Antimicrobial resistance monitoring of commensal *Enterococcus faecalis* in broiler breeders

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ABSTRACT *Enterococcus faecalis* (*E. faecalis*) has rapidly acquired resistance to multiple antimicrobials, and the antimicrobial resistance of *E. faecalis* from broiler breeders has been implicated in its vertical transmission to their offspring. The objective of this study was to investigate the antimicrobial resistance and genetic diversity of commensal *E. faecalis* isolated from the broiler breeder farms. Among a total of 229 *E. faecalis* isolates from 9 broiler breeder farms, the highest resistance rate was observed in tetracycline (78.2%), followed by doxycycline (58.1%) and erythromycin (43.7%), and the prevalence of antimicrobial resistance showed significant differences among the 9 broiler breeder farms ($P < 0.05$). The *tetM* gene (77.1%) and *ermB* gene (85.0%) were detected at the highest levels in 179 TE-and 100 E-resistant isolates, respectively. Twenty-four high-level gentamicin-resistant isolates carried *aac(6″)Ie-aph(2″)-la* gene, and 9 high-level ciprofloxacin-resistant isolates showed point mutations in both *gyrA* and *parC* genes. All high-level gentamicin-resistant or high-level ciprofloxacin-resistant isolates showed one of the two different virulence gene patterns, *ace-asa1-efaA-gelE* complex or *ace-efaA-gelE* complex. These results indicate that constant epidemiological monitoring at the breeder level is required to prevent the pyramidal transmission of antimicrobial-resistant *E. faecalis.*

Key words: *Enterococcus faecalis*, broiler breeder, poultry, antimicrobial resistance

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INTRODUCTION

Enterococci are normal inhabitants of the gastrointestinal tracts of animals and humans (Cauwerts et al., 2007; Choi and Woo, 2015). However, *Enterococcus faecalis* (***E. faecalis***) and *Enterococcus faecium*, which are of the greatest importance to human health, could cause nosocomial bacteremia, peritonitis, surgical wound infections, and urinary tract infections (Diarra et al., 2010; Han et al., 2011). In particular, *E. faecalis* has rapidly acquired resistance to multiple antimicrobials in the last several decades (Kim et al., 2018) and could transfer the antimicrobial resistance genes from animals to humans through the food chain (Aarestrup et al., 2000; Cauwerts et al., 2007; Han et al., 2011).

The broiler industry is vertically integrated from breeding flocks and hatcheries to feed mills, transportation divisions, and processing plants. Dierikx et al. (2013) and Ha et al. (2018) have implicated a vertical transmission of the bacterial isolates from broiler breeding chickens to their offspring. Antimicrobial resistance of the enterococci inhabiting the intestinal tract of the parent stock can result from the use of antimicrobials and can directly reflect the dissemination in commercial chicks through hatcheries which serve as a reservoir (Osman et al., 2018).

The Korea Animal Health Products Association reported that 154 tons of antimicrobials were sold for use in the poultry industry in 2017 (APQA, 2017). In particular, antimicrobial agents such as aminoglycoside, β-lactam, and fluoroquinolone have been widely used in the broiler industry in Korea (Kim et al., 2018). Although the Korean Veterinary Antimicrobial Resistance Monitoring is an ongoing program against the zoonotic and commensal bacteria isolated from the food-producing animals including broiler chicken since 2003 (APQA, 2017), the level of antimicrobial resistance at the broiler parent stage has not yet been studied. In this study, we

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investigated the antimicrobial resistance and genetic diversity of commensal *E. faecalis* isolated from the broiler breeder farms.

MATERIALS AND METHODS

Sampling

Fecal samples were collected from 9 broiler breeder farms including 94 flocks from 2016 to 2018 (Table 1) in accordance with the standards set by the National Poultry Improvement Plan (USDA, 2012). Briefly, approximately 10 g of fecal droppings were sampled from each of the 15 different locations in 2 divided areas per flock. All the samples were transported to the laboratory in a cooler, individually inoculated into 100 mL of buffered peptone water (BD Biosciences, Sparks, MD) and incubated for 18 to 24 h at 37°C.

Bacterial Isolation

Pre-enriched buffered peptone water was transferred to Enterococcosel broth (BD Biosciences) at a 1:10 ratio, and the broth was streaked onto Enterococcosel agar (BD Biosciences) after incubation for 18 to 24 h at 37°C. The suspected *Enterococcus* colonies were selected and subsequently confirmed as *E. faecalis* by using the polymerase chain reaction (**PCR**) method as previously described (Dutka-Malen et al., 1995; Kim et al., 2019). At least 3 suspicious colonies were randomly confirmed, and only one isolate was included if the isolates from the same flock showed the same antimicrobial susceptibility patterns. As a result, a total of 229 *E. faecalis* isolates were tested in this study (Table 1).

Antimicrobial Susceptibility Testing

All isolates were tested for resistance to 9 antimicrobial agents using the disc diffusion method on Mueller-Hinton agar (BD Biosciences), according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2013) as previously described (Kim et al., 2019). The antimicrobial discs (BD Biosciences) used were ampicillin (10 μg), chloramphenicol (30 μg), ciprofloxacin (**CIP**, 5 μg), doxycycline (**DOX**, 30 μg), erythromycin (**E**, 15 μg), penicillin (10 units), rifampin (5 μg), tetracycline (**TE**, 30 μg), and vancomycin (30 μg). Multidrug resistance (**MDR**) was defined as the acquired resistance to at least one agent in 3 or more antimicrobial classes.

The minimum inhibitory concentration (**MIC**) was determined by standard agar dilution methods on Muller-Hinton agar (BD Biosciences) and the MIC breakpoint values for high-level CIP (≥64 μg/mL) as previously described (Leavis et al., 2006). The high-level gentamicin (**HLG**, ≥500 μg/mL) and high-level streptomycin (**HLS**, ≥2,000 μg/mL) was defined by the CLSI (CLSI, 2013). The MIC breakpoint values for high-level kanamycin (**HLK**, ≥500 μg/mL) was determined according to European Committee on Antimicrobial Susceptibility Testing (2019). *Staphylococcus aureus* ATCC 25923 was used as quality control for the disk diffusion and MIC tests.

