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Original Article

Using *groEL* as the target for identification of *Enterococcus faecium* clades and 7 clinically relevant *Enterococcus* species

Wei-Wen Hung [a], Yen-Hsu Chen [b,c], Sung-Pin Tseng [d], Ya-Ting Jao [e], Lee-Jene Teng [f,], Wei-Chun Hung [g,h,*]**

[a] *Division of Endocrinology and Metabolism, Department of Internal Medicine, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan*
[b] *Graduate Institute of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan*
[c] *Division of Infectious Diseases, Department of Internal Medicine, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan*
[d] *Department of Medical Laboratory Science and Biotechnology, College of Health Sciences, Kaohsiung Medical University, Kaohsiung, Taiwan*
[e] *Infection Control Center, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan*
[f] *Department of Clinical Laboratory Sciences and Medical Biotechnology, National Taiwan University College of Medicine, Taipei, Taiwan*
[g] *Department of Microbiology and Immunology, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan*
[h] *Department of Medical Research, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan*

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Abstract *Background/Purpose:* Accurate identification is important for effective treatment because *Enterococcus* species have talents to cope with various antibiotics either by intrinsic resistance or by acquisition of mobile genetic elements. The *groEL* gene is a permissive target in identification of bacteria. We aimed to develop simple assays based on *groEL* for identification of enterococci.
Results: We continued our previous work and determined *groEL* gene sequences of *Enterococcus* species isolated from clinical specimens. Phylogenetic analysis based on *groEL* revealed that each strain clustered well with their reference strains (bootstrap value 100%), in which *Enterococcus faecium* and *Enterococcus gallinarum* could be split into two clades. The divergence of *E. faecium* was coincident with hospital-associated clade, known as clade A, and community-

* Corresponding author. Department of Microbiology and Immunology, College of Medicine, Kaohsiung Medical University, No. 100 Shi-Quan 1st Road, Kaohsiung, 807, Taiwan. Fax: +886 7 3212062.
** Corresponding author. Department of Clinical Laboratory Sciences and Medical Biotechnology, National Taiwan University College of Medicine, No. 1 Chang-Te Street, Taipei, 100, Taiwan. Fax: +886 2 23711574.
E-mail addresses: ljteng@ntu.edu.tw (L.-J. Teng), wchung@kmu.edu.tw (W.-C. Hung).

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associated clade, known as clade B. A PCR-restriction fragment length polymorphism (PCR-RFLP) assay was therefore designed to differentiate the two *E. faecium* clades, based on the specific RsaI cutting sites present in the two clades. To differentiate 7 clinical relevant *Enterococcus* species, the multiplex PCR assay was designed to identify *Enterococcus avium*, *Enterococcus casseliflavus*, *Enterococcus faecalis*, *E. faecium*, *E. gallinarum*, *Enterococcus hirae* and *Enterococcus raffinosus*. Specificity was tested with other *Enterococcus* species including *Enterococcus cecorum*, *Enterococcus durans* and *Enterococcus mundtii*. None of these bacterial species generated products of similar size to those of the seven *Enterococcus* species.

Conclusion: The simple PCR-RFLP and multiplex PCR assays on the basis of *groEL* gene provided an alternative way to identify *Enterococcus* species.

Introduction

Enterococcus species are Gram-positive, catalase-negative, facultatively anaerobic oval cocci and are part of the natural microbiota of the gastrointestinal tract in humans.[1] They have emerged as leading causes of healthcare-associated infections in recent years and also cause a variety of community-associated infection from soft tissue infections to intra-abdominal infections.[1,2] According to 2011–2014 U.S. National Healthcare Safety Network data, *Enterococcus* species were the top 10 most common pathogens of healthcare-associated infections.[3] Among the enterococcal infections, *Enterococcus faecalis* and *Enterococcus faecium* are the most encountered species, accounting up to 90% of the clinical isolates[4]; the remaining are caused by other species such as *Enterococcus avium*, *Enterococcus casseliflavus*, *Enterococcus gallinarum*, *Enterococcus hirae* and *Enterococcus raffinosus*, and the reported number of cases is progressively increasing.[4,5]

The resistance profile of enterococci is usually correlated with the species. *E. faecalis* is intrinsically resistant to quinupristin-dalfopristin, the combination of which is highly active against *E. faecium*.[6,7] Low level resistance to vancomycin caused by chromosomally encoded VanC is found in *E. casseliflavus* and *E. gallinarum*.[8] VanA and VanB, which display high level resistance to vancomycin, are predominantly acquired by *E. faecalis* and *E. faecium*.[1,8] Vancomycin-resistant *E. faecium* has increased dramatically worldwide at the turn of the century, and most of them belonged to clonal complex 17 (CC17).[9–12] Comparative genomics further demonstrate the two distinct clades of *E. faecium*: (i) clade A is hospital-adapted and is responsible for the global emergence of vancomycin-resistant enterococci (VRE), to which CC17 belongs; (ii) clade B is community-associated and rarely causes disease to humans.[13–15] Therefore, rapid and accurate identification of enterococci to species level or even to clades is important for current diagnosis and for effective treatment.

Identification of enterococci by conventional culture and biochemical tests is time-consuming and complicated.[16] Commercially standardized systems, which significantly reduce testing time such as API, Phoenix and VITEK 2, have been instead adopted in routine clinical microbiology. However, some problems in identification of enterococci with automated systems have been addressed, particularly for the identification of non-*faecalis* and non-*faecium* *Enterococcus* species.[17–21] Recently matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has been introduced and provides a rapid and feasible approach for identifying *E. faecalis*, *E. faecium*, and less encountered species including *E. avium*, *E. casseliflavus*, *Enterococcus durans*, *E. gallinarum*, *E. hirae* and *E. raffinosus*.[22–25] Nevertheless, the DNA-based approaches are still the reference methods to access identification agreement while discordant results are obtained with automated systems or MALDI-TOF MS.[24,26]

The 16S rRNA gene-based species-specific assay is the most common molecular approach method. However, difficulty to differentiate *E. faecium* from *Enterococcus mundtii* and *E. hirae*, as well as *E. casseliflavus* from *E. gallinarum* is noted, due to the high DNA sequence similarity.[27] Other molecular targets used for molecular diagnosis include ribosomal 16S–23S intergenic spacer regions,[28,29] D-alanine- D-alanine ligase gene,[30,31] beta subunit of RNA polymerase gene[32] and superoxide dismutase gene.[33,34] Direct sequencing or array technique based on the above target would be cost-ineffective or complicated[28,29,32,33]; whereas the multiplex PCR assays described above focus on limited *Enterococcus* species.[30,31,34]

The *groESL* genes (also known as *hsp*10/60), which encode 10-kDa (GroES) and 60-kDa (GroEL) heat shock proteins, are ubiquitous and evolutionarily highly conserved among bacteria.[35,36] Previously we developed a broad-range PCR-restriction fragment length polymorphism (PCR-RFLP) of *groESL* and direct sequencing of *groES* and spacer region to identify clinically relevant *Enterococcus* species.[37,38] The aim of the study was to develop simple PCR assays on the basis of *groEL* gene, providing an alternative way to identify frequently encountered *Enterococcus* species in clinical laboratory.

