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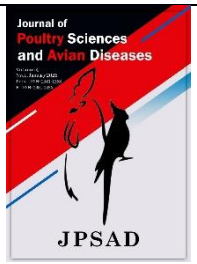

Molecular Characterization of Plasmid-Mediated Fluoroquinolone Resistance Genes (qnr, oqxAB, aac(6′)-Ib-cr, qepA) in Escherichia coli from Broiler Flocks with Colibacillosis in Northern Iran

Kimia Tahmasbi Ghasabsaraei[1], Habib Miri Ahoodashti[1], Saeed Shateri[1*], Majid Alipour[2], Mohammad Barari[3]

[1] Department of Veterinary Medicine, Bob.C., Islamic Azad University, Babol, Iran
[2] Department of Cell and Molecular Biology, Bob.C., Islamic Azad University, Babol, Iran
[3] Department of Avian Diseases, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

*** Corresponding author email address**: saeedshateri@iau.ir

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A B S T R A C T

Colibacillosis caused by avian pathogenic *Escherichia coli* (APEC) is a major health and stewardship concern in poultry. We conducted a study in Mazandaran Province, northern Iran, which was chosen because it is a major hub for broiler production and frequently reports veterinary cases of colibacillosis. From 106 diagnostic submissions with compatible necropsy lesions, 81 isolates of *Escherichia coli* were recovered using EMB culture and confirmed by IMViC. Plasmid-mediated quinolone resistance (PMQR) genes were screened by conventional PCR for *qnrA*, *qnrB*, *qnrS*, *oqxAB*, *qepA*, and *aac(6')-Ib-cr*, with one representative amplicon per target Sanger-confirmed. Fluoroquinolone susceptibility was assessed by Kirby–Bauer with ciprofloxacin, enrofloxacin, norfloxacin, and nalidixic acid. PMQR carriage was common: *oqxAB* in 53.1% of isolates and *qnrS* in 34.6%, with lower frequencies of *aac(6')-Ib-cr* (13.6%) and *qnrB* (4.9%); *qnrA* and *qepA* were not detected. Non-susceptibility in disk diffusion was highest for enrofloxacin (76.5%) and also high for nalidixic acid (60.5%), while ciprofloxacin was lowest (23.5%), indicating substantial but heterogeneous fluoroquinolone pressure in this setting. Genotype–phenotype discordance occurred: five PMQR-positive isolates were fully susceptible to all tested fluoroquinolones, whereas nine non-susceptible isolates lacked the screened PMQR genes, consistent with alternative mechanisms such as *gyrA*/*parC* mutations or non-*oqxAB* efflux. These Mazandaran-specific data link PMQR genotypes to clinically relevant phenotypes in a high-priority poultry region, providing a baseline for surveillance and targeted stewardship to curb empirical fluoroquinolone use while expanding monitoring of both plasmid-borne and chromosomal resistance.

Keywords: *Escherichia coli; broiler chickens; colibacillosis; plasmid mediated quinolone resistance; qnrS; oqxAB; aac(6')-Ib-cr; Iran*

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1 Introduction

Colibacillosis, caused by avian pathogenic *Escherichia coli* (APEC), is one of the most impactful bacterial problems in modern poultry production. It drives mortality, carcass condemnation, poor weight gain, and feed efficiency, as well as sustained treatment costs that erode margins in broiler operations (1, 2). Beyond economics, APEC and the mobile resistance elements it can carry raise clear One Health concerns because extraintestinal *Escherichia coli* lineages and their plasmids may circulate among birds, farms, and people who work with poultry or handle poultry products (3, 4).

Over the last two decades, fluoroquinolones have been widely used in poultry, sometimes for both metaphylaxis and treatment. That use has created strong selection pressure and has been accompanied by higher rates of non-susceptibility in *Escherichia coli* recovered from broilers and other birds across several regions (5, 6). Surveys from commercial flocks, retail meat, and wildlife that interface with poultry production repeatedly document this trend, indicating that resistance is not confined to a single point in the production cycle (7, 8). Although classical quinolone resistance is driven by chromosomal mutations in the quinolone resistance-determining regions of *gyrA/gyrB* and *parC/parE*, these mutations alone do not explain the speed or breadth of spread seen in poultry systems (9-11).