Detection of Antimicrobial Resistance and Virulence Genes

The presence of genes encoding resistance to E, including *ermA*, *ermB*, and *mef* genes; TE, including *tetL*, *tetM*, *tetO*, *Int-Tn*, and *tndX* genes; and aminoglycoside-modifying enzyme (**AME**), including *aac*(6′)-*Ie*–*aph*(2″)-*Ia*, *aph*(2″)-*Ib*, *aph*(2″)-*Ic*, *aph*(2″)-*Id*, *ant*(3″)-*Ia*, and *ant*(6)-*Ia* genes, were screened by PCR, using primers and conditions as previously described (Clark et al., 1999; Vakulenko et al., 2003; Kehrenberg and Schwarz, 2006; Di Cesare et al., 2013; Choi and Woo, 2015). Furthermore, the genes encoding the virulence factors—*ace*, *asa1*, *cylA*, *efaA*, *esp*, *gelE*, and *hyl*—were also identified by using PCR as previously described (Choi and Woo, 2013).

Detection of IS256-Flanking Pattern for aac(6′)Ie-aph(2″)-Ia

The presence of the insertion sequence IS*256*-flanking patterns was investigated in all the high-level gentamicin-resistant (**HLGR**) *E. faecalis* harboring *aac(6′)*

Table 1. Distribution of *Enterococcus faecalis* isolated from 9 broiler breeder farms for this study.

Farm	No. of positive flocks/no. of flocks tested (%)	No. of *E. faecalis* isolates[1]
I	9/12 (75.0)	28
II	9/9 (100.0)	25
III	13/14 (92.9)	31
IV	18/20 (90.0)	50
V	9/9 (100.0)	23
VI	9/9 (100.0)	24
VII	5/8 (62.5)	16
VIII	3/4 (75.0)	9
IX	9/9 (100.0)	23
Total	84/94 (92.4)	229

[1]If isolates from the same flock showed the same antimicrobial susceptibility patterns, only one isolate was included in this study.

Ie-aph(2″)-Ia. PCR using 2 primer pairs as reported by Watanabe et al. (2009) was performed to determine the IS*256*-flanking patterns.

Screening for Mutations in gyrA and parC

The detection of *gyrA* and *parC* genes in high-level CIP-resistance (**HLCR**) isolates was also assessed by PCR using the primers previously described (Kwak et al., 2013). The PCR products were purified using the PCR purification kit (Qiagen, Valencia, CA) and sequenced by the automatic sequencer (Cosmogenetech, Korea). The DNA sequences were compared with those deposited in the GeneBank accession no. AF060881 and AB017811 for *gyrA* and *parC* genes, respectively (El Amin et al., 1999).

Statistical Analysis

Statistical analysis was performed using the Statistical Package for Social Science version 25 (SPSS; IBM, Korea) and conducted with the chi-square test to identify statistically significant differences. Differences were considered significant at $P < 0.05$.

RESULTS

Antimicrobial Resistance Profile

The distribution of antimicrobial resistance to 12 antimicrobial agents in the 229 *E. faecalis* isolates from 9 broiler breeder farms is presented in Table 2. The highest resistance rate was observed to TE (78.2%), followed by DOX (58.1%), E (43.7%), rifampin (31.0%), HLS (16.2%), CIP (15.3%), HLK (14.0%), chloramphenicol (13.1%), and HLG (10.5%). The rates of antimicrobial resistance to penicillin and ampicillin were only 8.3 and 3.5%, respectively. None of the isolates were resistant to vancomycin. The prevalence of antimicrobial resistance also showed significant differences among the 9 broiler breeder farms ($P < 0.05$).

Distribution of TE- and E-Resistance Genes

The distribution of TE- and E-resistance genes is shown in Figure 1. The *tetM* and *tetO* genes were detected in 138 (77.1%) and 15 (8.4%) among the 179 TE-resistant isolates, respectively. One hundred and eight (60.3%) isolates carried both *tetM and tetL* genes. The *ermB* gene was detected in 85 (85.0%) among the 100 E-resistant isolates. None of the E-resistant isolates had *ermA* and *mef* genes. Among the 90 both TE- and E-resistant isolates, *tetM-tetL-ermB* gene combinations were detected in 75 (80.3%) isolates. Only one (1.1%) isolate carried *tetO-ermB* gene combinations. The transposon genes, *Int-Tn* and *tndx*, were not detected in any of the isolates, and the distribution of antimicrobial resistance genes showed a significant difference among the 9 broiler breeder farms ($P < 0.05$).

Characteristics of HLGR E. faecalis Isolates

The characteristics of 24 (10.5%) HLGR among the 229 *E. faecalis* isolates are shown in Table 3. The HLGR isolates were revealed in 6 broiler breeder farms, I (n = 6, 21.4%), II (n = 1, 4.0%), IV (n = 12, 24.0%), VI (n = 1, 4.2%), VII (n = 3, 18.8%), and IX (n = 1, 4.3%). All HLGR isolates carried the *aac(6″)Ie-aph(2″)-la* gene, and 2 isolates carried both *aac(6″)Ie-aph(2″)-la* and *ant(6)-Ia* genes. With respect to the distribution of IS*256*-flanking pattern, the pattern type C (50.0%) showed the highest prevalence, followed by type A (41.7%) and type D (8.3%). In particular, 6 (100%) isolates from farm I and 11 (91.7%) isolates from farm IV showed pattern type A and type C, respectively. Seventeen (70.8%) of the 24 HLGR *E. faecalis* isolates showed an MDR to 3 to 6 classes of antimicrobial agents. Two different virulence gene patterns, *ace-asa1-efaA-gelE* complex (54.2%) and *ace-efaA-gelE* complex (45.8%), were identified among the 24 HLGR *E. faecalis* isolates. None of the isolates carried the *cyl*, *esp*, and *hyl* genes.

