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ORIGINAL ARTICLE

Plasmid-mediated quinolone resistance in *Escherichia coli* isolates from commercial broiler chickens in Semnan, Iran

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ABSTRACT

Background and Objectives: Antibiotic resistance within the poultry sector presents a considerable health concern due to treatment inefficacy and resistance transmission to humans and the environment. The investigation of plasmid-mediated quinolone resistance (PMQR) in *Escherichia coli*, acknowledged for its role in advancing resistance, remains inadequately studied in Iranian poultry. This study aimed to evaluate PMQR gene prevalence as well as to determine correlation between resistance phenotype and genotype in *E. coli* obtained from poultry colibacillosis.

Materials and Methods: A collection of 100 *E. coli* isolates from the viscera of broilers suspected to colibacillosis was assessed. Using the Kirby-Bauer disk diffusion method, antimicrobial susceptibility tests were conducted for ofloxacin, nalidixic acid, levofloxacin, ciprofloxacin, and ampicillin. Additionally, PCR was employed to screen for *qnrS*, *qnrB*, and *aac(6)Ib-cr* genes.

Results: Among the analyzed *E. coli* isolates, 51% demonstrated resistance to at least one of the tested antibiotics, with 17% exhibiting resistance to four different antibiotics. Nalidixic acid displayed the highest resistance rate at 48%, while ampicillin had the lowest at 16%. PMQR genes were detected in 28% of the *E. coli* isolates, with *aac(6')-Ib-cr* being the most prevalent at 14%, followed by *qnrB* in 13%, and *qnrS* in 7%.

Conclusion: The study underscores the vital need for careful antibiotic usage in poultry to curb the emergence of antibiotic-resistant bacteria. The results illuminate the prevalence of PMQR genes and their association with resistance trends in Iranian poultry, forming a pivotal basis for forthcoming approaches to combat antibiotic resistance within the poultry sector.

Keywords: Anti-bacterial agents; Drug resistance; *Escherichia coli*; Poultry diseases; Quinolones

INTRODUCTION

Antibiotic resistance is a multifaceted ecosystem problem that threatens the interdependent humans, animals and environmental health linked together under the “One Health” framework (1). Addressing this challenge, the World Health Organization (WHO) has proposed the establishment of a worldwide monitoring system encompassing both veterinary and human medicine. The 2011 World Health Day centered around the theme "Antibiotic resistance: no action today, no cure tomorrow," was chosen with the intent of raising widespread consciousness among the global population (2).

Escherichia coli (E. coli), a member of the Enterobacteriaceae family, plays a significant role in the

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prevalence of foodborne infections and commonly takes up residence within the gastrointestinal tracts of poultry, animals, and humans. *E. coli* also is responsible for colibacillosis, an avian disease characterized by considerable morbidity, heightened mortality rates, diminished productivity, and the condemnation of carcasses. In the poultry industry, preventing substantial economic losses hinges upon the timely and effective use of antimicrobial treatments for afflicted flocks (3). Consequently, the emergence and proliferation of antimicrobial resistance among clinical strains of *E. coli* responsible for colibacillosis carry far-reaching implications for both economic stability and animal well-being. Furthermore, a mounting concern in public health circles is the potential for zoonotic transmission of resistance to critically important classes of antimicrobials, such as third-generation cephalosporins (3GC) and quinolones. It's worth noting that this type of resistance can be facilitated by conjugative plasmids, which possess the ability to transfer between lineages of *E. coli* present in both animals and humans (4).

Numerous antimicrobial agents of critical importance in human medicine such as quinolones are extensively employed within the global poultry industry for the prophylaxis and treatment of bacterial infections. Notably, these antimicrobial drugs are frequently administered as growth promoters or for the prevention and control of bacterial diseases. This prevailing practice, however, has been unequivocally associated with an escalation in antimicrobial resistance (AMR) among Avian Pathogenic *Escherichia coli* (APEC) and commensal *E. coli* strains (5). This resistance is driven by chromosomal mutations affecting DNA gyrase and DNA topoisomerase IV, along with the emergence of plasmid-mediated quinolone resistance (PMQR) genes in various Enterobacteriaceae species.