Plasmid-mediated quinolone resistance (PMQR) fills that gap. PMQR includes (i) target protection proteins encoded by *qnr* alleles (*qnrA, qnrB, qnrS*) that shield DNA gyrase and topoisomerase IV, (ii) the aminoglycoside acetyltransferase variant *aac(6')-Ib-cr* that partially inactivates ciprofloxacin and norfloxacin, and (iii) efflux determinants such as *qepA* (MFS) and *oqxAB* (RND) that reduce intracellular drug levels (12-14). On their own, these mechanisms often confer modest increases in MICs but facilitate stepwise selection of high-level chromosomal resistance and frequently travel with β-lactamases on conjugative plasmids (15). Poultry studies from multiple regions report these determinants alone and in combination, often on epidemic plasmid backbones (16-21). Consistent with this breadth of evidence, Iranian broiler data further underscore the local relevance of PMQR within national production systems (22-24).

Mazandaran Province in northern Iran hosts a dense, economically important broiler industry yet remains underrepresented in integrated assessments of the main PMQR classes within clinical colibacillosis. To inform stewardship and surveillance, we conducted a regional molecular investigation of APEC isolates from broiler flocks with necropsy-supported diagnoses of colibacillosis in Mazandaran. This study aimed to characterize, within a single study framework, the prevalence and distribution of target-protection genes (*qnrA, qnrB, qnrS*) along with the principal efflux and drug-modifying determinants (*oqxAB, qepA, aac(6')-Ib-cr*), using sequencing confirmation of representative amplicons for specificity. We interpret our findings in the context of the poultry literature from Asia, Europe, and beyond, and highlight practical implications for Iranian broiler production and regional One Health priorities.

2 Materials and Methods

2.1 Study design and sampling strategy

This molecular study was conducted in Mazandaran Province, northern Iran, from 2023 to 2024. Mazandaran was selected because it is a major broiler-producing region with frequent veterinary reports of colibacillosis, making it an appropriate setting for baseline surveillance of plasmid-mediated fluoroquinolone resistance (PMQR) in *Escherichia coli* (25).

Consecutive, non-duplicate diagnostic submissions were obtained from commercial broiler farms where field veterinarians observed clinical signs and necropsy lesions consistent with colibacillosis. A "suspect case" was defined as a flock with increased mortality and typical gross lesions at necropsy, including fibrinous pericarditis. From 106 suspect farm submissions (one per farm), pericardial exudate and sterile swabs of fibrinous pericarditis lesions were aseptically collected at necropsy, placed in transport medium, and processed the same day under biosafety level-2 conditions. To minimize clonality bias, a single *Escherichia coli* isolate per submission was advanced to downstream analyses. Planning targeted estimation of a single proportion (PMQR carriage) with 95% confidence. Assuming an anticipated prevalence of 30–60% and using the conservative worst-case scenario (p=0.50), ~80 isolates yield an approximate half-width of ±11% (26). To accommodate submissions that did not yield APEC, we aimed to enroll at least 100 suspect farm submissions.

2.2 Identification of Escherichia coli

Pericardial material was streaked onto eosin methylene blue (EMB) agar and aerobically incubated at 37°C for 18-

24 hours. Lactose-fermenting colonies with a metallic-green sheen were subcultured to purity and examined as Gram-negative rods. Presumptive isolates were confirmed as *Escherichia coli* by the IMViC panel (Indole positive, Methyl Red positive, Voges-Proskauer negative, Simmons citrate negative). Only biochemically confirmed *Escherichia coli* were used for molecular assays (27).

2.3 Fluoroquinolone susceptibility testing

Fluoroquinolone susceptibility was evaluated using the Kirby–Bauer disk diffusion method on Mueller–Hinton agar: overnight cultures of confirmed *Escherichia coli* were adjusted to a 0.5 McFarland standard, plates were uniformly inoculated, five µg ciprofloxacin, 10 µg norfloxacin, five µg enrofloxacin, and 30 µg nalidixic acid disks were applied, incubation proceeded at 37 °C for 18 h, and inhibition zone diameters were measured in millimeters with a digital caliper; quantitative zone diameters are reported for all isolates and analyzed in relation to PMQR genotype (28).

2.4 DNA extraction and PCR detection of PMQR genes

Template DNA was prepared from overnight cultures by boiling lysis (95 to 100 °C), followed by rapid chilling and centrifugation. The supernatant was then used as the template. Conventional PCR assays targeted plasmid-mediated quinolone resistance determinants, including target-protection genes (*qnrA*, *qnrB*, *qnrS*), the drug-modifying enzyme gene *aac(6')-Ib-cr*, and the efflux determinants *qepA* and *oqxAB*. Primer sequences, expected amplicon sizes, and gene-specific annealing temperatures are provided in Table 1. Each 25 µL reaction contained 1× PCR buffer with $MgCl_2$, 200 µM of each dNTP, 0.3 µM of each primer, 1 U Taq DNA polymerase, nuclease-free water, and 1–2 µL of template DNA. PCRs were run on a SensoQuest thermocycler (Germany) using Taq DNA Polymerase 2x Master Mix RED as the reaction master mix.