Characteristics of HLCR E. faecalis Isolates

The characteristics of 9 (3.9%) HLCR among the 229 *E. faecalis* isolates are shown in Table 4. The HLCR isolates were revealed in 5 broiler breeder farms, I (n = 1, 3.6%), III (n = 5, 20.0%), IV (n = 1, 3.2%), VI (n = 1, 4.2%), and IX (n = 1, 4.3%). All 9 isolates showed a point mutation at both *gyrA* and *parC* genes and MICs from 64 to 128 μg/mL to fluoroquionolones. One isolate showed resistance to 7 classes of antimicrobial agents and carried the AME gene, *ant(6)-Ia* gene. The 2 virulence gene patterns, *ace-efaA-gelE* complex (5 isolates) and *ace-asa1-efaA-gelE* complex (4 isolates), were only identified, but the *cyl*, *esp*, and *hyl* genes were not detected in any of the HLCR isolates.

DISCUSSION

The integrated production, processing, and distribution systems of the poultry industry can vertically transfer antimicrobial resistant bacteria from the breeding chickens to their offspring (Olsen et al., 2011; Kim et al., 2012; Dierikx et al., 2013; Seo et al., 2018). Although numerous studies on antimicrobial resistance in poultry have been reported, only a few studies have investigated the breeders (Fei et al., 2018). In this study, *E. faecalis* from broiler breeders showed high resistance to a variety of antimicrobials and suggested a potential role as reservoirs for the transmission of resistant isolates throughout the poultry industry. In particular, the resistance rate to TE (45.8–100%), DOX (12.5–91.3%), E (21.4–73.9%), HLS (0–47.8%), CIP (0–32.3%), HLK (0–31.3%), and HLG (0–24.0%) were observed, and the significant ($P < 0.05$) differences in between each of the 9 broiler breeder farms were found.

The different resistance rate to antimicrobial agents might be due to differences in the amount and frequency of antimicrobial agents used for disease prevention or

Table 2. Prevalence of antimicrobial resistance of 229 *Enterococcus faecalis* from 9 broiler breeder farms.

Farm (no. of isolates)	No. of antimicrobial resistance isolates (%)												
	AM	C	CIP	DOX	E	P	TE	RA	VA	HLC	HLG	HLK	HLS	MDR
I (28)	0 (0.0)	0 (0.0)	3 (10.7)	18 (64.3)	6 (21.4)	1 (3.6)	24 (85.7)	6 (21.4)	0 (0.0)	1 (3.6)	6 (21.4)	6 (21.4)	8 (28.6)	6 (21.4)
II (25)	1 (4.0)	2 (8.0)	5 (20.0)	20 (80.0)	15 (60.0)	2 (8.0)	23 (92.0)	3 (12.0)	0 (0.0)	0 (0.0)	1 (4.0)	1 (4.0)	1 (4.0)	7 (28.0)
III (31)	0 (0.0)	0 (0.0)	10 (32.3)	8 (25.8)	7 (22.6)	0 (0.0)	15 (48.4)	15 (48.4)	0 (0.0)	5 (16.1)	0 (0.0)	0 (0.0)	0 (0.0)	3 (9.7)
IV (50)	1 (2.0)	13 (26.0)	7 (14.0)	38 (76.0)	28 (56.0)	3 (6.0)	45 (90.0)	6 (12.0)	0 (0.0)	1 (2.0)	12 (24.0)	14 (28.0)	10 (20.0)	18 (36.0)
V (23)	0 (0.0)	11 (47.8)	0 (0.0)	21 (91.3)	17 (73.9)	2 (8.7)	23 (100.0)	4 (17.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	11 (47.8)	13 (56.5)
VI (24)	0 (0.0)	0 (0.0)	2 (8.3)	3 (12.5)	9 (37.5)	2 (8.3)	11 (45.8)	15 (62.5)	0 (0.0)	1 (4.2)	1 (4.2)	2 (8.3)	0 (0.0)	3 (12.5)
VII (16)	2 (12.5)	3 (18.8)	4 (25.0)	8 (50.0)	5 (31.3)	2 (12.5)	12 (75.0)	6 (37.5)	0 (0.0)	0 (0.0)	3 (18.8)	5 (31.3)	3 (18.8)	4 (25.0)
VIII (9)	0 (0.0)	0 (0.0)	0 (0.0)	2 (22.2)	3 (33.3)	0 (0.0)	5 (55.6)	7 (77.8)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (11.1)
IX (23)	4 (17.4)	1 (4.3)	4 (17.4)	15 (65.2)	10 (43.5)	7 (30.4)	21 (91.3)	9 (39.1)	0 (0.0)	1 (4.3)	1 (4.3)	4 (17.4)	4 (17.4)	9 (39.1)
Total (229)[1]	8 (3.5)	30 (13.1)	35 (15.3)	133 (58.1)	100 (43.7)	19 (8.3)	179 (78.2)	71 (31.0)	0 (0.0)	9 (3.9)	24 (10.5)	32 (14.0)	37 (16.2)	64 (27.9)

Abbreviations: AM, ampicillin; C, chloramphenicol; CIP, ciprofloxacin; DOX, doxycycline; E, erythromycin; HLC, high-level ciprofloxacin; HLG, high-level gentamicin; HLK; high-level kanamycin; HLS, high-level streptomycin; MDR, multidrug-resistance; P, penicillin; RA, rifampin; TE, tetracycline; VA, vancomycin.

[1]Differences in the prevalence of resistance between isolates from farms had chi-square P values of <0.05 for all drugs depicted except vancomycin.

therapeutic purposes in each farm (Rizzotti et al., 2005). In Korea, although a variety of antimicrobial agents are required to be administered only by veterinary prescription since 2013, broiler breeder farms should also adopt guidelines for the prudent use of antimicrobial agents throughout the broiler production process to reduce the emergence and spread of resistant strains with potentially serious effects (Jeon et al., 2019).