Materials and methods

Bacterial strains

The strains examined in this study include 14 reference strains and 203 clinical isolates. The 14 reference strains

were included *E. avium* ATCC 14025, *E. casseliflavus* ATCC 25788, *E. casseliflavus* ATCC 49996, *Enterococcus cecorum* ATCC 43198, *E. durans* ATCC 19432, *E. faecalis* ATCC 19433, *E. faecalis* ATCC 29212, *E. faecium* ATCC 19434, *E. faecium* ATCC 35667, *E. gallinarum* ATCC 49573, *E. hirae* ATCC 8043, *Enterococcus malodoratus* BCRC 17125, *E. mundtii* ATCC 43186 and *E. raffinosus* ATCC 49427 obtained from American Type Culture Collection (ATCC) or from Bioresource Collection and Research Center (BCRC). Clinical isolates, including 9 *E. avium*, 16 *E. casseliflavus*, 3 *E. cecorum*, 14 *E. faecalis*, 141 *E. faecium*, 9 *E. gallinarum*, 6 *E. hirae* and 5 *E. raffinosus* were collected from Bacteriology Laboratory, National Taiwan University Hospital between 1998 and 2003, and from Microbiology Laboratory, Kaohsiung Medical University Hospital during 2014. Species identification of *E. faecalis* and *E. faecium* were achieved by *E. faecalis*-specific PCR and *E. faecium*-specific PCR.[31,38] Other clinical isolates was confirmed by 16S rRNA gene sequencing or *groESL* sequencing. Antimicrobial susceptibility tests of *E. faecium* were performed by the standard agar dilution method according to the CLSI guidelines.[39]

Sequencing of the *groESL* genes in enterococci

Enterococcal genomic DNA was isolated and purified with a DNA isolation kit (Puregene), according to the instructions of the manufacturer. Degenerate PCR primers groES90F and groEL1500R, which are complementary to conserved regions of the *groES* and *groEL* genes, were designed and used to amplify and to determine the sequences of a 198-bp fragment of *groES* gene, the *groESL* spacer region, and a 1496-bp fragment of the *groEL* gene. PCR was carried out in a Veriti 96-Well Thermal Cycler (Applied Biosystems) with 30 cycles of denaturation (94 °C, 30 s), annealing (45—50 °C, 30 s) and extension (72 °C, 2 min) followed by a final extension step (72 °C, 5 min). The PCR products were purified and subsequently sequenced on an Applied Biosystem model 3100 sequencing system (Applied Biosystems) using the *Taq* BigDye-Deoxy Terminator cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions.

PCR-restriction fragment length polymorphism (PCR-RFLP)

To discriminate clades of *E. faecium*, the PCR products amplified from *E. faecium* by primers groES90F and groEL1500R were subsequently digested with the restriction enzyme RsaI (New England Biolabes). After incubation at 37 °C for 1 h, the DNA fragments were subjected to gel electrophoresis stained with ethidium bromide, and photographed under UV light.

Multilocus sequence typing (MLST)

MLST of *E. faecium* was performed as previously described.[40] The allele numbers of 7 loci (*adk*, *atpA*, *ddl*, *gdh*, *gyd*, *purK* and *pstS*) and sequence type (ST) were assigned according to the program at the MLST website (https://pubmlst.org/efaecium/). Clonal complex was determined by using eBURST (http://eburst.mlst.net/).[41]

rpoB sequencing

The 19 *E. faecium* isolates were chosen for *rpoB* sequencing. The nucleotide sequences of the primers used in this experiment and PCR condition were described previously.[32] A 740-bp fragment (nt 2404 to 3143 in the *rpoB*) was obtained for sequencing.

Multiplex PCR

Based on the determined sequences, one forward primer uni-groEL610F and seven species-specific reverse primers were designed to amplify a target region with a different amplicon size depending on the species (Table 1). The multiplex PCR was performed in a 25-μl volume containing the following: 1X PCR buffer (Takara), 0.25 mM dNTP mixture (for each deoxynucleoside triphosphate), 0.4 μM concentrations of primers Uni-groEL610F and avium1210R, 0.3 μM concentrations of primers raffi1020R, Egall831R2 and Eca930R, 0.2 μM concentrations of primers Ehi894R and fils740R, 0.1 μM concentrations of primer faecium1297R, 0.5 U of rTaq (Takara) and template DNA. The PCR was

Table 1 Primer sequences used in the multiplex PCR assay and the expected sizes of the products.

Primer	[a]Sequence (5′→3′)	Size (bp)	Amplified species
groESL amplification		1745	*Enterococcus* species
groES90F	GCNAAAGARAAACCNCAAAC		
groEL1500R	GAACGHGTHACTTTNGTTGG		
Multiplex PCR to differentiate enterococci			
Uni-groEL610F	ACWGAYAAYGAYAAAATGGAAGC		
flis-740R	AATGATCAATAGTGGACGGCT	135	*E. faecalis*
Egall831R2	TGGWGCTTTRACTGCYACG	222	*E. gallinarum*
Ehi894R	CACTGTTCCGCCTGTTAG	285	*E. hirae*
Eca930R	TTGCATCTTTCAACTCAAGG	322	*E. casseliflavus*
raffi1020R	GATCATTTCAACACGGTCAGTC	432	*E. raffinosus*
avium1210R	AGCAGCACGTGTTGAGTTTAAG	601	*E. avium*
faecium1297R	CGTCTCCTTCTGCTTCTACAG	688	*E. faecium*

[a] N = A + T + G + C; R = A + G; H = A + T + C; W = A + T; Y = A + T.

carried out with 30 cycles of denaturation (94 °C, 30 s), annealing (56 °C, 30 s), and extension (72 °C, 45 s) followed by a final extension step (72 °C, 6 min). The amplification products were then subjected to 2% agarose gel electrophoresis with 100 V for about 40 min, stained with ethidium bromide, and photographed under UV light.

Phylogenetic relationships

DNA sequences were aligned using DNAMAN software (Lynnon Corporation). The phylogenetic relationships among species were analyzed using the neighbor-joining method listed in the MEGA (molecular evolutionary genetic analysis) analytical package. For the neighbor-joining analysis, the distance between the sequences was calculated using Kimura's two-parameter model. Bootstrap values were obtained for 500 randomly generated trees.

