Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2025.249

RESEARCH ARTICLE

Genetic and phylogenetic characterization of fluoroquinolone-resistant *Escherichia coli* from farrowing to finishing in Korean pig farms

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ARTICLE HISTORY (25-627)

Received: June 30, 2025
Revised: August 17, 2025
Accepted: September 7, 2025
Published online: September 18, 2025

Key words:
Antimicrobial resistance
Escherichia coli
Fluoroquinolones
Plasmid-mediated quinolone resistance
Production stage
Swine

ABSTRACT

This study examined the phenotypic and molecular characteristics of FQ-resistant *E. coli* from different pig production stages (suckling, weaned, grower, finisher, and sow). Antimicrobial susceptibility, minimum inhibitory concentrations, quinolone resistance determinants, plasmid-mediated quinolone resistance (PMQR) genes, and multilocus sequence types (STs) were analyzed in 104 *E. coli* isolates collected from pig farms in Korea. To facilitate a systematic comparison, resistance was evaluated in a stage-stratified manner and integrated with molecular typing and clonal analysis. FQ resistance prevalence was high (28.6–66.7%) across all pig production stages. PMQR genes (predominantly *qnrD* and *qnrS*) were detected in 37.8% of isolates; all exhibited multidrug resistance (11–18 antimicrobials), and 41.2% possessed class I integrons. Over half of the FQ-resistant isolates (25 isolates) had enrofloxacin MICs > 256 µg/mL. ciprofloxacin MICs ranged from 8–256 µg/mL, while NOR resistance was consistently high, with MICs of 256 µg/mL. Ten distinct STs were identified, with ST5229 being the most prevalent (41.2%) across multiple production stages. Phylogenetic analysis revealed three clades: clade A (primarily ST5229) spanned multiple production stages, which indicates vertical transmission, while clades B and C indicated horizontal gene transfer. These findings emphasize the significance of integrated surveillance and responsible antimicrobial stewardship in mitigating the spread of FQ-resistant *E. coli* in pig production.

To Cite This Article: Do KH, Seo KW and Lee WK 2025. Genetic and phylogenetic characterization of fluoroquinolone-resistant *Escherichia coli* from farrowing to finishing in Korean pig farms. Pak Vet J. http://dx.doi.org/10.29261/pakvetj/2025.249

INTRODUCTION

Fluoroquinolones (FQs) are widely used in human and veterinary medicine (Seo *et al.,* 2022). The World Health Organization has classified FQs as critically important antimicrobials, highlighting their essential role in treating infections in both humans and animals (WHO). FQ resistance involves multiple genetic mechanisms (Seo *et al.,* 2022), including chromosomal mutations in genes encoding DNA gyrase and topoisomerase IV (Seo *et al.,* 2022), as well as plasmid-mediated quinolone resistance (PMQR) factors (Seo *et al.,* 2022). PMQR genes, though conferring low-level resistance, can spread horizontally among *Escherichia coli*, promoting mutant selection during FQ exposure (Seo *et al.,* 2022).

Pigs are a globally important food source, and pig farming constitutes a major sector of animal agriculture in many regions (Caekebeke *et al.*, 2020; Lee *et al.*, 2023). The production system involves several stages: piglets are born from sows and progress through suckling, weaning, growing, and finishing stages before slaughter (Zimmerman *et al.*, 2024). At each stage pigs encounter dietary modifications, immune fluctuations, and changes in husbandry practices, all of which can increase infection risk and antimicrobial usage (Do *et al.,*2020). Due to declining maternal antibodies and environmental and dietary changes, weaned piglets are particularly susceptible to infections, resulting in increased antimicrobial use and resistance rates (Zimmerman *et al.*, 2024). Once established, resistance can disseminate along the production chain through multiple routes, including direct between animals, contaminated feed or water, environmental surfaces, and farmworkers acting as mechanical vectors (Pan *et al.*, 2021). Consequently, it is important to investigate stage-specific resistance patterns and the molecular mechanisms of FQ resistance.

Although previous studies have investigated antimicrobial resistance in pigs, relatively few have systematically compared resistance across production stages. Moreover, previous work often lacked concurrent

analysis of resistance mechanisms, clonal relationships, and stage-stratified patterns within farms (Gibbons *et al.*, 2016; Do *et al.,* 2020; Hayer *et al.*, 2022; Seo *et al.*, 2023a). Variations in feed composition, husbandry conditions, and antimicrobial usage at each stage may influence the emergence and dissemination of resistant strains (Alexa *et al.*, 2019). Therefore, a detailed investigation of antimicrobial resistance across pig production stages is essential to understand how resistance develops in practical farm settings. The objective of this study was to systematically characterize FQs (ciprofloxacin [CIP], enrofloxacin [ENR], and norfloxacin [NOR]) resistance across swine production stages in Korea, integrating both molecular (mutations in *gyrA*, and *parC* and PMQR genes including *qnrA*, *qnrB*, *qnrS*, *qepA*, and *aac(6')-Ib-cr*) as and clonal analysis. This approach has not been fully explored in prior stage-specific surveillance studies.

