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Original Article

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Multiplex PCR analysis of virulence genes and their influence on antibiotic resistance in *Enterococcus* spp. isolated from broiler chicken

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Conflict of Interest
The authors declare no conflicts of interest.

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Conceptualization: Joh S. Investigation: Bae Y, Jeon E. Resources: Bae Y; Supervision: Kwon Y; Validaion: Kwon Y; Writing - original draft: Song H, Joh S.

ABSTRACT

Enterococcus spp. are opportunistic pathogens that cause lameness in broiler chickens, resulting in serious economic losses worldwide. Virulence of *Enterococcus* spp. is associated with several putative virulence genes including *fsr*, *efm*, *esp*, *cylA*, *cad1*, *ace*, *gelE*, and *asa1*. In this study, multiplex polymerase chain reaction (PCR) for the simultaneous detection of these virulence genes in *Enterococcus* spp. was developed, and detection limits for *E. faecium*, *E. faecalis*, and *E. hirae* were 64.0 pg/μL, 320.0 pg/μL, and 1.6 ng/μL DNA, respectively. Among 80 *Enterococcus* isolates tested, *efm* and *cad1* were detected in all 26 *E. faecium* samples, and only *cad1* was observed in *E. hirae*. Additionally, the presence of virulence genes in 25 *E. faecalis* isolates were 100% for *cad1*, 88.0% for *gelE*, 64.0% for *fsr*, 44.0% for *asa1*, 16.0% for *cylA*, and 4.0% for *esp*. No virulence genes were found in *E. gallinarum* isolates. A total of 49 isolates were resistant to tigecycline and to at least 2 different classes of antibiotics. The most prevalent resistance was to ciprofloxacin (73.5%), quinupristin/dalfopristin (55.1%), and tetracycline (49.0%). No strains were resistant to vancomycin or linezolid. This is the first multiplex PCR assay to simultaneously detect eight virulence genes in *Enterococcus* spp., and the method provides diagnostic value for accurate, rapid, and convenient detection of virulence genes. Additionally, we report the prevalence of virulence genes and antimicrobial resistance in *Enterococcus* isolates from commercial broiler chickens suffering lameness.

Keywords: Multiplex polymerease chain reaction; virulence genes; antimicrobial drug resistance; *Enterococcus* spp.; broiler chickens

INTRODUCTION

Enterococcus spp. are opportunistic pathogens that inhabit the intestines of many warm-blooded animals, including humans, and have been isolated from several sources such as food, plants, water, and soil [1]. *Enterococcus* spp. can infect birds through various routes including the respiratory system, gastrointestinal tract, and integument, particularly in poultry environments where birds are hatched, reared, or processed. After infection, bacteria circulate in the bloodstream and form abscesses that cause arthritis, osteomyelitis, spondylitis, and femoral head necrosis in broiler and broiler breeder flocks [2-4]. Virulence of *Enterococcus* spp. is associated with several genes including *fsr* (regulator of *gelE* expression),

efm (*E. faecium*-specific cell wall adhesin), *esp* (enterococcal surface protein), *cylA* (hemolysin), *cad1* (pheromone cAD1 precursor lipoprotein), *ace* (collagen-binding cell wall protein), *gelE* (gelatinase), *asa1* (aggregation substance), *acm* (surface-exposed antigen), *cpd1* (pheromone cPD1 lipoprotein), *efaAEfs* (endocarditis-specific antigen), and *sagA* (secreted antigen) [5,6]. These virulence factors are involved in attachment to target organs, colonization, invasion of host tissues, diminished host immune function, and the production of enzymes that increase the severity of infection and regulate extracellular production of toxins [7-9].

The use of antimicrobials to enhance the production of animals for human consumption of meat and eggs is leading to increased antimicrobial resistance worldwide. *Enterococcus* spp. acquire antibiotic resistance via transfer of plasmids and transposons, chromosomal exchange, and spontaneous mutations [6]. Consequently, the use of antibiotics for animal growth promotion exposes humans to antibiotic-resistant pathogens through food and contaminates the environment, which facilitates spreading.

Herein, we developed a multiplex polymerase chain reaction (PCR) method for simultaneous detection of 8 virulence genes in *Enterococcus* spp. and investigated potential virulence factors and antimicrobial resistance using *E. faecium*, *E. faecalis*, *E. hirae*, and *E. gallinarum* isolates from broiler chickens suffering lameness in 2 commercial poultry farms.

MATERIALS AND METHODS

Isolation of bacteria

Enterococcus spp. were isolated from various samples of 13 different flocks suffering from lameness in 2 commercial poultry farms over a period of 7 weeks in 2016. Broilers with lameness varied in age from 2 to 15 days. Swabs from liver, femur, and joint tissue were inoculated onto Enterococcosel agar and blood agar plates (BD Diagnostics, Germany) and cultivated at 37°C for 18–24 h. After the initial growth period, dark brown colonies on selective Enterococcosel agar medium were presumptively characterized as *Enterococcus* spp. and subcultured in brain heart infusion (BHI) agar for *Enterococcus* species-specific identification using 16s rRNA sequencing analysis and conventional biochemical testing, including Gram staining and Vitek2.