As TEs are relatively cheap and effective against a wide variety of microorganisms, they are frequently used in poultry (Brtková et al., 2011; Choi and Woo, 2015). Macrolides are also used in both human and veterinary medicine and may be important as an alternative therapy for the treatment of enterococcal infections in human (Cauwerts et al. 2007). In this study, 189 (82.5%) among the 229 isolates showed resistance to at least TE or E. The majority of the TE-resistant *E. faecalis* isolates harbored the *tetM* gene (77.1%) or carried both *tetM* and *tetL* genes (60.3%), and E-resistant isolates harbored the *ermB* gene (85.0%). The *tetM* are cytoplasmic proteins that protect the ribosomes from the action of TE (Giovanetti et al., 2003). The *tetL* efflux gene encodes membrane-associated proteins that export TE from the cell (Giovanetti et al., 2003; Choi and Woo, 2015). Kim et al., (2019) reported that resistance mediated by *tetM* and *tetL* was the most frequent in the isolates from chicken meat. The *ermB* gene also encodes an *erm* methylase that modifies the 23S rRNA for resistance to macrolides, which was the most common (Poeta et al., 2005; Kim et al., 2019). This study indicates that the antimicrobial resistance and distribution of genes are similar in isolates from commercial broiler farms (Nowakiewicz et al., 2017b). Agersø et al., 2006 reported that the *ermB* and *tetM* genes can be easily transferred by conjugative transposons. In contrast to the findings of Agersø et al. (2006), *E. faecalis* isolates, positive for *tetM* and *ermB* genes, were negative for the *Int-Tn* and *tndX* genes already reported as major determinants of Tn*916/1545* and Tn*5385* families in the this study, respectively. Although the extensive use of antimicrobials in commercial broiler farms can lead to resistance, another reason could also be the vertical transmission of *E. faecalis* isolates through the integrated broiler operation system from broiler breeding chickens to retail chicken meat (Dierikx et al., 2013; Ha et al., 2018).

The HLGR eliminates the synergistic bactericidal effect and causes a major reduction in efficient therapeutic options (Rosvoll et al., 2012). The HLGR *E. faecalis* isolates were characterized by the presence of the AME gene, *aac(6′)-Ie-aph(2″)-Ia*, encoding a bifunctional enzyme whose activity is related to the occurrence of resistance to all clinically available aminoglycosides but not S (Vakulenko et al., 2003; Niu et al., 2016; Nowakiewicz et al., 2017b). In this study, 24 (10.5%) of the 229 *E. faecalis* isolates showed HLGR, which is lower than that seen in previous investigations (40.7%) in chicken meat in Korea (Han et al., 2011). However, all HLGR *E. faecalis* isolates carried the *aac(6′)-Ie-aph(2″)-Ia* gene. Because the *aac(6′)-Ie-aph(2″)-Ia* gene is linked to Tn*5281* and is often located in plasmids

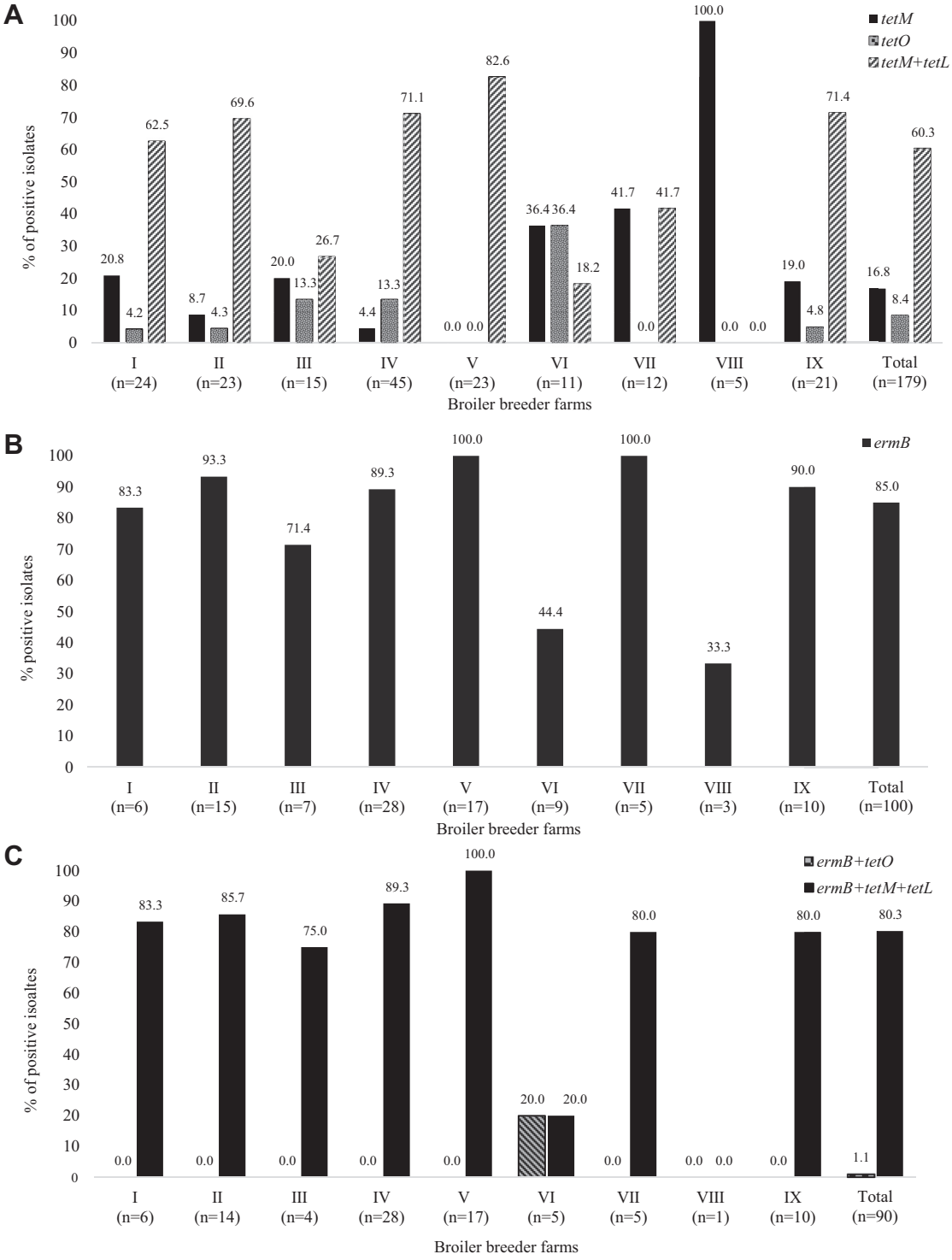

Figure 1. Distribution of resistance genes to tetracycline (A), erythromycin (B), and both tetracycline and erythromycin (C)-resistant *Enterococcus faecalis* isolated from 9 broiler breeder farms. The *tetL* gene for tetracycline resistance (A) and *ermA* and *mef* genes for erythromycin resistance (B) were not detected in any of the isolates. The distribution of antimicrobial resistance genes showed a significant difference among 9 broiler breeder farms ($P < 0.05$).

for facilitating cell-to-cell dissemination (Rosvoll et al. 2012), HLGR *E. faecalis* from breeders could also be the transmission to commercial chickens.

