Check for updates

Situational analysis on fluoroquinolones use and characterization of high-level ciprofloxacin-resistant *Enterococcus faecalis* by integrated broiler operations in South Korea

Yu Jin Lee[1], Hye-Ri Jung[1], Sunghyun Yoon[2], Suk-Kyung Lim[3] and Young Ju Lee[1]*

[1]College of Veterinary Medicine and Zoonoses Research Institute, Kyungpook National University, Daegu, Republic of Korea, [2]Division of Microbiology, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR, United States, [3]Bacterial Disease Division, Animal and Plant Quarantine Agency, Gimcheon-si, Gyeongsangbuk-do, Republic of Korea

Fluoroquinolones are classified as "critically important antimicrobials for human medicine"; however, their extensive use in livestock poses a significant health risk to humans as it leads to the rapid spread of antimicrobial resistance. This study confirmed that 40.0%−71.4% of the farms in three of the five integrated broiler operations were administered ciprofloxacin (CIP). Moreover, preventive purposes (60.9%), veterinarian prescriptions (82.6%), drinking water route (100%), and 1 to 3 days (82.6%) of age were significantly highest ($P < 0.05$). 194 high-level ciprofloxacin-resistant (HLCR) *Enterococcus faecalis* (*E. faecalis*) were found in 65 of 74 farms, and of which, the prevalence of *qnrA* (63.9%), *tetM* (60.3%), *ermB* (64.9%), *blaZ* (38.7%), and *catA* (34.0%) was significantly highest ($P < 0.05$). 154 (79.4%) isolates showed MDR, and the distribution of MDR was significantly differences among the operations ($P < 0.05$). All HLCR *E. faecalis* possessed double mutations in *gyrA* and *parC*, and S83I/S80I (90.7%) mutations were most commonly identified. Interestingly, the distribution of isolates with MICs $\geq$ 512 for both CIP and moxifloxacin was significantly higher in CIP−administered farms (56.5%) than in non-CIP−administered farms (41.4%) ($P < 0.05$). Also, the prevalence of strong or moderate biofilm formers in HLCR *E. faecalis* was significantly higher than that of weak and no biofilm formers ($P < 0.05$). HLCR *E. faecalis* were heavily distributed in the broiler farms in Korea; therefore, it is necessary to minimize the prevalence of resistant bacteria *via* structural management regulations such as cleaning and disinfection of farm environments.

KEYWORDS

high level ciprofloxacin resistance, *Enterococcus faecalis*, broiler, Korea, biofilm, antimicrobial resistance genes, multidrug resistance, mutation

1. Introduction

Antimicrobials play a critical role in the prevention and treatment of disease in humans and animals. However, the extensive use of antimicrobials poses a significant health risk to humans, leading to the rapid spread of antimicrobial resistance (1). Fluoroquinolones, including ciprofloxacin (CIP), are classified as "critically important antimicrobials for human medicine" by the World Health Organization (2), but they have been widely used for treatment and prevention of livestock because of their strong antibacterial activity against gram-negative bacteria, gram-positive bacteria, and mycoplasma (3). Fluoroquinolones inhibit DNA replication by inhibiting bacterial DNA topoisomerase (*parC* and *parE* subunits) and DNA gyrase (*gyrA* and *gyrB* subunits), which act as essential enzymes in bacteria (4). Therefore, single and double point mutations in the quinolone resistance determining region (QRDR) of these subunits reduce susceptibility and lead to high resistance to fluoroquinolones in the future (5, 6).

Because the risk promoting fluoroquinolones-resistant bacteria could be harmful to humans, US FDA has finally decided to withdraw approval of enrofloxacin for use in poultry in 2005 (7, 8), and in Ireland, fluoroquinolones have been prescribed in <1% of antimicrobials for livestock since 2009 (9). Moreover, in Australia, fluoroquinolones have never been approved for food production animals due to strict regulations on antimicrobial use. Although the manufacture and import of enrofloxacin in the poultry industry in Korea have been only banned since October 2021 (10), the rate of resistance to CIP, a major metabolic product of enrofloxacin *in vivo*, increased by ∼4.5% in 2021 compared with that in 2020 (10).

In Korea, 96.4% of the broilers are produced in integrated operations (11). However, antimicrobial resistance patterns may differ by operation depending on biosecurity and hygiene practices, vaccine programs, and antimicrobial applications (12). Therefore, this study was conducted to analyze the current status of fluoroquinolones-based prescriptions and characterize high-level CIP-resistant (HLCR) *E. faecalis* from five major integrated broiler operations which produce 50.2% of chicken meat in Korea (11).

2. Materials and methods

2.1. Data source

A situational analysis of CIP use was performed using a questionnaire on CIP administration patterns during the broiler grow-out period. The questionnaire areas included purpose, proponent, route, age, and duration of CIP administration.

2.2. Sample collection

Fecal and dust samples from 74 farms of five integrated broiler operations were collected in 2021 according to the standard set of Processing and Ingredients Specification of Livestock Products by the Ministry of Food and Drug Safety (2018) (13). Briefly, approximately 10 g of the dust was swabbed using a sterile gauze moistened with sterile double-strength skim milk, and feces were sampled to disposable boots in five different sites from each flock. All samples were placed in whirl pak bags (Whirl-Pak, Nasco, Madison, WI) and transported to the laboratory at 4°C.

2.3. Bacterial identification

The isolation and identification of *E. faecalis* were performed following the standard microbiological protocols published by the Ministry of Food and Drug Safety (2018). In brief, each dust and fecal samples were cultured in 90 mL of buffered peptone water (BPW; BD Biosciences, San Jose, CA, USA) for 18–24 h at 37°C. The pre-enriched BPW was transferred to Enterococcosel broth (BD Biosciences) at a ratio of 1:10 and streaked onto Enterococcosel agar (BD Biosciences) after incubation at 37 °C for 18–24 h. At least three presumptive *Enterococcus* spp. colonies were selected from each sample, and *E. faecalis* was confirmed using polymerase chain reaction (PCR) with a specific primer for *ddl*, as described previously (14). If two or more isolates from the same farm showed the same antimicrobial susceptibility pattern, only one isolate was selected. A total of 351 *E. faecalis* isolates were analyzed in this study.

