# Molecular characterization of fluoroquinolone-resistant *Escherichia coli* from broiler breeder farms

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**ABSTRACT** Fluoroquinolones (**FQs**) have been used effectively antimicrobial agents of choice for treatment of various infections caused by *E. coli* and FQs-resistance of *E. coli* from broiler breeders has been implicated in its vertical transmission to their offspring. The objective of this study investigated the phenotypic and genotypic characteristics of FQ-resistant *E. coli* isolates from broiler breeder farms in Korea. A total of 106 FQ-resistant *E. coli* isolates were tested in this study and all isolates had mutations in quinolone resistance determining regions; all (100%) had mutations in *gyrA*, 89 (84.0%) had mutations in *parE*, 8 (7.5%) isolates showed the mutations with *parC* and *parE*, and none had mutations in *gyrB*. The predominant mutation type was double mutation in *gyrA* (S83L and D87N), and all FQ-resistant *E. coli* isolates that had mutations in *parC* or *parE* also had double mutations in *gyrA*. Especially, FQ-resistant *E. coli* isolates which possessed double mutations in *gyrA* in combination with double mutations in *parC* or single mutations in both *parC* and *parE* were shown high levels of minimum inhibitory concentrations rage. Of the 23 plasmid-mediated quinolone resistance (PMQR)-positive *E. coli* isolates, *qnrS* was detected in 10 (9.4%) isolates, and followed by *qnrA* (7 isolates, 6.6%), *qnrB* (4 isolates, 3.8%), and *aac(6′)-Ib-cr* (2 isolates, 1.9%). Sixteen (69.6%) of the 23 PMQR-positive *E. coli* isolates harbored class 1 integrons with four different gene cassette arrangements and total of 9 plasmid replicon types were also identified in 23 PMQR-positive *E. coli* isolates. This is the first study to investigate the prevalence and characteristics of FQ-resistant and PMQR-positive *E. coli* isolated from the broiler breeder in Korea; it supports that constant monitoring and studies at the broiler breeder level are required to prevent the pyramidal transmission of FQ-resistant *E. coli*.

**Key words:** *Escherichia coli*, fluoroquinolone, antimicrobial resistance, broiler parent stock, PMQR

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## INTRODUCTION

Fluoroquinolones (**FQs**) have been used effectively antimicrobial agents of choice for treatment of various infections caused by *E. coli* or other gram-negative bacteria. Because of clinical importance in both human and animal medicine, the World Health Organization has classified FQs as ‘‘critically important antimicrobials (WHO, 2017). However, the continuous use of FQs in livestock can lead to the emergence and maintenance of FQ-resistant bacteria, and it is considered a significant public health threat (Wasyl et al., 2013, Xu et al., 2015). Especially, since enrofloxacin have been introduced to the poultry industry in Korea in 1987, FQ-resistant *E. coli* have developed over the time (Hu et al. 2017; Seo and Lee, 2020).

FQ-resistance is mainly due to chromosomal mutations that alter the drug target enzymes DNA gyrase (*gyrA* and *gyrB*) and DNA topoisomerase IV (*parC* and *parE*) (Jacoby, 2005). Moreover, 3 different plasmid-mediated quinolone resistance (**PMQR**) determinants have been described: the *qnr* genes that protect the DNA gyrase and topoisomerase IV from quinolone inhibition, the *aac(6')-Ib-cr* gene that an aminoglycoside acetyltransferase that confers reduced susceptibility to ciprofloxacin, and the *qepA* gene that the major facilitator superfamily-type quinolone efflux pump decreasing susceptibility to quinolones ( Liu et al., 2012). Although PMQR genes confer low-level resistance to FQ, they can facilitate the selection of mutations in gyrase and topoisomerase genes which results in high-level FQ-resistance (Yang et al., 2008).



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The broiler industry has a pyramidal structure in which grandparent stock on the top through breeding chickens parent stock that produce eggs for the produce the broiler chickens on the bottom. In this structure, antimicrobial resistant bacteria and drug-resistance genes can be vertically transmitted through the broiler breeding chain. Although studies from several countries have documented the prevalence and characteristics of FQ-resistance in commercial broiler level (Taylor et al., 2008; Abdi-Hachesoo et al., 2017; Nishikawa et al., 2019), there is still limited information regarding the molecular characteristics of FQ-resistant and PMQR-positive isolates at the broiler breeding level. Therefore, this study investigated the phenotypic and genotypic characteristics of FQ-resistant *E. coli* isolates from broiler breeder farms in Korea.