MATERIALS AND METHODS

Bacterial isolates: Between 2021 and 2022, fecal samples were collected from 20 pig farms across diverse regions of South Korea, representing nationwide production settings. Fecal samples (about 10 g) were collected using sterile gauze swabs from 15 locations per farm to ensure standardization and minimize stress (Seo *et al.*, 2023a). Samples were transported to the laboratory within one day with ice packs. Samples were streaked onto MacConkey agar (BD, CA, USA) and incubated overnight at 37°C. From each plate, pink colonies were selected and subjected to PCR for confirmation of *E. coli*, as previously described (Seo *et al.*, 2023b). A total of 104 *E. coli* isolates (21 each from suckling piglets, weaned piglets, finisher pigs, and sows; 20 from grower pigs) were obtained from these samples. To minimize potential bias, samples for each production stage were collected in a balanced manner across the 20 farms.

Antimicrobial susceptibility testing: All *E. coli* isolates were tested for antimicrobial susceptibility using the disc diffusion method, following the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2023) . The antimicrobial agents tested included ampicillin (10 µg), amoxicillin-clavulanic acid (20/10 µg), cefazolin (30 µg), cefoxitin (30 µg), ceftiofur (30 µg), ceftazidime (30 µg), cefepime (30 µg), gentamicin (10 µg), streptomycin (10 µg), kanamycin (30 µg), oxytetracycline (30 µg), tetracycline (30 µg), florfenicol (30 µg), chloramphenicol (30 µg), nalidixic acid (30 µg), CIP (5 µg), sulfisoxazole (250 µg), and trimethoprim-sulfamethoxazole (1.25/23.75 µg).

Minimum inhibitory concentrations: Minimum inhibitory concentrations (MICs) for CIP, NOR, and ENR were determined following CLSI guidelines (CLSI, 2023). MIC values were interpreted using the CLSI-defined breakpoints (CLSI, 2023). For quality control, *E. coli* ATCC 25922 was used.

Molecular analysis: PCR was conducted to amplify the quinolone resistance-determining regions (QRDRs) of the *gyrA* and *parC* genes to identify mutations in 45 FQ-resistant *E. coli* isolates, following previously established protocols (Bai *et al.*, 2012). PCR products were sequenced and aligned using the BLAST.

PMQR genes (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6')-Ib-cr*, *qepA*) were identified through PCR and sequencing (Seo *et al.,* 2022). PMQR-positive isolates were further screened for resistance genes associated with β-lactamases (bla_{TEM}, bla_{SHV}, bla_{OXA}, and bla_{CTX-M} families), tetracyclines (*tetA*, *tetB*, and *tetC*), chloramphenicols (*cmlA* and *catA1*), sulfonamides (*sul1* and *sul2*), and aminoglycosides (*aac(3)-II* and *ant(2'')-I*). Additionally, class I and II integrons (*intl1* and *intl2*) were investigated, and gene cassettes within integron-positive isolates were characterized. PCR products were purified and sequenced as previously described (Yoon *et al.*, 2020). Homology of gene cassettes was assessed using BLAST analysis.

Multilocus sequence typing: Multilocus sequence typing (MLST) on seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) was performed on all PMQR-positive isolates. Sequence types were assigned using the *E. coli* MLST database (http://pubmlst.org/biqsdb?db=pubmlst_ecoli_achtman_seqdef).

Phylogenetic analysis: Concatenated sequences of the seven housekeeping genes used for MLST were aligned using MEGA X software (Do *et al.*, 2022). A phylogenetic tree was constructed using the neighbor-joining method with 1,000 bootstrap replicates. Isolates with similar PMQR genes and resistance profiles were grouped into three clades, supported by robust bootstrap values (≥ 70%).

Network analysis: To explore potential clonal relationships among isolates, a threshold-based adjacency network was constructed. MLST allele profiles of the seven housekeeping genes were used to calculate pairwise Manhattan distances, reflecting the total number of differing alleles between isolates (Nakano *et al.,* 2023) . A distance threshold of 130 was applied to define connections between isolates, and an adjacency matrix was generated for network visualization. The resulting graph was visualized using the igraph package (version 2.1.1) in RStudio (version 2024.12.0+467).

Statistical analysis: All statistical analyses were performed using SPSS version 27.0 (SPSS Inc., Chicago, IL). Resistance differences were analyzed by ANOVA with Duncan post hoc tests (p-value < 0.05).