2.4. Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) of CIP and moxifloxacin (MOX) were determined by standard agar dilution methods with Mueller–Hinton agar (BD Biosciences) according to the guidelines of the Clinical and Laboratory Standards Institute (15). The breakpoint for HLCR was set at $\geq 64 \mu g/mL$ against CIP (16). Moreover, based on the guidelines of the Clinical and Laboratory Standards Institute (15), antimicrobial resistance of all HLCR *E. faecalis* isolates was determined using the disc diffusion method with the following discs (BD Biosciences): ampicillin (10 μg), chloramphenicol (30 μg), ciprofloxacin (CIP, 5 μg), doxycycline (30 μg), erythromycin (15 μg), penicillin (10 units), rifampin (5 μg), tetracycline (30 μg), and vancomycin (30 μg). Multidrug resistance (MDR) was defined as the acquired non-susceptibility to at least one agent in three or more antimicrobial classes (17).

2.5. Identification of mutations in QRDRs

PCR was performed to amplify *gyrA* and *parC* in HLCR *E. faecalis* isolates using primers and conditions described previously (18). The amplified DNA was purified by a PCR purification kit (Bioneer Inc., Seoul, Republic of Korea) and sequenced using an automatic sequencer (Cosmogenetech, Seoul, Republic of Korea). The sequences were confirmed with those in the GenBank nucleotide database using the Basic Local Alignment Search Tool (BLAST) program available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST).

2.6. Detection of antimicrobial resistance genes

DNA extraction was performed using the boiling method as described previously (19). The presence of resistance genes associated with fluoroquinolones (*qnrA*, *qnrB*, *qnrD*, and *qnrS*), β-lactams (*blaZ*, and *mecA*), phenicols (*cfr*, *catA*, and *catB*), glycopeptides (*vanA*), macrolides (*ermA*, *ermB*, and *ermC*), tetracyclines (*tetL*, *tetM*, and *tetO*), and rifamycin (*rpoB*) was detected using PCR as described previously (20–29).

2.7. Biofilm formation assay

All HLCR *E. faecalis* were determined for biofilm formation by using the standard microtiter plate test, as described previously (30). All HLCR isolates were cultured overnight on Brain–Heart Infusion agar (BD Biosciences, Sparks, MD, USA) at 37°C. Then, 500 µL of bacterial suspension adjusted for turbidity with 0.5 McFarland standard was inoculated into 5 mL of tryptic soy broth (BD Biosciences) with 0.25% (wt/vol) glucose, and 200 µL of the mixture was transferred into three wells of a 96-well microtiter plate. After overnight incubation at 37°C, the 96-well microtiter plates were washed with phosphate–buffered saline (BD Biosciences) to remove the planktonic bacteria. After air-drying, the attached bacteria were fixed with 200 µL of methanol for 15 min, and the bacterial biomass was quantified by measuring the absorbance at 595 nm (OD_{595}) after staining with crystal violet (1% wt/vol) for 10 min and destaining with 50% ethanol-50% glacial acetic acid solution. The ability to form biofilms was classified as negative ($OD_{595} < 0.12$), weak ($0.12 \leq OD_{595} < 0.24$), moderate ($0.24 \leq OD_{595} \leq 0.48$), and strong ($OD_{595} > 0.48$). To verify the analysis, *E. faecalis* OG1RF and *E. faecalis* ATCC 29212 were used as reference strains for weak and no biofilm formers, respectively. A sterile medium was used as the contamination control as described previously (31, 32).

2.8. Statistical analysis

Statistical analysis was performed using Pearson's chi-square tests and Fisher's exact tests with Bonferroni correction in Statistical Package for the Social Science version 25 (IBM, Korea). Significant differences were considered at $P < 0.05$.

3. Results

3.1. Analysis of the prescription status of CIP

The situational analysis of the prescription status of CIP in 74 farms of five integrated broiler operations is shown in Table 1. Among the five integrated broiler operations, the prevalence of CIP–administered farms was significantly highest in operation D (71.4%), followed by operations E (50.0%) and B (40.0%) ($P < 0.05$). No farms administered CIP in operations A and C. In the analysis for the purpose of CIP administration, operation D

was significantly higher for the prevention (80.0%) than treatment (20.0%), whereas operation E were significantly higher for the treatment (60.0 %) than prevention (40.0 %) ($P < 0.05$). There were no significant differences between the rates of prevention and treatment in operation B. CIP administration *via* veterinarians (82.6%) and drinking water route (100%) was significantly highest ($P < 0.05$). Moreover, CIP was administered significantly highest at 1 to 3 days of age (82.6%), and significantly higher on duration of CIP administration in 2 days (43.5%) to 3 days (39.1%) ($P < 0.05$).

3.2. Distribution of HLCR *E. faecalis* and antimicrobial resistance genes

The distribution of HLCR *E. faecalis* and antimicrobial resistance genes in 74 farms of five integrated broiler operations are shown in Table 2. HLCR *E. faecalis* were found in 65 (87.8%) of 74 farms. In particular, HLCR *E. faecalis* were significantly higher at 95.0% and 100% in farms of operations B and E, respectively ($P < 0.05$). In addition, HLCR *E. faecalis* was found in a total of 194 (55.3%) of 351 *E. faecalis* isolates, which was significantly highest in farms of operation B ($P < 0.05$). In the distribution of fluoroquinolones resistance genes of HLCR *E. faecalis*, the prevalence of *qnrA* (63.9%) and *qnrB* (57.7%) genes were significantly higher than that of *qnrD* (20.1%) and *qnrS* (18.0%) ($P < 0.05$). In particular, *qnrA* showed a significantly high prevalence in the farms of four operations A (66.7%), B (60.7%), D (78.9%), and E (73.3%) except for operation C (45.0%) ($P < 0.05$), and *qnrB* was found in 50.0%−66.7% of farms in all five operations without significant differences.

All HLCR *E. faecalis* also showed the highest resistance to tetracyclines (82.0%), followed by macrolides (79.4%), β-lactams (52.1%), phenicols (43.3%), rifamycin (32.5%), and glycopeptides (1.0%) without no significant differences among the five integrated broiler operations. Moreover, the prevalence of *tetM* (60.3%), *ermB* (64.9%), *blaz* (38.7%), and *catA* (34.0%) was significantly highest among tetracyclines, marcrolides, β-lactams and phenicols resistance-related genes, respectively ($P < 0.05$). MDR was found in 154 (79.4%) of 194 HLCR *E. faecalis* isolates; however, the distribution of MDR was significantly highest in operation B (87.5%) and lowest in operations A (73.3%) and C (70.0%) ($P < 0.05$).