Plasmid-mediated quinolone resistance (PMQR) genes are systematically categorized into three classes based on their distinct mechanisms of action. Exemplars of these classes encompass various qnr alleles (such as *qnrA, qnrB, qnrS, qnrC,* and *qnrD*), efflux pump genes (including *oqxAB, qepA*), and a specific variant of aminoglycoside acetyltransferase denoted as *aac-(6')-Ib-cr.* This systematic categorization contributes to a nuanced understanding of the diverse mechanisms through which PMQR genes manifest resistance, thereby enhancing the comprehension within the realm of antimicrobial research (5). The *qnr* gene family, including *qnrA, qnrB,* and *qnrS,* plays a significant role in quinolone resistance among strains like *E. coli* and *K. pneumoniae* (6, 7). These genes produce proteins that shield enzymes from quinolones, impacting their effectiveness. The presence of *qnr* genes can lead to a substantial increase in the minimum inhibitory concentration (MIC) against quinolones, reducing their potency. This evolving landscape of resistance has implications for treatment efficacy and global public health concerns (8).

The global demand for poultry meat is steadily rising among consumers, leading to a continuous growth in Iran's poultry industry, which stands as the second-largest in the country. To enhance poultry production and prevent infections, the industry commonly incorporates feed supplements, including antimicrobials, into avian rations (9). Antimicrobial use in this context raises concerns about the emergence of resistant bacterial strains. Despite this, there remains limited information about the prevalence and genotypic attributes of antibiotic-resistant bacteria in both human and food-animal environments in Iran. This highlights the importance of closely monitoring and controlling antimicrobial resistance in veterinary settings to mitigate its impact on public health and maintain the effectiveness of vital antimicrobial agents. Thus, the objective of this study was to investigate the extent of quinolone antibiotic resistance and to evaluate the prevalence of *qnr* genes (*qnrS*, *qnrB*), and *aac(6)Ib-cr* in *E. coli* isolates derived from broilers diagnosed with colibacillosis in Semnan, Iran.

MATERIALS AND METHODS

Isolation of *Escherichia coli*. From October 2018 to April 2019, a total of 150 *E. coli* isolates were collected from broiler farms and veterinary laboratories in Semnan, Iran. The isolates were recovered from the liver, heart, and air sacs lesions of birds suspected to have colibacillosis. All isolates were subjected to standard biochemical tests, including Gram stain, oxidase test, Indol test, methyl red test, citrate test, Voges-Proskauer tests, triple sugar iron test, and urea agar test, to identify them as *E. coli.* Subsequently, all confirmed isolates were preserved in BHI broth (Merk; Germany) with 15% glycerol at -20°C until further procedures.

Antimicrobial susceptibility testing. All isolates were subjected to antimicrobial susceptibility tests using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar (Himedia, India). The testing was conducted with overnight cultures standardized to 0.5 McFarland, following the guidelines provided by the Clinical Laboratory Standards Institute (2020). *E. coli* ATCC25922 was employed as a quality control reference. The selection of antimicrobials for the study was based on the most commonly used quinolone drugs in Iran. The tested antibiotics comprised ofloxacin (5 µg), nalidixic acid (30 µg), levofloxacin (5 µg), and ciprofloxacin (5 µg) (MASTDisks®, UK). Additionally, ampicillin (10 µg) was also included in the evaluation.

Detection of PMQR genes. Individual colonies on EMB agar were inoculated in 3ml Luria-Bertani broth at 37°C, overnight. Among the isolates obtained from the same farm with similar antibiotic susceptibility characteristics, only one isolate was randomly selected and examined. 30 µl of bacterial culture was added to 270 µl of TE buffer (10mM Tris-Hcl, 1mM EDTA[pH= 8.0]) and boiled for 10 min. After heating, they were put immediately on ice for 5 min. The output solution was centrifuged at 12,000 rpm for 5 min and the supernatant was kept at -20°C for use as the DNA template (10). Amplification of *qnrS*, *qnrB* and *aac(6)Ib-cr* was performed in a 20µL reaction mixture using PCR master mix (Takapou Zist Co, Iran) and specific primers described in Table 1 (11). Briefly, in all PCR reactions, a mixture contained 10 µL of 2× master mix (supplied with dNTPs, Taq DNA polymerase, $MgCl_2$), 2 µL of genomic DNA, 0.5 µL (20pmol) of each primer and distilled water in a total volume of 20 µL. The cycling conditions for each polymerase chain reaction and all primers used in this section were described in Table 1. PCR products were loaded on 1.5% agarose gels with DNA Green Viewer Safe DNA gel stain. The gels were photographed under UV light after electrophoresis.

