Molecular characterization of high-level gentamicin-resistant Enterococcus faecalis from chicken meat in Korea

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**Abstract**

Because the intrinsically antimicrobial-resistant Enterococcus has acquired high-level aminoglycoside resistance genes, treating enterococcal infections is difficult. In this study, of the 101 food-borne Enterococcus faecalis isolates collected from retail chicken meat between 2003 and 2010, 11 high-level gentamicin-resistant (HLGR) E. faecalis isolates (MICs > 2,048 μg/mL) were found. Molecular characterization was performed to determine the basis of this resistance. All HLGR E. faecalis isolates encoded aac(6')-le-aph(2')-Ia and harbored at least 3 virulence traits in the osa1, esp, gcl, efaA, ace, and cyA genes. Pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) were performed to characterize their molecular epidemiology. A total of 8 sequence types (STs), including 3 novel STs, were identified (ST35, ST82, ST116, ST202, ST300, ST403, ST407, and ST420). ST82, which is associated with amyloid arthropathy in poultry, was the most prevalent ST among HLGR E. faecalis isolates (4 out of 11 isolates, 36.4%); all other STs were identified in the isolates as well. The STs of food-borne HLGR E. faecalis in this study have been confirmed as corresponding to clinical isolates in the MLST database (DB), except for ST300 and the new STs. Three out of 11 isolates belonged to CC116, including ST116, ST407, and ST420. This study characterized HLGR E. faecalis isolates and provided evidence for the spread of HLGR E. faecalis with virulence factors to chicken sources in Korea. The emergence of food-borne HLGR E. faecalis suggests that chicken could be a potential source of transmission of antimicrobial resistance and virulence factors.

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1. Introduction

Enterococci have recently been described as important nosocomial pathogens having multi-drug resistance (MDR) through intrinsic and acquired resistance to various antibiotics (Bonten et al., 2001; Deshpande et al., 2007). Among them, high-level gentamicin-resistant enterococci (MIC ≥ 2,000 μg/mL) have made the clinical treatment of infections difficult (Leavis et al., 2006). The combination of a cell wall-active agent (ampicillin, penicillin, or vancomycin) and gentamicin has been used to treat enterococcal infection (Del Campo et al., 2000). However, this combination treatment is not applicable for cases of infections caused by enterococci with a high level of aminoglycoside resistance. Therefore, the development of appropriate therapies to combat the emergence of high-level gentamicin-resistant (HLGR) enterococci has been difficult.

High-level resistance to gentamicin is caused by the bifunctional 6'-aminoglycoside acetyltransferase and 2' aminoglycoside phosphotransferase (AAC6'-APH2'), which reduce the effect of aminoglycosides (Udo et al., 2004), with the exception of streptomycin, which is modified by the 6-nucleotidyltransferase (ANT6) (Chow, 2000). HLGR Enterococcus faecalis was first isolated from hospitalized patients with endocarditis in France in 1979. These bacteria cause human infections, and hence, several studies have focused on HLGR enterococci harboring the aac(6')-le-aph(2')-Ia gene from a variety of sources, e.g., clinical specimens, food-producing animals, and food. In most cases, the aac(6')-le-aph(2')-Ia gene has been found on Tn5281 (Hodel-Christian, Murray, Hodel-Christian and Murray, 1991), which can be located either on a plasmid or in chromosomal DNA (Hegstad et al., 2010; Kreft et al., 1992). In addition, enterococci are well known for their potential to exchange genetic information via an inducible pheromone-responsive conjugation system (Clewell, 1990). This system is also known to function in the food matrix or gastrointestinal tract (Huycke et al., 1992). Antimicrobial resistance genes can be simultaneously transferred via conjugation.

**Abbreviations:** ANTS, 6-nucleotidyltransferase; ATCC, American type culture collection; AME, Aminoglycoside-modifying enzyme; AM, Ampicillin; AMR, Antimicrobial resistance; AAC6’-APH2’, Bifunctional 6'-aminoglycoside acetyltransferase and 2' aminoglycoside phosphotransferase; BHI, Brain heart infusion; C, Chloramphenicol; Animals, Ciprofloxacin; CLSI, Clinical laboratory and standards institute; CC, Clonal complex; DLVs, Double locus variants; E, Erythromycin; Waters et al., Gentamicin; HLGR, High-level gentamicin resistance; MICs, Minimal inhibitory concentrations; MDR, Multi-drug resistance; MLST, Multilocus sequence typing; NARMP, National antimicrobial resistance management program; NCBI, National center for biotechnology information; OECD, Organization for economic cooperation and development; P, Penicillin; PCR, Polymerase chain reaction; PFGE, Pulsed-field gel electrophoresis; RA, Rifampin; SLVs, Single locus variants; S, Streptomycin; Coque et al., Teicoplanin; TE, Tetracycline; VA, Vancomycin; VRE, Vancomycin-resistant Enterococcus.