Table 1. Primers used for PCR detection of plasmid-mediated quinolone resistance determinants in Escherichia coli

Target gene	Sequence (5′–3′)	Amplicon (bp)	Reference
oqxAB-F	CCG CAC CGA TAA ATT AGT CC	313 bp	(29)
oqxAB-R	GGC GAG GTT TTG ATA GTG GA		
aac(6')-Ib-cr-F	TTG GAA GCG GGG ACG GAM	260 bp	(30)
aac(6')-Ib-cr-R	ACA CGG CTG GAC CAT A		
qepA-F	GCA GGT CCA GCA GCG GGT AG	218 bp	(31)
qepA-R	CTT CCT GCC CGA GTA TCG TG		
qnrA-F	ATT TCT CAC GCC AGG ATT TG	516 bp	(29)
qnrA-R	GAT CGG CAA AGG TTA GGT CA		
qnrB-F	GAT CGT GAA AGC CAG AAA GG	469 bp	(29)
qnrB-R	ACG ATG CCT GGT AGT TGT CC		
qnrS-F	ACG ACA TTC GTC AAC TGC AA	417 bp	(32)
qnrS-R	TAA ATT GGC ACC CTG TAG GC		

Thermocycling for the *qnrA/B/S* assays consisted of an initial denaturation at 95 °C for 5 min; 35 cycles of 95 °C for 45 s, 53 °C for 45 s, and 72 °C for 60 s; a final extension at 72 °C for 10 min; and a hold at 4 °C. For *oqxAB*, *qepA*, and *aac(6')-Ib-cr*, cycling consisted of an initial denaturation at 95 °C for 15 min; 30 cycles of 94 °C for 30 s, 63 °C for 30 s, and 72 °C for 90 s; a final extension at 72 °C for 10 min; and a hold at 4 °C.

Each PCR run included a no-template negative control (NTC) to monitor reagent and workflow contamination. Amplicons were resolved on 1.5% Merck agarose in 1× TAE, run at approximately 100 V for 45–60 min, stained with ethidium bromide or SYBR-Safe, and visualized under UV/blue light. A 100-bp DNA ladder was used for sizing; bands at the expected sizes were interpreted as positive.

Sequencing confirmation

A representative subset of positives for each target was purified (Silica column) and subjected to Sanger sequencing. Chromatograms were quality-checked and trimmed in Chromas; bidirectional reads were assembled when available. Consensus sequences were queried against the NCBI database using BLASTn; matches with high coverage and ≥ 99% identity to the corresponding PMQR references were accepted as confirmatory (16, 17).

2.5 Statistical analysis

The isolate served as the unit of analysis. For each plasmid-mediated quinolone resistance determinant (*qnrA, qnrB, qnrS, oqxAB, qepA, aac(6')-Ib-cr*), prevalence was

calculated as the proportion of PCR-positive isolates among those tested for that determinant and is reported as a percentage with two-sided 95% confidence intervals computed by the exact (Clopper–Pearson) method. Statistical summaries were generated in IBM SPSS Statistics version 26.0, and percentages were reported to one decimal place.

3 Results

3.1 *Bacterial isolation, identification, and fluoroquinolone susceptibility*

A total of 106 diagnostic submissions from commercial broiler flocks with necropsy lesions compatible with colibacillosis were processed, of which 81 yielded *Escherichia coli*. Isolation was based on growth of lactose-fermenting colonies with a metallic green sheen on EMB agar, followed by biochemical confirmation using the IMViC scheme, namely Indole positive, Methyl Red positive, Voges–Proskauer negative, and Simmons citrate negative, which is consistent with *Escherichia coli* and was used as the entry criterion for molecular analysis.

Fluoroquinolone susceptibility testing by disk diffusion (ciprofloxacin five µg, enrofloxacin five µg, norfloxacin 10 µg, nalidixic acid 30 µg) is summarized in Table 2. Overall non-susceptibility (I+R) was highest for enrofloxacin (76.5%) and nalidixic acid (60.5%), moderate for norfloxacin (35.8%), and lowest for ciprofloxacin (23.5%).