Nucleotide sequence accession numbers

The *groEL* nucleotide sequences determined in this study were submitted to GenBank under accession numbers MH109095 to MH109130.

Results

Revised *groESL* broad-range PCR-RFLP

We previously developed a *groESL* broad-range PCR-RFLP to differentiate *Enterococcus* species.[38] In the present study, PCR amplification was performed with revised primer pair groES90F and groEL1500R to improve efficacy of PCR. The PCR amplicons were subsequently digested by RsaI to differentiate *Enterococcus* species as previously described.[38] Unexpectedly, two *E. faecium* strains, C4 and 81, showed atypical RFLP pattern, which was not consistent with that of other *E. faecium* strains such as ATCC 19434^T and ATCC 35667 (Fig. 1). RsaI digested the 1744-bp PCR product from most *E. faecium* strains into 126-bp, 148-bp, 235-bp, 254-bp, 354-bp and 627-bp fragments, but digested the PCR product of the two *E. faecium* strains C4 and 81 into 80-bp, 126-bp, 174-bp, 236-bp, 354-bp and 775-bp

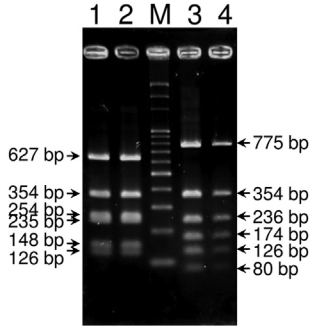

Fig. 1. RFLP analysis of *groEL* gene amplicons from *E. faecium*. PCR products were digested with RsaI and separated by electrophoresis through a 2% agarose gel. Lane 1: *E. faecium* ATCC 19434; lane 2: *E. faecium* ATCC 35677; lane 3: *E. faecium* C4; lane 4: *E. faecium* 81; lane M: 100-bp DNA ladder, the bottom line is 100 bp.

fragments. BLAST analysis revealed that the *groESL* sequences of C4 and 81 showed the highest similarities to the community-associated strains, also called clade B *E. faecium*.[13,42,43] Computer-assisted pattern analysis of *groESL* sequences showed that the PCR-RFLP patterns of 14 reference clade B *E. faecium* strains (listed in Table 2) were identical to that of C4 and 81.

Two clades of the *E. faecium* strains

In addition to C4 and 81, we randomly chose 17 strains and compared with ATCC 19434^T, ATCC 35667 and 14 clade B *E. faecium* strains by MLST analysis to clarify the phylogenetic relationships of the *E. faecium* strains of our collection. Strain information was listed in Table 2. The phylogenetic tree based on concatenated sequences of 7 housekeeping genes for *E. faecium* MLST indicated that the 35 strains clustered into two distinct groups, corresponding to clade A and clade B *E. faecium* (Fig. 2). The 17 randomly chosen strains and two ATCC reference strains formed a clade, in which V22 and V52 was a subclade (bootstrap value 100%). Except strain 89, the other 16 strains belonged to CC17, which was reported to be the most important genetic complex of global spreading hospital-derived isolates and was assigned to clade A *E. faecium*.[13,42,43] For C4 and 81, they were clustered together with reference clade B *E. faecium* strains and formed a stable clade (bootstrap value 100%). In addition, gene sequence analysis revealed that a thymine at base-pair 61 out of 1569-bp 16S rRNA gene was observed in C4 and 81, corresponding to the clade B-specific change that previously reported.42 The results further supported that the strains showing atypical PCR-RFLP pattern were compatible with clade B *E. faecium* strains.

groEL and *rpoB* gene sequencing of *E. faecium*

To evaluate the discriminative power in identifying the two clades of *E. faecium*, *groEL* and *rpoB* gene sequences were determined for the two ATCC reference strains and the 19 strains in our collection or were obtained from GenBank for the 14 clade B *E. faecium* strains. Comparisons of nucleotide identities among clade A and clade B *E. faecium* strains were shown in Table 3. Most strains within the same clade shared >99% sequence identity, except for *rpoB* gene within clade B strains, which was due to strain C4 sharing 99.7—100% sequence identity with clade A strains but only 98.3—98.8% sequence identity with other clade B strains. For the *groEL* (positions 1 to 1427), a total of 60 variations were observed and would be classified into 9 *groEL* sequence types: Groups I to IV indicated the sequences found in various strains, and singletons 1 to 5 indicated unique sequences found in only one strain as noted in Table 2. Table 4 listed the pairwise nucleotide identities and numbers of different nucleotides for the 9 *groEL* sequences types. For inter-clade sequence identity, *groEL* gene showed 96.4—97.2% in DNA sequence identity, quite lower than intra-clade sequence identity (99.0—100%) (Tables 3 and 4). For *rpoB* gene, inter-clade sequence identity was 98.2—100% in DNA sequence identity or was 98.2—98.9% if strain C4 was excluded (Table 3). Therefore, *groEL* gene has better discriminative power in

Table 2 Characteristics of *E. faecium* strains used for *groESL* and *rpoB* gene sequencing and MLST analysis.