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http://dx.doi.org/10.1016/j.ijfoodmicro.2013.02.016
to bacteria with other virulence traits, e.g., aggregation substances, endocellular structures, and hemolysin—cytolysin production functions (Eaton and Gasson, 2001; Hegstad et al., 2010; Kreft et al., 1992).

The resistance rate in Korea had been recorded as high in a previous WHO report (2000). Several studies of food-producing animals and food products have shown high levels of antimicrobial resistance in Korea (Jung et al., 2007; Lim et al., 2007). Therefore, our goal was to investigate and molecularly analyze HLGR E. faecalis harboring AMEs and virulence traits in retail chicken meats isolated between 2003 and 2010 in Korea.

2. Materials and methods

2.1. Bacterial isolates and identification

Between 2003 and 2010, a total of 101 E. faecalis isolates were collected from retail chicken meat through the National Antimicrobial Resistance Management Program (NARMP) in Korea. The presence of E. faecalis was confirmed using the VITEK-2 Compact system (BioMérieuxVitek, Inc., Hazelwood, MO, USA) and the Thermal iCycler system (Bio-Rad, Hercules, CA, USA) to perform polymerase chain reactions (PCRs) targeting the ddi gene, which encodes D-Ala-D-Ala ligase (Dutka-Malen et al., 1995). Of these 101 isolates, E. faecalis was selected to identify strains that were highly resistant to gentamicin using the disk diffusion method. The gentamicin resistance level was confirmed using the minimum inhibitory concentrations (MICs) test according to Clinical Laboratory Standards Institute guidelines (CLSI) (CLSI, 2011).

2.2. Assessment of antibiotic susceptibility

HLGR food-borne E. faecalis isolates were screened for phenotypic resistance against 11 antimicrobials using the disk diffusion method. The following disks were used (BD Sensi-disc, Becton Dickinson, Germany): ampicillin (10 μg), amoxicillin (30 μg), teicoplanin (30 μg), erythromycin (15 μg), tetracycline (30 μg), ciprofloxacin (5 μg), rifampin (5 μg), chloramphenicol (30 μg), lincomycin (30 μg), and streptomycin (300 μg). In addition, MIC values for gentamicin and streptomycin were determined using the agar dilution method with brain heart infusion (BHI) at concentrations ranging from 256 to 2048 μg/mL (serial 2-fold dilutions). All susceptibility tests were conducted as recommended by the CLSI guidelines (CLSI, 2011). E. faecalis ATCC 29212, Staphylococcus aureus ATCC 25923, and E. faecalis ATCC 51299 were used as control strains. Susceptibility results were interpreted according to CLSI standards.

2.3. Detection of high-level gentamicin and streptomycin resistance and virulence determinants

In HLGR isolates, AME genes, including aac(6′)-Ile–aph(3′)-Ie, aph(2′)-Ib, aph(2′)-Ic, aph(2′)-Ia, ant(3′)-Ia, and ant(6)-Ia, were screened using PCR. In addition, virulence genes, including asa1, esp, gelE, efaA, ace, cylA, and hyl, were investigated to access the virulence potential of HLGR E. faecalis isolates. To verify the amplification of the correct band, reference strains were used, including 2 E. faecalis strains (for esp and hyl), provided by the Korean Centers for Disease Control and Prevention, E. faecalis ATCC 29212 (for asa1, gelE, efaA, ace, and cylA), and E. faecalis ATCC 51299 (for aac(6′)-Ile–aph(3′)-Ie, and ant(6)-Ia). All DNA sequences were analyzed by Macrogen Inc. (Seoul, Korea). Results were matched on the basis of sequence homology according to the GenBank database using the BLAST search engine of the National Center for Biotechnology Information (NCBI). Primer sequences and PCR conditions used in this study are shown in Table 1.