3.3. Detection of mutations in the QRDRs of HLCR *E. faecalis*

The presence of mutations in *gyrA* and *parC* and distribution of MICs in 194 HLCR *E. faecalis* isolates are shown in Table 3. All HLCR *E. faecalis* isolates possessed double mutations in *gyrA* and *parC*. Moreover, S83I/S80I (90.7%) mutations were most commonly identified. The distribution of isolates with MICs $\geq$ 512 for both CIP and MOX was significantly higher in CIP–administered farms (56.5%) than in non-CIP–administered farms (41.4%) ($P < 0.05$).

TABLE 1 Analysis of the prescription status of ciprofloxacin (CIP) by five integrated broiler operations.

Variables	No. of farms included / No. of CIP–administered farms by five integrated broiler operations (%)					
	A	B	C	D	E	Total
No. of CIP–administered farms/No. of farms tested (%)	$0/15\ (0.0)_c$	$8/20\ (40.0)_b$	$0/15\ (0.0)_c$	$10/14\ (71.4)_a$	$5/10\ (50.0)_b$	$23/74\ (31.1)$
Purpose of CIP administration						
Prevention		$4/8\ (50.0)_b$		$8/10\ (80.0)_a^A$	$2/5\ (40.0)_b^B$	$14/23\ (60.9)^A$
Treatment		$4/8\ (50.0)_a$		$2/10\ (20.0)_b^B$	$3/5\ (60.0)_a^A$	$9/23\ (39.1)^B$
Who proposed CIP						
Veterinarian		$7/8\ (87.5)_a^A$		$9/10\ (90.0)_a^A$	$3/5\ (60.0)_b^A$	$19/23\ (82.6)^A$
Veterinary drug dealer		$1/8\ (12.5)^B$		$2/10\ (20.0)^B$	$2/5\ (40.0)^B$	$5/23\ (21.7)^B$
Others*		$0/8\ (0.0)^C$		$0/10\ (0.0)^C$	$0/5\ (0.0)^C$	$0\ (0.0)^C$
Route of CIP administration						
Drinking water		$8/8\ (100.0)^A$		$10/10\ (100.0)^A$	$5/5\ (100.0)^A$	$23/23\ (100.0)^A$
Feed		$1/8\ (12.5)_a^B$		$0/10\ (0.0)_b^B$	$0/5\ (0.0)_b^B$	$1/23\ (4.3)^B$
Age of CIP administration (days)						
$1\leq\ \leq3$		$6/8\ (75.0)^A$		$9/10\ (90.0)^A$	$4/5\ (80.0)^A$	$19/23\ (82.6)^A$
$4\leq\ \leq6$		$2/8\ (25.0)^B$		$1/10\ (10.0)^B$	$1/5\ (20.0)^B$	$4/23\ (17.4)^B$
$15\leq\ \leq18$		$0/8\ (0.0)_b^C$		$0/10\ (0.0)_b^C$	$1/5\ (20.0)_a^B$	$1/23\ (4.3)^C$
Duration of CIP administration (days)						
1		$1/8\ (12.5)_a^B$		$2/10\ (20.0)_a^B$	$0/5\ (0.0)_b^B$	$3/23\ (13.0)^B$
2		$5/8\ (62.5)_a^A$		$3/10\ (30.0)_b^B$	$2/5\ (40.0)_b^A$	$10/23\ (43.5)^A$
3		$2/8\ (25.0)_b^B$		$5/10\ (50.0)_a^A$	$2/5\ (40.0)_{a,b}^A$	$9/23\ (39.1)^A$
4		$0/8\ (0.0)_b^C$		$0/10\ (0.0)_b^C$	$2/5\ (40.0)_a^A$	$2/23\ (8.7)^B$

The superscript letter represents significant differences of the column, while the subscript letter represents significant differences of the row ($P < 0.05$).

*Others included farm owners and pharmaceutical company employees.

3.4. Distribution of biofilm-forming ability of HLCR *E. faecalis*

The distribution of biofilm-forming ability of 194 HLCR *E. faecalis* isolates is shown in Table 4. Although there were no significant differences between CIP–administered farms and non-CIP–administered farms, the prevalence of strong or moderate biofilm formers (60.5% and 60.0%, respectively) in all HLCR *E. faecalis* showed significantly higher than that of weak and no biofilm formers ($P < 0.05$).

4. Discussion

Enterococci are harmless to healthy individuals, but are considered opportunistic pathogens associated with hospital-acquired infections in humans and animals (33). In particular, the ability to cause infection is critical to avoid the action of most commonly used antimicrobials. Recently, antimicrobial-resistant enterococci isolated from poultry farms have become a problem in many countries, including Korea (34–37), because antimicrobial-resistant enterococci can easily spread within pathogenic strains or between pathogenic and non-pathogenic strains (38). Moreover,

resistance to fluoroquinolones, which are critically important in human medicine, has been continuously reported worldwide (39).

Antimicrobial resistance in enterococci is described as intrinsic resistance found within a species genome as well as acquired resistance by obtaining new genetic material or acquiring sporadic mutations in intrinsic genes (40, 41). In particular, the prevalence of acquired fluoroquinolones-resistant enterococci has increased with the increased use of fluoroquinolones (36, 42). In this study, CIP was administered in 23 (31.1%) of 74 broiler farms of five integrated broiler operations. Two integrated broiler operations did not use CIP, but the other three used CIP for prevention and treatment, mostly under the proposal of veterinarian. Moreover, the prevalence of 1 to 3 days of age (82.6%) was the highest among the age of CIP administration in the three integrated broiler operations. It is related to the high incidence of colibacillosis in young chicks causing mortality in Korea (43). CIP was also mainly administered *via* drinking water for 2 or 3 days because it is easier than injection administration, but it has a disadvantage that the time when the drug appears varies depending on individual even within the same flock (44).

