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Original Article

Vancomycin-variable enterococci in sheep and cattle isolates and whole-genome sequencing analysis of isolates harboring *vanM* and *vanB* genes

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Abstract

Background: Vancomycin resistance encoded by the *vanA*/*B*/*M* genes in enterococci is clinically important because of the transmission of these genes between bacteria. While vancomycin resistance is determined by detecting only *vanA* and *vanB* genes by routine analyses, failure to detect *vanM* resistance causes vancomycin resistance to be overlooked, and clinically appropriate treatment cannot be provided. **Aims:** The study aimed to examine the presence of *vanM*-positive enterococcal isolates in Ankara, Turkey, and to have detailed information about them with sequence analyses. **Methods:** Caecal samples were collected from sheep and cattle during slaughter at different slaughterhouses in Ankara, Turkey. Enterococci isolates were identified, confirmed, and analyzed for the presence of *vanA*/*B*/*M* genes. Antibiotic resistance profiles of isolates were determined by the broth microdilution method. A whole genome sequence analysis of the isolates harboring the *vanM* and *vanB* genes was performed. **Results:** 13.7% of enterococcal isolates were determined as *Enterococcus faecium* and *Enterococcus faecalis*. 15% of these isolates contained *vanB*, and 40% were *vanM*-positive. S98b and C32 isolates were determined to contain 16 CRISPR-Cas elements. 80% of the enterococci isolates were resistant to nitrofurantoin and 15% to ciprofloxacin. The first *vanM*-positive vancomycin-variable enterococci (VVE) isolates from food-producing animals were identified, and the S98b strain has been assigned to Genbank with the accession number CP104083.1. **Conclusion:** Therefore, new studies are needed to facilitate the identification of *vanM*-resistant enterococci and VVE strains.

Key words: CRISPR-Cas, Vancomycin-variable enterococci, *vanM*

Introduction

Enterococci are bacteria common in the gastrointestinal tract of both humans and animals. They have received great attention for their ability to harbor antibiotic resistance genes and their potential to transfer these genes to other bacteria, thus contributing to the global problem of antibiotic resistance. Understanding the carriage of enterococci in food animals holds significant implications for public health. First and foremost, the potential transmission of antibiotic-resistant enterococci from animals to humans raises concerns about the spread of antibiotic resistance genes. This could lead to reduced effectiveness of antibiotics in treating infections in both animals and humans, ultimately compromising medical interventions (Leclercq, 1997; Zaheer *et al*., 2020). By the Commission Implementation Decision No. 2020/1729/EU on monitoring and reporting antimicrobial resistance in zoonotic and commensal bacteria, it is expected that *Enterococcus faecalis* and *Enterococcus faecium* isolates will be isolated from cecum samples taken from bovine animals during slaughter (EU, 2020).

Vancomycin-resistant enterococci (VRE) are recognized as significant public health pathogens due to limited treatment options (Lee *et al*., 2022). VRE clones are considered a serious threat, with an estimated 54,500 cases in hospitalized patients and 5,400 deaths per year (CDC, 2019). Vancomycin resistance in enterococci is associated with multiple *van* genes, including *vanA*/*B*/*C*/*D*/*E*/*F*/*G*/*L*/*M*/*N* (Ahmed and Baptiste, 2018). Vancomycin resistance, mostly seen in *E. faecium* and *E. faecalis*, is encoded by *vanA*/*B*/*M* genes and is clinically important because of the transmission of these genes between bacteria (Lee *et al*., 2022). Besides, only *vanA* and *vanM* genes are reported to cause high levels of vancomycin and teicoplanin resistance (Ahmed and Baptiste, 2018). So far, *vanM*-type VREs have only been reported from China, Japan, and Singapore, and their clinical isolation has increased rapidly in China (Chen *et al*., 2015; Hashimoto *et al*., 2019; Sun *et al*., 2019). Moreover, in Shanghai, *vanM*-type VRE has been

reported to be more common than *vanA*-type (Chen *et al*., 2015).

These data indicate that the global spread of *vanM*-type VRE is a critical public health problem. However, there is no direct detection method for VRE strains, including *vanM* strains, other than molecular methods (Yan *et al*., 2022). To prevent the transmission of vancomycin resistance to other pathogens and spread to the environment, detecting vancomycin-resistant *E. faecium* and *E. faecalis* is crucial for public health (Zhou *et al*., 2020). Considering their resistance profiles and transmissibility, there is insufficient information on *vanM*-resistant enterococci. As stated in other studies, the determination of *vanM*-type vancomycin resistance in enterococcal isolates in our country as well as in the world is essential, especially in terms of comparing resistance profiles and interactions with data obtained from different geographical regions (Zhou *et al*., 2020; Yan *et al*., 2022).