## MATERIALS AND METHODS

### Sampling

Feces and dust were sampled from nine broiler breeding farms including 69 flocks (20 wk of age) between 2016 and 2018 in accordance with the standards set by the National Poultry Improvement Plan (United States Department of Agriculture USDA, 2011). Briefly, 15 different spots were swabbed per flock in order to collect 10 g of dust sample using surgical gauze moistened with 12 mL of sterile double strength skim milk (Fluka, Neu-Ulm, Germany). Approximately 10 g of feces were also sampled from 15 different locations. Samples were transported to the laboratory in a cooler and stored at 4°C until use.

### Bacterial Identification

The samples were individually inoculated into 225 mL of mEC (Merck, Darmstadt, Germany) and incubated at 37°C for 20 to 24 h. Pre-enriched mEC was streaked onto MacConkey agar (BD Biosciences, Sparks, MD) plates and incubated at 37℃ for 24 h. Five typical colonies selected from each sample were identified by PCR as previously described (Candrian et al., 1991), and plated on Mueller-Hinton agar (BD Biosciences) plates supplemented with 4 μg/mL ciprofloxacin (Sigma-Aldrich, St. Louis, MO) to select FQ-resistant *E. coli*. If isolates of the same origin showed the same antimicrobial susceptibility patterns, only one isolate was randomly chosen and included in the analysis. As a result, a total of 106 FQ-resistant *E. coli* were tested in this study (Table 1).

### Antimicrobial Susceptibility Testing

All FQ-resistant *E. coli* isolates were investigated for their antimicrobial resistance with the disc diffusion test using the following discs (BD Biosciences): amoxicillin-clavulanate (20/10 μg), ampicillin (10 μg), cefazolin (30 μg), cephalothin (30 μg), cefadroxil (30 μg), cefoxitin (30 μg), chloramphenicol (30 μg), gentamicin (10 μg), imipenem (10 μg), nalidixic acid (30 μg), tetracycline (30 μg), and trimethoprim-sulfamethoxazole (1.25/23.75 μg). Minimum inhibitory concentrations (**MICs**) ranging from 0.06 to 512 mg/L to nalidixic acid, ciprofloxacin, and enrofloxacin (Sigma-Aldrich) were determined using standard agar dilution methods according to recommendations of the Clinical & Laboratory Standards Institute (CLSI, 2015, 2020). *E. coli* ATCC 25922 was included as a quality control. Multidrug-resistance (**MDR**) was defined as acquired resistance to at least one agent in 3 or more antimicrobial classes (Magiorakos et al., 2012).

### Identification of Mutations in QRDRs and Detection of PMQRs

PCR was carried out to amplify the target genes (*gyrA, gyrB, parC*, and *parE*) in quinolone resistance determining regions (**QRDRs**) to identify mutations in 106 FQ-resistant *E. coli* isolates using primers and conditions described previously (Fendukly et al., 2003; Dutta et al., 2005; Bai et al., 2012). The PCR products were purified using GFX PCR DNA and the Gel band purification kit (Amersham Bioscience, Freiburg, Germany), and sequenced by automatic sequencer (Cosmogenetech, Seoul, Korea). The sequences were confirmed with those in the GenBank nucleotide database using the Basic Local Alignment Search Tool (BLAST) program available through the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST). PMQR genes (*qnrA, qnrB, qnrC, qnrD, qnrS, aac(6')-Ib-cr*, and *qepA*) were also detected by PCR amplification and sequencing analysis, as described in previous studies (Yu et al., 2015).