RESULTS

Antimicrobial resistance: The analysis of antimicrobial resistance across different production stages in pigs is summarized in Table 1. Regardless of production stage, ampicillin and streptomycin exhibited the highest resistance rates at 78.8% followed by cefazolin (75.0%), chloramphenicol (74.0%), and sulfisoxazole (72.1%). Among the various stages, weaned piglets demonstrated the highest levels of antimicrobial resistance. Ampicillin resistance was particularly higher in weaned piglets (85.7%), compared to suckling piglets (81.0%), grower pigs (80.0%), finisher pigs (71.4%), and sows (76.2%). Similarly, streptomycin resistance was highest in weaned

Table 1: Antimicrobial resistance of *Escherichia coli* from each production stage of pigs tested in Korea

Antimicrobial subclasses[1)]	No. of resistant isolates (Antimicrobial resistance %)					
Antimicrobial agents	Suckling (n = 21)	Weaned (n = 21)	Grower (n = 20)	Finisher (n = 21)	Sows (n = 21)	Total (n = 104)
Aminopenicillins						
Ampicillin	17 (81.0%)^{ab}	18 (85.7%)^{b}	16 (80.0%)^{ab}	15 (71.4%)^{a}	16 (76.2%)^{ab}	82 (78.8%)
BL/BLI combinations						
AMC	2 (9.5%)^{ab}	3 (14.3%)^{b}	2 (10.0%)^{ab}	2 (9.5%)^{ab}	1 (4.8%)^{a}	10 (9.6%)
PTZ	3 (14.3%)	3 (14.3%)	2 (10.0%)	2 (9.5%)	3 (14.3%)	13 (12.5%)
1st cephalosporins						
Cefazolin	16 (76.2%)	17 (81.0%)	15 (75.0%)	15 (71.4%)	15 (71.4%)	78 (75.0%)
2nd cephalosporins						
Cefuroxime	2 (9.5%)	3 (14.3%)	2 (10.0%)	2 (9.5%)	2 (9.5%)	11 (10.6%)
Cefoxitin	1 (4.8%)	2 (9.5%)	1 (5.0%)	1 (4.8%)	1 (4.8%)	6 (5.8%)
3rd cephalosporins						
Ceftiofur	6 (28.6%)^{a}	10 (47.6%)^{b}	6 (30.0%)^{a}	5 (23.8%)^{a}	4 (19.0%)^{a}	31 (29.8%)
Cefotaxime	2 (9.5%)	3 (14.3%)	2 (10.0%)	2 (9.5%)	1 (4.8%)	10 (9.6%)
Ceftazidime	1 (4.8%)	2 (9.5%)	2 (10.0%)	1 (4.8%)	2 (9.5%)	8 (7.7%)
4th cephalosporins						
Cefepime	1 (4.8%)^{a}	2 (9.5%)^{a}	2 (10.0%)^{a}	1 (4.8%)^{a}	0 (0.0%)^{b}	6 (5.8%)
Aminoglycosides						
Gentamicin	6 (28.6%)^{a}	9 (42.9%)^{c}	5 (25.0%)^{ab}	5 (23.8%)^{ab}	3 (14.3%)^{b}	28 (26.9%)
Streptomycin	18 (85.7%)^{a}	18 (85.7%)^{a}	15 (75.0%)^{ab}	15 (71.4%)^{b}	16 (76.2%)^{ab}	82 (78.8%)
Kanamycin	13 (61.9%)^{a}	13 (61.9%)^{a}	10 (50.0%)^{ab}	10 (47.6%)^{b}	7 (33.3%)^{c}	53 (51.0%)
Tetracyclines						
Oxytetracycline	13 (61.9%)	13 (61.9%)	12 (60.0%)	12 (57.1%)	12 (57.1%)	62 (59.6%)
Tetracycline	12 (57.1%)	12 (57.1%)	11 (55.0%)	12 (57.1%)	12 (57.1%)	59 (56.7%)
Tigecycline	3 (14.3%)	2 (9.5%)	2 (10.0%)	2 (9.5%)	2 (9.5%)	11 (10.6%)
Phenicols						
Florfenicol	13 (61.9%)^{a}	14 (66.7%)^{ab}	13 (65.0%)^{ab}	16 (76.2%)^{b}	13 (61.9%)^{a}	69 (66.3%)
Chloramphenicol	15 (71.4%)	15 (71.4%)	14 (70.0%)	17 (81.0%)	16 (76.2%)	77 (74.0%)
Quinolones						
Nalidixic acid	10 (47.6%)^{a}	13 (61.9%)^{b}	10 (50.0%)^{ab}	9 (42.9%)^{ac}	7 (33.3%)^{c}	49 (47.1%)
Fluoroquinolones						
Ciprofloxacin	9 (42.9%)^{a}	14 (66.7%)^{b}	8 (40.0%)^{ac}	8 (38.1%)^{ac}	6 (28.6%)^{c}	45 (43.3%)
Sulfonamides						
Sulfisoxazole	14 (66.7%)	16 (76.2%)	14 (70.0%)	15 (71.4%)	16 (76.2%)	75 (72.1%)
SXT	12 (57.1%)	14 (66.7%)	13 (65.0%)	12 (57.1%)	12 (57.1%)	63 (60.6%)
Lipopeptides						
Colistin	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Carbapenems						
Meropenem	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)