Table 2. Fluoroquinolone disk diffusion results for Escherichia coli isolates (n=81). Values are n (%) by category (S, I, R).

Antibiotic (disk)	Susceptible n (%)	Intermediate n (%)	Resistant n (%)
Enrofloxacin (5 µg)	19 (23.5)	7 (8.6)	55 (67.9)
Nalidixic acid (30 µg)	32 (39.5)	12 (14.8)	37 (45.7)
Norfloxacin (10 µg)	52 (64.2)	5 (6.2)	24 (29.6)
Ciprofloxacin (5 µg)	62 (76.5)	2 (2.5)	17 (21.0)

3.2 *Detection of plasmid-mediated quinolone resistance determinants*

All 81 *Escherichia coli* isolates were assayed for the predefined plasmid-mediated quinolone resistance targets. Among target-protection genes, *qnrA* was 0/81 (0.0%; 95% CI, 0.0 to 4.5), *qnrB* was 4/81 (4.9%; 95% CI, 1.4 to 12.2), and *qnrS* was 28/81 (34.6%; 95% CI, 24.3 to 46.0). Among efflux and drug-modifying determinants, *qepA* was 0/81 (0.0%; 95% CI, 0.0 to 4.5), *oqxAB* was 43/81 (53.1%; 95% CI, 41.7 to 64.3), and *aac(6')-Ib-cr* was 11/81 (13.6%; 95% CI, 7.0 to 23.0). These findings indicate that *oqxAB* was the most frequently detected marker in this cohort, that *qnrS* accounted for roughly one-third of isolates, that *aac(6')-Ib-cr* and *qnrB* were detected at lower frequencies, and that *qnrA* and *qepA* were not detected under the conditions of this study. Per-gene prevalence with exact 95% confidence intervals is shown in Figure 1.

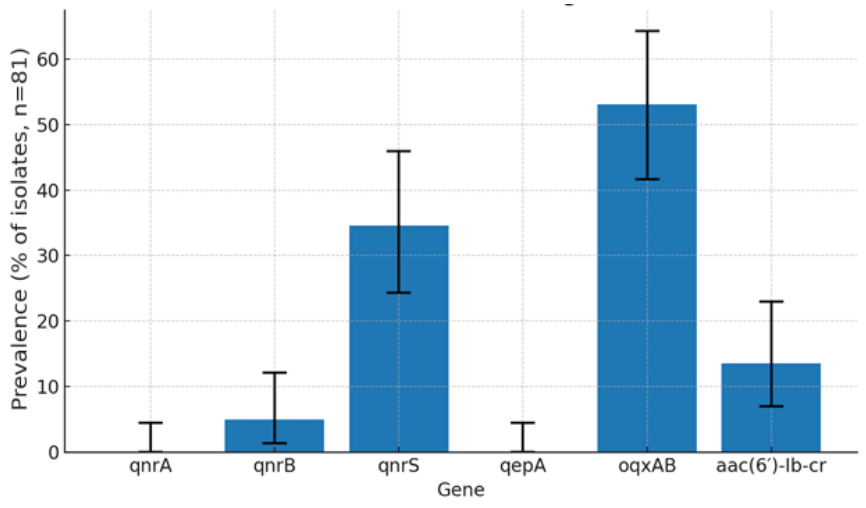

Figure 1. Prevalence of PMQR determinants among Escherichia coli isolates from broiler colibacillosis cases in Mazandaran (n=81). Y-axis: Prevalence (% of isolates, n=81). Bars show percentage per gene with exact 95% confidence intervals (error bars).

3.3 *PMQR genotypes and their relationship with fluoroquinolone susceptibility*

Overall, 43/81 (53.1%) isolates carried at least one PMQR determinant (the "PMQR-positive set"). At the isolate level (Table 3), PMQR-positive strains were enriched among non-susceptible categories, particularly for enrofloxacin and nalidixic acid, mirroring the cohort-level antibiogram. Genotype–phenotype discordance was observed in both directions. Five PMQR-positive isolates harbored one or more determinants yet remained fully susceptible to all four fluoroquinolones by disk diffusion. Conversely, nine isolates showed non-susceptibility to ≥1 fluoroquinolone but were negative for all screened PMQR genes, consistent with alternative mechanisms such as *gyrA*/*parC* mutations or other efflux systems beyond *oqxAB*. Detailed isolate-level gene profiles and corresponding resistance patterns appear in Table 3.