**Table 1.** Distribution of 106 ciprofloxacin-resistant *E. coli* isolated from 9 broiler breeder farms.

| | Broiler breeder farms | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | I | II | III | IV | V | VI | VII | VIII | IX | Total |
| No. of flocks tested | 6 | 9 | 10 | 17 | 7 | 7 | 5 | 3 | 5 | 69 |
| No. of positive flocks (%) | 5 (83.3) | 8 (88.9) | 9 (90.0) | 13 (76.5) | 7 (100.0) | 5 (71.4) | 4 (80.0) | 3 (100.0) | 4 (80.0) | 58 (84.1) |
| No. fluoroquinolone-resistant *E. coli*[1] | 9 | 12 | 18 | 22 | 14 | 10 | 8 | 6 | 7 | 106 |
| No. of PMQR-positive *E. coli*[2] | 2 | 4 | 5 | 5 | 2 | 2 | 0 | 0 | 3 | 23 |

[1]If several isolates from same origin showed the same antimicrobial susceptibility patterns, only one isolate was included.
[2]PMQR, plasmid-mediated quinolone resistance.

### Plasmid Replicon Typing and Detection of Integrons and Gene Cassettes

For plasmid replicon typing and detection of integrons and gene cassettes, PCR was performed using DNA extracted from 23 PMQR-positive *E. coli* isolates. The primers used in this study targeted 18 different replicons (Johnson et al., 2007) and class 1 and 2 integrons (Ng et al., 1999; Sáenz et al., 2004). Gene cassettes were tested for integron-positive isolates (Ng et al., 1999; Sáenz et al., 2004). The PCR products of the gene cassettes were sequenced as described above.

### Transfer of Resistance Genes by Conjugation

To determine the transferability of PMQR genes, conjugation assays were performed using the broth mating method, with *E. coli* J53 used as the recipient as previously described (Tamang et al., 2012). Transconjugants were selected on MacConkey agar (BD Biosciences) plates containing sodium azide (100 $\mu$g/mL; Sigma-Aldrich) and ampicillin or tetracycline (100 $\mu$g/mL; Sigma-Aldrich). All transconjugants were tested for the presence of PMQR genes, as described above.

### Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) was performed on PMQR-positive *E. coli* isolates by digesting the genomic DNA using the *Xba*I restriction enzyme (Takara Bio Inc., Shiga, Japan) according to the standard protocol of the Center for Disease Control and Prevention and CHEF-MAPPER apparatus (Bio-Rad Laboratories, Hercules, CA), as previously described (Liu et al., 2007). Gel images were analyzed using Info-Quest FP software ver. 4.5 (Bio-Rad). The dice coefficient was used to calculate similarity, and the similarity matrix was expressed graphically by an unweighted average linkage.

## RESULTS

### Antimicrobial Resistance Profile

The antimicrobial resistance patterns of FQ-resistant *E. coli* isolated from broiler breeder farms is shown in Figure 1. FQ-resistant *E. coli* isolates showed the highest resistance to quinolones (100.0%) and cephems (100.0%) followed by penicillins (90.6%), tetracyclines (90.6%), folate pathway inhibitors (77.4%), phenicols (72.6%), $\beta$-lactam/$\beta$-lactamase inhibitor combinations (25.8%), aminoglycosides (13.2%), and carbapenems (5.7%). Also, all FQ-resistant *E. coli* isolates were identified as having MDR against 3 to 10 classes of antimicrobial agents. The rate of resistance to 8 antimicrobial classes was the highest at 34.0% and 1 (0.9%) FQ-resistant *E. coli* isolate showed resistance to 10 classes.

### Presence of Amino Acid Substitutions in QRDRs in FQ-Resistant E. coli

All 106 FQ-resistant *E. coli* isolates showed the mutation in *gyrA*. But, 89 (84.0%) isolates showed the mutation with *parE*, and 8 (7.5%) isolates showed the mutations with *parC* and *parE*, simultaneously (Table 2). The high *gyrA* amino acid substitutions were S83L (99 isolates, 93.4%) and D87N (75 isolates, 70.8%), and 75 isolates showed double mutations of S83L and D87N. The highest *parC* substitution were S80I (74 isolates, 69.8%), but 25 isolates also showed double mutations of S80I and E84A. In *parE* mutations, I464F (5 isolates) and S458A (3 isolates) were observed. The *gyrB* mutations were not detected in any of the isolates in this study. MICs range of isolates with double mutations in *gyrA* were relatively higher than those of other isolates with single mutations in *gyrA*. Especially, FQ-resistant *E. coli* isolates with a high level of MICs rage (≥64 mg/L for ciprofloxacin and ≥128 mg/L for enrofloxacin) were shown to carry double mutations in *gyrA* in combination