Strain	[a]MLST profile	ST	CC17	Clade	[b]Alloted group based on *groEL*	Accession number of *groEL*	[c]Resistance	Reference
B6	15-1-1-1-1-20-1	203	Yes	A	II	—	E, G	This study
B7	1-1-1-1-1-1-1	17	Yes	A	I	—	C, E, G	This study
B8	7-1-35-1-5-1-1	340	Yes	A	I	—	None	This study
C4	6-6-4-4-3-3-27	812	No	B	Singleton 1	MH109108	None	This study
C5	7-1-1-1-1-7-1	359	Yes	A	I	—	ND	This study
E09	1-1-1-1-1-1-1	17	Yes	A	II	—	E, S	This study
E36	1-1-1-1-1-1-1	17	Yes	A	II	—	None	This study
V18	7-1-1-1-5-7-1	262	Yes	A	I	—	E, G, V	This study
V19	7-1-1-1-5-7-1	262	Yes	A	I	—	E, G, V	This study
V22	1-1-1-1-1-3-1	NT1	Yes	A	I	—	E, V	This study
V52	1-1-1-1-1-3-1	NT1	Yes	A	I	—	E, V	This study
V68	15-1-1-1-1-1-1	78	Yes	A	I	MH109129	E, S, V	This study
V77	1-1-1-1-1-1-1	17	Yes	A	II	—	E, V	This study
81	25-8-8-8-10-10-6	296	No	B	IV	MH109127	None	This study
89	9-2-1-3-1-7-5	NT2	No	A	Singleton 2	MH109130	E	This study
92	1-1-1-1-1-7-1	202	Yes	A	I	—	E, S	This study
94	1-1-1-1-1-7-1	202	Yes	A	I	—	E, S	This study
4780—1	7-1-1-1-5-1-1	18	Yes	A	I	—	E, G, S	This study
9091—2	7-1-1-1-5-7-1	262	Yes	A	I	—	E, G, S, V	This study
ATCC 19434	4-3-1-27-1-1-1	160	Yes	A	II	AY417582	ND	Teng et al.[38]
ATCC 35667	4-3-1-27-1-1-1	160	Yes	A	Singleton 3	AY417584	ND	Teng et al.[38]
1_141_733	25-13-9-15-10-9-6	327	No	B	III	NZ_GG688465	ND	Montealegre et al.[14]
com12	11-13-18-17-10-9-6	107	No	B	III	NZ_GG670308	ND	Montealegre et al.[14]
com15	5-8-14-22-8-26-6	583	No	B	Singleton 4	NZ_GG670328	ND	Montealegre et al.[14]
E980	13-8-8-8-6-10-6	94	No	B	IV	NZ_ABQA01000018	ND	Montealegre et al.[14]
EnGen0003	5-13-9-15-8-37-6	163	No	B	III	NZ_KB029693	ND	Montealegre et al.[14]
EnGen0015	13-8-8-8-6-17-6	61	No	B	IV	NZ_KB029496	ND	Montealegre et al.[14]
EnGen0026	25-8-8-8-10-10-6	296	No	B	IV	NZ_KB029917	ND	Montealegre et al.[14]
EnGen0028	13-8-8-23-6-28-11	75	No	B	IV	NZ_KB029699	ND	Montealegre et al.[14]
EnGen0029	13-8-10-23-6-29-11	77	No	B	IV	NZ_KB029708	ND	Montealegre et al.[14]
EnGen0033	24-8-8-8-6-10-11	299	No	B	IV	NZ_KB029905	ND	Montealegre et al.[14]
EnGen0042	13-8-8-8-6-40-6	289	No	B	IV	NZ_KB029865	ND	Montealegre et al.[14]
EnGen0047	25-13-18-17-10-19-6	328	No	B	III	NZ_KB029981	ND	Montealegre et al.[14]
EnGen0056	25-13-9-15-10-19-6	327	No	B	Singleton 5	NZ_KB029968	ND	Montealegre et al.[14]
LCT-EF90	13-8-10-23-6-28-11	76	No	B	IV	NZ_JH636586	ND	Montealegre et al.[14]

[a] Order of 7 loci of MLST: *atpA*, *ddl*, *gdh*, *purK*, *gyd*, *pstS*, *adk*.
[b] Identical *groEL* sequences were allotted into groups I to IV; unique *groEL* sequences were indicated as singletons 1 to 5.
[c] For gentamicin and streptomycin, only high-level resistance (>500 μg/ml and >2000 μg/ml, respectively) are listed. Abbreviation: C, chloramphenicol; E, erythromycin; G, gentamicin; S, streptomycin; V, vancomycin. None, susceptible to antibiotics tested; ND, not determined in this study.

differentiating clade A and clade B *E. faecium* compared with *rpoB* gene.

Phylogenetic analysis of *Enterococcus* species based on *groEL* gene

Nearly full-length of *groEL* nucleotide sequences (positions 1 to 1427) for 77 strains of 12 different *Enterococcus* species were determined to evaluate the applicability of the *groEL* sequence in *Enterococcus* species identification. The 30 *E. faecium* strains with identical sequences were indicated groups I to IV respectively (noted in Table 2) instead of listing all of the strain numbers to condense the phylogenetic tree without changing the topology. The lowest inter-species DNA sequence identity was 78.6% (*E. cecorum* vs. *Enterococcus gilvus*), while the highest identity was 88.5% (*E. raffinosus* vs. *E. malodoratus*). The phylogenetic tree based on *groEL* was presented in Fig. 3. Isolates of the same species clustered together and formed stable groups (bootstrap value 100%). Moreover, strains of *E. faecium* and *E. gallinarum* could be further divided into two clades, respectively. For *E. gallinarum* strains, the intra-clade sequence variation was 99.8—100% in DNA sequence identity; the inter-clade sequence variation was 95.0—95.4% in DNA sequence identity (66—71 different nucleotides), which was lower than that for *E. faecium*.

Multiplex PCR to differentiate seven *Enterococcus* species

Based on the determined *groEL* sequences, one universal forward primer and seven species-specific primers were