Table 3. Isolate-level PMQR genotypes and fluoroquinolone phenotypes for 43 gene-positive Escherichia coli from broiler colibacillosis in Mazandaran (2023–2024); genotypes include qnrB, qnrS, oqxAB, aac(6′)-Ib-cr, and resistance profiles reflect disk diffusion to ciprofloxacin (CIP), enrofloxacin (ENR), norfloxacin (NOR), and nalidixic acid (NAL); "None" denotes full susceptibility to all four agents.

Antibiotic resistance profile (disk diffusion)	*aac(6′)-Ib-cr*	*oqxAB*	*qnrS*	*qnrB*	Isolate
ENR, NAL	–	+	+	–	P-0003
ENR	–	+	–	–	P-0005
CIP, ENR, NAL	+	+	–	+	P-0008
NOR, ENR, NAL	–	+	+	–	P-0009
None	–	+	–	–	P-0011
CIP, ENR	–	+	+	–	P-0013
ENR, NOR	–	+	–	–	P-0014
NAL	–	+	–	–	P-0017
CIP, ENR, NOR, NAL	–	+	+	–	P-0019
ENR	+	+	–	–	P-0022
ENR, NAL	–	+	+	–	P-0023
ENR, NOR	–	+	–	–	P-0025
CIP, ENR, NOR	–	+	–	+	P-0029
ENR, NAL	+	+	+	–	P-0032
ENR	–	+	–	–	P-0034
ENR, NOR, NAL	–	+	+	–	P-0035
None	–	+	–	–	P-0038
ENR, CIP	–	+	+	–	P-0040
ENR, NAL	+	+	–	–	P-0041
CIP, ENR, NAL	–	+	+	–	P-0042
ENR, NOR	–	+	–	–	P-0044
ENR	–	+	–	–	P-0047
CIP, NAL	–	+	+	–	P-0052
ENR, NOR, NAL	–	+	–	–	P-0056
CIP, ENR	–	+	+	–	P-0059
ENR, NOR	+	+	–	–	P-0060
ENR, NAL	–	+	+	–	P-0064
ENR, CIP, NAL	–	+	–	–	P-0068
NOR	–	+	+	–	P-0073
ENR	–	+	–	–	P-0076
CIP, ENR, NOR, NAL	–	+	+	–	P-0078
ENR	+	+	–	–	P-0082
ENR, NAL	–	+	+	–	P-0085
None	–	+	–	–	P-0088
CIP, ENR, NOR	–	+	+	–	P-0091
ENR	–	+	–	–	P-0092
ENR, NAL	–	+	+	–	P-0095
CIP	–	+	–	–	P-0098
ENR, NOR, NAL	–	+	–	+	P-0100
ENR, NAL	–	+	+	–	P-0101
None	+	+	–	–	P-0104
ENR, NOR	–	+	+	–	P-0105
None	–	+	–	–	P-0106

3.4 Sequencing confirmation

For analytical specificity, one representative amplicon from each positive target (*qnrB*, *qnrS*, *oqxAB*, and *aac(6')-Ib-cr*) was purified and subjected to Sanger sequencing. All sequences showed ≥99% identity to their respective reference targets on BLASTn, confirming the identity of each assay's amplification product.

4 Discussion

In this regional analysis of *Escherichia coli* from broiler flocks with necropsy-confirmed colibacillosis in Mazandaran, northern Iran, *oqxAB* and *qnrS* were the predominant plasmid-mediated quinolone resistance markers, while *aac(6')-Ib-cr* and *qnrB* occurred at lower levels, and *qnrA*/*qepA* were absent. This distribution is consistent with reports from commercial poultry systems, where *oqxAB* and *qnrS* are frequently detected. Studies from China and Korea have documented widespread *oqxAB* and substantial *qnr* carriage across birds, retail meat, and farm environments (1, 33). The absence of *qepA* and the low rate of *qnrB* in animal-origin surveys, where these genes are uncommon, contrasts *with qnrS*, which is typically the most frequent target-protection allele in avian isolates. (6, 13, 16). Our PMQR is broadly consistent with recent poultry cohorts in Europe and Asia, where *oqxAB* and *qnrS* are recurrent and *qnrA*/*qepA* remain infrequent (19, 21).