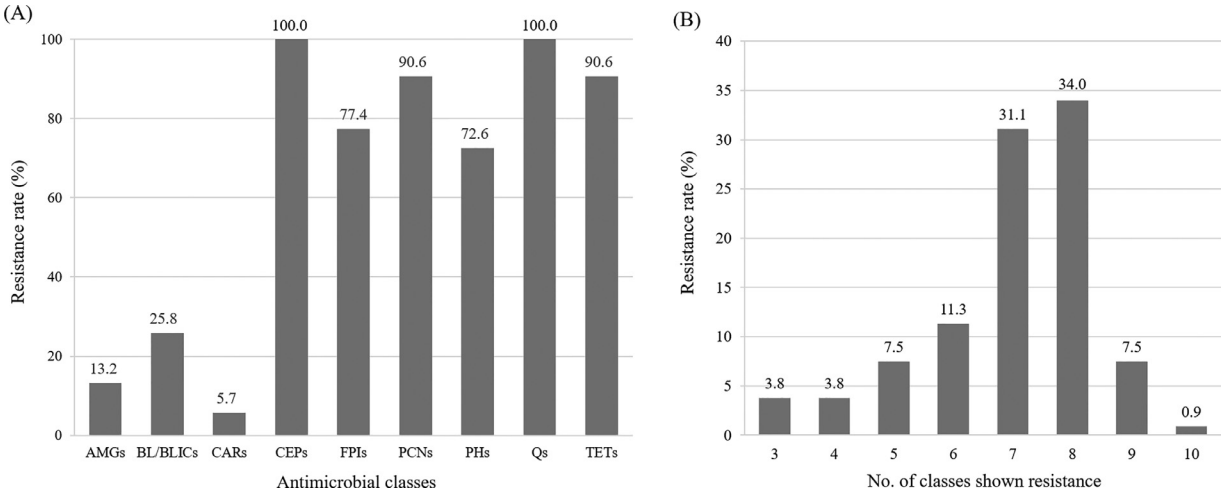


**Figure 1.** Antimicrobial resistance classes (A) and spectrum (B) of 106 fluoroquinolone-resistant *E. coli* isolated from broiler breeder farms. Abbreviations: AMGs, aminoglycosides; BL/BLICs, $\beta$-lactam/$\beta$-lactamase inhibitor combinations; CARs, carbapenems; CEPs, cephems; FPIs, folate pathway inhibitors; PCNs, penicillins; PHs, phenicols; Qs, quinolones; TETs, tetracyclines.

**Table 2.** Amino acid changes in the QRDRs, MICs and PMQR determinants of 106 fluoroquinolone-resistant *E. coli* isolates.

| QRDR mutations[1] | | | | MICs rage (μg/mL)[2] | | | | |
|---|---|---|---|---|---|---|---|---|
| *gyrA* | *gyrB* | *parC* | *parE* | NA | CIP | ENR | PMQR genes (No. of isolates)[3] | No. of fluoroquinolone-resistant *E. coli* (%) |
| S83L/D87N | WT | S80I/E84A | I464F | >512 | 256 | 256 | - | 4 (3.8) |
| S83L/D87N | WT | S80I/E84G | WT | >512 | 64-128 | 128-256 | *qnrA* (4), *qnrB* (2) | 21 (19.8) |
| S83L/D87N | WT | S80I | S458A | >512 | 128 | 128 | *aac(6′)-Ib-cr* (2) | 2 (1.9) |
| S83L/D87Y | WT | S80I | S458A | >512 | 64 | 128 | *qnrS* (1) | 1 (0.9) |
| S83L/D87N | WT | S80R | I464F | >512 | 64 | 128 | - | 1 (0.9) |
| S83L/D87N | WT | S80I | WT | >512 | 16-64 | 16-64 | *qnrS* (4), *qnrA* (2), *qnrB* (2) | 37 (34.9) |
| S83L/D87Y | WT | S80I | WT | >512 | 16-32 | 32-64 | *qnrS* (3) | 9 (8.5) |
| S83L/D87N | WT | S80R | WT | >512 | 32 | 32-64 | *qnrS* (2) | 10 (9.4) |
| S83L/D87Y | WT | S80R | WT | >512 | 16-32 | 32 | *qnrA* (1) | 7 (6.6) |
| S83I/D87E | WT | WT | WT | >512 | 8 | 32 | | 5 (4.7) |
| S83I | WT | WT | WT | >512 | 4 | 8 | - | 2 (1.9) |
| S83L | WT | WT | WT | >512 | 4 | 4-8 | - | 7 (6.6) |
| | | | | 8 | <0.06 | <0.06 | | ATCC 25922 |

