results

A total of 259 *E. coli* strains; 94 from broiler chickens, 66 from sheep, 52 from cattle and 47 from dogs were isolated from collected samples. According to antimicrobial susceptibility tests, the number and percentages of ciprofloxacin and nalidixic acid resistant *E. coli* isolates and their distribution among animal origins are presented in Table 2.

Among all 259 *E. coli* isolates, *qnr*B and *qnr*S genes were not observed, whereas *qnr*A gene was found in 5 (5.3%) of the 94 chicken isolates. MIC values and susceptibility to nalidixic acid and ciprofloxacin according to CLSI breakpoints of these 5 isolates are shown in Table 3. Among all 5 *qnr*A positive isolates, one isolate (Isolate 4) exhibited no resistance to either of the quinolones but two isolates (Isolate 1 and 2) were found to be resistant to both quinolones.

Table 1. Primers used for amplification of *qnr* genes and PCR product.
Tablo 1. *qnr* genlerini çoğaltmak için kullanılan primerler ve PCR ürün büyüklükleri.

Gene	Primer	Sequence (5'-3')	Size	Reference
*qnr*A	QP1	GATAAAGTTTTTCAGCAAGAGG	593 bp	Jacoby et al. (8)
	QP2	ATCCAGATCGGCAAAGGTTA		
*qnr*B	QnrB-A	GATCGTGAAAGCCAGAAAGG	469 bp	Gay et al. (4)
	QnrB-B	ACGATGCCTGGTAGTTGTCC		
*qnr*S	QnrS-A	ACGACATTCGTCAACTGCAA	417 bp	Gay et al. (4)
	QnrS-B	TAAATTGGCACCCTGTAGGC		

Table 2. Distribution of ciprofloxacin and nalidixic acid resistant strains among animal origins according to susceptibility test results. n: number of strains; CIP: ciprofloxacin; NAL: nalidixic acid.
Tablo 2. Antibiyotik duyarlılık test sonuçlarına göre siprofloksasin ve nalidiksik asit dirençli suşların hayvan kökenleri arasındaki dağılımı. n: suş sayısı; CIP: siprofloksasin; NAL: nalidiksik asit.

Quinolone	Chicken (n: 94)	Sheep (n: 66)	Cattle (n: 52)	Dog (n: 47)	Total (n: 259)
CIP	47 (50.0%)	3 (4.5%)	5 (9.6%)	3 (6.4%)	58 (22.4%)
NAL	55 (58.5%)	12 (18.2%)	15 (28.8%)	5 (10.6%)	87 (33.6%)

Table 3. MIC values and quinolone susceptibility of 5 *qnr*A positive chicken isolates according to CLSI breakpoints. R: resistant; I: intermediate; S: susceptible; CIP: ciprofloxacin; NAL: nalidixic acid.
Tablo 3. CLSI'nın belirlediği değerlere göre 5 *qnr*A pozitif tavuk izolatının MİK değerleri ve kinolon duyarlılıkları. R: dirençli; I: orta; S: duyarlı; CIP: siprofloksasin; NAL: nalidiksik asit.

Quinolone	MICs (µg/ml)				
	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5
CIP	4 (R)	16 (R)	0.5 (S)	0.5 (S)	0.5 (S)
NAL	64 (R)	512 (R)	32 (R)	8 (S)	16 (I)

Discussion and Conclusion

This study aimed to investigate the prevalence of nalidixic acid and ciprofloxacin resistance and the presence of *qnr*A, *qnr*B and *qnr*S genes in 259 *E. coli* strains isolated from animals of different origin.

In the present study, among 259 *E. coli* isolates, 58 (22.4%) isolates and 87 (33.6%) isolates were found resistant to ciprofloxacin and nalidixic acid, respectively, but only 5 (5.3%) chicken isolates were found *qnr*A positive by PCR. It is already known that *qnr* genes increases resistance to fluoroquinolones and nalidixic acid (12, 15, 16). This result and the high rate of *qnr*A, *qnr*B and *qnr*S negative but nalidixic acid and ciprofloxacin resistant *E. coli* strains lead us to consider other known ways of antimicrobial resistance mechanisms, such as chromosomal mutations in genes encoding DNA gyrase and topoisomerase IV.

Previous studies from Asia, United States and Europe exhibited the prevalence of *qnr* gene in humans as 0.3-11% of the investigated strains which varies in different regions of the world, and revealed that *qnr* gene in conjugative plasmids confers resistance to quinolones (9, 11, 20, 21). In Turkey, there is no data about PMQR in *E. coli* strains isolated from animals, despite few studies in humans. In 2008, Öktem et al. (14) found 5 (6.3%) *qnr*A positive strains (1 *qnr*A positive *E. coli* and 4 *qnr*A positive *K. pneumoniae*) among extended-spectrum β-lactamase-positive 34 *E. coli* and 44 *K. pneumoniae* strains isolated from the blood cultures of clinical patients. Whereas Nazik et al. (13) investigated *qnr*A, *qnr*B, *qnr*S and *aac(6')-lb-cr* genes in 694 *E. coli* strains isolated from humans, and found only 3 (0.4%) *qnr*A positive *E. coli* strains. In the current study, PMQR determinant of *qnr*A gene was found in 5 (1.9%) out of 259 *E. coli* isolates of different animals. It can be said that the PMQR gene prevalence rates of this study performed with animal isolates and the others performed with human isolates are compatible with the general prevalence rates taken from other regions of the world (9, 11, 20, 21).

There are several reports from other countries of the world, which have investigated PMQR in veterinary clinical *E. coli* isolates. Yue et al. (23) investigated PMQR genes of *qnr*A, *qnr*B, *qnr*S, *aac(6')-Ib-cr* in 232 poultry and swine clinical isolates of *E. coli* and found 14 (6%) *qnr* positive isolates of which 3 were pig, 2 were duck, and 8 goose isolates but none were *qnr*A positive. Also in 2009, Kuo et al. (10) investigated 660 *E. coli* strains isolated from pigs and chickens for the prevalence of *qnr* (*qnr*A, *qnr*B and *qnr*S) and *qep*A genes. Kuo et al. (10) detected only 12 (3.33%) and 6 (2%) *qnr*S gene positive *E. coli* 360 pig and 300 chicken isolates, respectively. Fortini et al. (3), detected 12 *qnr*S1, 3 *qnr*B19, 1 *qnr*B10 PMQR determinants, but none of the isolates were *qnr*A positive in 162 *E. coli* strains isolated from healthy chickens and pigs. Huang et al. (7), evaluated 532 *E. coli* chicken isolates for PMQR determinants of *qnr* gene and found 4 (0.75%) *qnr*A positive strains. All these reports indicated the importance of PMQR, despite the negative or low prevalence of *qnr*A gene. In comparison with the *qnr*A gene prevalence in human isolates, animal isolates shows low prevalence rates except the current study, which was found 5 (5.3%) in 94 *E. coli* chicken isolates.

In Turkey, there is no report on the determination of quinolone resistance in *E. coli* strains in veterinary medicine. There is only one study, where Savasan et al. (18) investigated chicken *Campylobacter* isolates for quinolone resistance by agar dilution method. This is the first and only report from Turkey that investigated the PMQR determinant of *qnr* gene in *E. coli* strains isolated from different animals. This study revealed that the PMQR, which was previously reported only from human *E. coli* isolates, could also be detected in animal isolates.

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