DNA extraction

Total genomic DNA was isolated from 80 different bacterial species using a QIAamp DNA mini kit (Qiagen, Germany) according to the manufacturer's instructions.

Oligonucleotide primer design

Specific primers were designed based on published *fsr* (GenBank accession No. JN246675), *efm* (GenBank accession No. AF097414), *esp* (GenBank accession No. AH013271 and JF826520), *cylA* (GenBank accession No. JQ794947 and KY613925), *cad1* (GenBank accession No. AF421355, NC017960, CP003504, and AOSM01000007), *ace* (GenBank accession No. AF159247 and KY613927), *gelE* (GenBank accession No D85393, KT598464, EU423275, and KY613931), and *asa1* (GenBank accession No. KT598461, KY613929, and U91527) gene sequences using OligoAnalyzer (Integrated DNA Technologies, USA). Primer specificity was confirmed by BLAST searches against the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/). The eight pairs of primers and amplicon sizes are listed in **Table 1**.

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Table 1. Sequences of oligonucleotide primers used to detect putative virulence genes in *Enterococcus* isolates

Virulence gene	Gene description	Primer	Sequence (5′ to 3′)	Amplicon size (bp)
fsr	Regulator of *gelE* and *sprE* expression	Forward primer	CAA GGC ACT ATT TCT TAC TTA GG	1,016
		Reverse primer	AGC GCA TAA ATC AAC CAA G	
efm	*E. faecium*-specific cell wall adhesin	Forward primer	GAA AAG TTG TCA GTC GTG G	818
		Reverse primer	TGT TTG TGA CAA ACC TTC ATG	
esp	Enterococcal surface protein	Forward primer	CAT CTT TGA TTC TTG GTT GTC G	695
		Reverse primer	GTT ATA GGT ACG TAT GTT GCA TCA	
cylA	Hemolysin	Forward primer	GAG TTA GAT GAA TAT GGT CAT GGT	521
		Reverse primer	AGA AAC TAG CGA TGT AGG GTA ATA	
cad1	Pheromone cAD1 precursor lipoprotein	Forward primer	TTC CAA AAC TAC GCA CAA CA	423
		Reverse primer	CTT TTT CAG CAG CAT TCA CTA ATT	
ace	Collagen-binding cell wall protein	Forward primer	ATA GAA ACG GAT TTC GGA ACA G	298
		Reverse primer	TCA AAC TCG GCA AGT GAA ATA T	
gelE	Gelatinase	Forward primer	TAT GAC AAT GCT TTT TGG GAT G	208
		Reverse primer	GCA CCC GAA ATA ATA TAA CCC	
asa1	Aggregation substance	Forward primer	AAC AAG CTT GGT CTG TGT ATC	168
		Reverse primer	TCT TCC CCT TTC TTG TTA TGA AC	

Multiplex PCR

PCR assays (20 μL) were conducted using PCR premix (iNtRON Biotechnology, Seongnam, Korea). Each reaction mixture contained 2 μL bacterial genomic DNA, 0.1 μM of each primer for *fsr*, *efm*, *esp*, *cylA*, *gelE*, and *asa1*, 0.16 μM of each primer for *cad1* and *ace*, 2.5 mM of each dNTP, 1.5 mM $MgCl_2$, 10× reaction buffer, and 1 U Taq polymerase. The thermal cycler (Eppendorf, Hamburg, Germany) was programmed for initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and then final elongation at 72°C for 7 min. PCR products were analyzed under UV light after separation by 1.5% agarose gel electrophoresis.

Determining the multiplex PCR detection limit

The detection limit of multiplex PCR assays was determined by testing 5-fold serial dilutions of bacterial DNA of *E. faecalis* (field isolate) containing virulence genes *fsr*, *esp*, *cylA*, *cad1*, *ace*, *gelE*, and *asa1*. Similarly, the sensitivity for detecting the pheromone cAD1 precursor lipoprotein gene and *E. faecium*-specific cell wall adhesin gene was tested using *E. hirae* (field isolate) possessing the *cAD1* gene and *E. faecium* (field isolate) harboring *efm* and *cAD1* genes, respectively.

Sequencing method

To confirm the exact virulence genes, all PCR products amplified by multiplex and simplex PCR were purified using a QIAquick Gel Extraction Kit (Qiagen) and sequencing was performed by Cosmogenetech Co. (Korea). Nucleotide sequences were analyzed by BLAST searching against the NCBI database.

Screening of virulence genes in *Enterococcus* spp.

Multiplex PCR was used to analyze eight virulence genes in 26 *E. faecalis*, 25 *E. faecium*, 20 *E. hirae*, and 9 *E. gallinarum* isolates from liver, femur and joint tissues of broiler chickens suffering lameness.