RESULTS

Antimicrobial susceptibility. Out of the 150 samples collected, 50 isolates were obtained from the same farms and exhibited entirely similar resistance profiles. Consequently, these 50 isolates were excluded from the investigation process. Among the 100 *E. coli* isolates analyzed, 51 of them (51%) demonstrated resistance to at least one of the tested antibiotics, as indicated by the inhibition zone observed in the disk diffusion method. Notably, 17% of the samples exhibited resistance to four different antibiotics.

Among the antibiotics investigated, nalidixic acid exhibited the highest level of resistance rate at 48%, while the lowest resistance rate was observed for ampicilin, which stood at 16%. More detail is described in Table 2.

Determination of PMQR harboring *E. coli.* PMQR genes were detected among 28 (28%) of *E. coli* isolates (Table 3). Among the identified PMQR determinants, the most prevalent was the *aac(6')-Ib-cr* gene, found in 14 (14%) isolates, followed by

Table 2. Resistance to quinolone antibiotics in *E. coli* isolates.

Antibiotic	Resistance (R) (n=100)	Intermediate (I) (n=100)	Sensitive (S) (n=100)
Nalidixic acid	48%	2%	50%
Levofloxacin	25%	9%	66%
Ciprofloxacin	35%	4%	61%
Ofloxacin	21%	8%	71%
Ampicillin	16%	11%	73%

Table 1. Primers used in the study, their sequence, and expected product size (11)

Gene	Primer sequence (5′ -3′)	Amplicon size (bp)	Annealing tem (◦C)
qnrS	F: GCAAGTTCATTGAACAGGGT R: TCTAAACCGTCGAGTTCGGCG	428	52
qnrB	F: GGCTGTCAGTTCTATGATCG R: GAGCAACGATGCCTGGTAG	488	52
aac(6')-Ib-cr	F: TTG GAA GCG GGG ACG GAM R: ACG CGG CTG GAC CAT A	260	54

Table 3. Phenotypic and genotypic characteristics of the 28 PMOR genes harboring *E. coli* isolates from poultry colibacilosis

	Isolate	*qnrS*	*qnrB*	*aac(6')-Ib-cr*	Antibiotic resistance profile
1	N.2		+		OFX.CIP.NAL
2	N.6	+			NAL
3	N.11			+	-
4	N.16	+	+		OFX.CIP.LEV.NAL
5	N.17		+	+	OFX.CIP.LEV.NAL
6	N.19			+	OFX.CIP.LEV.NAL
7	N.26	+	+		OFX.CIP.LEV.NAL
8	N.28			+	CIP.LEV.NAL
9	N.32			+	CIP.NAL
10	N.39			+	CIP.NAL
11	N.43			+	OFX. CIP.NAL
12	N.44			+	OFX.CIP.LEV.NAL
13	N.46	+	+		NAL.AMP
14	N.47		+		NAL.AMP
15	N.51			+	CIP.NAL
16	N.54		+		OFX.CIP.LEV.NAL
17	N.70		+		-
18	N.74			+	CIP.LEV.NAL
19	N.76		+		OFX.LEV.NA
20	N.77			+	-
21	N.79			+	CIP.NAL
22	N.84			+	LEV.AMP
23	N.85		+		NAL.AMP
24	N.90			+	-
25	N.95	+	+		-
26	N.96		+		NAL
27	N.97	+			-
28	N.100	+	+		-

qnrB present in 13 (13%) isolates, and *qnrS* in 7 (7%) isolates. Furthermore, 6 isolates carried two different PMQR genes, with the following combinations: *qnrB + qnrS* in 5 isolates, *qnrB + aac(6')-Ib-cr* in 1isolates, and co-existance of *qnrB* and *aac(6')-Ib-cr* genes was not observed.