The genetic diversity was related to the organization of IS*256* at both ends of this gene (Kilbi et al., 2006; Watanabe et al., 2009). In this study, IS*256*-flanking pattern types C (50%), A (41.7%), and D (8.3%) were detected, and the prevalence of individual types was different depending on each farm. Although Watanabe et al. (2009) reported that IS*256*-flanking pattern type C was more prevalent than other patterns in the hospital Enterococci isolates, no relationship between the IS*256*-flanking patterns and resistance level to aminoglycoside was evident till now, nor the presence of genes for

Table 3. Characteristics of 24 high-level gentamicin-resistant *Enterococcus faecalis* isolated from 9 broiler breeder farms.

Farm	Strains	AME gene	IS256-*flanking* pattern	Antimicrobial resistance phenotype	Antimicrobial resistance genotype	Virulence factor	MICs (μg/mL)		
							G	K	S
I	143	*aac(6′)Ie-aph(2″)-la*	A	DOX-HLG-HLK-TE	*tetM, tetL*	*ace, asa1, efaA, gelE*	>2,048	>2,048	<256
I	225	*aac(6′)Ie-aph(2″)-la*	A	DOX-E-HLG-HLK-TE	*ermB, tetM, tetL*	*ace, efaA, gelE*	2,048	>2,048	<256
I	227	*aac(6′)Ie-aph(2″)-la*	A	DOX-E-HLG-HLK-TE	*ermB, tetM, tetL*	*ace, efaA, gelE*	2,048	>2,048	<256
I	228	*aac(6′)Ie-aph(2″)-la*	A	DOX-E-HLG-HLK-TE	*ermB, tetM, tetL*	*ace, efaA, gelE*	2,048	>2,048	<256
I	224	*aac(6′)Ie-aph(2″)-la*	A	HLG-HLK-RA	NT	*ace, efaA, gelE*	>2,048	>2,048	<256
I	226	*aac(6′)Ie-aph(2″)-la*	A	HLG-HLK-RA	NT	*ace, efaA, gelE*	>2,048	>2,048	<256
II	167	*aac(6′)Ie-aph(2″)-la, ant(6)-Ia*	D	C-DOX-E-HLG-HLK-HLS-TE	*ermB, tetM, tetL*	*ace, asa1, efaA, gelE*	1,024	>2,048	>2,048
IV	103	*aac(6′)Ie-aph(2″)-la*	C	AM-C-HLG-HLK-HLS	NT	*ace, asa1, efaA, gelE*	>2,048	>2,048	>2,048
IV	95	*aac(6′)Ie-aph(2″)-la*	C	C-DOX-E-HLG-HLK-HLS-TE	*ermB, tetM, tetL*	*ace, asa1, efaA, gelE*	>2,048	>2,048	>2,048
IV	97	*aac(6′)Ie-aph(2″)-la*	C	C-DOX-E-HLG-HLK-HLS-TE,	*ermB, tetM, tetL*	*ace, asa1, efaA, gelE*	>2,048	>2,048	>2,048
IV	98	*aac(6′)Ie-aph(2″)-la*	C	C-DOX-E-HLG-HLK-HLS-TE	*ermB, tetM, tetL*	*ace, asa1, efaA, gelE*	>2,048	>2,048	>2,048
IV	99	*aac(6′)Ie-aph(2″)-la*	C	C-DOX-E-HLG-HLK-HLS-TE	*ermB, tetM, tetL*	*ace, asa1, efaA, gelE*	>2,048	2,048	2,048
IV	101	*aac(6′)Ie-aph(2″)-la*	C	C-DOX-E-P-HLG-HLK-RA-TE	*ermB, tetM, tetL*	*ace, asa1, efaA, gelE*	>2,048	>2,048	>2,048
IV	102	*aac(6′)Ie-aph(2″)-la*	C	C-DOX-E-HLG-HLK-HLS-TE	*ermB, tetM, tetL*	*ace, asa1, efaA, gelE*	>2,048	>2,048	>2,048
IV	105	*aac(6′)Ie-aph(2″)-la*	C	C-DOX-E-HLG-HLK-TE	*ermB, tetM, tetL*	*ace, asa1, efaA, gelE*	>2,048	>2,048	>2,048
IV	108	*aac(6′)Ie-aph(2″)-la*	C	C-DOX-E-HLG-HLK-HLS-TE,	*ermB, tetM, tetL*	*asa1, efaA, gelE*	>2,048	>2,048	>2,048
IV	112	*aac(6′)Ie-aph(2″)-la*	C	C-DOX-E-HLG-HLK-HLS-TE	*ermB, tetM, tetL*	*ace, asa1, efaA, gelE*	>2,048	>2,048	>2,048
IV	96	*aac(6′)Ie-aph(2″)-la*	C	DOX-HLG-HLK-RA-TE	*tetM, tetL*	*ace, efaA, gelE*	>2,048	>2,048	<256
IV	92	*aac(6′)Ie-aph(2″)-la, ant(6)-Ia*	D	C-DOX-E-HLG-HLK-HLS- TE	*ermB, tetM, tetL*	*ace, asa1, efaA, gelE*	>2,048	>2,048	>2,048
VI	1	*aac(6′)Ie-aph(2″)-la*	A	DOX-HLG-HLK-TE	*tetM, tetL*	*ace, asa1, efaA, gelE*	>2,048	>2,048	<256
VII	212	*aac(6′)Ie-aph(2″)-la*	A	CIP-HLG-HLK-RA	NT	*ace, efaA, gelE*	>2,048	>2,048	<256
VII	211	*aac(6′)Ie-aph(2″)-la*	A	HLG-HLK-RA	NT	*ace, efaA, gelE*	>2,048	>2,048	<256
VII	210	*aac(6′)Ie-aph(2″)-la*	C	HLG-HLK-RA	NT	*ace, efaA, gelE*	>2,048	>2,048	<256
IX	229	*aac(6′)Ie-aph(2″)-la*	A	HLG-HLK-RA	NT	*ace, efaA, gelE*	>2,048	>2,048	<256

Abbreviations: AM, ampicillin; C, chloramphenicol; CIP, ciprofloxacin; DOX, doxycycline; E, erythromycin; G, gentamicin; HLGR, high-level gentamicin; HLK; high-level kanamycin; HLS, high-level streptomycin; K, kanamycin; NT, not tested; P, penicillin; RA, rifampin; S, streptomycin; TE, tetracycline.