Antibiotic susceptibility testing

The 16 antimicrobial minimal inhibitory concentrations (MICs) of *Enterococcus* isolates were determined using the Sensititre automated system (Trek Diagnostic Systems, USA) according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2013). Ampicillin,

chloramphenicol, ciprofloxacin, daptomycin, erythromycin, florfenicol, gentamicin, kanamycin, linezolid, quinupristin-dalfopristin, salinomycin, streptomycin, tetracycline, tigecycline, tylosin, and vancomycin antibiotics were tested with Sensititre plates. *E. faecalis* ATCC 29212 and *S. aureus* ATCC 25923 strains were used as quality controls.

RESULTS

Development of multiplex PCR

Multiplex PCR assays were conducted using various concentrations of each primer pair to optimize the PCR conditions. Increasing the primer concentrations for *cad1* and *ace* genes facilitated the production of the expected PCR products at low DNA template concentrations. Multiplex PCR mixtures containing *efm* and *cAD1* genes in *E. faecium* genomic DNA generated 2 bands on 1.5% agarose gels: an 818 bp band for *efm* and a 423 bp band for *cad1* (**Fig. 1A**, lane 1). Samples containing *fsr*, *esp*, *cylA*, *cad1*, *ace*, *gelE*, and *asa1* genes in *E. faecalis* genomic DNA yielded 7 bands: 1,016 bp for *fsr*, 695 bp for *esp*, 521 bp for *cylA*, 423 bp for *cad1*, 298 bp for *ace*, 208 bp for *gelE*, and 168 bp for *asa1* (**Fig. 1B**, lane 10). Similarly, a multiplex PCR mixture containing only *E. hirae* genomic DNA amplified the *cad1* gene (**Fig. 1A**, lane 19).

Sequencing of PCR products

The eight amplicons were successfully amplified in eight single PCRs containing each gene-specific primer pair, as indicated by the expected PCR products on 1.5% agarose gels (**Fig. 1A**, lanes 2–8; 1B, lanes 11–18; and 1C, lanes 20–27). All PCR products amplified in multiplex and single PCRs were confirmed by DNA sequencing, and sequence analysis showed that both multiplex and single PCR sequences were identical in the amplified regions (data not shown).

Detection limit of multiplex PCR

To examine the detection limit of optimized multiplex PCR, genomic DNA from *E. faecium* (containing *efm* and *cAD1* genes), *E. faecalis* (containing *fsr*, *esp*, *cylA*, *cad1*, *ace*, *gelE*, and *asa1* genes), and *E. hirae* (containing the *cad1* gene) was prepared at DNA concentrations ranging from 12.8 pg to 200 ng. Multiplex PCR using the eight gene-specific primer pairs achieved detection limits of 64.0 pg/μL, 320.0 pg/μL, and 1.6 ng/μL DNA for *E. faecium*, *E. faecalis*, and *E. hirae*, respectively (**Fig. 2**).

Screening of virulence genes in *Enterococcus* spp.

Optimized multiplex PCR was conducted to confirm the presence of the 8 virulence genes (*fsr*, *efm*, *esp*, *cylA*, *cad1*, *ace*, *gelE*, and *asa1*) in 80 *Enterococcus* strains isolated from clinical samples. Virulence gene profiles for *E. faecium*, *E. faecalis*, *E. hirae*, and *E. gallinarum* isolates are shown in **Table 2**. All 26 *E. faecium* isolates were positive for the *E. faecium*-specific cell wall adhesin gene *efm* and the pheromone cAD1 precursor lipoprotein gene cAD1. In contrast, the *fsr*, *esp*, *cylA*, *ace*, *gelE*, and *asa1* genes were not detected. Among the 25 *E. faecalis* isolates, positive results were obtained for 16 (64.0%) isolates for *fsr*, 1 (4.0%) for *esp*, 4 (16.0%) for *cylA*, 22 (88.0%) for *gelE*, and 11 (44.0%) for *asa1*. Additionally, *cAD1* and *ace* genes were positive in all *E. faecalis* isolates. Although all *E. hirae* isolates were positive for *cAD1*, no other virulence genes were found in *E. hirae* isolates. All 8 virulence factors were negative in 9 *E. gallinarum* isolates (**Table 2**). No significant difference in the prevalence of *fsr*, *efm*, *esp*, *cylA*, *cad1*, *ace*, *gelE*, or *asa1* was observed between isolates from farms A or B.

Antibiotic susceptibility

The MICs for 22 *E. faecium*, 22 *E. faecalis*, 3 *E. hirae*, and 2 *E. gallinarum* isolates were tested, and resistance breakpoints are presented in **Table 3**. All 49 isolates were resistant to tigecycline, while none were resistant to linezolid or vancomycin. Among the 16 antimicrobial agents tested, resistance to daptomycin (77.3% in *E. faecium* and 100% in *E. gallinarum*), erythromycin (50% in *E. faecalis* and 66.7% in *E. hirae*), ciprofloxacin (100% in *E. faecium*, 54.6% in *E. faecalis*, and 66.7% in *E. gallinarum*), quinupristin-dalfopristin (95.5% in *E. faecalis*), tetracycline (63.8% in *E. faecalis* and 100% in *E. gallinarum*), and tylosin (40.9% in *E. faecalis* and 66.7% in *E. hirae*) was most frequent among *Enterococcus* isolates. In addition, low levels of resistance to