In this study, HLCR *E. faecalis* were isolated in 65 (87.8%) of 74 farms. Farms of operations A and C were not administered CIP at all, but the distribution of HLCR *E. faecalis* isolates in these two

TABLE 2   Distribution of high-level ciprofloxacin-resistant (HLCR) *E. faecalis* and antimicrobial resistance genes by five integrated broiler operations.

Resistance gene	No. of isolates carried gene / No. of HLCR *E. faecalis* isolates by five integrated broiler operations (%)					
	A	B	C	D	E	Total
No. (%) of farms with HLCR *E. faecalis* isolates / No. of farms tested (%)	11/15 (73.3)$_b$	19/20 (95.0)$_a$	12/15 (80.0)$_b$	13/14 (92.9)$_{a,b}$	10/10 (100.0)$_a$	65/74 (87.8)
No. of HLCR *E. faecalis* isolates / No. of *E. faecalis* isolates (%)	30/68 (44.1)$_b$	56/81 (69.1)$_a$	40/79 (50.6)$_b$	38/65 (58.5)$_{a,b}$	30/58 (51.7)$_{a,b}$	194/351 (55.3)
qnrA	20/30 (66.7)$_a$	34/56 (60.7)$_{a,b}$	18/40 (45.0)$_b$	30/38 (78.9)$_a$	22/30 (73.3)$_a$	124/194 (63.9)^A
qnrB	17/30 (56.7)	30/56 (53.6)	20/40 (50.0)	25/38 (65.8)	20/30 (66.7)	112/194 (57.7)^A
qnrD	3/30 (10.0)$_b$	11/56 (19.6)$_{a,b}$	8/40 (20.0)$_{a,b}$	9/38 (23.7)$_{a,b}$	8/30 (26.7)$_a$	39/194 (20.1)^B
qnrS	5/30 (16.7)	8/56 (14.3)	8/40 (20.0)	6/38 (15.8)	8/30 (26.7)	35/194 (18.0)^B
No. (%) of tetracyclines resistant HLCR *E. faecalis* isolates	25/30 (83.3)	48/56 (85.7)	34/40 (85.0)	29/38 (76.3)	23/30 (76.7)	159/194 (82.0)
tetL	12/30 (40.0)$_{a,b}$	25/56 (44.6)$_a$	10/40 (25.0)$_b$	12/38 (31.6)$_{a,b}$	10/30 (33.3)$_{a,b}$	69/194 (35.6)^B
tetM	20/30 (66.7)	32/56 (57.1)	22/40 (55.0)	25/38 (65.8)	18/30 (60.0)	117/194 (60.3)^A
tetO	1/30 (3.3)	0/56 (0.0)	0/40 (0.0)	0/38 (0.0)	0/30 (0.0)	1/194 (0.5)^C
No. (%) of macrolides resistant HLCR *E. faecalis* isolates	22/30 (73.3)	48/56 (85.7)	30/40 (75.0)	30/38 (78.9)	24/30 (80.0)	154/194 (79.4)
ermA	1/30 (3.3)	1/56 (1.8)	2/40 (5.0)	3/38 (7.9)	3/30 (10.0)	10/194 (5.2)^B
ermB	19/30 (63.3)	40/56 (71.4)	24/40 (60.0)	25/38 (65.8)	18/30 (60.0)	126/194 (64.9)^A
ermC	0/30 (0.0)	0/56 (0.0)	0/40 (0.0)	0/38 (0.0)	0/30 (0.0)	0/194 (0.0)^B
No. (%) of β-lactams resistant HLCR *E. faecalis* isolates	13/30 (43.3)	22/56 (55.4)	16/40 (50.0)	15/38 (50.0)	14/30 (60.0)	101/194 (52.1)
blaZ	10/30 (33.3)	23/56 (41.1)	15/40 (37.5)	14/38 (36.8)	13/30 (43.3)	75/194 (38.7)^A
mecA	2/30 (6.7)	1/56 (1.8)	2/40 (5.0)	0/38 (0.0)	1/30 (3.3)	6/194 (3.1)^B
No. (%) of phenicols resistant HLCR *E. faecalis* isolates	10/30 (33.3)	24/56 (42.9)	18/40 (45.0)	16/38 (42.1)	16/30 (53.3)	84/194 (43.3)
cfr	2/30 (6.7)	2/56 (3.6)	0/40 (0.0)	2/38 (5.3)	0/30 (0.0)	6/194 (3.1)^B
catA	12/30 (40.0)	18/56 (35.7)	13/40 (45.0)	12/38 (47.4)	11/30 (36.7)	66/194 (34.0)^A
catB	2/30 (6.7)	1/56 (1.8)	0/40 (0.0)	2/38 (5.3)	1/30 (3.3)	6/194 (3.1)^B
No. (%) of rifamycin resistant HLCR *E. faecalis* isolates	10/30 (33.3)	16/56 (28.6)	15/40 (37.5)	12/38 (31.6)	10/30 (33.3)	63/194 (32.5)
rpoB	10/30 (33.3)	13/56 (23.2)	13/40 (32.5)	9/38 (23.7)	8/30 (26.7)	53/194 (27.3)
No. (%) of glycopeptides resistant HLCR *E. faecalis* isolates	1/30 (3.3)	0/56 (0.0)	0/40 (0.0)	0/38 (0.0)	1/30 (3.3)	2/194 (1.0)
vanA	0/30 (0.0)	0/56 (0.0)	0/40 (0.0)	0/38 (0.0)	0/30 (0.0)	0/194 (0.0)
No. (%) of multidrug resistant HLCR *E. faecalis* isolates	22/30 (73.3)$_b$	49/56 (87.5)$_a$	28/40 (70.0)$_b$	30/38 (78.9)$_{a,b}$	25/30 (83.3)$_{a,b}$	154/194 (79.4)

The superscript letter represents significant differences of the column, while the subscript letter represents significant differences of the row ($P < 0.05$).

farms showed a high prevalence of 73.3% and 80.0%, respectively. Burbarelli et al. (45) reported that antimicrobial–resistant bacteria could survive and spread throughout the breeding period regardless of CIP administration unless cleaning and disinfection of the broiler environment is followed for farm hygiene. In particular, antimicrobial–resistant bacteria in feces, which are generally considered a natural reservoir, have the potential to remain in the environment or bind to dust and spread throughout the farm (46–48).