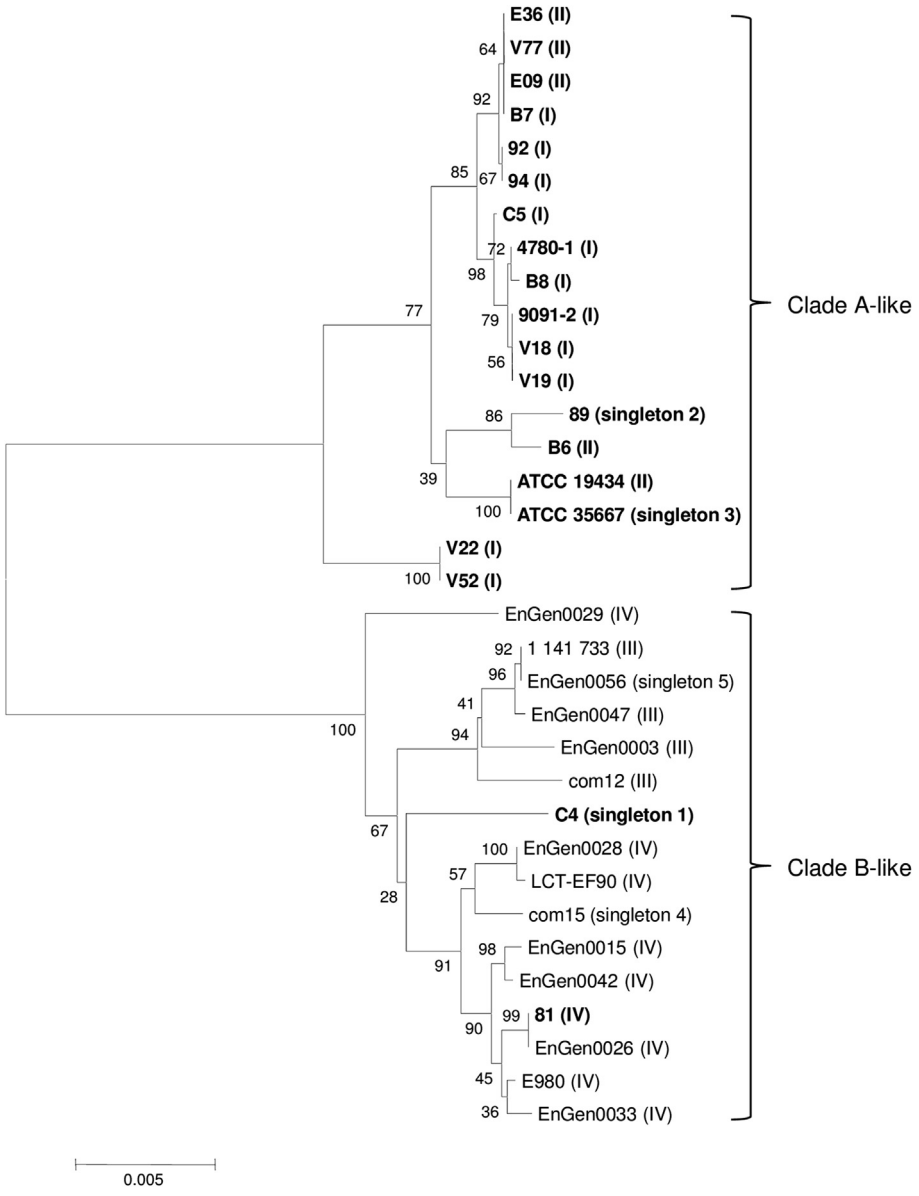

Fig. 2. Neighbor-joining tree showing the relationships among *E. faecium* based on concatenated sequences of 7 housekeeping genes of MLST. Numbers at nodes are confidence levels expressed as percentages of occurrence in 500 bootstrapped resamplings. MLST type of the strain determined in this study was shown in bold. Parentheses indicate the groups or singletons based on the *groEL* sequence as described in Table 2 and Fig. 3. The scale bar indicated 0.005 substitutions per nucleotide position.

Table 3 *groEL* and *rpoB* gene sequence identity among clade A and clade B *E. faecium* strain.

E. faecium strains	% Identity (no. of different nucleotides)	
	[a]*groEL*	[b]*rpoB*
Clade A to clade A	99.7—100 (0—4)	99.5—100 (0—3)
Clade B to clade B	99.0—100 (0—14)	98.3—100 (0—11)
Clade A to clade B	96.4—97.2 (40—52)	98.2—100 (0—12)

[a] The length of the sequence determined was 1427 bp, corresponding to nt 1 to 1427 in the *groEL* (whole length is 1626 bp).

[b] The length of the sequence determined was 660 bp, corresponding to nt 2461 to 3120 in the *rpoB* (whole length is 3627 bp).

designed to detect seven clinically relevant enterococcal species including *E. avium*, *E. casseliflavus*, *E. faecalis*, *E. faecium*, *E. gallinarum*, *E. hirae* and *E. raffinosus* (Table 1). Different sizes of PCR amplification products ranging from 135 bp to 688 bp were produced according to species-specific primers. Fig. 4 illustrated the multiplex PCR-amplified products with the *Enterococcus* ATCC reference strains following 2% agarose gel electrophoresis. Only the corresponding strains showed the expected PCR amplification products. Using strains other than the 7 *Enterococcus* species such as *E. cecorum*, *E. durans* and *E. mundtii* as template would not generate any PCR products (Fig. 4).

To assess the reproducibility of the multiplex PCR assay, we extended our analysis to include 203 additional enterococcal clinical isolates, including *E. avium*, *E.*

Table 4 The nucleotide sequence identities among the 9 *groEL* sequence types of the *E. faecium* strains.

[a]Groups or singletons	Clade	% nucleotide sequence similarity (lower left) and numbers of different nucleotides (upper right)								
		I	II	III	IV	S1	S2	S3	S4	S5
I	A	—	1	50	41	41	3	3	43	51
II	A	99.9	—	49	40	40	2	2	42	50
III	B	96.5	96.6	—	11	12	51	51	13	1
IV	B	97.1	97.2	99.2	—	5	42	42	6	12
S1	B	97.1	97.2	99.2	99.6	—	42	42	7	13
S2	A	99.8	99.9	96.4	97.1	97.1	—	4	44	52
S3	A	99.8	99.9	96.4	97.1	97.1	99.7	—	44	52
S4	B	97.0	97.1	99.1	99.6	99.5	96.9	96.9	—	14
S5	B	96.4	96.5	99.9	99.2	99.1	96.4	96.4	99.0	—

[a] Groups were indicated I to IV and singletons were indicated S1 to S5 as noted in Table 2.

casseliflavus, *E. cecorum*, *E. faecalis*, *E. faecium*, *E. gallinarum*, *E. hirae* and *E. raffinosus*. Complete agreement was obtained with the species-specific primers used in the present assay for all isolates examined.

Discussion

In this study, we used *groEL* as gene target to differentiate the two *E. faecium* clades. The *groEL* gene displayed 96.4—97.2% for inter-clade nucleotide identities of *E. faecium* and >99% intra-clade nucleotide identities (Tables 3 and 4), indicating that the *groEL* sequence variation is conserved in the two *E. faecium* clades. Previous study showed the inter-clade nucleotide identities of *E. faecium* were 96.2—96.9% for concatenated MLST sequences and 93.9—95.6% in the average nucleotide identity (ANI) analysis achieved by whole genome sequencing.[43] The *groEL* sequence identity between the two *E. faecium* clades was overlapping to that of concatenated MLST sequences but slightly higher than ANI values, implying the similar discriminatory power between *groEL* and MLST. The *groEL* gene is one of the 1423 core genome MLST (cgMLST) target genes to differentiate clade A and clade B *E. faecium*.[44] The *groES* gene, which is adjacent to *groEL* and forms an operon with *groEL* gene, has been chosen for phylogenetic analysis of the concatenated 100 gene sequences because of the clade distinction power.[42] Therefore, the *groEL* gene is a representative marker in screening two *E. faecium* clades.

Only two strains C4 and 81 were identified to be clade B out of 141 *E. faecium* clinical isolates. This is not surprising because clade A, particular CC17, is predominant in the hospital infection and usually confers resistance to multiple antibiotics[13]; clade B is dominant in human gastrointestinal commensal, rarely causes disease and is usually susceptible to antibiotics.[13,42,43] The two clade B strains were susceptible to the five antibiotics tested (Table 2), the same with previously reported. Unexpectedly, the C4 strain was identified to be clade B *E. faecium* by MLST and *groESL* gene sequencing, disagreement with the result of *rpoB* gene sequencing (Fig. 2 and Table 3). *E. faecium* genome is highly plastic. Howden et al. reported that a total of 1.3 Mb (44%) of the chromosome underwent recombination event.[45] Even four MLST genes are located in the recombination hot spots.[46] Therefore, the discrepancy may be due to recombination event occurring in *rpoB* gene.