On the other hand, vancomycin-variable enterococci (VVE) have been reported as the current problem with *Enterococcus* spp.; VVE strains have a *van*-positive genotype but a susceptible phenotype and can transform into a resistant phenotype during vancomycin selection in the cell (Wagner *et al*., 2021). VVE isolates harboring *vanA* or *vanB* genes have been reported and these clones have caused different hospital outbreaks. VVE is detectable only by molecular methods and cannot be cultured in media containing selective vancomycin. This makes VVE detection significant in preventing hospital outbreaks and providing effective antibiotic therapy for infected patients (Hammerum *et al*., 2019).

In recent years, it has been demonstrated that Clustered Regularly Interspaced Short Palindromic Repeats-related or CRISPR-Cas system, an adaptive immune system, basically targets and inactivates foreign genetic elements that cause invasion into the bacterial host cell (Krause *et al*., 2022). CRISPR-Cas system is defined as an obstacle to horizontal gene transfer because bacteria with the CRISPR-Cas system are more resistant to the invasion of genetic materials. It is predicted that it can be used to prevent antimicrobial resistance in microorganisms (Palmer and Gilmore, 2010).

This study aimed to investigate *vanA*, *vanB*, and *vanM*-type vancomycin resistance and CRISPR-Cas loci in enterococcal isolates in Turkey and to determine phenotypically the resistance profiles of different antibiotic groups. Our study has potential importance in providing the first information about *vanM*-type vancomycin resistance and CRISPR-Cas loci in enterococcal isolates in Turkey.

Materials and Methods

Sampling

Due to the Commission Implementation Decision No. 2020/1729/EU, 240 (120 cattle and 120 sheep) caecal samples were collected during the monthly visits to two different slaughterhouses in Ankara, Turkey after the evisceration process. The sample size used in the study was calculated with the following formula.

$$n = \frac{Z^2 P(1-P)}{d^2}$$

Where,
n: Sample size
Z: Z statistic for a level of confidence
P: Expected prevalence (if the expected prevalence is 20%, then P=0.2)
d: Precision (if the precision is 5%, then d=0.05)

The formula specified by Daniel *et al*. (1999) was used to determine the sample size. In this formula, Z=1.96, d=0.05, d=0.05, and P=0.2 [prevalence of vancomycin-resistant enterococci in Turkey was determined as 20% (Onaran *et al*., 2019)] were used. The sample size was determined as 240.

Samples were taken to the laboratory under aseptic conditions within cool boxes and analyzed on the same day. Sample collection dates of *vanM*-positive isolates are given in Table 1.

Enterococcus spp. isolation

Enterococcus spp. isolation was performed according to Domig *et al*. (2003). Ten g of the samples were weighed and mixed with 90 ml of Buffered Peptone Water (Merck, 107228), homogenized in a stomacher (Blender easyMix, Biomerieux, France) for two min. Aliquots of 0.1 ml were streaked onto Slanetz-Bartley Agar (SB, Oxoid CM0377A), and incubated for 24-48 h at 37°C aerobically. After incubation, typical red/pink colored colonies were selected, enriched in Brain Heart Infusion broth (BHI, Merck 110493), and stored at -80°C with 20% glycerol. Confirmation and identification of *Enterococcus* spp. were determined by the conditions and primers of the polymerase chain reaction (PCR) as shown in Table 2. *E. faecalis* ATCC 29212, *E. faecalis* ATCC 47077 (CRISPR1-Cas+), *E. faecium* tetM 7003, *E. faecium* ATCC 51559 (*vanA*+), and *E. faecalis* ATCC 51299 (*vanB*+) strains were used as positive controls.

Detection of *van* genes and CRISPR-Cas loci

PCR analysis to identify *vanA*, *vanB* (Kariyama *et al*., 2000), and *vanM* (Di Francesco *et al*., 2021) genes, CRISPR1-Cas, CRISPR2, and CRISPR3-Cas loci (Palmer and Gilmore, 2010) of the CRISPR-Cas system in *E. faecalis* and *E. faecium* isolates were performed using PCR conditions and primers as shown in Table 2. A total volume of 30 µL PCR master mix was prepared with 10 × Taq PCR buffer, 2 mM $MgCl_2$, 0.5 U of Taq DNA polymerase (Ep0402, Thermo, Lithuania), 0.2 mM of each dNTP (R0192, Thermo, Lithuania), and 0.2 µM of each primer (Sentebio, Turkey). Following the thermal cycling process, end products were run in 1.5% (w/v) agarose gels.