Moreover, in this study, the antimicrobial resistance genes *qnrA* and *qnrB*, which are related to fluoroquinolones resistance, were present in 63.9% and 57.7%, respectively, of the distribution of HLCR *E. faecalis* in the five integrated broiler operations. In particular, the prevalence of *qnrA* in HLCR *E. faecalis* isolates in operation A was 66.7%, but the prevalence of *qnrA* in operation C was 45.0%, which was significantly lowest in these operations, even though CIP was not administered in operation A and C. It is thought that environmental cleaning and the use of CIP, and it is

TABLE 3 Characteristics of amino acid changes in *gyrA* and *parC* and distribution of MICs in 194 high-level ciprofloxacin-resistant (HLCR) *E. faecalis* from 74 broiler farms of five integrated broiler operations.

Amino acid change		MIC (µg/mL)[a]		No. (%) of HLCR *E. faecalis* isolates		
gyrA	*parC*	CIP	MOX	CIP−administered farms ($n = 124$)	Non−CIP−administered farms ($n = 70$)	Total ($n = 194$)[b]
S83I	S80I	$\geq$512	$\geq$512	70 (56.5)$_a$	29 (41.4)$_b$	99 (51.0)
		256	$\geq$512	17 (13.7)	14 (20.0)	31 (16.0)
		$\geq$512	256	11 (8.9)	11 (15.7)	22 (11.3)
		256	256	6 (4.8)	6 (8.6)	12 (6.2)
		256	128	2 (1.6)	3 (4.3)	5 (2.6)
		128	256	2 (1.6)	0 (0.0)	2 (1.0)
		128	128	2 (1.6)	0 (0.0)	2 (1.0)
		128	64	1 (0.8)	0 (0.0)	1 (0.5)
		64	64	1 (0.8)	1 (1.4)	2 (1.0)
S83F	S80I	$\geq$512	256	5 (4.0)	2 (2.9)	7 (3.6)
		256	256	2 (1.6)	1 (1.4)	3 (1.5)
		256	128	2 (1.6)	0 (0.0)	2 (1.0)
		128	64	2 (1.6)	1 (1.4)	3 (1.5)
S83V	S80I	$\geq$512	256	1 (0.8)	0 (0.0)	1 (0.5)
S83Y	S80I	$\geq$512	256	0 (0.0)	1 (1.4)	1 (0.5)
S83I	S84K	256	256	0 (0.0)	1 (1.4)	1 (0.5)

[a]MIC, Minimum Inhibitory concentrations; CIP, ciprofloxacin; MOX, moxifloxacin.
[b]All isolates possessed double mutations in *gyrA* and *parC*.
The subscript letter represents significant differences of the row ($p < 0.05$).

necessary to continuously investigate the distribution of these genes in farms that do not use CIP.

The plasmids carrying mobile resistance genes are easily transferable from one bacteria to another by conjugation, sharing genetic information (49, 50). Moreover, the externalization of antimicrobials *via* efflux pumps responsible for the outflow of the antimicrobials can simultaneously confer resistance to several antimicrobials, and their overexpression can lead to high levels of resistance and MDR (51–53). In this study, HLCR *E. faecalis* isolated from five broiler integrated operations were mostly resistant to tetracyclines (82.0%), macrolides (79.4%), β-lactams (52.1%), phenicols (43.3%), and rifamycin (32.5%), without significant differences among the operations. In addition, the prevalence of *tetM*, *ermB*, *blaZ*, *catA*, and *rpoB*, which are related to resistance to tetracyclines, macrolides, β-lactams, and phenicols, was 60.3%, 64.9%, 38.7%, and 34.0%, respectively, without significant differences among the operations. However, the distribution of MDR was significantly highest in operation B (87.5%) and lowest in operations A (73.3%) and C (70.0%). These results are consistent with the fact that the distribution of HLCR *E. faecalis* isolates was significantly highest in farms of operation B and lowest in farms of operations A and C. Moreover, this is consistent with the reports that the efflux pump is overexpressed as the number of HLCR isolates increases (54, 55), which is thought to be related to the increased distribution of MDR.

The mechanism of resistance to fluoroquinolones includes the formation of mutations in the two bacterial essential

TABLE 4 Distribution of biofilm-forming ability in 194 high-level ciprofloxacin-resistant (HLCR) *E. faecalis* from 74 broiler farms of five integrated broiler operations.

Biofilm former	No. (%) of HLCR *E. faecalis* isolates		
	CIP–administered farms ($n = 124$)	Non-CIP–administered farms ($n = 70$)	Total ($n = 194$)
No biofilm former	19 (15.3)[B]	10 (14.3)[B]	29 (14.9)[B]
Weak biofilm former	30 (24.2)[B]	18 (25.7)[B]	48 (24.7)[B]
Strong or moderate biofilm former	75 (60.5)[A]	42 (60.0)[A]	117 (60.3)[A]

The superscript letter represents significant difference of the column ($p < 0.05$).

enzymes, DNA gyrase and DNA topoisomerase (56). DNA gyrase comprises *gyrA* and *gyrB* subunits, and DNA topoisomerase comprises *parC* and *parE* subunits. In particular, HLCR is associated with double mutations of *gyrA* and *parC* (57). Kim et al. (36) reported that all tested HLCR enterococci isolates showed double mutations in *gyrA* and *parC*, and the double mutations were the most frequently identified in S83I/S80I at 94.1%. Similarly, all HLCR *E. faecalis* isolates in this study

possessed double mutations in *gyrA* and *parC*. In particular, 90.7% of HLCR isolates showed S83I/S80I mutations. Moreover, 99 (56.3%) of 176 HLCR *E. faecalis* with S83I/S80I mutations showed MICs of ≥ 512 for CIP and MOX. However, the distribution of these HLCR *E. faecalis* with MIC of ≥ 512 for both CIP and MOX in S83I/S80I mutations was found to be significantly higher in CIP–administered farms (56.5%) than in non–CIP–administered farms (41.4%). These results indicate that higher CIP administration leads to higher MIC values, just as fluoroquinolones-resistant enterococci increase with increasing fluoroquinolones use.