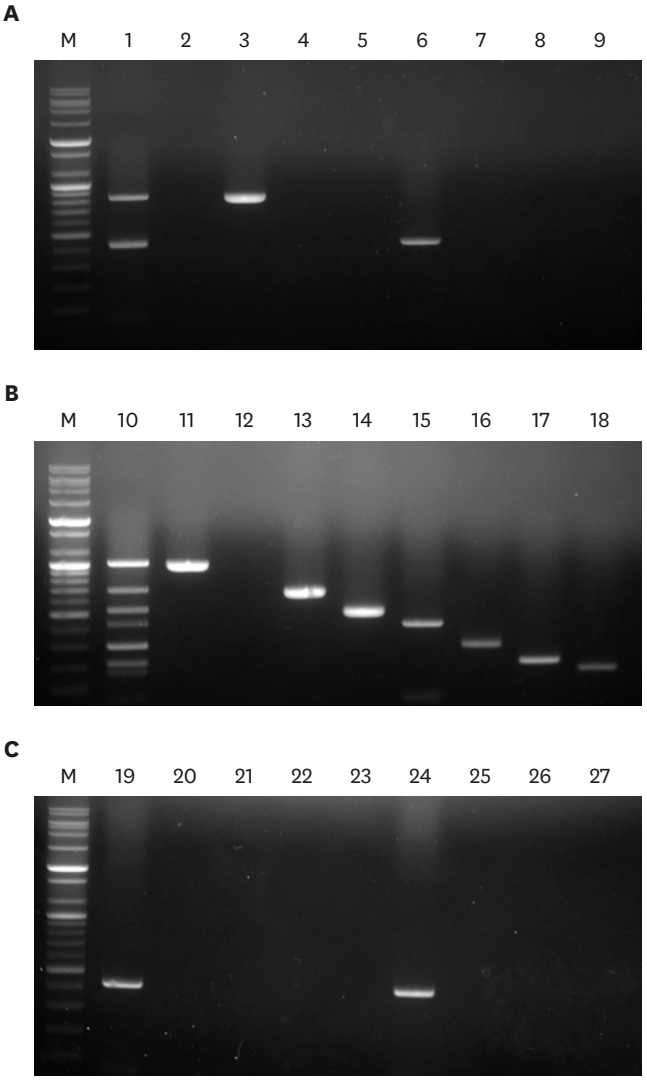

Fig. 1. Validation of eight primer pairs by simplex and multiplex PCR assays. To confirm that primer pairs correctly amplified their respective regions they were evaluated in simplex and multiplex PCR assays using (A) *E. faecium*, (B) *E. faecalis*, and (C) *E. hirae* genomic DNA isolated from broiler chickens suffering lameness. Lane M, 100 bp DNA markers; lane 1, multiplex PCR with *E. faecium* genomic DNA; lane 10, multiplex PCR with *E. faecalis* genomic DNA; lane 19, multiplex PCR with *E. hirae* genomic DNA; lanes 2, 11, and 20, simplex PCR with only *fsr* primers; lanes 3, 12, and 21, simplex PCR with only *efm* primers; lanes 4, 13, and 22, simplex PCR with only *esp* primers; lanes 5, 14, and 23, simplex PCR with only *cylA* primers; lanes 6, 15, and 24, simplex PCR with only *cad1* primers; lanes 7, 16, and 25, simplex PCR with only *ace* primers; lanes 8, 17, and 26, simplex PCR with only *gelE* primers; lanes 9, 18, and 27, simplex PCR with only *asa1* primers.
PCR, polymerase chain reaction.

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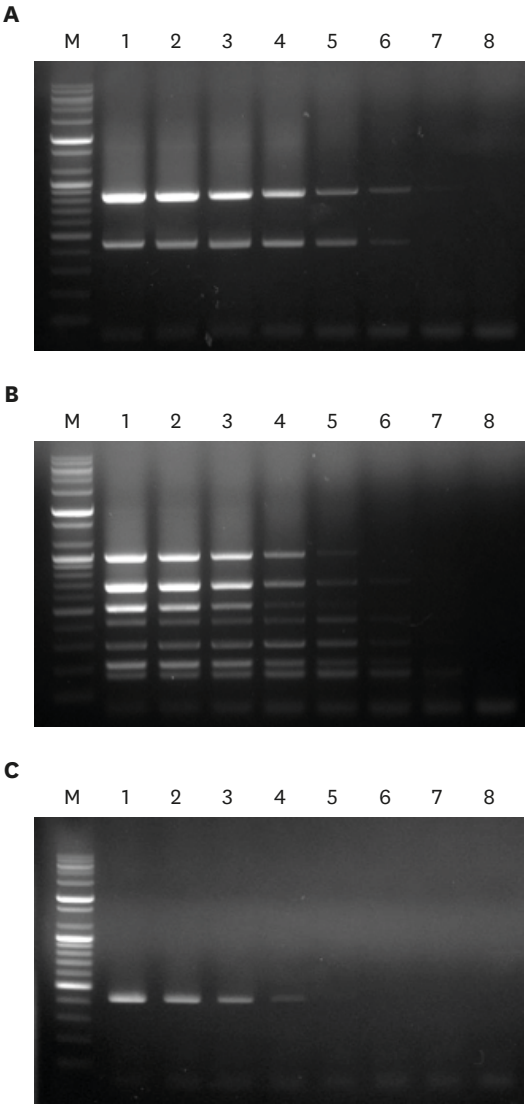