Table 4. Characteristics of 9 high-level ciprofloxacin-resistant *Enterococcus faecalis* isolated from 9 broiler breeder farms.

Farm	Strains	Amino acid change		Antimicrobial resistance phenotype	Antimicrobial resistance genotype	Virulence factor	MICs (μg/mL)	
		gyrA	*parC*				Ciprofloxacin	Enrofloxacin
I	148	S83I	S80I	HLC	NT	*ace, asa1, efaA, gelE*	64	128
III	63	S83I	S80I	HLC	NT	*ace, efaA, gelE*	64	128
III	65	S83I	S80I	HLC	NT	*ace, asa1, efaA, gelE*	64	64
III	67	S83I	S80I	HLC	NT	*ace, asa1, efaA, gelE*	64	64
III	82	S83I	S80I	HLC-E	*ermB*	*ace, efaA, gelE*	64	128
III	62	S83I	S80I	HLC-E-RA-TE	*ermB*	*ace, efaA, gelE*	64	64
IV	100	S83Y	S80I	C-HLC-DOX-E-HLK-HLS-TE	*ermB, tetM, tetL, ant(6)-Ia*	*ace, asa1, efaA, gelE*	64	64
VI	131	S83I	S80I	HLC-E-HLK	*ermB*	*ace, efaA, gelE*	64	64
IX	161	S83I	S80I	HLC-DOX-TE	*tetM, tetL*	*ace, efaA, gelE*	64	64

Abbreviations: C, chloramphenicol; CIP, ciprofloxacin; DOX, doxycycline; E, erythromycin; HLC, high-level ciprofloxacin; HLK; high-level kanamycin; HLS, high-level streptomycin; NT, not tested; RA, rifampin; TE, tetracycline.

virulence determination. Therefore, the significance of IS*256*-flanking patterns for HLGR is still unknown (Kilbi et al., 2006; Watanabe et al., 2009). The *ant(6′)-Ia* gene, which is the most common AME gene among the HLS-resistant isolates (Udo et al., 2004), was detected in 2 (10%) of 24 HLGR isolates in this study.

Fluoroquinolone resistance is essentially mediated by the alteration of target enzymes such as DNA gyrase and topoisomerase IV encoded by the *gyrA* gene and *parC* gene, respectively (Petersen and Jensen, 2004; Lee et al., 2005; Oyamada et al., 2006). In this study, 35 (15.3%) of the 229 *E. faecalis* isolates showed resistance to CIP, which is lower than those reported in chicken meat (43.8%) in Korea (Kim et al., 2018). However, the prevalence of HLCR isolates (25.7%) was similar to that of chicken meat (24.9%) in Korea (Kim et al., 2018). Although the results showed a low resistance rate to fluoroquinolones in broiler breeder farms, the use of CIP has been increasing in the poultry industry in Korea (APQA, 2017). Therefore, the occurrence of HLCR isolates in broiler breeder farms might be continuously increased.

Seventeen (70.8%) of the 24 HLGR *E. faecalis* and 3 (33.3%) of the 9 HLCR *E. faecalis* isolates showed MDR. Among both these MDR isolates, the most common resistance pattern included resistance to TE, E, and aminoglycosides, which is widely used in the poultry industry in Korea (APQA, 2017). Similar resistance profiles in MDR isolates with their corresponding genotypes within and between the farms and the occurrence of the same profiles in different farms may be an indication of the ease of spread of enterococcus strains (Nowakiewicz et al., 2017a). This study also suggest that the different resistance levels and patterns in MDR isolates may be related to the use of diverse antimicrobials during poultry production on each farm (Fei et al., 2018; Li et al., 2018).

Virulence factors contribute to the pathogenesis of enterococcal infections through the mediation of adhesion, colonization, and invasion into the host tissues, modulation of the host immunity, and extracellular production of enzymes and toxins, which enhance the severity of the infection (Strateva et al., 2016). In this study, *ace* (collagen-binding protein), *efaA* (cell wall–associated protein involved in immune evasion), and *gelE* (gelatinase) were present in all HLCR and HLGR isolates. These results are similar to that of *E. faecalis* isolated from poultry in a previous study (Olsen et al., 2011). Therefore, this study indicates that *E. faecalis* isolates from broiler breeders could be a reservoir of genes important for the pathogenic potential of the poultry industry involving the transmission of antimicrobial related genes. This is the first study to investigate the prevalence and characteristics of antimicrobial resistant *E. faecalis* isolated from broiler breeders in Korea. Our findings indicate that the genotypic characteristics of *E. faecalis* from commercial broilers have recently emerged in broiler breeders. Therefore, constant epidemiological monitoring and studies at the breeder level are required to prevent the pyramidal transmission of antimicrobial-resistant *E. faecalis*.

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REFERENCES

Aarestrup, F. M., Y. Agerso, P. Gerner-Smidt, M. Madsen, and L. B. Jensen. 2000. Comparison of antimicrobial resistance phenotypes and resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* from humans in the community, broiler, and pigs in Denmark. Diagn. Microbiol. Infect. Dis. 37:127–137.

Animal and Plant Quarantine Agency (APQA) 2017. National Antimicrobial Resistance Monitoring Program. Animal and Plant Quarantine Agency, Gimcheon, Republic of Korea.

Agersø, Y., A. G. Pedersen, and F. M. Aarestrup. 2006. 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. 57:832–839.