Data were expressed as number of isolates (%). Different lowercase subscript letters (a, b, and c) indicate significant differences among each production stage ($P<0.05$). [1)] BL/BLI combinations: β-lactam / β-lactamase inhibitor combinations; AMC: amoxicillin-clavulanic acid; PTZ: piperacillin-tazobactam; SXT: trimethoprim-sulfamethoxazole

piglets (85.7%), compared to grower pigs (75.0%), finisher pigs (71.4%), and sows (76.2%). Ceftiofur resistance in weaned piglets was 47.6%, significantly higher than in all other production stages (suckling piglets: 28.6%, grower pigs: 30.0%, finisher pigs: 23.8%, and sows: 19.0%). CIP resistance was also notably higher in weaned piglets at 66.7%, compared to suckling piglets (42.9%), grower pigs (40.0%), finisher pigs (38.1%), and sows (28.6%).

Characteristics of antimicrobial resistance in FQ-resistant E. coli: Among the 104 *E. coli* isolates, 45 were identified as FQ-resistant. Of these, 44 carried at least one amino acid substitution in the QRDRs of *gyrA* and/or *parC*, with the majority exhibiting double mutations (Table 2). The remaining isolate (0387) carried the *qnrS* gene but did not harbor any mutations in *gyrA* or *parC*. The most prevalent alterations included S83L and D87N substitutions in *gyrA* and S80I and E84A in *parC*. Several strains showed combined mutations, such as S83L/D87N in *gyrA* and S80I/E84A in *parC*, which corresponded to high MIC values > 256 μg/mL for both CIP and ENR.

Among the 45 FQ-resistant isolates, 17 (37.8%) harbored PMQR genes. The highest detection rate of PMQR genes was observed in isolates from weaned piglets (23.8%), followed by finisher pigs (19.0%), grower pigs (15.0%), sows (14.3%), and suckling piglets (9.5%). The *qnrD* gene was the most frequently detected (nine isolates), followed by *qnrS* (seven isolates), *qnrB* (two isolates), and *aac(6')-Ib-cr* (one isolate). In suckling piglets, the *qnrD* gene was identified in two isolates. Among weaned piglets, *qnrB*, *qnrD*, *qnrS*, and *aac(6')-Ib-cr* were detected in one, three, one, and one isolate, respectively. In grower pigs, *qnrD* was present in one isolate and *qnrS* in two isolates. Among finisher pigs, *qnrB* was identified in one isolate, while *qnrS* was detected in three isolates. In sows, *qnrD* was found in three isolates and *qnrS* in one isolate.

More than half of the isolates (25 isolates) displayed MICs > 256 μg/mL for ENR, with MIC_{50} and MIC_{90} values of 256 μg/mL and >256 μg/mL, respectively. CIP MICs varied widely (8 to >256 μg/mL), with most isolates exhibiting high-level resistance (MIC_{50}: 32 μg/mL; MIC_{90}: 256 μg/mL). NOR resistance was consistently high (MIC_{50}: 256 μg/mL; MIC_{90}: 256 μg/mL), although a few isolates showed lower MICs (< 8 μg/mL). ENR resistance frequently exceeded 256 μg/mL in isolates from weaned piglets, grower pigs, and finisher pigs. In contrast, CIP exhibited a broader MIC range (8 to > 256 μg/mL) across all production stages. NOR resistance remained high, except in several grower and finisher isolates with MICs of 1-8 μg/mL.

Table 2: Amino acid changes in the QRDRs and prevalence of PMQR genes in 45 fluoroquinolone-resistant *E. coli* isolates from each production stage of pigs tested in Korea