The predominance of *oqxAB* has practical implications because this RND-family efflux system can reduce intracellular levels of multiple agents beyond fluoroquinolones. It may also be co-selected by non-antibiotic pressures encountered in poultry production, broadening the ecological niches that favor persistence (5). The substantial *qnrS* signal indicates that target protection remains a significant contributor to decreased quinolone susceptibility in clinical broiler isolates, and the presence of *aac(6')-Ib-cr* in a smaller subset suggests additional modification of ciprofloxacin and norfloxacin in the population (9, 12, 34). These patterns are consistent with the elevated non-susceptibility we observed for enrofloxacin and nalidixic acid, supporting the view that PMQR provides a low-level scaffold that facilitates selection of high-level chromosomal resistance under repeated drug exposure (35). Although we did not characterize β-lactam resistance, prior work has shown that PMQR genes can share plasmids with extended-spectrum β-lactamases, creating opportunities for co-selection under either antibiotic class and compounding the stewardship challenge in integrated operations (15).

Our findings add province-specific context to the growing Iranian literature on PMQR in broiler systems. The detection of PMQR determinants in commercial broilers from Semnan has already established a national signal, and the current data from Mazandaran indicate that efflux, dominated by *oqxAB*, and target protection via *qnrS* are also prominent in northern production, which argues for broader inter-provincial monitoring within a single analytic framework (22, 23). The concurrent phenotypic data also highlight that some isolates lacking detectable PMQR genes still exhibit fluoroquinolone non-susceptibility, indicating an additional contribution from chromosomal *gyrA*/*parC* mutations. The study's strengths include a uniform diagnostic case definition based on necropsy findings, a single analytical unit per farm to limit clonality bias, standardized PCR conditions across all isolates, and sequencing confirmation of representative amplicons for specificity. The cross-sectional design, lack of species-specific PCR and APEC virulence-gene screening, absence of positive controls for *qnrA*/*qepA*, and no sequencing of quinolone–resistance–determining regions limit the inference on chromosomal backgrounds and clinical interpretation. The single-province scope may also constrain generalizability to settings with different antimicrobial-use practices. These limitations define priorities for future work, yet do not alter the core description of PMQR distribution in clinical Escherichia coli from broiler colibacillosis cases in Mazandaran (29).

From a One Health perspective, the coexistence of robust *oqxAB* and substantial *qnrS* detection in broiler colibacillosis isolates supports targeted stewardship that limits empirical fluoroquinolone use, prioritizes diagnostics and susceptibility testing, and strengthens biosecurity and vaccination strategies that reduce the need for antimicrobial therapy (5, 33). In our work, genotype–phenotype mismatches (five PMQR-positive but fully susceptible isolates and nine non-susceptible isolates lacking the screened PMQR genes) further support the coupling of routine antibiograms with molecular assays and periodic MIC testing to capture chromosomal mechanisms, such as *gyrA*/*parC* and non-*oqxAB* efflux activity. Given evidence of PMQR within birds, farm environments, and along retail pathways in several regions, a provincial surveillance program that pairs PMQR screening with ESBL markers, plasmid typing, and periodic whole-genome sequencing would provide early warning for emerging plasmid backgrounds and help align farm-level interventions (for example, indication-based prescribing, withdrawal-interval

adherence, and litter/manure management) with public health priorities (3, 4).

5 Conclusion

Clinical *Escherichia coli* from broiler colibacillosis in Mazandaran, northern Iran, showed a PMQR profile dominated by oqxAB and qnrS, with lower detection of aac(6′)-Ib-cr and qnrB, and no detection of qnrA or qepA. This distribution aligns with recent poultry reports from Asia and Europe and complements Iranian data from Semnan, indicating that PMQR determinants are now entrenched in national broiler production. The findings underscore the importance of prudent fluoroquinolone use, enhanced farm-level prevention, and coordinated surveillance that links PMQR with plasmid and β-lactam resistance markers to inform effective antimicrobial stewardship and One Health interventions in the poultry sector.

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Conflict of Interest

The authors declare no competing interests.

Author Contributions

The authors confirm sole responsibility for the following: study conception and design, data collection, analysis and interpretation of results, and manuscript preparation. S.S. and M.A. conceived and designed the study; K.T.G. coordinated field sampling and bacteriology; H.M.A. performed molecular assays and sequencing; M.B. curated data and performed the statistical analysis, also drafted the manuscript; all authors critically revised the text, approved the final version, and agree to be accountable for all aspects of the work.

Data Availability Statement

All authors are ready to provide the available data to readers upon request via email or any other communication platform.

Ethical Considerations

All procedures involving animals were reviewed and approved by the Islamic Azad University of Babol under approval code IR.IAU.AMOL.REC.1403.176. Samples consisted of post-mortem diagnostic submissions from commercial broilers; no live animal procedures were conducted.

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