Brtková, A., M. Revallová, and H. Bujdáková. 2011. Detection of tetracycline and macrolide resistance determinants in enterococci of animal and environmental origin using multiplex PCR. Folia. Microbiol. (Praha) 56:236–240.

Cauwerts, K., A. Decostere, E. M. De Graef, F. Haesebrouck, and F. Pasmans. 2007. High prevalence of tetracycline resistance in Enterococcus isolates form broilers carrying the erm (B) gene. Avian Pathol. 36:395–399.

Choi, J. M., and G. J. Woo. 2013. Molecular characterization of high-level gentamicin-resistant Enterococcus faecalis from chicken meat in Korea. Int. J. Food Microbial. 165:1–6.

Choi, J. M., and G. J. Woo. 2015. Transfer of tetracycline resistance genes with aggregation substance in food-borne *Enterococcus faecalis*. Curr. Microbiol. 70:476–484.

Clark, N. C., Ø. Olsvik, J. M. Swenson, C. A. Spiegel, and F. C. Tenover. 1999. Detection of a streptomycin/spectinomycin adenylyltransferase gene (*aadA*) in *Enterococcus faecalis*. Antimicrob. Agents Chemother. 43:157–160.

Clinical and Laboratory Standards Institute (CLSI) 2013. Performance Standards for Antimicrobial Susceptibility Testing, M100-S23. Clinical and Laboratory Standards Institute, Wayne, PA.

Di Cesare, A. D., G. M. Luna, C. Vignaroli, S. Pasquaroli, S. Tota, P. Paroncini, and F. Biavasco. 2013. Aquaculture can promote the presence and spread of antibiotic-resistant enterococci in marine sediments. PLoS One 26:e62838.

Diarra, M. S., H. Rempel, J. Champagne, L. Masson, J. Pritchard, and E. Topp. 2010. Distribution of antimicrobial resistance and virulence genes in *Enterococcus* spp. and characterization of isolates from broiler chickens. Appl. Environ. Microbiol. 76:8033–8043.

Dierikx, C. M., J. V. D. Goot, H. E. Smith, A. Kant, and D. J. Mevius. 2013. Presence of ESBL/AmpC-producing *Escherichia coli* in the broiler production pyramid: a descriptive study. PLoS One 8:e97005.

Dutka-Malen, S., S. Evers, and P. Courvalin. 1995. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J. Clin. Microbiol. 33:24–27.

El Amin, N., S. Jalal, and B. Wretlind. 1999. Alterations in *gyrA* and *parC* associated with fluoroquinolone resistance in *Enterococcus faecium*. Antimicrob. Agents Chemother. 43:947–949.

EUCAST. 2019. European Committee on Antimicrobial Susceptibility Testing. Breakpoint Tables for Interpretation of MICs and Zone Diameters. Version 9.0. Accessed Jan. 2019. http://www.eucast.org.

Fei, X., K. Yin, C. Yin, Y. Hu, J. Li, Z. Zhou, Y. Tian, S. Geng, X. Chen, Z. Pan, Q. Li, and X. Jiao. 2018. Analysis of prevalence and molecular typing reveal the spread of antimicrobial-resistant *Salmonella* infection across two breeder chicken farms. Poult. Sci. 97:4374–4383.

Giovanetti, E., A. Brenciani, R. Lupidi, M. C. Roberts, and P. Varaldo. 2003. Presence of the *tet*(O) gene in erythromycin- and tetracycline-resistant strains of *Streptococcus pyogenes* and linkage with either the *mef*(A) or the *erm*(A) gene. Antimicrob. Agents Chemother. 47:2844–2849.

Ha, J. S., K. W. Seo, Y. B. Kim, M. S. Kang, C. Song, and Y. J. Lee. 2018. Prevalence and characterization of *Salmonella* in two integrated broiler operations in Korea. Ir. Vet. J. 71:3.

Han, D., T. Unno, J. Jang, K. Lim, S.-N. Lee, G. Ko, M. J. Sadowsky, and H.-G. Hur. 2011. The occurrence of virulence traits among high-level aminoglycosides resistant Enterococcus isolates obtained from feces of humans, animals, and birds in South Korea. Int. J. Food Microbiol. 144:387–392.

Jeon, H. Y., K. W. Seo, Y. B. Kim, D. K. Kim, S. W. Kim, and Y. J. Lee. 2019. Characteristics of third-generation cephalosporin-resistant Salmonella from retail chicken meat produced by integrated broiler operations. Poult. Sci. 98:1766–1774.

Kehrenberg, C., and S. Schwarz. 2006. Distribution of florfenicol resistance genes fexA and cfr among chloramphenicol-resistant Staphylococcus isolates. Antimicrob. Agents Chemother. 50:1156–1163.

Kim, M. S., T.-H. Lim, J.-H. Jang, D.-H. Lee, B.-H. Lee, B.-Y. Kim, J.-H. Kwon, S.-W. Choi, J.-Y. Noh, Y.-H. Hong, S.-B. Lee, S.-Y. Yang, H.-J. Lee, J.-B. Lee, S.-Y. Park, I.-S. Choi, and C.-S. Song. 2012. Prevalence and antimicrobial resistance of *Salmonella* species isolated from chicken meats produced by different integrated broiler operations in Korea. Poult. Sci. 91:2370–2375.

Kim, Y. B., H. J. Seo, K. W. Seo, H. Y. Jeon, D. K. Kim, S. W. Kim, S. K. Lim, and Y. J. Lee. 2018. Characteristics of high-level ciprofloxacin-resistant enterococcus faecalis and enterococcus faecium from retail chicken meat in Korea. J. Food Prot. 81:1357–1363.

Kim, Y. B., K. W. Seo, H. Y. Jeon, S. K. Lim, H. W. Sung, and Y. J. Lee. 2019. Molecular characterization of erythromycin and tetracycline-resistant Enterococcus faecalis isolated from retail chicken meats. Poult. Sci. 98:977–983.

Kilbi, N., K. B. Slama, A. Masmoudi, S. Gharbi, F. Ruiz-Larrea, C. Fendri, A. Boudabous, and C. Torres. 2006. Diversity of structures carrying the aac(6')-aph(2") gene in clinical Enterococcus faecalis and Enterococcus faecium strains isolated in Tunisia. J. Chemother. 18:353–359.