Production stages	Isolates	Amino acid change[1)]		PMQR gene[2)]	MIC (μg/mL)[3)]		
		gyrA	*parC*		CIP	ENR	NOR
Suckling	0627	S83L / D87N	S80I / E84A	*qnrD*	32	128	256
	0689	S83L / D87N	S80I	*qnrD*	8	128	8
	0272	S83L / D87N	S80I / E84A	-	64	> 256	256
	0650	S83L / D87N	S80I / E84A	-	64	> 256	256
	0988	S83L / D87N	S80I / E84A	-	32	256	256
	0128	S83L / D87N	S80I	-	16	> 256	256
	0224	S83L / D87N	S80I	-	8	>256	256
	0078	D87N	S80I	-	16	256	256
	0247	D87N	S80I	-	16	256	256
Weaned	0316	S87L / D87N	S80I	*qnrD:aac(6')-Ib-cr*	32	>256	256
	0109	S83L / D87N	S80I	*qnrB*	128	>256	256
	0575	S83L / D87N	S80I	*qnrD*	16	256	256
	0691	S83L / D87N	S80I	*qnrD*	16	128	256
	0652	S83L / D87N	S80I	*qnrS*	16	256	256
	0706	S83L / D87N	S80F / E84G	-	256	> 256	256
	0759	S83L / D87N	S80I	-	64	> 256	256
	0179	S83L / D87N	S80I	-	32	> 256	256
	0295	S83L / D87N	S80I	-	32	> 256	256
	0081	S83L / D87N	S80I	-	32	256	256
	0745	S83L / D87N	S80I	-	16	128	8
	0060	S83L / D87N	S80I	-	128	> 256	256
	0274	S83L / D87N	S80I	-	64	> 256	256
	0642	S83L / D87G	S80R	-	32	128	256
Grower	0009	S83L / D87N	S80I / E84G	*qnrD*	256	256	256
	0578	S83L / D87N	S80I / E84A	*qnrS*	> 256	> 256	256
	0255	S83L / D87N	S80I / E84G	*qnrS*	> 256	> 256	256
	0197	S83L / D87Y	S80I	-	256	256	256
	0036	S83L / D87N	S80I	-	256	128	256
	0603	S83L / D87N	S80I	-	256	128	8
	1287	S83L / D87N	S80I	-	256	128	8
	1111	S87L	S80I	-	32	32	2
Finisher	0723	S83L	S80I / E84A	*qnrB*	32	> 256	256
	0655	S83L / D87N	S80I	*qnrS*	256	64	4
	0965	S87L	WT	*qnrS*	64	32	1
	0387	WT	WT	*qnrS*	16	16	1
	1220	S83L / D87N	S80R	-	256	128	256
	0125	S83L / D87G	S80R	-	64	32	2
	0645	S83L / D87N	S80I	-	256	128	256
	0611	D87N	S80R	-	256	128	8
Sow	0657	S83L	S80I	*qnrD:qnrS*	8	128	8
	0683	S83L / D87N	S80I	*qnrD*	16	128	8
	0412	S83L / D87N	S80I	*qnrD*	8	256	256
	0303	S83L / D87N	S80I	-	16	256	256
	0995	S83L	S80F	-	16	128	256
	1091	WT	S80I	-	32	128	256

[1)] WT: wild-type; [2)] -: not detected; [3)] MIC: minimum inhibitory concentration; CIP: ciprofloxacin; ENR: enrofloxacin; NOR: norfloxacin. For quality control, *E. coli* ATCC 25922 showed MIC values of CIP: < 0.125 μg/mL, ENR: < 0.125 μg/mL, NOR: 0.5 μg/mL.

Antimicrobial resistance characteristics of PMQR-harboring *E. coli*: The phenotypic and genotypic characteristics of the 17 PMQR-harboring FQ-resistant *E. coli* isolates are presented in Fig. 1. Across all production stages, 10 STs were identified. ST5229 was the most prevalent (seven isolates), followed by ST6786 (two isolates). Other STs, including ST10, ST75, ST117, ST410, ST542, ST744, ST1642, and ST3014, were each represented by a single isolate. ST5229 was the most frequently detected ST overall, spanning four production stages (suckling piglets, weaned piglets, grower pigs, and sows).

Phylogenetic analysis revealed three distinct clades of PMQR-harboring *E. coli* isolates, with each clade demonstrating similar antimicrobial resistance phenotypes. Clade A (isolates 0627, 0689, 0691, 0412, and 0683) included isolates from multiple production stages, such as suckling piglets, weaned piglets, and sows. Clade B (isolates 0109, 0652, 0723, 0009, 0655, and 0657) comprised isolates from weaned piglets, grower pigs, finisher pigs, and sows. Clade C (isolates 0965, 0316, 0387, 0255, 0575, and 0578) contained isolates from weaned piglets, grower pigs, and finisher pigs.

All PMQR-positive isolates exhibited multidrug resistance (MDR), with resistance to 11–18 different antimicrobials. The β-lactamase genes bla_{CTX-1} and bla_{CTX-15} were detected in 12 and six isolates, respectively, across all production stages. The aminoglycoside resistance gene *aac(3)-II* was identified in eight isolates. For tetracycline resistance, *tetA* and *tetB* were detected in 10 and three isolates, respectively. The sulfonamide resistance genes *sul1* and *sul2* were present in three and 14 isolates, respectively. The chloramphenicol resistance genes *cmlA* and *catA1* were identified in seven and one isolate, respectively. Among the 17 PMQR-harboring *E. coli* isolates, seven (41.2%) carried class I integrons, with four distinct gene cassette arrangements identified: *aadA22*, *aadA12:linF*, *aadA12:aadA15:aadA2-linF*, and *dfrA12-OrfF-aadA2: dhfrXII-aadA2*.