Determination of minimum inhibitory concentrations (MICs) of antibiotics

The broth microdilution method was used to determine MICs of vancomycin, teicoplanin, linezolid,

Table 1: Sample collection dates, species, and *van* gene distribution of *vanM*-positive isolates and sequence results according to BLAST analysis

Isolate (cattle/sheep)	Date of sample collection	*tuf*	Efa/Efec	*vanA*/*vanB*	*vanM*	SANGER/WGS
C1a	20.02.2020	+	Efa	-	*vanM*	SANGER: *E. faecium* strain N56454 chromosome, complete genome, Ident. 100.00%, GenBank: CP040904.1
C32	24.06.2020	+	Efa	*vanB*	*vanM*	WGS: *E. faecalis* strain CQ025 chromosome, complete genome, Ident. 100.00%, GenBank: CP098418.1
C33a	24.06.2020	+	Efec	*vanB*	*vanM*	WGS: *E. faecium* strain ef332 plasmid plas3, complete sequence, Ident. 100.00%, GenBank: CP058894.1 *E. faecium* strain VRE chromosome, complete genome, Ident. 100.00%, GenBank: CP046077.1
S14a	10.03.2021	+	Efec	-	*vanM*	SANGER: *E. faecium* strain VVEswe-R chromosome, complete genome, Ident. 84.21%, GenBank: CP041261.3
S23a	10.03.2021	+	Efec	-	*vanM*	SANGER: *E. faecium* strain VVEswe-R chromosome, complete genome, Ident. 94.38%, GenBank: CP041261.3
S31a	10.03.2021	+	Efec	-	*vanM*	SANGER: *E. faecium* strain VVEswe-R chromosome, complete genome, Ident. 91.67%, GenBank: CP041261.3
S73b	28.03.2021	+	Efec	-	*vanM*	SANGER: *E. faecium* strain VVEswe-R chromosome, complete genome, Ident. 89.34%, GenBank: CP041261.3
S98b	28.03.2021	+	Efec	*vanB*	*vanM*	WGS: *E. faecium* strain VVEswe-R chromosome, complete genome, Ident. 100.00%, GenBank: CP041261.3

Efa: *E. faecalis*, and Efec: *E. faecium*

Table 2: Primers and annealing temperatures of PCR assays

Target	Sequence (5´-3´)	Predicted product size	Annealing temp (°C)	Reference
tuf	TACTGACAAACCATTCATGATG AACTTCGTCACCAACGCGAAC	112	54	Ke *et al*. (1999)
$ddl_{E.faecalis}$	ATCAAGTACAGTTAGTCTTTATTAG ACGATTCAAAGCTAACTGAATCAGT	941	54	Kariyama *et al*. (2000)
$ddl_{E.faecium}$	TTGAGGCAGACCAGATTGACG TATGACAGCGACTCCGATTCC	658	54	Cheng *et al*. (1997)
vanA	CATGAATAGAATAAAAGTTGCAATA CCCCTTTAACGCTAATACGATCAA	1030	54	Evers *et al*. (1993)
vanB	GTGACAAACCGGAGGCGAGGA CCGCCATCCTCCTGCAAAAAA	433	54	Handwerger *et al*. (1992)
vanM	GGCAGAGATTGCCAACAACA AGGTAAACGAATCTGCCGCT	425	56	Nomura *et al*. (2018)
CRISPR1-Cas	CAGAAGACTATCAGTTGGTG CCTTCTAAATCTTCTTCATAG	783	55	Palmer and Gilmore (2010)
CRISPR2	CTGGCTCGCTGTTACAGCT GCCAATGTTACAATATCAAACA	Variable	55	Palmer and Gilmore (2010)
CRISPR3-Cas	GCTGAATCTGTGAAGTTACTC CTGTTTTGTTCACCGTTGGAT	258	50	Palmer and Gilmore (2010)