Fig. 2. Evaluation of multiplex PCR detection limits using genomic DNA from (A) *E. faecalis* (harboring *fsr*, *esp*, *cylA*, *cad1*, *ace*, *gelE*, and *asa1*), (B) *E. faecium* (harboring *efm* and *cad1*), and (C) *E. hirae* (harboring *cad1*). Lane M, 100 bp DNA markers; lane 1, 200.0 ng/μL DNA; lane 2, 40.0 ng/μL DNA; lane 3, 8.0 ng/μL DNA; lane 4, 1.6 ng/μL DNA; lane 5, 320.0 pg/μL DNA; lane 6, 64.0 pg/μL DNA; lane 7, 12.8 pg/μL DNA; lane 8, negative control. PCR, polymerase chain reaction.

chloramphenicol, kanamycin, and streptomycin were observed. Multi-resistance patterns of isolates are shown in **Table 4**. All *Enterococcus* isolates were resistant to at least 2 different classes of antimicrobials, with 43 (87.8%) of 49 strains being resistant to 3 or more antimicrobials.

DISCUSSION

Many *Enterococcus* spp. from various sources including humans, animals, birds, and the environment are generally considered harmless, although some can become opportunistic pathogens by acquiring antibiotic resistance and putative virulence genes from other bacteria through horizontal gene transfer [10-15]. Herein, the pathogenicity of isolates was evaluated by screening for the presence of virulence factors, and a multiplex PCR method

Table 2. Prevalence of virulence genes in 80 enterococcal isolates from liver, joint, and femur samples from commercial broiler chickens suffering lameness

Species	Farm	Origin	Number of virulence genes (%)							
			fsr	*efm*	*esp*	*cylA*	*cad1*	*ace*	*gelE*	*asa1*
E. faecium (n = 26)	A	Liver (n = 3)	0 (0.0)	3 (100.0)	0 (0.0)	0 (0.0)	3 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
		Joint (n = 7)	0 (0.0)	7 (100.0)	0 (0.0)	0 (0.0)	7 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
		Femur (n = 5)	0 (0.0)	5 (100.0)	0 (0.0)	0 (0.0)	5 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
	B	Liver (n = 3)	0 (0.0)	3 (100.0)	0 (0.0)	0 (0.0)	3 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
		Joint (n = 4)	0 (0.0)	4 (100.0)	0 (0.0)	0 (0.0)	4 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
		Femur (n = 4)	0 (0.0)	4 (100.0)	0 (0.0)	0 (0.0)	4 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Total		0 (0.0)	26 (100.0)	0 (0.0)	0 (0.0)	26 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
E. faecalis (n = 25)	A	Liver (n = 1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100.0)	1 (100.0)	1 (100.0)	0 (0.0)
		Joint (n = 6)	4 (66.7)	0 (0.0)	1 (16.7)	2 (33.3)	6 (100.0)	6 (100.0)	6 (100.0)	3 (50.0)
		Femur (n = 5)	4 (80.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (100.0)	5 (100.0)	5 (100.0)	4 (80.0)
	B	Liver (n = 5)	2 (40.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (100.0)	5 (100.0)	2 (40.0)	1 (20.0)
		Joint (n = 6)	4 (66.7)	0 (0.0)	0 (0.0)	2 (33.3)	6 (100.0)	6 (100.0)	6 (100.0)	3 (50.0)
		Femur (n = 2)	2 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (100.0)	2 (100.0)	2 (100.0)	0 (0.0)
	Total		16 (64.0)	0 (0.0)	1 (4.0)	4 (16.0)	25 (100.0)	25 (100.0)	22 (88.0)	11 (44.0)
E. hirae (n = 20)	A	Liver (n = 7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	7 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
		Joint (n = 3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
		Femur (n = 8)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	8 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
	B	Liver (n = 0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
		Joint (n = 2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
		Femur (n = 0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Total		0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	20 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
E. gallinarum (n = 9)	A	Liver (n = 0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
		Joint (n = 3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
		Femur (n = 0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	B	Liver (n = 3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
		Joint (n = 2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
		Femur (n = 1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Total		0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

was developed for the simultaneous detection of 8 enterococcal virulence genes (*fsr*, *efm*, *esp*, *cylA*, *cad1*, *ace*, *gelE*, and *asa1*). All primer pairs were validated using simplex PCR before being employed in multiplex PCR and only 1 amplified product of the expected size was observed in each simplex PCR as expected. In addition, we confirmed that each primer pair amplified the correct putative virulence gene by DNA sequencing.