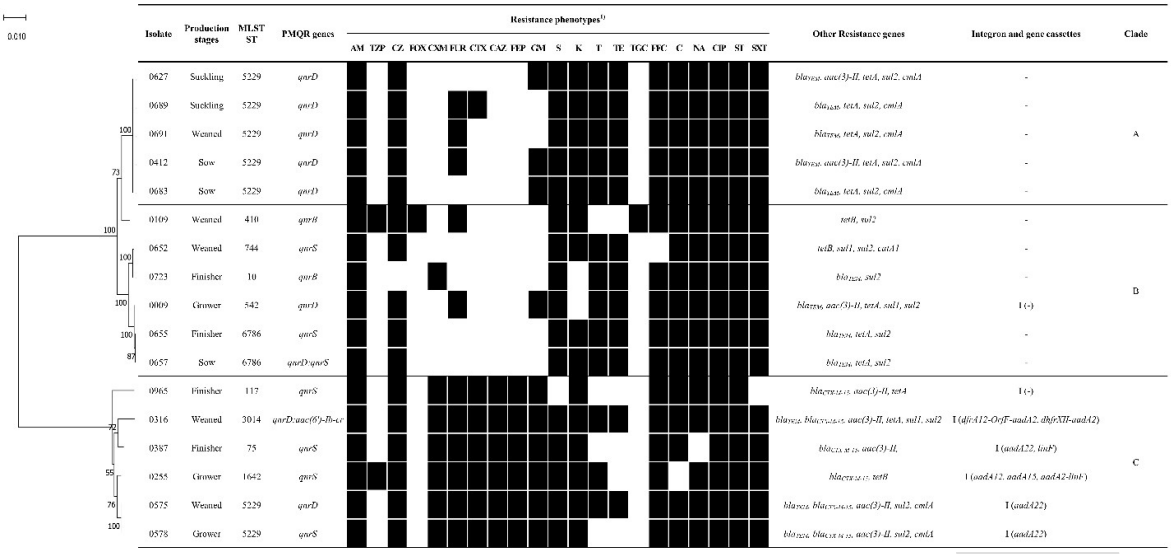

Fig. 1: Neighbor-joining MLST phylogenetic tree and resistance profiles of 17 PMQR-harboring *Escherichia coli* from each production stage of pigs in Korea. The black squares indicate resistance to each antimicrobial agent. The "–" indicates not detected. AM: ampicillin; AMC: amoxicillin-clavulanic acid; TZP: piperacillin-tazobactam; CZ: cefazolin; CXM: cefuroxime; FOX: cefoxitin; FUR: ceftiofur; CTX: cefotaxime; CAZ: ceftazidime; FEP: cefepime; GM: gentamicin; S: streptomycin; K: kanamycin; T: oxytetracycline; TE: tetracycline; TGC: tigecycline; FFC: florfenicol; C: chloramphenicol; NA: nalidixic acid; CIP: ciprofloxacin; ST: sulfisoxazole; SXT: trimethoprim-sulfamethoxazole.

Network analysis of STs among the 17 PMQR-harboring *E. coli* isolates revealed distinct patterns of clonal relationships across production stages (Fig. 2). The most prominent ST, ST5229, was identified across multiple production stages, including suckling piglets, weaned piglets, grower pigs, and sows. ST5229 and its related variants formed a highly interconnected cluster, which also included ST10, ST75, ST410, and ST1642, showing close associations with ST5229 based on network connections.

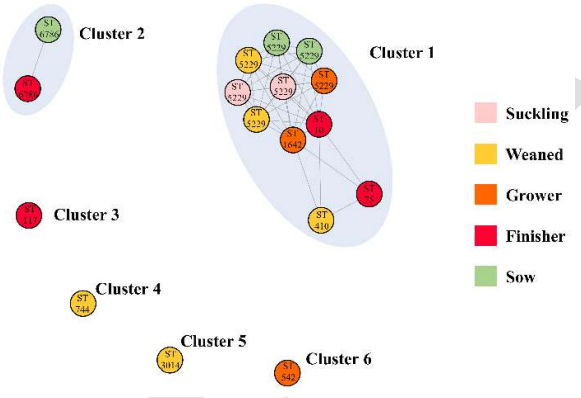

Fig. 2: Network analysis of sequence types of 17 PMQR-harboring *Escherichia coli* isolates from different pig production stages. Each node is labeled with the corresponding STs and colored by each production stage (pink: suckling piglets, yellow: weaned piglets, orange: grower pigs, red: finishing pigs, and green: sows). Connecting lines indicate clonal relatedness among STs based on shared alleles. Each oval encloses a group of closely related isolates.

DISCUSSION

Increasing use of FQs has raised concerns about the emergence and dissemination of FQ-resistant *E. coli* strains across different production stages. In this context, the aim of this study was to identify the phenotypic characteristics of *E. coli* resistance and investigate the molecular basis of FQ-resistance across different pig production stages.