The 9 *E. gallinarum* strains could be further divided into two clades based on *groEL* sequence, similar to *E. faecium* (Fig. 3). The inter-clade *groEL* sequence similarity of *E. gallinarum* was lower than that of *E. faecium* (95.0—95.4% vs. 96.4—97.2%). The divergence of *E. faecium* clades represent a good example how gut commensal adapts to hospital environment by the acquisition of new metabolic capabilities and by the virtue of rendering many antibiotics.[13,47,48] However, to the best of the authors' knowledge there are no reports exploring the diversification of *E. gallinarum*. Further studies are needed to trace evolution of *E. gallinarum*.

Little is known about the clinical epidemiology of non-*faecalis* and non-*faecium* enterococcal infection. Tan et al. reported that prevalence of enterococcal bacteremia caused by non-*faecalis* and non-*faecium* enterococci was 9.6% during 2000—2008 at National Taiwan University Hospital, and the top five species were *E. casseliflavus* (n = 59), *E. gallinarum* (n = 58), *E. avium* (n = 45), *E. hirae* (n = 9) and *E. raffinosus* (n = 9) in 182 of these patients.[4] *E. durans* (n = 2), *E. cecorum* (n = 2) and *Enterococcus canintestini* (n = 1) were less encountered.[4] As a result, we developed the multiplex PCR to differentiate *E. faecalis*, *E. faecium* and the top five non-*faecalis* and non-*faecium* species described above. Because automated system such as VITEK2 and Phoenix is less efficient in detection of *Enterococcus* species other than *E. faecalis* and *E. faecium*,[17—21] the multiplex PCR we developed, which efficiently differentiated the 7 most commonly encountered *Enterococcus* species, would be beneficial for decreasing the rate of misidentification.

In this study, we developed a PCR-RFLP assay based on *groESL* gene to screen two *E. faecium* clades, and a multiplex PCR assay targeting *groEL* gene to identify 7 clinically relevant *Enterococcus* species including *E. avium*, *E. casseliflavus*, *E. faecalis*, *E. faecium*, *E. gallinarum*, *E. hirae* and *E. raffinosus*. These assays are easy to perform

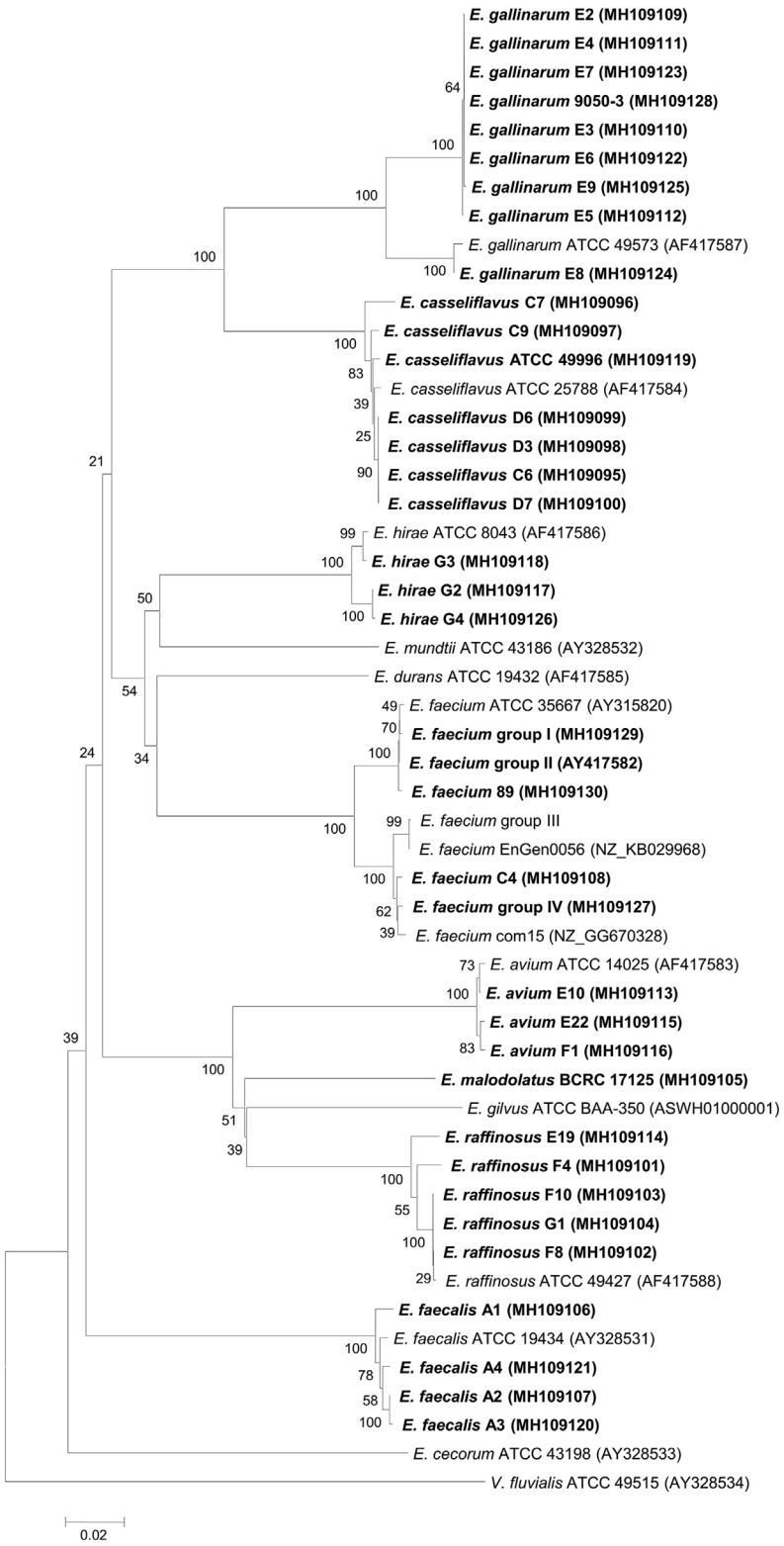

Fig. 3. Neighbor-joining tree showing the relationships among *Enterococcus* species based on *groEL* nucleotide sequences (nt 1 to 1427). Numbers at nodes are confidence levels expressed as percentages of occurrence in 500 bootstrapped resamplings. The *groEL* sequences determined in this study were shown in bold. *E. faecium* strains showing identical *groEL* sequences were allotted into group I to IV (also noted in Table 2). *V. fluvialis* ATCC 49515 was used as an outgroup. The scale bar indicated 0.02 substitutions per nucleotide position. GenBank accession numbers are given in parentheses.