2.4. Pulsed-field gel electrophoresis (PFGE)

On the basis of the PulseNet standard protocol, modified PFGE was applied using the CHEF Mapper system (Bio-Rad) as described previously by Saeedi et al. (2002). Briefly, HLGR E. faecalis cultures were grown overnight at 37 °C on tryptic soy agar (TSA). Bacterial colonies were suspended in cell suspension buffer (10 mm Tris, 1 mM EDTA). Bacterial suspensions were mixed with an equal volume of low-melting agarose (2%; AMERCO, Solon, OH, USA). The plug was applied using the CHEF Mapper system (Bio-Rad) as described previously by Saeedi et al. (2002). Briefly, HLGR E. faecalis cultures were grown overnight at 37 °C on tryptic soy agar (TSA). Bacterial colonies were suspended in cell suspension buffer (10 mm Tris, 1 mM EDTA). Bacterial suspensions were mixed with an equal volume of low-melting agarose (2%; AMERCO, Solon, OH, USA). The plug was applied using the CHEF Mapper system (Bio-Rad) as described previously by Saeedi et al. (2002).

### Table 1

<table>
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<th>Description</th>
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<th>Sequence primer sequence (5′ → 3′)</th>
<th>Size (bp)</th>
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<td>348</td>
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<td>Virulence determinants</td>
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<td>cylA</td>
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<td>efaA</td>
<td>R: CTACTAACACACAGCTAACTG</td>
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conditions: gradient 6.0 V/cm at 14 °C for 20 h with pulse times ramped from an initial start time of 1 s to a final start time of 20 s in 0.5 × Tris/borate/EDTA buffer. The agarose gel was stained using ethidium bromide and visualized using the Gel Doc system (Bio-Rad). Cluster analysis of the PFGE results was conducted to determine the relatedness of HLGR \textit{E. faecalis} isolates using InfoQuest FP software, version 4.5 (Bio-Rad), with the Dice co-efficient and unweighted pair group method with arithmetic mean (UPGMA). Optimization settings for the dendrogram were 0.5% with a band tolerance of 0.1%.

### 2.5. Multilocus sequence typing (MLST)

MLST of HLGR \textit{E. faecalis} isolates was performed as presented on the multilocus sequence website (http://efaecalis.mlst.net/). Seven housekeeping genes (\textit{gdh}, \textit{gyd}, \textit{pstS}, \textit{gki}, \textit{aroE}, \textit{xpt}, and \textit{yqiI}) were amplified using PCR. The sequences of the purified products were analyzed by Macrogen, Inc. Sequences, PCR conditions, and primers are available on the Macrogen website, including allelic profiles. Allele numbers were assigned by comparing the sequence at each locus to known alleles available on the \textit{E. faecalis} MLST site. The sequence type (ST) was determined through a combination of 7 alleles. New STs demonstrated in the present study were deposited in the database. STs of HLGR \textit{E. faecalis} isolates were further analyzed to determine the clonal complex (CC) using the e-BURST version 3 software. Single locus variants (SLVs) and double locus variants (DLVs) were observed within the CC. Finally, the results of PFGE and MLST were compared and analyzed.

### 3. Results

#### 3.1. Profiles of HLGR \textit{E. faecalis} isolates from chicken meat

Of the 101 \textit{E. faecalis} isolates collected from retail chicken meat, 11 \textit{E. faecalis} isolates (10.9%) were resistant to high-level gentamicin (MIC > 2048 \textmu g/mL). Seven HLGR isolates showed a high level of streptomycin resistance (MIC > 2048 \textmu g/mL). Three isolates showed the same antimicrobial resistance pattern against gentamicin, streptomycin, tetracycline, and erythromycin. In general, HLGR isolates were resistant to tetracycline, erythromycin, chloramphenicol, and ciprofloxacin. The results of this analysis are presented in Fig. 1.

#### 3.2. Distribution of AMEs and virulence traits in HLGR \textit{E. faecalis} isolates

All HLGR \textit{E. faecalis} isolates carried the \textit{aac(6')-le-aph(2')-Ia} gene; no other genes corresponding to gentamicin resistance, e.g., \textit{aph(2')-Ib, aph(2')-Ic}, or \textit{aph(2')-Id}, were observed. Seven isolates were resistant to streptomycin and harbored only the \textit{ant(6)-la} gene. However, 3 high-level streptomycin-resistant (HLSR) \textit{E. faecalis} isolates contained no AME genes, which corresponds to phenotypic resistance, whereas 1 isolate was susceptible to streptomycin despite the facts that it was carrying \textit{ant(6)-la} genes. Virulence determinants in \textit{E. faecalis} were detected in most isolates examined in this study. All these isolates shared the same virulence profile. Overall, 4 virulence genes (\textit{gelE} and \textit{eafA}, 100%; \textit{asa1}, 90.9%; \textit{ace}, 72.7%) were commonly detected. The cydA gene was detected in a clone showing some genetic relatedness. No isolates contained the \textit{hyl} or \textit{esp} genes (Fig. 1).