To evaluate the sensitivity of multiplex PCR, DNA isolated from *E. faecium* harboring *efm* and *cAD1* genes, *E. faecalis* harboring *fsr*, *esp*, *cylA*, *cad1*, *ace*, *gelE*, and *asa1* genes, and *E. hirae* harboring the *cad1* gene was tested at different concentrations. The detection limits of *E. faecium*, *E. faecalis*, and *E. hirae* were 64.0 pg/μL, 320.0 pg/μL, and 1.6 ng/μL DNA, respectively. Moreover, multiplex PCR was carried out using 5 μL heat-treated suspensions from individual colonies as DNA template, and the sensitivity results mirrored the detection limits obtained using genomic DNA isolated with a DNA extraction kit (data not shown). Thus, the multiplex PCR method for the 8 virulence genes was shown to be a reliable and rapid alternative for phenotypic testing and simplex PCR.

Furthermore, the multiplex PCR method was applied to screen 26 *E. faecium*, 25 *E. faecalis*, 20 *E. hirae*, and 9 *E. gallinarum* isolates from broiler chickens suffering lameness for the presence of these virulence genes, and the results showed that the assay could detect all 8 virulence genes in *Enterococcus* spp. isolates. Additionally, all 26 *E. faecium* isolates were positive for *efm*, whereas all *E. faecalis* isolates were positive for ace. Among the virulence genes analyzed, the sex pheromone cAD1 precursor lipoprotein gene *cad1*, which is related to the secretion of signaling molecules for intercellular communication, was detected in all 26 *E. faecium*,

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Table 3. Distribution of antimicrobial MICs among 22 *E. faecium*, 22 *E. faecalis*, 3 *E. hirae*, and 2 *E. gallinarum* isolates from broiler chickens suffering lameness

CLSI subclass	Antimicrobials	Species	MIC_{50}	MIC_{90}	Resistance (%)	Total resistance (%)	Number of strains with MIC (μg/mL)*													
							0.25	0.5	1	2	4	8	16	32	64	128	256	512	1,024	2,048	> 2,048
Penicillins	AMP	*E. faecium*	1	4	1 (4.6)	1 (2.0)			18	1	2		1								
		E. faecalis	1	1	0 (0.0)				22												
		E. hirae	1	2	0 (0.0)				2	1											
		E. gallinarum	1	1	0 (0.0)				2												
Phenicols	CHL	*E. faecium*	8	> 32	3 (13.6)	7 (14.3)						18	1	3							
		E. faecalis	8	> 32	3 (13.6)							13	6	3							
		E. hirae	8	> 32	1 (33.3)							2		1							
		E. gallinarum	8	8	0 (0.0)							2									
	FFN	*E. faecium*	4	> 32	3 (13.6)	4 (8.2)					18	1		3							
		E. faecalis	4	8	1 (4.6)						17	4		1							
		E. hirae	4	4	0 (0.0)						3										
		E. gallinarum	4	4	0 (0.0)						2										
Lipopeptides	DAP†	*E. faecium*	8	16	17 (77.3)	21 (42.9)				1	4	14	2	1							
		E. faecalis	2	4	1 (4.6)				2	11	8	1									
		E. hirae	2	8	1 (33.3)				1	1		1									
		E. gallinarum	8	8	2 (100)							2									
Macrolides	ERY	*E. faecium*	2	> 64	5 (22.7)	18 (36.7)			3	10	4				5						
		E. faecalis	2	> 64	11 (50.0)				8	3				1	10						
		E. hirae	32	> 64	2 (66.7)				1					1	1						
		E. gallinarum	1	1	0 (0.0)				2												
Aminoglycosides	GEN	*E. faecium*	128	128	1 (4.6)	3 (6.1)										20	1				1
		E. faecalis	128	128	1 (4.6)											21				1	
		E. hirae	128	1,024	1 (33.3)											2			1		
		E. gallinarum	128	128	0 (0.0)											2					
	KAN	*E. faecium*	128	1,024	3 (13.6)	10 (20.4)										15	4		1		2
		E. faecalis	128	> 2,048	6 (27.3)											16				1	5
		E. hirae	128	> 2,048	1 (33.3)											2					1
		E. gallinarum	128	128	0 (0.0)											2					
	STR	*E. faecium*	128	128	2 (9.1)	11 (22.4)										20				1	1
		E. faecalis	128	> 2,048	8 (36.4)											14					8
		E. hirae	128	> 2,048	1 (33.3)											2					1
		E. gallinarum	128	128	0 (0.0)											2					
Fluoroquinolone	CIP	*E. faecium*	4	> 16	22 (100)	36 (73.5)					11	7	4								
		E. faecalis	4	> 16	12 (54.6)					10	2		10								
		E. hirae	16	> 16	2 (66.7)			1					2								
		E. gallinarum	1	2	0 (0.0)				1	1											

(continued to the next page)

Table 3. (Continued) Distribution of antimicrobial MICs among 22 *E. faecium*, 22 *E. faecalis*, 3 *E. hirae*, and 2 *E. gallinarum* isolates from broiler chickens suffering lameness