Kwak, Y. G., Q. C. Truong-Bolduc, H. B. Kim, K.-H. Song, E. S. Kim, and D. C. Hooper. 2013. Association of *norB* overexpression and fluoroquinolone resistance in clinical isolates of *Staphylococcus aureus* from Korea. J. Antimicrob. Chemother. 68:2766–2772.

Leavis, H. L., R. J. L. Willems, J. Top, and M. J. M. Bonten. 2006. 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. 44:1059–1064.

Lee, J. K., Y. S. Lee, Y. K. Park, and B. S. Kim. 2005. Mutations in the *gyrA* and *parC* genes in ciprofloxacin-resistant clinical isolates of *Acinetobacter baumannii* in Korea. Microbiol. Immunol. 49:647–653.

Li, X., S. S. Aly, Z. Su, R. V. Pereira, D. R. Williams, P. Rossitto, J. D. Champagne, J. Chase, T. Nguyen, and E. R. Atwill. 2018. Phenotypic antimicrobial resistance profiles of E. coli and Enterococcus from daily cattle in different management units on a central California daily. Clin. Microbiol. 7:311.

Niu, H., H. Yu, T. Hu, G. Tian, L. Zhang, X. Guo, H. Hu, and Z. Wang. 2016. The prevalence of aminoglycoside-modifing enzyme and virulence genes among enterococci with high-level aminoglycoside resistance in inner Mongolia, China. Braz. J. Microbiol. 47:691–696.

Nowakiewicz, A., G. Ziółkowska, A. Trościańczyk, P. Zięba, and S. Gnat. 2017a. Determination of antimicrobial resistance of Enterococcus strains isolated from pigs and their genotypic characterization by method of amplification of DNA fragments surrounding rare restriction sites (ADSRRS fingerprinting). J. Med. Microbiol. 66:175–183.

Nowakiewicz, A., G. Ziólkowska, A. Trościańczyk, P. Zięba, and S. Gnat. 2017b. Determination of resistance and virulence genes in *Enterococcus faecalis* and *E. faecium* strains isolated from poultry and their genotypic characterization by ADSRRS-fingerprinting. Poult. Sci. 96:986–996.

Olsen, R. H., H. C. Schønheyder, H. Christensen, and M. Bisgaaard. 2011. *Enterococcus faecalis* of human and poultry origin share virulence genes supporting the zoonotic potential of *E. faecalis*. Zoonoses Public Health 59:256–263.

Osman, K. M., A. D. Kappell, M. Elhadidy, F. Elmougy, W. A. A. El-Ghany, A. Orabi, A. S. Mubarak, T. M. Dawoud, H. A. Hemeg, I. M. I. Moussa, A. M. Hessain, and H. M. Y. Yousef. 2018. Poultry hatcheries as potential reservoirs for antimicrobial-resistant *Escherichia coli*: a risk to public health and food safety. Sci. Rep. 8:5859.

Oyamada, Y., H. Ito, M. Inoue, and J. Yamagishi. 2006. Topoisomerase mutations and efflux are associated with fluoroquinolone resistance in Enterococcus faecalis. J. Med. Microbiol. 55:1395–1401.

Petersen, A., and J. B. Jensen. 2004. Analysis of *gyrA* and *parC* mutations in enterococci from environmental samples with reduced susceptibility to ciprofloxacin. FEMS Microbiol. Lett. 231:73–76.

Poeta, P., D. Costa, Y. Sáenz, N. Klibi, F. Ruiz-Larrea, J. Rodrigues, and C. Torres. 2005. Characterization of antibiotic resistance genes and virulence factors in faecal enterococci of wild animals in Portugal. J. Vet. Med. B. Infect. Dis. Vet. Public Health 52:396–402.

Rizzotti, L., D. Simeoni, P. Cocconcelli, S. Gazzola, F. Dellaglio, and S. Torriani. 2005. Contribution of enterococci to the spread of antibiotic resistance in the production chain of swine meat commodities. J. Food Prot. 68:955–965.

Rosvoll, T. C., B. L. Lindstad, T. M. Lunde, K. Hegstad, B. Aasnaes, A. M. Hammerum, C. H. Lester, G. S. Simonsen, A. Sundsfjord, and T. Pedersen. 2012. Increased high-level gentamicin resistance in invasive *Enterococcus faecium* is associated with *aac(6′)-Ie-aph(2″)-Ia*-encoding transferable megaplasmids hosted by major

hospital-adapted lineages. FEMS Immunol. Med. Microbiol. 66:166–176.

Seo, K. W., Y. B. Kim, H. Y. Jeon, S. K. Lim, and Y. J. Lee. 2018. Comparative genetic characterization of third-generation cephalosporin-resistant *Escherichia coli* from chicken meat produced by integrated broiler operations in South Korea. Poult. Sci. 97:2871–2879.

Strateva, T., D. Atanasova, E. Savov, G. Petrova, and I. Mitov. 2016. Incidence of virulence determinants in clinical *Enterococcus faecalis* and *Enterococcus faecium* isolates collected in Bulgaria. Braz. J. Infect. Dis. 20:127–133.

Udo, E. E., N. Al-Swith, P. John, L. E. Jacob, and S. Mohanakrishnan. 2004. Characterization of high-level aminoglycoside-resistant enterococci in Kuwait hospitals. Microb. Drug Resist. 10:139–145.

United States Department of Agriculture (USDA) 2012. National Poultry Improvement Plan and Auxiliary Provisions. APHIS Publication, Washington, D C.

Vakulenko, S. B., S. M. Donabedian, M. Anatoliy, M. J. Zervos, S. A. Lerner, J. W. Chow, and A. M. Voskresenskiy. 2003. Multiplex PCR for detection of aminoglycoside resistance genes in Enterococci. Antimicrob. Agents Chemother. 47:1423–1426.

Watanabe, S., N. Kobayashi, D. Quiñones, S. Nagashima, N. Uehara, and N. Watanabe. 2009. Genetic diversity of enterococci harboring the high-level gentamicin resistance gene *aac(6')-Ie-aph(2'')-Ia* or *aph(2'')-Ie* in a Japanese hospital. Microb. Drug Resist. 15:185–194.