Distribution of PMQR harboring *E. coli* among phenotypically resistance isolates. The distribution of PMQR genes in isolates that showed phenotypic resistance to each of the antibiotics under investigation is presented in Table 4. In the isolates resistant to nalidixic acid, the genes *qnrB* and *aac(6')-Ib-cr* were more prevalent. The same pattern was observed in the strains resistant to ciprofloxacin, levofloxacin, and ofloxacin. Out of 28 isolates possessing the genes, seven isolates did not exhibit resistance to any of the antibiotics. Among these seven isolates, even though two possessed both genes, no phenotypic resistance to the antibiotics under investigation was observed. In contrast, in 11 out of 17 isolates (11%) that exhibited simultaneous resistance to four antibiotics, none of the investigated genes were detected.

Table 4. Distribution of PMQR genes among phenotypically resistant isolates.

Antibiotic	Resistance (R) (n=100)	*qnrB*	*qnrS*	*aac(6)Ib-cr*
Nalidixic acid	48%	20%	8.3%	25%
Levofloxacin	25%	20%	8%	23%
Ciprofloxacin	35%	14.2%	5.71%	28%
Ofloxacin	21%	28%	9%	14%
Ampicillin	16%	18%	6.25%	6.25%

DISCUSSION

The increasing development of bacterial resistance to quinolone antibiotics is becoming a significant issue in both human and animal contexts. Importantly, animals or their derived products entering the human food supply chain have the potential to introduce antibiotic-resistant bacteria, which poses threats to human well-being (12).

Interestingly, several investigations have identified poultry as a focal point with notably elevated levels of resistance to quinolone antibiotics (13). The significance of Iran's poultry industry as a crucial protein source underscores the urgency of addressing this issue. Given the noticeable overuse of quinolones within this industry, our study was designed to specifically investigate the prevalence of resistance in *E. coli* strains responsible for poultry colibacillosis (9). Our analysis encompassed five key antibiotics: ofloxacin, nalidixic acid, levofloxacin, ciprofloxacin, and ampicillin. The results emphasized a clear pattern, showing that nalidixic acid had the highest rates of resistance, while ampicillin demonstrated the lowest levels of resistance.

To gain comprehensive insights into the prevalence of quinolone resistance in Iran, we conducted an extensive literature review.

Our investigation concentrated on recent studies

examining antibiotic resistance in *E. coli* isolates derived from poultry and ready-to-eat poultry products in different regions of Iran. The analysis revealed a troubling trend. The prevalence of resistance to two pivotal quinolone antibiotics, nalidixic acid and ciprofloxacin, emerges as a consistent and troubling observation. Across multiple studies, resistance rates have consistently fallen within the range of 66% to 100% (14-17). While this alone is worrisome, it's crucial to highlight that the increase in quinolone resistance is not limited to *E. coli* in poultry; it also extends to other pathogenic bacteria originating from poultry sources. This concerning phenomenon is substantiated by a study conducted by Mousavinafchi et al. in 2022. Their investigation of various *Campylobacter* spp. isolated from poultry meat unveiled a disconcerting reality: every examined sample exhibited resistance to both nalidixic acid and ciprofloxacin (18). Sahebkar and Khademi's (2020) comprehensive study involved a meticulous systematic review and meta-analysis, delving into the analysis of 34 scholarly papers concerning the prevalence of antimicrobial susceptibility patterns among *Campylobacter* species within Iran. Their rigorous investigation unveiled a noteworthy trend, indicating a substantial prevalence of fluoroquinolone-resistant *Campylobacter* species across the country (19). Additional support for this argument comes from an extensive meta-analysis conducted by Waze et al. which illuminates resistance patterns among various *Salmonella* serotypes sourced from animals in Iran (20).