CLSI subclass	Antimicrobials	Species	MIC_{50}	MIC_{90}	Resistance (%)	Total resistance (%)	Number of strains with MIC (μg/mL)*													
							0.25	0.5	1	2	4	8	16	32	64	128	256	512	1,024	2,048	> 2,048
Oxazolidinones	LZD	*E. faecium*	4	4	0 (0.0)	0 (0.0)				7	15										
		E. faecalis	2	4	0 (0.0)					16	6										
		E. hirae	2	4	0 (0.0)					2	1										
		E. gallinarum	4	4	0 (0.0)						2										
Streptogramins	SYN	*E. faecium*	2	4	5 (22.7)	27 (55.1)			9	8	3	2									
		E. faecalis	8	16	21 (95.5)					1	2	16	3								
		E. hirae	2	4	1 (33.3)					2	1										
		E. gallinarum	2	2	0 (0.0)					2											
Glycylcyclines	TGC‡	*E. faecium*	1	4	22 (100.0)	49 (100.0)		5	14	3											
		E. faecalis	2	16	22 (100.0)			1	7	14											
		E. hirae	0.5	4	3 (100.0)			2	1												
		E. gallinarum	1	2	2 (100.0)				2												
Tetracyclines	TET	*E. faecium*	2	> 128	7 (31.8)	24 (49.0)				14	1			1	1	5					
		E. faecalis	64	128	14 (63.8)					8				1	5	8					
		E. hirae	2	> 128	1 (33.3)					2						1					
		E. gallinarum	64	64	2 (100.0)										2						
	TYLT	*E. faecium*	4	> 64	5 (22.7)	16 (32.7)				9	2	6			5						
		E. faecalis	4	> 64	9 (40.9)					9	4				9						
		E. hirae	64	> 64	2 (66.7)					1					2						
		E. gallinarum	2	2	0 (0.0)					2											
Ionophore coccidiostats	SAL	*E. faecium*	2	4	1 (4.6)	1 (2.0)				19	1	1	1								
		E. faecalis	2	4	0 (0.0)					19	3										
		E. hirae	4	4	0 (0.0)					1	2										
		E. gallinarum	2	2	0 (0.0)					2											
Glycopeptides	VAN	*E. faecium*	2	2	0 (0.0)	0 (0.0)				21		1									
		E. faecalis	2	2	0 (0.0)					22											
		E. hirae	2	2	0 (0.0)					3											
		E. gallinarum	8	8	0 (0.0)							2									

MIC, minimal inhibitory concentration; CLSI, Clinical and Laboratory Standards Institute; AMP, ampicillin; CHL, chloramphenicol; FFN, florfenicol; DAP, daptomycin; ERY, erythromycin; GEN, gentamicin; KAN, kanamycin; STR, streptomycin; CIP, ciprofloxacin; LZD, linezolid; SYN, quinupristin/dalfopristin; TGC, tigecycline; TET, tetracycline; TYLT, tylosin; SAL, salinomycin; VAN, vancomycin.
*Solid and dashed lines indicate the breakpoints of intermediate and complete resistance established by CLSI, respectively; †The susceptibility breakpoint has only been established for daptomycin (≤ 4 μg/mL). In this study, isolates with an MIC ≥ 8 μg/mL were classified as resistant; ‡The susceptibility breakpoint has only been established for tigecycline (≤ 0.25 μg/mL). In this study, isolates with an MIC ≥ 0.5 μg/mL were classified as resistant.

Table 4. Distribution of antimicrobial resistance patterns among 49 *Enterococcus* isolates from liver, joint, and femur samples from broilers suffering lameness

Resistance patterns	Number of strains with antimicrobial resistance												Total (%)
	E. faecium			*E. faecalis*			*E. hirae*			*E. gallinarum*			
	Liver	Joint	Femur	Liver	Joint	Femur	Liver	Joint	Femur	Liver	Joint	Femur	
TGC-CIP	2		1										6 (12.2)
TGC-SYN				1	1								6 (12.2)
TGC-DAP									1				6 (12.2)
TGC-CIP-DAP	2	4	3										19 (38.8)
TGC-CIP-KAN		1											19 (38.8)
TGC-SYN-TET				1	2	2							19 (38.8)
TGC-SYN-CIP				2									19 (38.8)
TGC-DAP-TET											1	1	19 (38.8)
TGC-CIP-DAP-TET			2										7 (14.3)
TGC-CIP-DAP-SYN		1	1										7 (14.3)
TGC-SYN-CIP-TET						2							7 (14.3)
TGC-CIP-ERY-TYLT									1				7 (14.3)
TGC-SYN-TYLT-CIP-ERY						1							2 (4.1)
TGC-SYN-ERY-STR-TET					1								2 (4.1)
TGC-CIP-DAP-TET-SYN-ERY		1											3 (6.1)
TGC-SYN-CIP-TYLT-ERY-DAP						1							3 (6.1)
TGC-SYN-CIP-TYLT-ERY-CHL						1							3 (6.1)
TGC-CIP-TET-SYN-ERY-TYLT-STR		1			1								6 (12.2)
TGC-SYN-TYLT-ERY-STR-TET-KAN					2								6 (12.2)
TGC-CIP-STR-ERY-KAN-SYN-TET					1								6 (12.2)
TGC-CIP-STR-ERY-KAN-TYLT-CHL					1								6 (12.2)
TGC-CIP-DAP-TET-ERY-TYLT-CHL-FFN		1											2 (4.1)
TGC-SYN-CIP-TET-STR-ERY-TYLT-KAN					1								2 (4.1)
TGC-CIP-DAP-TET-SYN-ERY-TYLT-CHL-FFN-SAL			1										2 (4.1)
TGC-CIP-ERY-TYLT-SYN-STR-TET-CHL-KAN-GEN								1					2 (4.1)
TGC-SYN-CIP-TET-STR-ERY-TYLT-KAN-CHL-FFN-GEN				1									1 (2.0)
TGC-CIP-DAP-TET-SYN-ERY-TYLT-STR-CHL-FFN-KAN-GEN-AMP	1												1 (2.0)
Total	5	9	8	5	10	7	0	1	2	0	1	1	49