meropenem, erythromycin, chloramphenicol, ciprofloxacin, tetracycline, ampicillin, sulfamethoxazole-trimethoprim, fosfomycin, nitrofurantoin, and tobramycin antibiotics (Merck, Germany) (EUCAST, 2022a). Antibiotics were selected from 10 different antibiotic classes to detect multidrug-resistant (MDR) strains. The turbidity of overnight cultures at 37°C from Tryptic Soy broth (TSB, Merck 105459) prepared with cation-calibrated Mueller Hinton broth (caMHB, Merck 90922) was equivalent to the 0.5 McFarland standard. Vancomycin, teicoplanin, linezolid, erythromycin, chloramphenicol, ciprofloxacin, tetracycline, ampicillin, nitrofurantoin MIC values for enterococci were interpreted according to EUCAST v.12.0 guideline (EUCAST, 2022b) and erythromycin, fosfomycin, chloramphenicol, tetracycline MIC values were interpreted according to the Performance Standards for Antimicrobial Susceptibility Testing, 33rd Edition (CLSI, 2023). The lowest concentration at which the antimicrobial agent inhibited the growth of a strain was considered the MIC value (µg/ml) after 18-24 h of incubation at 37±2°C. *E. faecalis* ATCC 29212 was used as a quality control strain.

Sanger and whole genome sequencing (WGS) analysis of *vanM* harboring isolates

Eight *vanM*-positive isolates were selected for Sanger sequencing, and three *vanB* and *vanM*-positive *Enterococci* strains (*E. faecalis* C32, *E. faecium* C33a, and S98b) were selected for whole genome sequencing

(Table 1). For Sanger sequencing, BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, United States) and ABI 3730XL Sanger sequencing device (Applied Biosystems, Foster City, CA) were used. Reads obtained with *vanM* primers were performed using the CAP contig assembly algorithm via BioEdit software. For whole genome sequencing, bacterial genomic DNA was isolated using Zymo Research Quick-DNA TM Bacterial Miniprep Kit (Zymo Research, United States). DNA quantity and quality were measured fluorometrically and spectrophotometrically. DNA concentration was evaluated as 270.9 ng/µL and the total volume was 50 µL. The sequencing was performed by Illumina NovaSeq 6000 (Illumina, United States) as paired-end 2 × 150 base reads and the sequencing library was prepared using Nextera XT DNA Library Preparation Kit (Illumina, United States). Raw reads (FASTQ) were quality checked by FASTQC and trimmed by Trimmomatic v0.32 (Bolger *et al*., 2020). Then, the raw reads' quality was checked, and contamination, adapter sequences, and low-quality reads were removed. Demultiplexing and low-quality read filtering were performed via CLC Genomics Workbench (Qiagen, US). Then, clean reads were mapped with map reads to the reference module as the following parameters:
Mismatch cost = 3
Insertion cost = 3
Deletion cost = 3
Length fraction = 0.5
Similarity fraction = 0.8

Clean data were mapped on the *E. faecalis* EnGen0336 (GenBank assembly accession: GCA_ 000393015.1) and *E. faecium* ASM973400v2 reference genomes and reference-based consensus genomes were assembled. Genes in the consensus genome were identified using the CLC genomics workbench tool based on the reference genome annotation file. The OmicsBox tool was used to annotate these putative genes.

Additional acquired antibiotic resistance genes were screened with a minimal alignment coverage of Cheng 90% in ResFinder (Bortolaia *et al*., 2020) and the comprehensive antibiotic resistance database (CARD) (Alcock *et al*., 2020).

Results

Enterococci isolates

Within the scope of the study, the *tuf* gene was determined in 146 of 240 samples. In total, 59/120 (49%) of the cattle and 87/120 (72.5%) of the sheep samples were confirmed as *Enterococcus* spp.; Overall, 20 out of 146 (13.7%) *Enterococcus* spp. isolates were determined as *E. faecium* and *E. faecalis*. Among them, seven of the cattle isolates were confirmed as *E. faecalis*, two of them as *E. faecium*, and 11 of the sheep isolates were detected as *E. faecium*.

van genes and CRISPR-Cas loci from *E. faecalis* and *E. faecium* isolates

While three (15%) of *E. faecium* and *E. faecalis* isolates were confirmed as positive for *vanB* and eight (40%) were found to have the *vanM* resistance gene, it was observed that all of the isolates with the *vanB* gene also carried the *vanM* gene, but none of the isolates were positive for the *vanA* gene. Species and *van* gene distribution of *vanM*-positive isolates are given in Table 1. The phylogenetic tree of the isolates isolated in this study was drawn using reference strains vanA_ AY648698, vanB_AB374546, and vanM_FJ349556 from NCBI (National Center for Biotechnology Information) (Fig. 1). We could not identify CRISPR1-Cas, CRISPR2, and CRISPR3-Cas loci in our isolates.