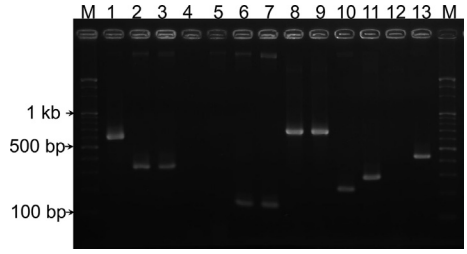

Fig. 4. Multiplex PCR among the *Enterococcus* species following 2% agarose gel electrophoresis. Lane M, 100-bp DNA ladder; lane 1, *E. avium* ATCC 14025; lane 2, *E. casseliflavus* ATCC 25788, lane 3, *E. casseliflavus* ATCC 49996; lane 4, *E. cecorum* ATCC 43198; lane 5, *E. durans* ATCC 19432; lane 6, *E. faecalis* ATCC 19433; lane 7, *E. faecalis* ATCC 29212; lane 8, *E. faecium* ATCC 19434; lane 9, *E. faecium* ATCC 35667; lane 10, *E. gallinarum* ATCC 49573; lane 11, *E. hirae* ATCC 8043; lane 12, *E. mundtii* ATCC 43186; and lane 13, *E. raffinosus* ATCC 49427.

and offer an alternative way to currently used automated methods.

Conflicts of interest

All authors declare no conflicts of interest.

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References

1. Arias CA, Murray BE. The rise of the *Enterococcus*: beyond vancomycin resistance. *Nat Rev Microbiol* 2012;**10**:266—78.
2. Chiu CC, Lin TC, Wu RX, Yang YS, Hsiao PJ, Lee Y, et al. Etiologies of community-onset urinary tract infections requiring hospitalization and antimicrobial susceptibilities of causative microorganisms. *J Microbiol Immunol Infect* 2017;**50**:879—85.
3. Weiner LM, Webb AK, Limbago B, Dudeck MA, Patel J, Kallen AJ, et al. Antimicrobial-resistant pathogens associated with healthcare-associated infections: summary of data reported to the national healthcare safety network at the centers for disease control and prevention, 2011—2014. *Infect Control Hosp Epidemiol* 2016;**37**:1288—301.
4. Tan CK, Lai CC, Wang JY, Lin SH, Liao CH, Huang YT, et al. Bacteremia caused by non-*faecalis* and non-*faecium Enterococcus* species at a medical center in Taiwan, 2000 to 2008. *J Infect* 2010;**61**:34—43.
5. Prakash VP, Rao SR, Parija SC. Emergence of unusual species of enterococci causing infections, South India. *BMC Infect Dis* 2005;**5**:14.
6. Singh KV, Weinstock GM, Murray BE. An *Enterococcus faecalis* ABC homologue (Lsa) is required for the resistance of this species to clindamycin and quinupristin-dalfopristin. *Antimicrob Agents Chemother* 2002;**46**:1845—50.
7. Hollenbeck BL, Rice LB. Intrinsic and acquired resistance mechanisms in *Enterococcus*. *Virulence* 2012;**3**:421—33.
8. Faron ML, Ledeboer NA, Buchan BW. Resistance mechanisms, epidemiology, and approaches to screening for vancomycin-resistant *Enterococcus* in the health care setting. *J Clin Microbiol* 2016;**54**:2436—47.
9. Top J, Willems R, Blok H, de Regt M, Jalink K, Troelstra A, et al. Ecological replacement of *Enterococcus faecalis* by multiresistant clonal complex 17 *Enterococcus faecium*. *Clin Microbiol Infect* 2007;**13**:316—9.
10. Leavis HL, Bonten MJ, Willems RJ. Identification of high-risk enterococcal clonal complexes: global dispersion and antibiotic resistance. *Curr Opin Microbiol* 2006;**9**:454—60.
11. Kuo AJ, Shu JC, Liu TP, Lu JJ, Lee MH, Wu TS, et al. Vancomycin-resistant *Enterococcus faecium* at a university hospital in Taiwan, 2002—2015: fluctuation of genetic populations and emergence of a new structure type of the Tn*1546*-like element. *J Microbiol Immunol Infect* 2018;**51**:821—8. https://doi.org/10.1016/j.jmii.2018.08.008.
12. Ye JJ, Shie SS, Cheng CW, Yang JH, Huang PY, Wu TS, et al. Clinical characteristics and treatment outcomes of vancomycin-resistant *Enterococcus faecium* bacteremia. *J Microbiol Immunol Infect* 2018;**51**:705—16. https://doi.org/10.1016/j.jmii.2017.08.025.
13. Lebreton F, van Schaik W, McGuire AM, Godfrey P, Griggs A, Mazumdar V, et al. Emergence of epidemic multidrug-resistant *Enterococcus faecium* from animal and commensal strains. *MBio* 2013;**4**, e00534-13.
14. Montealegre MC, Singh KV, Murray BE. Gastrointestinal tract colonization dynamics by different *Enterococcus faecium* clades. *J Infect Dis* 2016;**213**:1914—22.
15. Gao W, Howden BP, Stinear TP. Evolution of virulence in *Enterococcus faecium*, a hospital-adapted opportunistic pathogen. *Curr Opin Microbiol* 2018;**41**:76—82.
16. Willey BM, Jones RN, McGeer A, Witte W, French G, Roberts RB, et al. Practical approach to the identification of clinically relevant *Enterococcus* species. *Diagn Microbiol Infect Dis* 1999;**34**:165—71.
17. Eisner A, Gorkiewicz G, Feierl G, Leitner E, Kofer J, Kessler HH, et al. Identification of glycopeptide-resistant enterococci by VITEK 2 system and conventional and real-time polymerase chain reaction. *Diagn Microbiol Infect Dis* 2005;**53**:17—21.
18. Abele-Horn M, Hommers L, Trabold R, Frosch M. Validation of VITEK 2 version 4.01 software for detection, identification, and classification of glycopeptide-resistant enterococci. *J Clin Microbiol* 2006;**44**:71—6.
19. Brigante G, Luzzaro F, Bettaccini A, Lombardi G, Meacci F, Pini B, et al. Use of the Phoenix automated system for identification of *Streptococcus* and *Enterococcus* spp. *J Clin Microbiol* 2006;**44**:3263—7.
20. Kawalec M, Kedzierska J, Gajda A, Sadowy E, Wegrzyn J, Naser S, et al. Hospital outbreak of vancomycin-resistant enterococci caused by a single clone of *Enterococcus raffinosus* and several clones of *Enterococcus faecium*. *Clin Microbiol Infect* 2007;**13**:893—901.
21. Fang H, Ohlsson AK, Ullberg M, Ozenci V. Evaluation of species-specific PCR, Bruker MS, VITEK MS and the VITEK 2 system for the identification of clinical *Enterococcus* isolates. *Eur J Clin Microbiol Infect Dis* 2012;**31**:3073—7.
22. Kassim A, Pfluger V, Premji Z, Daubenberger C, Revathi G. Comparison of biomarker based Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) and conventional methods in the identification of clinically relevant bacteria and yeast. *BMC Microbiol* 2017;**17**:128.
23. Clark AE, Kaleta EJ, Arora A, Wolk DM. Matrix-assisted laser desorption ionization-time of flight mass spectrometry: a fundamental shift in the routine practice of clinical microbiology. *Clin Microbiol Rev* 2013;**26**:547—603.