TGC, tigecycline; CIP, ciprofloxacin; SYN, quinupristin/dalfopristin; DAP, daptomycin; KAN, kanamycin; TET, tetracycline; ERY, erythromycin; TYLT, tylosin; STR, streptomycin; CHL, chloramphenicol; FFN, florfenicol; SAL, salinomycin; GEN, gentamicin; AMP, ampicillin.

25 *E. faecalis*, and 20 *E. hirae* isolates. Interestingly, aggregation substances encoded by sex pheromone plasmids are known to mediate aggregation between bacterial cells and facilitate transfer of plasmids [16]. Hemolysin secreted by bacteria damages cell membranes and facilitates the infection process [16,17].

The distribution of *asa1* and *cylA* among *E. faecalis* isolates was 44% and 16%, respectively. The *gelE* gene is believed to enhance the survivability of *Enterococcus spp.* in extra-intestinal environments, and this gene was found in 88% of the 25 *E. faecalis* isolates. Gelatinase activity is known to be co-controlled by *gelE* and *fsr* genes, and lack of *fsr* affects the production of gelatinase [18,19]. The *fsr* gene product, which hydrolyzes gelatin, casein, hemoglobin, and other bioactive peptides, was detected in 64% of *E. faecalis* isolates. Among *E. faecalis* isolates harboring the *esp* gene, which contributes to enterococcal biofilm formation, resistance to environmental stresses, and adhesion to eukaryotic cells, only 1 of 25 isolates was positive fpr *esp*. Meanwhile, *fsr*, *esp*, *cylA*, *ace*, *gelE*, and *asa1* genes were not detected in 26 *E. faecium* isolates, and *fsr*, *esp*, *cylA*, *cad1*, *ace*, *gelE*, and *asa1* genes were detected in 2 of 25 *E. faecalis* isolates. No virulence factors were found in any of the 9 *E. gallinarum* isolates.

Enterococcus spp. are widespread, resistant to various antimicrobial agents, and have the ability to rapidly acquire resistance to various antimicrobial agents [5,20]. Herein, all 49 *Enterococcus* isolates were resistant to tigecycline, followed by ciprofloxacin (73.5%), quinupristin/dalfopristin (55.1%), tetracycline (49.0%), daptomycin (42.9%), erythromycin (36.7%), and

tylosin (32.7%). Resistance to linezolid and vancomycin was not observed in any of the *E. faecium*, *E. faecalis*, *E. hirae*, or *E. gallinarum* isolates. Among the 49 *Enterococcus* isolates, 22 *E. faecium* isolates were resistant to ciprofloxacin (100.0%) and daptomycin (77.3%).

Moreover, *E. faecalis* displayed 95.5%, 63.8%, 54.6%, and 50.0% resistance to high levels of quinupristin/dalfopristin, tetracycline, ciprofloxacin, and erythromycin, respectively. Additionally, 66.7% of *E. hirae* isolates were resistant to tylosin, ciprofloxacin, and erythromycin. Resistance to daptomycin, tetracycline, and tigecycline was 100% in all *E. gallinarum* isolates.

Comparison of antimicrobial agents revealed that the frequency of resistance to daptomycin and ciprofloxacin was much higher in *E. faecium* (77.3% and 100.0%, respectively) than in *E. faecalis* (4.6% and 54.6%, respectively). Similarly, resistance to erythromycin, streptomycin, quinupristin/dalfopristin, and tetracycline was higher in *E. faecalis* (50.0%, 36.4%, 95.5%, and 63.8%, respectively) than *E. faecium* (22.7%, 9.1%, 22.7%, and 31.8%, respectively). Multidrug resistance was observed in the majority of isolates, and the prevalence of multidrug resistance (resistant to at least 3 antimicrobials) was 19/22 (86.4%) for *E. faecium*, 20/22 (90.9%) for *E. faecalis*, 2/3 (66.7%) for *E. hirae*, and 2/2 (100.0%) for *E. gallinarum*.

To the best of our knowledge, this report is the first to provide detailed antibiotic resistance patterns for *Enterococcus* spp. isolated from commercial broiler chickens suffering lameness. The developed multiplex PCR method may have diagnostic value for the reliable, cost- and time-effective detection of 8 putative virulence genes in *Enterococcus* spp. simultaneously using a single assay.

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