#### 3.3. Molecular epidemiology of HLGR in \textit{E. faecalis} by PFGE

Genetic relationships between HLGR \textit{E. faecalis} isolated from retail chicken meat were examined using PFGE analysis (SmaI digestion) with a homology cut-off value of 80%. As shown in Fig. 1, ST82 clones harboring a similar phenotype/genotype profile showed high homology, although the isolates were found in different years. In particular, EF506-FC-KF03 and EF576-FC-KF04 were complete matches according to genomic analysis. Differences in these 2 variants were identified using PFGE on the basis of the results of ST82 \textit{E. faecalis} (Fig. 1). Other STs (ST35, ST116, and ST202) and new STs, including ST403, ST407 (SLV of ST116), and ST420 (SLV of ST116), showed diversity in their genetic profiles.

#### 3.4. Clonal lineages identified by MLST e-BURST

Eleven HLGR \textit{E. faecalis} isolates were assigned to 8 different STs, including 3 novel types (ST403, ST407, and ST420). ST82 was observed in 4 isolates (36.3%) at the highest frequency, followed by ST35, ST116, ST202, ST300, and the new types ST403, ST407, and ST420 from each isolate. The 3 novel types, ST403, ST407, and ST420, were registered in the e-BURST MLST database. ST403 was found to be closely related to ST29, sharing all alleles, except for the \textit{gdh} gene. In addition, ST403 shared all alleles with ST244 and ST416, except for the \textit{yqiI} and \textit{pstS} genes. The novel type ST403 is the founder of CC403 and is related to some SLVs (assigned as ST29, ST244, ST292, and ST416). Two other new types, ST407 and ST420, shared all alleles with ST116, except for 1 gene for each isolate (\textit{pstS} or \textit{aroE}, respectively). Clustering of these types with e-BURST revealed the presence of CC21 (ST202), CC116 (ST116, ST407, and ST420), CC403 (ST403), and the singlet (ST35, ST82, and ST300) (Fig. 2).

### 4. Discussion

\textit{Enterococcus} strains isolated from poultry meat are problematic due to their high rate of antimicrobial resistance (Koluman et al.,...
Nevertheless, only a few studies have systematically investigated *E. faecalis* isolates originating from chicken, despite the high degree of relatedness to human isolates (Hayes et al., 2003; Jung et al., 2007). Therefore, we characterized and molecularly analyzed HLGR *E. faecalis* isolates collected from retail chicken meat during the period of 2003–2010 in Korea. All HLGR *E. faecalis* isolates from retail chicken meat showed a high prevalence of virulence genes and MDR.

The HLGR *E. faecalis* isolates characterized in this study showed high prevalence of virulence genes. *E. faecalis* strains that possess potential virulence factors could be a matter of concern. The virulence factors detected in this study, e.g., hemolysin-cytolysin production and the capacity for adhesion, help to efficiently share their genetic elements, including transmissible antibiotic resistance. However, the *hyl* and *esp* genes were not detected. The precise roles of these genes in *Enterococcus* are not completely clear, but they could be considered as a remarkable virulence marker causing human infections (Fisher and Phillips, 2009). The HLGR *E. faecalis* isolates of our study collected from chicken meat did not carry the *hyl* and *esp* genes; however, they appear to expand to other hosts and harbored inducible pheromones and other virulence factors. Coburn et al. (2007) have reported that *E. faecalis* could acquire *esp* or *hyl* genes through an inducible pheromone-responsive conjugation system.