Biofilms of bacteria protect them from host immunological responses, phagocytosis, and antimicrobials (58). The ability to form biofilms is related to the ability of bacteria to adhere to a surface and form a layer, and the density of the layer indicates the strength of the biofilms (59). In addition, the thicker the layer, the less effective the antimicrobials against bacteria, suggesting a correlation between antimicrobial resistance and biofilm-forming ability (60). Al-Shammary (61) reported that 93.8% of MDR *E. faecalis* isolates from raw milk samples were biofilm formers. Sadek and Koriem (62) also reported that all MDR *E. faecalis* isolates from dairy products were biofilm formers. In this study, 165 (85.0%) of 194 HLCR *E. faecalis* isolates showed strong or moderate biofilm-forming ability. However, there were no significant differences between the isolates from CIP–administered farms and non–CIP–administered farms. Therefore, these results suggest that if already classified as HLCR, isolates can have strong and moderate biofilm-forming ability regardless of CIP administration, and the increased biofilm density also allows infections to persist chronically despite antimicrobial treatment strategies for broiler diseases. In this study, it was confirmed that HLCR *E. faecalis* were heavily distributed in the broiler operations in Korea, therefore, it is necessary to minimize the prevalence of resistant bacteria *via* structural management regulation, such as cleaning and disinfection of farm environments, and reduce the use of antimicrobials *via* prescriptions based on scientific disease evidence rather than disease prevention.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

YuL, SK-L, and YoL conceived of the presented idea. YuL, H-RJ, and SY developed the theory and performed the experiments. YuL and H-RJ verified the analysis. YuL wrote the manuscript with supervision from S-KL and YoL. All authors contributed to the article and approved the submitted version.

Funding

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Hidron AI, Edwards JR, Patel J, Horan TC, Sievert DM, Pollock DA, et al. Antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the national healthcare safety network at the centers for disease control and prevention, 2006–2007. *Infect Control Hosp Epidemiol.* (2008) 29:996–1011. doi: 10.1086/591861

2. World Health Organization. *WHO list of Critically Important Antimicrobials for Human Medicine (WHO CIA list).* (2019). Available online at: https://www.who.int/publications/i/%0Aitem/9789241515528 (accessed May 25, 2022).

3. Pham TDM, Ziora ZM, Blaskovich MAT. Quinolone antibiotics. *Medchemcomm.* (2019) 10:1719–39. doi: 10.1039/c9md00120d

4. Khan T, Sankhe K, Suvarna V, Sherje A, Patel K, Dravyakar B, et al. gyrase inhibitors: Progress and synthesis of potent compounds as antibacterial agents. *Biomed Pharmacother.* (2018) 103:923–38. doi: 10.1016/j.biopha.2018.04.021

5. Urushibara N, Suzaki K, Kawaguchiya M, Aung MS, Shinagawa M, Takahashi S, et al. Contribution of type II topoisomerase mutations to fluoroquinolone resistance in enterococcus faecium from Japanese clinical setting. *Microb Drug Resist.* (2018) 24:1–7. doi: 10.1089/mdr.2016.0328

6. Esfahani S, Ahmadrajabi R, Mollaei H, Saffari F. Co-incidence of type II topoisomerase mutations and efflux expression in high fluoroquinolone resistant Enterococcus faecalis isolated from urinary tract infections. *Infect Drug Resist.* (2020) 13:553–9. doi: 10.2147/IDR.S237299

7. Crawford L. Senior management perceptions of project management competence. *Int J Proj Manag.* (2005) 23:7–16. doi: 10.1016/j.ijproman.2004.06.005

8. U.S. Food and Drug Administration. Available online at: https://www.fda.gov/animal-veterinary/recalls-withdrawals/withdrawal-enrofloxacin-poultry (accessed May 25, 2022).

9. Beechinor JG. Report on consumption of veterinary antimicrobial drugs in Ireland in 2009. *Ir Med J.* (2011) 104:1–3.

10. Animal and plant quarantine agency. Enrofloxacin. (2021). Available online at: https://www.qia.go.kr/popup_search/Search.jsp (accessed July 7, 2022).

11. Korea Agricultural Marketing Information Service. *Report of Domestic Distribution.* Naju: Korea Agro-Fisheries & Food Trade Coporation. (2020). p. 251–77.

12. Davies R, Wales A. Antimicrobial resistance on farms: a review including biosecurity and the potential role of disinfectants in resistance selection. *Compr Rev Food Sci Food Saf.* (2019) 18:753–74. doi: 10.1111/1541-4337.12438

13. Processing Standards and Ingredient Specifications for Livestock Products. Chongju, Korea. *Minist Food Drug Saf.* (2020). Available online at: http://www.foodsafetykorea.go.kr/foodcode/01_02.jsp?idx=%0A263 (accessed May 25, 2022).

14. Dutka-Malen S, Evers S, Courvalin P. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. *J Clin Microbiol.* (1995) 33:1434. doi: 10.1128/jcm.33.5.1434-1434.1995

15. CLSI. *M100-ED29: 2021 Performance Standards for Antimicrobial Susceptibility Testing, 30th Edition.* Wayne, PA: Centers for Disease Control. (2020). p. 50–1.

16. Leavis HL, Willems RJL, Top J, Bonten MJM. High-level ciprofloxacin resistance from point mutations in gyrA and parC confined to global hospital-adapted clonal lineage CC17 of enterococcus faecium. *J Clin Microbiol.* (2006) 44:1059–64. doi: 10.1128/JCM.44.3.1059-1064.2006

17. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect.* (2012) 18:268–81. doi: 10.1111/j.1469-0691.2011.03570.x

18. El Amin N, Jalal S, Wretlind B. Alterations in gyrA and parC associated with fluoroquinolone resistance in Enterococcus faecium. *Antimicrob Agents Chemother.* (1999) 43:947–9. doi: 10.1128/aac.43.4.947

19. Bakshi CS, Shah DH, Verma R, Singh RK, Malik M. Rapid differentiation of Mycobacterium bovis and Mycobacterium tuberculosis based on a 127-kb fragment by a single tube multiplex-PCR. *Vet Microbiol.* (2005) 109:211–6. doi: 10.1016/j.vetmic.2005.05.015