Minimum inhibitory concentrations (MIC) of antibiotics

Resistance profiles of 20 *E. faecium* ve *E. faecalis* isolates against 13 antimicrobial agents were determined. MIC values of the isolates are shown in Table 3. In this study, the isolates listed from top to bottom C1a-C32 were identified as *E. faecalis*, and those listed as C33a-S120 were identified as *E. faecium* (Table 3). The MIC values of antibiotics were specified as resistant or

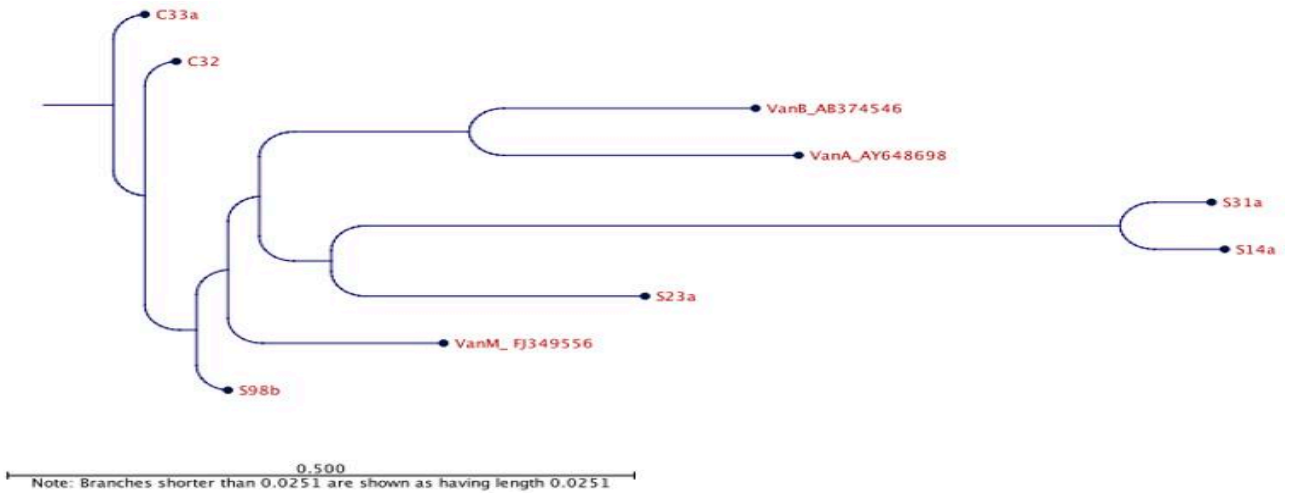

Fig. 1: Phylogenetic tree of this study isolates

susceptible based on breakpoints in the EUCAST v 12.0 guideline (EUCAST, 2022b) and CLSI guideline (CLSI, 2023). Among *E. faecium* and *E. faecalis* isolates, 16 (80%) of them were observed resistant to nitrofurantoin, 7 (35%) to tetracycline, 3 (15%) to ciprofloxacin, 2 (10%) to erythromycin, and 1 (5%) to chloramphenicol. All of the isolates were found susceptible to ampicillin, fosfomycin, linezolid, teicoplanin, and vancomycin.

Bioinformatics results of *vanM* harboring isolates

According to WGS analysis and relevant databases, virulence factors and antimicrobial resistance genes determined in *E. faecalis* C32, *E. faecium* C33a, and S98b strains are listed in Tables 4, 5, and 6. On the other hand, when Sanger and WGS sequence results were analysed in BLAST by NCBI, it was found that most of the *E. faecium* isolates (5/6, 83.3%) and S98b strain matched the VVEswe-R chromosome with ≥84.21% and 100% identity, respectively. S98b strain was also assigned to GenBank with accession number CP104083.1 as a *vanM*-harboring VVE strain. According to the BLAST search, sequence results of *vanM*-positive isolates are given in Table 1.

Discussion

Enterococci have been reported in several recent studies from food-producing animals, as 69.7% (Gião *et al*., 2022), and 57% (Samad *et al*., 2022), similar to the prevalence of *Enterococcus* spp. in our study (60.8%). *E. faecium* was detected to be the dominant species as 13/146 (8.9%) in our study, as well, while *E. hirae* (85.6%) and *E. faecalis* (54.5%) were reported as the

Table 3: MIC table of Enterococci isolates (mg/L)