[1]QRDR, quinolone-resistance determining region; WT, wild type.
[2]MICs, minimum inhibitory concentrations; NA, nalidixic acid; CIP, ciprofloxacin; ENR, enrofloxacin.
[3]PMQR, plasmid-mediated quinolone resistance; -, not detected.

with mutations in *parC*. PMQR genes were detected in 23 (21.7%) of the 106 FQ-resistant *E. coli* isolates. The *qnrS* was detected in 10 isolates (9.4%), and followed by *qnrA* (7 isolates, 6.6%), *qnrB* (4 isolates, 3.8%), and *aac(6′)-Ib-cr* (2 isolates, 1.9%).

## Characteristics of PMQR-Positive E. coli

The phenotypic and genotypic characteristics of the 23 PMQR-positive isolates among the 106 FQ-resistant *E. coli* isolates are shown in Figure 2. Sixteen (69.6%) isolates were found to have class 1 integrons, with the following 4 types of gene cassettes, *dfrA1* (6 isolates), *dfrA17* (3 isolate), *aadA2* (2 isolate), and *dfrA1+ aadA1* (1 isolate). Four isolates did not carry any of the gene cassettes. A total of 9 plasmid replicon types were also identified in 23 PMQR-positive *E. coli* isolates. The most common plasmid replicon was FIB (12 isolates, 52.2%), followed by FIA (9 isolates, 39.1%). Transferability of PMQR genes was only identified in ten (43.5%) isolates among 23 PMQR-positive *E. coli* isolates.

## PFGE Analysis

In determination of the epidemiological genetic relationships by PFGE (Figure 2), 18 PFGE patterns showing 85% similarity were observed in 23 PMQR-positive *E. coli* isolates. In particular, isolates that included 3 PFGE patterns (PEP003, PEP011, and PEP018) were