20. Predari SC, Ligozzi M, Fontana R. Genotypic identification of methicillin-resistant coagulase-negative staphylococci by polymerase chain reaction. *Antimicrob Agents Chemother.* (1991) 35:2568–73. doi: 10.1128/AAC.35.12.2568

21. Okamoto R, Okubo T, Inoue M. Detection of genes regulating β-lactamase production in Enterococcus faecalis and Staphylococcus aureus. *Antimicrob Agents Chemother.* (1996) 40:2550–4. doi: 10.1128/aac.40.11.2550

22. Aarestrup FM, Agerso Y, Gerner-Smidt P, Madsen M, Jensen LB. Comparison of antimicrobial resistance phenotypes and resistance genes in Enterococcus faecalis and Enterococcus faecium from humans in the community, broilers, and pigs in Denmark. *Diagn Microbiol Infect Dis.* (2000) 37:127–37. doi: 10.1016/S0732-8893(00)00130-9

23. Kariyama R, Mitsuhata R, Chow JW, Clewell DB, Kumon H. Simple and reliable multiplex PCR assay for surveillance isolates of vancomycin-resistant enterococci. *J Clin Microbiol.* (2000) 38:3092–5. doi: 10.1128/jcm.38.8.3092-3095.2000

24. Agersø Y, Pedersen AG, Aarestrup FM. Identification of Tn5397-like and Tn916-like transposons and diversity of the tetracycline resistance gene tet(M) in enterococci from humans, pigs and poultry. *J Antimicrob Chemother.* (2006) 57:832–9. doi: 10.1093/jac/dkl069

25. Robicsek A, Strahilevitz J, Sahm DF, Jacoby GA, Hooper DC. qnr prevalence in ceftazidime-resistant enterobacteriaceae isolates from the United States. *Antimicrob Agents Chemother.* (2006) 50:2872–4. doi: 10.1128/AAC.01647-05

26. Cavaco LM, Hasman H, Xia S, Aarestrup FM. qnrD, a novel gene conferring transferable quinolone resistance in Salmonella enterica serovar Kentucky and Bovismorbificans strains of human origin. *Antimicrob Agents Chemother.* (2009) 53:603–8. doi: 10.1128/AAC.00997-08

27. Zenteno-Cuevas R, Zenteno JC, Cuellar A, Cuevas B, Sampieri CL, Riviera JE, et al. Mutations in rpoB and katG genes in mycobacterium isolates from the Southeast of Mexico. *Mem Inst Oswaldo Cruz.* (2009) 104:468–72. doi: 10.1590/S0074-02762009000300012

28. Di Cesare A, Luna GM, Vignaroli C, Pasquaroli S, Tota S, Paroncini P, et al. Aquaculture can promote the presence and spread of antibiotic-resistant enterococci in marine sediments. *PLoS ONE.* (2013) 8:e62838. doi: 10.1371/journal.pone.0062838

29. Osman KM, Badr J, Orabi A, Elbehiry A, Saad A, Ibrahim MDS, Hanafy MH. Poultry as a vector for emerging multidrug resistant Enterococcus spp: First report of vancomycin (van) and the chloramphenicol–florfenicol (cat-fex-cfr) resistance genes from pigeon and duck faeces. *Microb Pathog.* (2019) 128:195–205. doi: 10.1016/j.micpath.2019.01.006

30. Schlüter S, Franz CMAP, Gesellchen F, Bertinetti O, Herberg FW, Schmidt FRJ. The high biofilm-encoding bee locus: a second pilus gene cluster in enterococcus faecalis? *Curr Microbiol.* (2009) 59:206–11. doi: 10.1007/s00284-009-9422-y

31. Montealegre MC, La Rosa SL, Roh JH, Harvey BR, Murray BE. The Enterococcus faecalis EbpA Pilus protein: Attenuation of expression, biofilm formation, and adherence to fibrinogen start with the rare initiation codon ATT. *MBio.* (2015) 6:15. doi: 10.1128/mBio.00467-15

32. Zheng JX, Bai B, Lin ZW, Pu ZY, Yao WM, Chen Z, et al. Characterization of biofilm formation by Enterococcus faecalis isolates derived from urinary tract infections in China. *J Med Microbiol.* (2018) 67:60–7. doi: 10.1099/jmm.0.000647

33. Cattoir V. The multifaceted lifestyle of enterococci: genetic diversity, ecology and risks for public health. *Curr Opin Microbiol.* (2022) 65:73–80. doi: 10.1016/j.mib.2021.10.013

34. Maasjost J, Mühldorfer K, De Jäckel SC, Hafez HM. Antimicrobial susceptibility patterns of enterococcus faecalis and enterococcus faecium isolated from poultry flocks in Germany. *Avian Dis.* (2015) 59:143–8. doi: 10.1637/10928-090314-RegR

35. Bortolaia V, Espinosa-Gongora C, Guardabassi L. Human health risks associated with antimicrobial-resistant enterococci and Staphylococcus aureus on poultry meat. *Clin Microbiol Infect.* (2016) 22:130–40. doi: 10.1016/j.cmi.2015.12.003

36. Kim Y. Bin, Seo HJ, Seo KW, Jeon HY, Kim DK, Kim SW, et al. Characteristics of high-Level ciprofloxacin-Resistant enterococcus faecalis and enterococcus faecium from retail chicken meat in Korea. *J Food Prot.* (2018) 81:1357–63. doi: 10.4315/0362-028X.JFP-18-046

37. Rajendiran S, Veloo Y, Thahir SSA, Shaharudin R. Resistance towards critically important antimicrobials among enterococcus faecalis and e. faecium in poultry farm environments in Selangor, Malaysia. *Antibiotics.* (2022) 11:1118. doi: 10.3390/antibiotics11081118

38. Sparo M, Delpech G, Allende NG. Impact on public health of the spread of high-level resistance to gentamicin and vancomycin in enterococci. *Front Microbiol.* (2018) 9:1–10. doi: 10.3389/fmicb.2018.03073

39. Hayer SS, Casanova-Higes A, Paladino E, Elnekave E, Nault A, Johnson T, et al. Global Distribution of fluoroquinolone and colistin resistance and associated resistance markers in *Escherichia coli* of Swine Origin—A systematic review and meta-analysis. *Front Microbiol.* (2022) 13:1–13. doi: 10.3389/fmicb.2022.834793