The elevated resistance to nalidixic acid appears to be primarily attributed to the extensive utilization of this antibiotic in the management of diverse infections (21). This notion underscores a critical consideration: nalidixic acid, positioned as the pioneering antibiotic in its category, has played a pivotal role in combating *E. coli* infections for a span exceeding five decades. However, this prolonged and widespread deployment has engendered selective pressures, ultimately culminating in a notable surge in resistance to this once-potent therapeutic agent (7).

While the prevalence of ampicillin resistance was found to be comparatively lower in our current investigation when contrasted with other antibiotics, notable examples from the literature highlight the severity of this issue. For instance, in a study carried out by Rahman et al. a staggering 100% and 97% of *E. coli* isolates from broiler and laying poultry meat demonstrated resistance to ampicillin, respectively (2). Similarly, Gorbani et al. investigated a total of 70 identified *E. coli* strains, comprising 35 avian pathogenic *E. coli* (APEC) and 35 uropathogenic *E. coli* (UPEC) isolates. These strains were isolated from cases of avian colibacillosis and human urinary tract infections (UTIs). The study findings revealed that ampicillin demonstrated the highest resistance rates among all *E. coli* isolates, reaching 84.20% (22). Another study by Zarei et al. focusing on *E. coli* isolated from raw chicken meat samples, found that over 82% of the samples exhibited resistance to ampicillin (23). The potential explanation for the comparatively reduced resistance to ampicillin in comparison to other antibiotics could be attributed to insights provided by local veterinarians. They suggest that the decline in ampicillin's efficacy in past years has led to a decrease in its utilization.

Another remarkable observation in this study, is that all isolates displaying resistance to both ciprofloxacin and ofloxacin also exhibited resistance to nalidixic acid. In contrast, certain strains demonstrated resistance solely to nalidixic acid. This implies that the resistance mechanisms for ciprofloxacin and ofloxacin likely resemble the mechanism governing nalidixic acid resistance, whereas resistance to nalidixic acid might stem from diverse mechanisms. It's important to note that these observations are based on our study results, and we do not make definitive claims but rather provide insights based on our findings.

The emergence of antibiotic resistance is closely associated with the existence of genes that confer resistance. In this investigation, we explored the occurrence of three specific PMQR genes and their correlation with observed phenotypic resistance. The findings revealed that the prevalence of each of the *qnrB* and *qnrS*, *aac(6')-Ib-cr* genes stood at 13%, 7%, and 14%, respectively. Within five isolated instances, the coexistence of *qnrS + qnrB* genes was detected, while in one isolate, the combination of *qnrB + aac(6')-Ib-cr* genes was observed. However, the presence of all three genes simultaneously was not witnessed in any of the isolates.

Despite numerous research efforts focusing on the prevalence of PMQR in *E. coli* isolated from various human infections across different regions of Iran (24-26), the extent of information regarding *E. coli* sourced from poultry remains limited. Consistent with the findings of the present study, Ferria et al.

reported a comparable pattern. Their research emphasized that within Enterobacteriaceae isolates originating from human, animal, and environmental sources, the predominance of *qnrB* genes outshines that of other *qnr* genes (27). Similar to this study Rasoulinasab et al. reported the most frequent genes was *aac(6')-Ib-cr* in ST131 isolates (25). In the investigation conducted by Ghorbani et al. the *qnrA* gene was detected in 14% of APEC strains (22). Additionally, Pourhossein et al. documented the presence of *qnrB* and *qnrS* genes in *E. coli* isolated from poultry feces in the northern region of Iran (28). However, in a broader international context, Röderova et al. carried out an extensive investigation in the Czech Republic. They collected a total of 1050 *E. coli* isolates, which comprised 303 samples from human sources, 156 from chickens, 105 from turkeys, 114 from rooks, and 372 from wastewater samples. Among these, PMQR genes were identified in 262 (25%) isolates. The highest prevalence was recorded in isolates sourced from commercially sold turkey meat, where 49% of the isolates tested positive, closely followed by human patients at 32%. Among the detected PMQR determinants, the *qnrS1* gene exhibited the highest occurrence, being identified in 146 (56%) isolates, succeeded by *aac(6')-Ib-cr* in 77 (29%) isolates, *qnrB19* in 41 (16%) isolates, and *qnrB1* in 9 (3%) isolates (5).