MLST was conducted to identify the lineage of food-borne *E. faecalis* isolates harboring *aac(6′)-Ie-aph(2″)-Ia*, which provides important information with regard to the distribution and population of enterococcal species. In this study, 1 ST from *E. faecalis* was determined to be ST35. This sequence type has been identified in retail chicken meat in Portugal (Freitas et al., 2009) and pork from Denmark (Jesper Larsen et al., 2010). Our data confirmed the origin of this ST and that it is a universal ST in poultry. However, recently, the ST35 clone identified as porcine in origin carried a high level of gentamicin resistance (Jesper Larsen et al., 2010), suggesting that the ST clone may have an increased tendency to acquire AMEs. In addition, ST116 has been found in 1 isolate. Quiñones first examined the ST of *E. faecalis* found in a hospitalized patient in Cuba (Dianelys Quiñones and Nagashima, 2009). Interestingly, in this study, CC116 clones were often found in food-borne *E. faecalis* expressing a high level of gentamicin resistance. By referring to MLST data, CC116 appears to consist primarily of human hosts carrying pathogens (Dianelys Quiñones and Nagashima, 2009). ST275, a SLV of ST116, was identified in *E. faecalis* from human feces in the USA in 1994. Subsequently, ST39 and ST45 were detected in patients in Spain, and in 2009, ST374 was isolated from sewage. In this study, ST116, ST407, and ST420 carrying AMEs and virulence genes of CC116 were detected in retail chicken meat. This finding could indicate that virulent variants of ST116 have expanded to food as well as environments. In this study, and in contrast to the original ST116 clone possessing low antibiotic resistance, our ST116 clone displayed MDR with various virulence factors; furthermore, the CC116 clones were distinguishable according to their PFGE patterns. However, they had a similar profile with regard to antimicrobial resistance and virulence traits. Of them, the EFS206-FC-KF06 (ST407) isolate showed no susceptibility to streptomycin although the isolate harbored the *anti(6)-Ia* gene. A possible reason for this silent phenomenon may be a false-positive result that leads to discordance between phenotypic and genotypic resistance data through horizontal gene transfer (Klare et al., 2007).

In contrast, the novel type ST403 was found to be a predicted founder by forming CC403 and shown to be linked to some SLVs (assigned as ST29, ST244, ST292, and ST416). Until now, only 2 ST403 variants were found in poultry; however, ST244 and ST416 were also found in patients, according to the MLST database. Thus, CC403 clones might have become accustomed to human and poultry hosts. Similarly, ST300 appears to be undergoing host expansion through SLV and DLV, which have been obtained in humans. In addition, ST202 was also found in Korea. This clone has been shown to display a broad host spectrum and is the predicted subgroup founder of CC21, which is a major *E. faecalis* CC (Ruiz-Garbajosa et al., 2006). The ST202 identified in this
study was highly resistant to multiple antimicrobial compounds, while CC21 clones typically showed less antibiotic resistance than isolates from other CCs (Freitas et al., 2009).

Notably, 4 of the 11 isolates were found to be ST82 (Fig. 1). Previous studies have extensively examined the epidemiology and characteristics of this ST82 clone in E. faecalis (Petersen et al., 2008). ST82 has been shown to be associated with amyloid arthropathy (Petersen et al., 2008, 2009). The ST82 clones isolated in this study were highly resistant to gentamicin and carried the aac(6’)-Ie-aph(2’)-Ia gene with a high level of streptomyacin resistance, which is related to the presence of the cydA gene. We observed similar profiles only for ST82, implying that specific clones were disseminated. As reported by Petersen et al. (2009), E. faecalis ST82 has caused outbreaks of amyloid arthropathy in poultry not only in Denmark but also in other countries. Interestingly, the period of the E. faecalis ST82 outbreak corresponded to the time of our ST82 clones were isolated in Korea. Therefore, it could be possible that several chicken meat products infected with amyloid arthropathy were circulated in food markets. Incidentally, ST82 was also found in a blood sample from a hospitalized patient with amyloid infection in Poland. Thus, our results imply that there might be a potential risk of amyloid arthropathy infection via the food chain. We further confirmed their clonality with high homology based on PFGE results, supporting that clonal expansion can be explained by vertical transmission among live poultry and migratory birds (Petersen et al., 2009).

E. faecalis commonly causes life-threatening infections in humans. Furthermore, infections by antibiotic-resistant E. faecalis, e.g., VRE or HLGCRE, have become an increasing problem. In a previous study, HLGCR Enterococcus faecalis isolated from food sources in Korea. In addition, ST82 was shown to be a food-borne E. faecalis clone. Importantly, our results examining food-borne HLGCR E. faecalis indicate that the food chain is a potential route of enterococcal infection in humans. Further investigations examining pathogenic factors in animal food should be continuously pursued.

Acknowledgments

This study was supported by grants from the National Antimicrobial Resistance Management Program (NARMIP) of the Korean Food and Drug Administration. We thank the Korea University Food Safety Hall and Institute of Food and Biomedicine Safety for allowing the use of their equipments and facilities.

References