40. Hollenbeck BL, Rice LB. Intrinsic and acquired resistance mechanisms in enterococcus. *Virulence.* (2012) 3:421–33. doi: 10.4161/viru.21282

41. Rózańska H, Lewtak-Piłat A, Kubajka M, Weiner M. Occurrence of enterococci in mastitic cow's milk and their antimicrobial resistance. *J Vet Res.* (2019) 63:93–7. doi: 10.2478/jvetres-2019-0014

42. Miller WR, Munita JM, Arias CA. Mechanisms of antibiotic resistance in enterococci. *Expert Rev Anti Infect Ther.* (2014) 12:1221–36. doi: 10.1586/14787210.2014.956092

43. Oh JY, Kang MS, Kim JM, An BK, Song EA, Kim JY, et al. Characterization of Escherichia coli isolates from laying hens with colibacillosis on 2 commercial egg-producing farms in Korea. *Poult Sci.* (2011) 90:1948–54. doi: 10.3382/ps.2011-01509

44. Landoni MF, Albarellos G. The use of antimicrobial agents in broiler chickens. *Vet J.* (2015) 205:21–7. doi: 10.1016/j.tvjl.2015.04.016

45. Burbarelli MF de C, Polycarpo G. do V, Lelis KD, Granghelli CA, Pinho ACC de, Queiroz SRA, et al. The effects of two different cleaning and disinfection programs on broiler performance and microbiological status of broiler houses. *Poult Sci.* (2017) 96:3188–98. doi: 10.3382/ps/pex153

46. Casey JA, Kim BF, Larsen J, Price LB, Nachman KE. Industrial food animal production and community health. *Curr Environ Heal reports.* (2015) 2:259–71. doi: 10.1007/s40572-015-0061-0

47. Woolhouse M, Ward M, Van Bunnik B, Farrar J. Antimicrobial resistance in humans, livestock and the wider environment. *Philos Trans R Soc B Biol Sci.* (2015) 370:83. doi: 10.1098/rstb.2014.0083

48. Yang Y, Xie X, Tang M, Liu J, Tuo H, Gu J, et al. Exploring the profile of antimicrobial resistance genes harboring by bacteriophage in chicken feces. *Sci Total Environ.* (2020) 700:1–9. doi: 10.1016/j.scitotenv.2019.134446

49. Choi JM, Woo GJ. Transfer of tetracycline resistance genes with aggregation substance in food-borne enterococcus faecalis. *Curr Microbiol.* (2015) 70:476–84. doi: 10.1007/s00284-014-0742-1

50. Wallace RL, Bulach DM, Jennison A V, Valcanis M, McLure A, Smith JJ, et al. Molecular characterization of Campylobacter spp. recovered from beef, chicken, lamb and pork products at retail in Australia. *PLoS ONE.* (2020) 15:1–18. doi: 10.1371/journal.pone.0236889

51. Alcalde-Rico M, Hernando-Amado S, Blanco P, Martínez JL. Multidrug efflux pumps at the crossroad between antibiotic resistance and bacterial virulence. *Front Microbiol.* (2016) 7:1–14. doi: 10.3389/fmicb.2016.01483

52. Masi M, Réfregiers M, Pos KM, Pagès JM. Mechanisms of envelope permeability and antibiotic influx and efflux in Gram-negative bacteria. *Nat Microbiol.* (2017) 2:1. doi: 10.1038/nmicrobiol.2017.1

53. Vergalli J, Atzori A, Pajovic J, Dumont E, Malloci G, Masi M, et al. The challenge of intracellular antibiotic accumulation, a function of fluoroquinolone influx versus bacterial efflux. *Commun Biol.* (2020) 3:1–12. doi: 10.1038/s42003-020-0929-x

54. Martins M, Mccusker MP, Viveiros M, Couto I, Fanning S, Pagès J-M, et al. A simple method for assessment of MDR Bacteria for over-expressed efflux pumps. *Open Microbiol J.* (2013) 3:72–82. doi: 10.2174/1874285801307010072

55. Nishino K, Yamasaki S, Nakashima R, Zwama M, Hayashi-Nishino M. Function and inhibitory mechanisms of multidrug efflux pumps. *Front Microbiol.* (2021) 12:1–13. doi: 10.3389/fmicb.2021.737288

56. Mohammed HHH, Abuo-Rahma GE-DAA, Abbas SH, Abdelhafez E-SMN. Current trends and future directions of fluoroquinolones. *Curr Med Chem.* (2019) 26:3132–49. doi: 10.2174/0929867325666180214122944

57. Werner G, Fleige C, Ewert B, Laverde-Gomez JA, Klare I, Witte W. High-level ciprofloxacin resistance among hospital-adapted enterococcus faecium (CC17). *Int J Antimicrob Agents.* (2010) 35:119–25. doi: 10.1016/j.ijantimicag.2009.10.012

58. González JF, Hahn MM, Gunn JS. Chronic biofilm-based infections: skewing of the immune response. *Pathog Dis.* (2018) 76:1–7. doi: 10.1093/femspd/fty023

59. Machado TS, Pinheiro FR, Soares L, Andre P, Freire R, Pereira A, et al. virulence factors found in nasal colonization and infection of methicillin-resistant staphylococcus aureus (MRSA) isolates and their ability to form a biofilm. *Toxins.* (2020) 13:14. doi: 10.3390/toxins13010014

60. Benbelaïd F, Khadir A, Abdoune MA, Bendahou M, Muselli A, Costa J. Antimicrobial activity of some essential oils against oral multidrug-resistant Enterococcus faecalis in both planktonic and biofilm state. *Asian Pac J Trop Biomed.* (2014) 4:463–72. doi: 10.12980/APJTB.4.2014C1203

61. Al-Shammary AHA. Run-off patterns of vancomycin resistant enterococci (VRE clones) in cows raw milk and imported milk powders at baghdad markets. *Iraqi J Vet Med.* (2019) 43:65–70. doi: 10.30539/iraqijvm. v43i2.532

62. Sadek OA, Koriem AM. Multidrug resistance and virulence factors of enterococci isolated from milk and some dairy desserts. *J Food Qual Hazards Cont.* (2022) 9:215–25. doi: 10.18502/jfqhc.9. 4.1137