Therefore, as evidenced by the existing literature, the prevalence of antibiotic resistance genes varies across different studies and geographical regions. The quantity and manner of antibiotic consumption within animal herds stand out as key determinants influencing the antibiotic resistance pattern and the occurrence of distinct types of resistance genes in various geographical areas (2).

A noteworthy observation from the current study is that among the 28 isolates with at least one gene, 7 isolates surprisingly exhibited no resistance to any of the antibiotics. Intriguingly, despite two of these isolates harboring two genes, they did not exhibit any phenotypic resistance. This underscores that the mere presence of resistance genes does not independently induce antibiotic resistance. Instead, the interplay of additional factors alongside these genes plays a pivotal role in the development of antibiotic resistance. In contrast, in 11 isolates out of 17 isolates (11%) that displaying resistance to all four quinolone antibiotics concurrently, none of the examined genes were found. This raises the prospect that resistance to any of these four antibiotics might be driven by alternative mechanisms or other genes associated with quinolone resistance plasmids. Hence, additional comprehensive research is required to reveal the precise mechanism behind antimicrobial resistance in *E. coli* isolates in Iran.

The concerning aspect is that, antibiotic resistance among commonly employed antibiotics within the poultry sector not only presents obstacles in managing prevalent bacterial infections many of which lack preventive vaccines but also extends its impact to human populations through the food chain, thereby compounding the challenges faced by the medical community. The transfer of resistance from poultry to humans emphasizes the interconnected nature of health challenges. Consequently, the mounting resistance within this antibiotic family is contributing to a surge in treatment-resistant human infections across diverse contexts. Abundant evidence strongly indicates that the excessive application of antimicrobial agents in animals raised for food production could potentially contribute to the emergence of antimicrobial resistance in bacteria associated with human health. The gravity of this concern lies in the direct link between the prevalence of quinolone resistance in livestock and its subsequent impact on human well-being. This concern is further substantiated by the findings of an extensive study conducted by Kenyon (2021). Using Spearman's correlation analysis, they assessed whether the national-level occurrence of fluoroquinolone resistance in human infections caused by *Acinetobacter baumannii, Escherichia coli, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa* was connected to the application of quinolones in animals raised for food production. The results demonstrated a positive correlation between the prevalence of fluoroquinolone resistance in all four species and the utilization of quinolones in food animals (29).

There is limited information regarding antibiotic usage in Iranian chicken farming. Apart from the antimicrobial resistance (AMR) genes that can currently be identified using existing resources, there could be additional AMR genes that become apparent in upcoming research endeavors. As such, it is crucial to closely monitor antibiotic resistance in livestock products and establish well-considered guidelines for the proper and accurate administration of antibiotics. This encompasses the observance of a defined interval during which antibiotic usage is suspended

before the slaughtering process, aimed at preventing the proliferation of antibiotic resistance in the environment and the transference of resistance to humans.

CONCLUSION

Our primary objective was to characterize plasmid-mediated *E. coli* isolates obtained from commercial broilers suspected of colibacillosis in Semnan, Iran. We also investigated the connections between phenotypic and genotypic susceptibility. In general, the highest resistance was observed against nalidixic acid, while resistance to ampicillin was the lowest. Furthermore, 17% of the isolates displayed resistance to four antibiotics simultaneously, with similar and higher resistance rates observed for *qnrB* and *aac(6')-Ib-cr* genes compared to *qnrS*. Lack of information regarding the history of antibiotic consumption in the herds from which samples were taken may limit the comprehensive understanding of the factors influencing antibiotic resistance patterns in the studied poultry population. This situation raises concerns in Iran, where healthcare facilities and antibiotic surveillance are still in developmental stages. Our findings underscore the urgency of creating new antibiotics with potent effectiveness against PMQR-producing bacteria. Simultaneously, promoting the rational utilization of antibiotics in livestock, alongside adopting safe practices for food handling and proper cooking, becomes pivotal in diminishing or eliminating the risk posed by antibiotic-resistant pathogenic bacteria originating from raw foods.

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