In this study, antimicrobial resistance was frequently observed across all pig production stages, with weaned piglets showing significantly higher resistance to ceftiofur (47.6%) and CIP (66.7%) than other stages. The higher resistance rates in weaned piglets may be linked to increased antimicrobial use during this stage, which is often necessitated by heightened disease susceptibility due to weaning stress and immune immaturity (Guo *et al.*, 2021). Conversely, the lower resistance rates in finisher pigs and sows may be due to reduced use of antimicrobials - finisher pigs often undergo withdrawal periods prior to slaughter, and sows typically require fewer treatments once they are reproductively stable - although further investigation is warranted (Raasch *et al.*, 2020). The high resistance rate to CIP in weaned piglets (66.7%) is particularly concerning, as FQs were classified as critically important antimicrobials by the World Health Organization (Seo *et al.,* 2022). Such resistant strains can spread resistance determinants along the farm-to-fork continuum, potentially impacting food safety and contributing to broader environmental contamination (Patel *et al.,* 2016; Cheng *et al.*, 2020).

Among the 45 FQ-resistant *E. coli* isolates analysed, amino acid substitutions in both *gyrA* and *parC* were consistently observed. These findings align with reports that DNA gyrase is the primary FQ target in gram-negative bacteria, with topoisomerase IV as a secondary target (Seo *et al.,* 2022). The results of this study demonstrate that double mutations in *gyrA* and *parC* are associated with high-level FQ resistance, as demonstrated by high MIC values (≥ 256 µg/mL) for ENR and NOR in isolates such as 0272, 0650, 0578, and 0723. Conversely, isolates without double mutations (e.g., 0387 and 0657) showed lower MICs, consistent with earlier studies (Seo *et al.,* 2022).

While double mutations in QRDRs are typically required for FQ resistance (Ruiz, 2003), this study revealed that the presence of PMQR genes can circumvent this requirement. For instance, isolates 0965 and 1091 exhibited FQ resistance despite carrying only a single mutation in *gyrA* or *parC*. Moreover, isolate 0387 showed resistance without any *gyrA* or *parC* mutations, harboring the *qnrS* gene, underscoring the significant role of PMQR genes in FQ resistance. Among the 45 FQ-resistant *E. coli* isolates, 17 (37.8%) harbored PMQR genes. The *qnrD* gene was the most prevalent (nine isolates), followed by *qnrS* (seven isolates), *qnrB* (two isolates), and *aac(6')-Ib-cr* (one isolate). These PMQR variants have been detected in *E. coli* from swine in Europe (Koster *et al.*, 2023), the United States (Hayer *et al.*, 2020), China (Wu *et al.*, 2019), and Korea (Seo *et al.,* 2022). The highest detection rate of PMQR genes (23.8%) was observed in weaned piglets, indicating heightened exposure or selective pressure during this stage (Seo *et al.*, 2023b).

PMQR genes frequently co-occurred with other resistance genes, such as bla_{TEM}, $bla_{CTX\text{-}M}$, *tetA*, and *sul2*, contributing to the emergence of MDR *E. coli* isolates (Do *et al.*, 2020). This co-occurrence underscores the necessity for comprehensive farm-level interventions, combining robust surveillance with prudent antimicrobial stewardship, to effectively control resistance (Pokharel *et al.,* 2020). All PMQR-positive isolates showed MDR to 11–18 antimicrobials, raising concerns for animal and public health (Abdalla *et al.*, 2021; Do *et al.,* 2021; Barros *et al.*, 2023).

Beyond clonal dissemination, horizontal transfer mediated by mobile genetic elements such as integrons also plays a crucial role in shaping resistance dynamics. Integrons rapidly disseminate resistance genes within bacterial populations (Seo *et al.,* 2022; Seo *et al.*, 2023a, 2023b). Four distinct gene cassette arrangements were identified among seven class I integron-positive isolates, enabling extensive genetic exchange and the emergence of novel resistance profiles (Seo *et al.,* 2022; Seo *et al.*, 2023a, 2023b). These diverse gene cassette arrangements contributed to resistance against several non-FQ antimicrobials, including aminoglycosides (*aadA* variants), lincosamides (*linF*), and trimethoprim (*dfrA12*, *dhfrXII*) (Seo *et al.*, 2023a). Moreover, the co-occurrence of these non-FQ resistance genes can lead to co-selection, where the use of unrelated antimicrobials inadvertently maintains FQ-resistant strains in the population (Guo *et al.*, 2021). This indicates the importance of prudent antimicrobial administration across all drug classes, as selective pressure from one agent may preserve resistance determinants conferring FQ resistance.