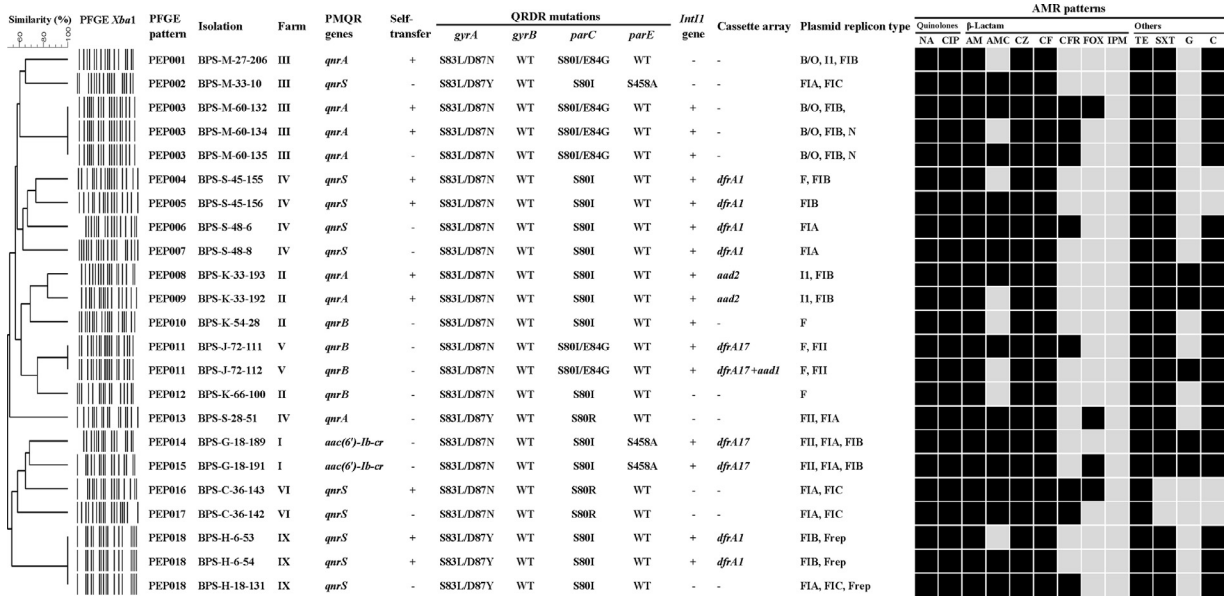


**Figure. 2.** Pulsed-field gel electrophoresis patterns of *Xba*I-digested total DNA of 23 PMQR-positive *E. coli* isolated from broiler breeder farms. The black color indicates that the trait is present, and the gray color indicates that the trait is absent. Self-transfer of carrying PMQR genes in conjugation experiments. Abbreviations: AM, ampicillin; AMC, amoxicillin-clavulanate; AMR, antimicrobial resistance; C, chloramphenicol; CIP, ciprofloxacin; CZ, cefazolin; CF, cephalothin; CFR, cefadroxil; FOX, cefoxitin; G, gentamicin; IPM, imipenem; NA, nalidixic acid; PMQR, plasmid-mediated quinolone resistance; SXT, sulfamethoxazole/trimethoprim; QRDR, quinolone-resistance determining region; TE, tetracycline.

originated from the same broiler breeder farm with the same antimicrobial resistance genes, QRDR mutation, and plasmid replicon types, and showed similar antimicrobial resistance patterns.

## DISCUSSION

FQs are highly effective antimicrobial class with many advantages including high oral absorption, large volume of distribution, and broad-spectrum antimicrobial activity ( Patel and Goldman, 2016). In Korea, the mass medication of poultry with FQs is still permitted, and the sale volume of enrofloxacin is the highest among all antimicrobials (APQA, 2017). However, resistance to FQs has emerged following their widespread use in poultry farms; thus, FQ-resistant *E. coli* isolates can be spread in poultry production pyramid (Seo and Lee, 2020). In this study, 106 FQ-resistant *E. coli* isolates showed co-resistance to cephems (100.0%) penicillins (90.6%), and tetracyclines (90.6%). Especially, all isolates showed MDR against more than 3 antimicrobial agents, and nine isolates showed resistance to more than 9 classes. These results are consistent with those of recent studies showing co-association of resistance to other classes of antimicrobials and high MDR rates among FQ-resistant *E. coli* (Mitra et al., 2019, Seo and Lee, 2020). It is because FQ-resistant *E. coli* has plasmids harboring resistant genes to diverse classes of antimicrobials including PMQR genes (Mitra et al., 2019).

In this study, all FQ-resistant *E. coli* isolate showed amino acid exchanges at *gyrA*. Especially, isolates that had *parC* and *parE* mutations also had double mutations in *gyrA*. Heisig et al. reported that because the DNA gyrase activity is more sensitive to quinolones than that of DNA topoisomerase IV, *gyrA* becomes the primary target of quinolones and *parC* and *parE* are second (Heisig, 1996). Also, previous studies showing that mutations in the *parC* and/or *parE* are closely related to double mutations in the *gyrA* (Khodursky et al., 1995; Breines et al., 1997). Moreover, FQ-resistant *E. coli* isolates had mutations at codons 83 (Ser) and 87 (Asp) in *gyrA* and at codon 80 (Ser) in *parC* in the QRDRs, and the most common type of amino acid substitution were S83L and D87N in *gyrA* and S80I in *parC* as previous studies (Yang et al., 2004; Uchida et al., 2010; Yang et al., 2010). Also, MICs range of isolates with double mutations in *gyrA* were relatively higher than those of other isolates with single mutations in *gyrA*. Vila et al., (1994) reported that high-level resistance towards FQ is found if a second mutation accumulates in *gyrA*. Especially, FQ-resistant *E. coli* isolates which possessed double mutations in *gyrA* in combination with double mutations in *parC* or single mutations in both *parC* and *parE* were shown high levels of MICs rage. These results were consistent with previous studies that the total number of point mutations in QRDR has been associated with the increased FQ-resistance levels ( Liu et al., 2012; Hu et al. 2017).