24. Rychert J, Burnham CA, Bythrow M, Garner OB, Ginocchio CC, Jennemann R, et al. Multicenter evaluation of the Vitek MS matrix-assisted laser desorption ionization-time of flight mass spectrometry system for identification of Gram-positive aerobic bacteria. *J Clin Microbiol* 2013;**51**:2225—31.
25. Moon HW, Lee SH, Chung HS, Lee M, Lee K. Performance of the Vitek MS matrix-assisted laser desorption ionization time-of-flight mass spectrometry system for identification of Gram-positive cocci routinely isolated in clinical microbiology laboratories. *J Med Microbiol* 2013;**62**:1301—6.
26. Christ APG, Ramos SR, Cayo R, Gales AC, Hachich EM, Sato MIZ. Characterization of *Enterococcus* species isolated from marine recreational waters by MALDI-TOF MS and Rapid ID API(R) 20 Strep system. *Mar Pollut Bull* 2017;**118**:376—81.
27. Ryu H, Henson M, Elk M, Toledo-Hernandez C, Griffith J, Blackwood D, et al. Development of quantitative PCR assays targeting the 16S rRNA genes of *Enterococcus* spp. and their application to the identification of *Enterococcus* species in environmental samples. *Appl Environ Microbiol* 2013;**79**:196—204.
28. Tung SK, Teng LJ, Vaneechoutte M, Chen HM, Chang TC. Identification of species of *Abiotrophia*, *Enterococcus*, *Granulicatella* and *Streptococcus* by sequence analysis of the ribosomal 16S-23S intergenic spacer region. *J Med Microbiol* 2007;**56**:504—13.
29. Tung SK, Teng LJ, Vaneechoutte M, Chen HM, Chang TC. Array-based identification of species of the genera *Abiotrophia*, *Enterococcus*, *Granulicatella*, and *Streptococcus*. *J Clin Microbiol* 2006;**44**:4414—24.
30. Kariyama R, Mitsuhata R, Chow JW, Clewell DB, Kumon H. Simple and reliable multiplex PCR assay for surveillance isolates of vancomycin-resistant enterococci. *J Clin Microbiol* 2000;**38**:3092—5.
31. Cheng S, McCleskey FK, Gress MJ, Petroziello JM, Liu R, Namdari H, et al. A PCR assay for identification of *Enterococcus faecium*. *J Clin Microbiol* 1997;**35**:1248—50.
32. Drancourt M, Roux V, Fournier PE, Raoult D. *rpoB* gene sequence-based identification of aerobic Gram-positive cocci of the genera *Streptococcus*, *Enterococcus*, Gemella, *Abiotrophia*, and *Granulicatella*. *J Clin Microbiol* 2004;**42**:497—504.
33. Poyart C, Quesnes G, Trieu-Cuot P. Sequencing the gene encoding manganese-dependent superoxide dismutase for rapid species identification of enterococci. *J Clin Microbiol* 2000;**38**:415—8.
34. Jackson CR, Fedorka-Cray PJ, Barrett JB. Use of a genus- and species-specific multiplex PCR for identification of enterococci. *J Clin Microbiol* 2004;**42**:3558—65.
35. Hemmingsen SM, Woolford C, van der Vies SM, Tilly K, Dennis DT, Georgopoulos CP, et al. Homologous plant and bacterial proteins chaperone oligomeric protein assembly. *Nature* 1988;**333**:330—4.
36. Viale AM, Arakaki AK, Soncini FC, Ferreyra RG. Evolutionary relationships among eubacterial groups as inferred from GroEL (chaperonin) sequence comparisons. *Int J Syst Bacteriol* 1994;**44**:527—33.
37. Tsai JC, Hsueh PR, Lin HM, Chang HJ, Ho SW, Teng LJ. Identification of clinically relevant *Enterococcus* species by direct sequencing of *groES* and spacer region. *J Clin Microbiol* 2005;**43**:235—41.
38. Teng LJ, Hsueh PR, Wang YH, Lin HM, Luh KT, Ho SW. Determination of *Enterococcus faecalis groESL* full-length sequence and application for species identification. *J Clin Microbiol* 2001;**39**:3326—31.
39. Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial susceptibility testing. Twenty-seventh informational supplement M100-S27*. Wayne, PA, USA: CLSI; 2017.
40. Homan WL, Tribe D, Poznanski S, Li M, Hogg G, Spalburg E, et al. Multilocus sequence typing scheme for *Enterococcus faecium*. *J Clin Microbiol* 2002;**40**:1963—71.
41. Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *J Bacteriol* 2004;**186**:1518—30.
42. Galloway-Pena J, Roh JH, Latorre M, Qin X, Murray BE. Genomic and SNP analyses demonstrate a distant separation of the hospital and community-associated clades of *Enterococcus faecium*. *PLoS One* 2012;**7**, e30187.
43. Palmer KL, Godfrey P, Griggs A, Kos VN, Zucker J, Desjardins C, et al. Comparative genomics of enterococci: variation in *Enterococcus faecalis*, clade structure in *E. faecium*, and defining characteristics of *E. gallinarum* and *E. casseliflavus*. *MBio* 2012;**3**, e00318-11.
44. de Been M, Pinholt M, Top J, Bletz S, Mellmann A, van Schaik W, et al. Core genome multilocus sequence typing scheme for high-resolution typing of *Enterococcus faecium*. *J Clin Microbiol* 2015;**53**:3788—97.
45. Howden BP, Holt KE, Lam MM, Seemann T, Ballard S, Coombs GW, et al. Genomic insights to control the emergence of vancomycin-resistant enterococci. *MBio* 2013;**4**:e00412—3.
46. Raven KE, Reuter S, Reynolds R, Brodrick HJ, Russell JE, Torok ME, et al. A decade of genomic history for healthcare-associated *Enterococcus faecium* in the United Kingdom and Ireland. *Genome Res* 2016;**26**:1388—96.
47. Lebreton F, Manson AL, Saavedra JT, Straub TJ, Earl AM, Gilmore MS. Tracing the enterococci from Paleozoic origins to the hospital. *Cell* 2017;**169**:849—61.
48. Van Tyne D, Gilmore MS. Friend turned foe: evolution of enterococcal virulence and antibiotic resistance. *Annu Rev Microbiol* 2014;**68**:337—56.