Among the 17 PMQR-harboring *E. coli* isolates, 10 STs were identified, with ST5229 being the most prevalent (41.2%). ST5229 was detected in suckling piglets, weaned piglets, grower pigs, and sows, indicating its widespread presence across production stages. This ST, associated with FQ resistance, has been detected in swine where antimicrobial use is prevalent (Seo *et al.*, 2023a). Meanwhile, ST10, ST75, ST117, and ST6786 were detected in finisher pigs and have been reported in both human clinical and environmental samples, raising concerns about potential cross-species transmission (Seo *et al.*, 2023a). Since finisher pigs are at the stage just before slaughter, FQ-resistant isolates detected in them could contaminate meat products, posing a significant public health concern (Yun *et al.*, 2021).

Analysis of the phylogenetic relationships among the 17 PMQR-harboring isolates revealed three distinct clades, indicating how both vertical and horizontal gene transfer contribute to FQ resistance in pig farms. Clade A, which was predominantly composed of ST5229, was distributed across multiple stages. As shown in Fig. 1, these isolates cluster closely with high bootstrap support, indicating vertical clonal dissemination (Caneschi *et al.*, 2023). This highlights the importance of early-stage interventions to limit the spread of specific clones throughout the production chain. In contrast, clades B and C comprised diverse STs, each displaying similar resistance phenotypes. This similarity is consistent with horizontal gene transfer, particularly the acquisition of PMQR genes and/or class I integrons (Seo *et al.,* 2022). Recognizing these vertical and horizontal transmission is important for designing effective farm-level strategies that address all possible routes of resistance dissemination.

The network analysis (Fig. 2) highlighted a highly interconnected cluster, with ST5229 serving as a central hub surrounded by other STs, including ST10, ST75, ST410, and ST1642. These STs have been linked to MDR and have been isolated from various animal species, including pigs, cattle, poultry, and humans (Seo *et al.*, 2023a). Such extensive distribution implies that resistance spread is not isolated to a single production stage, but rather intertwined across species and farms (Seo *et al.*, 2023a). The diversity within this network further emphasizes both vertical and horizontal exchange of PMQR genes facilitated by plasmids or integrons (Caneschi *et al.*, 2023). To address these challenges, interventions should focus on the entire network of related strains by enhancing biosecurity measures, improving stage-specific surveillance, and ensuring robust antimicrobial stewardship across all production phases (Mencía-Ares *et al.*, 2021).

However, the study had certain limitations. The exclusive focus on lactose-fermenting *E. coli* may have missed atypical resistant strains. Additionally, lack of farm-level treatment data and the cross-sectional nature of sampling constrain causal interpretations. The lack of data concerning farm-level variables, including but not limited to antimicrobial use history, biosecurity practices, herd size, and recent outbreaks, complicates the exclusion of potential confounding of stage-related resistance differences by farm effects. Given that PMQR genes are key of horizontal dissemination, the present study focused its clonal analysis exclusively on PMQR-positive isolates. However, this emphasis may have overlooked the existence of other resistant lineages devoid of PMQR determinants. These lineages warrant consideration in subsequent studies to ensure a comprehensive understanding of the subject. In addition, the analysis was limited to *gyrA* and *parC*, excluding other QRDR loci such as *gyrB* and *parE*, which have also been implicated in FQ resistance. This restriction may have constrained the comprehensive assessment of mutation profiling. Moreover, because only one colony was selected per sample, the intra-sample diversity of resistant clones may have been underestimated. This suggests the need for multiple-colony or metagenomic

approaches in future studies. In addition, due to the cross-sectional nature of the sampling conducted during 2021–2022, fluctuations in resistance that might have been associated with seasonality or outbreaks could not be evaluated. To adequately address these issues, longitudinal monitoring is imperative to capture temporal variations. Finally, the relatively small sample size may compromise the study's ability to represent the broader nationwide context, underscoring the necessity for larger, longitudinal studies to ensure the generalizability.

Conclusions: This study investigated the molecular characteristics of FQ-resistant *E. coli* across different pig production stages. High levels of FQ resistance were observed across all stages, with weaned piglets exhibiting the highest resistance rates. All FQ-resistant isolates exhibited MDR, encompassing several antimicrobial classes and underscoring the complexity of resistance management. Mutations in *gyrA* and *parC* were frequently identified, along with PMQR genes and other resistance determinants. MLST revealed identical STs across stages, suggesting both horizontal and vertical transmission. These findings could support the design of stage-targeted stewardship strategies and inform integrated resistance surveillance in swine production systems.

Acknowledgements: This work was supported by the Animal and Plant Quarantine Agency, Ministry of Agriculture, Food, and Rural Affairs, Republic of Korea (Grant Number Z-1543061-2021-23-02).

Authors contribution: Conceptualization: Do KH, Seo KW, Lee WK; Methodology: Do KH; Validation: Do KH; Formal analysis: Do KH; Investigation: Do KH; Resources: Do KH, Seo KW; Writing-Original Draft: Do KH; Writing-Review & Editing: Seo KW, Lee WK; Visualization: Do KH; Supervision: Seo KW, Lee WK; Project administration: Seo KW, Lee WK; Funding acquisition: Lee WK.

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