In this study, 23 (21.7%) of the 106 FQ-resistant *E. coli* isolates detected PMQR genes. The prevalence of PMQR genes in FQ-resistant *E. coli* was considerably higher than that in a commercial broiler farm in Korea (17.8%) (Seo and Lee, 2020). These findings indicate that PMQR genes had already disseminated in broiler breeder and that the risk of PMQR spread in broiler production systems was considerable. Also, PMQR-positive *E. coli* isolates were carried 4 types of PMQR genes, *qnrS, qnrA, qnrB* and *aac(6′)-Ib-cr*. These PMQR variants have been previously detected in *E. coli* from livestock, including in healthy animals and retail meats in United States (Pereira et al., 2020), Taiwan (Kuo et al., 2009), Czech (Röderova et al., 2017), and China (Yu et al., 2015), as well as from commercial broiler farms and chicken meat in Korea (Seo and Lee, 2019, 2020).

Class 1 integrons can act as vectors that transfer and dissemination of antimicrobial resistance genes among bacteria and carry gene cassettes, which harbor antimicrobial resistance genes (Fluit and Schmitz, 2004). In this study, 16 (69.6%) PMQR-positive *E. coli* isolates contained class 1 integrons and 12 isolates have gene cassette that contains *aadA* or *dfrA* or both genes. In previous studies, *aadA* and *dfrA* gene were related resistance to antimicrobials such as aminoglycosides and trimethoprim and isolates harboring the *aadA* or *dfrA* or both genes showed higher antimicrobial resistance rates (Seo and Lee, 2018). Therefore, integrons in PMQR-positive isolates from broiler breeder can have acquired the mobile genetic elements of antimicrobial resistance, which could become a serious public health concern. Also, 10 transconjugants identified in this study carried the same PMQR genes of the donor strains, demonstrating that PMQR-positive *E. coli* isolates may be transferred clonally to humans through contaminated food products of poultry origin, leading to treatment failure in humans.

Plasmids are important genetic elements responsible for the dissemination of antimicrobial resistance through horizontal gene transfer (Thomas and Nielsen, 2005; Yang et al., 2015). Especially, IncFIA and IncFIB replicons are reported as the most common types found in *E. coli* from humans and animals (Carattoli, 2009, Mitra et al., 2019, Son et al., 2019, Seo et al., 2020), and this was seen in this study. These plasmid replicons, which encode factors involved in iron uptake, toxin production, enzymes, and a variety of resistance genes, for example, PMQR genes, are widely spread in Enterobacteriaceae (Carattoli, 2009). Furthermore, other plasmid replicons such as IncFIC, IncFII, IncFrep, IncI1, IncB/O, and IncN identified in this study have also been previously reported (Carattoli, 2009, Poirel et al., 2011, Mitra et al., 2019, Son et al., 2019). Our results indicate that plasmid replicon types that are able to confer the antimicrobial resistance function to bacteria are common in PMQR-positive *E. coli* isolated from broiler breeder farms. Also, epidemiological relationships among the PMQR-positive isolates were examined by PFGE analysis in this study. Eight (34.8%) isolates included 3 PFGE patterns identified the same QRDR mutation, PMQR genes, plasmid replicon types, and

originated from the same PS farm, respectively. This results indicate the possibility that similar PFGE pattern isolates may contribute to clonal expansion and horizontal transmission as previously described (Tamang et al., 2014; Jo and woo 2016). This is the first study to investigate the prevalence and characteristics of FQ-resistant and PMQR-positive *E. coli* isolated from the broiler breeder in Korea; it supports that constant monitoring and studies at the broiler breeder level are required to prevent the pyramidal transmission of FQ-resistant *E. coli*.

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## DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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