Samples (cattle/sheep)	VAN	TEI	LNZ	AMP	MP	ERT	STX	TET	CPL	FM	CIP	N	TOB
C1a	1.5	1	4	8	64	0.06	8	0.12	8	64	0.5	512	8
C2a	0.75	0.5	1	2	16	0.5	8	64	2	32	4	512	32
C3b	1	0.5	2	1	4	0.5	16	64	2	32	0.5	512	32
C13	1.5	0.5	0.75	0.12	4	2	4	0.25	2	32	0.5	256	32
C14a	1.5	0.5	0.25	0.25	1	0.5	8	0.5	4	64	1	256	32
C14b	1	0.5	1	0.5	2	0.5	0.5	4	2	32	2	64	16
C32	1	0.25	1.5	2	0.5	0.06	0.06	32	0.5	32	0.06	512	32
C33a	0.75	0.5	0.12	8	32	512	16	64	8	32	2	256	32
C67	0.75	0.5	1.5	4	1	4	0.25	0.12	4	32	1	256	32
S2a	1	0.75	2	2	8	0.5	16	64	8	32	1	128	8
S9a	0.75	0.5	0.5	0.5	2	2	1	0.25	2	64	2	256	16
S14a	0.75	0.5	2	2	16	0.5	16	0.25	2	32	1	64	32
S19	1.5	0.75	1.5	2	128	0.12	0.06	0.25	4	64	8	512	64
S23a	1	0.5	0.5	4	32	0.5	0.25	0.12	4	32	0.5	1024	8
S29a	1.5	0.5	1.5	4	32	0.5	16	32	4	64	8	128	32
S31a	1	0.25	1.5	4	16	0.5	2	0.12	4	64	0.5	512	4
S63	1	0.5	1	4	32	0.5	16	0.12	8	64	8	64	64
S73b	0.75	0.25	1	8	16	0.5	0.25	0.5	2	32	2	256	64
S98b	1	0.5	2	2	32	1024	16	32	32	0.25	4	128	32
S120	0.75	0.5	0.5	0.5	2	2	1	0.25	2	64	2	64	16

Efa: *E. faecalis*, Efec: *E. faecium*, VAN: Vancomycin, TEI: Teicoplanin, LNZ: Linezolid, AMP: Ampicillin, MP: Meropenem, ERT: Erythromycin, STX: Sulfamethoxazole-Trimethoprim, TET: Tetracycline, CPL: Chloramphenicol, FM: Fosfomycin, CIP: Ciprofloxacin, N: Nitrofurantoin, and TOB: Tobramycin

Table 4: Antimicrobial resistance genes and virulence factors common in *E. faecalis* C32, *E. faecium* C33a, and *E. faecium* S98b strains, according to whole genome sequencing analysis

Antimicrobial resistance genes	Function	Reference	Virulence factors	Function	Reference
vanB and *vanM*	Vancomycin resistance	Ahmed and Baptiste (2018)	*spxA*	Regulator in oxidative stress tolerance and virulence	Kajfasz *et al*. (2012)
liaXF	Daptomycin resistance	Ota *et al*. (2021)	*gshAB*	Glutathione synthesis was capable of protecting cells from acid stress	Patel *et al*. (1998)
trmBDFLO	Tigecycline-related-methyltransferase	Osei Sekyere *et al*. (2016)	*gap*	Essential component of the glycolytic pathway	Calvez *et al*. (2008)
fusA	Fusidic acid resistance	Gupta *et al*. (2022)	*rpiA* and *glnA*	Surface penetration	Ramos *et al*. (2019)
ileS	Mupirocin resistance	Dallo *et al*. (2023)	*recA* and *polA*	DNA damage response	Weaver and Reedy (2006)
murABCDGQ	Fosfomycin resistance	Xin *et al*. (2022)	*pta*	Phosphotransacetylase activity	Walsh *et al*. (2022)
msrAB	Macrolide resistance	Singh *et al*. (2001)	*dltABCD*	D-alanylation of lipoteichoic acid	Paganelli *et al*. (2012)
rpoBCDEZ	Rifampicin resistance	Urusova *et al*. (2022)	*yajC*	Stabilization of protein in bacterial surface	Jiang (2020)
rplA-X	Linezolid resistance	Zarzecka *et al*. (2022)	*frr*	Ribosome-recycling factor	Qayyum *et al*. (2019)
celB	Endonuclease colicin E2	de Carvalho *et al*. (2020)	*secAYEG*	ATP-dependent molecular motor	Meining *et al*. (2006)
efrAB	ABC multidrug efflux pump	Lee *et al*. (2003)	*pgmB*	β-phosphoglucomutase	Van Tyne *et al*. (2019)
mprF	Multiple peptide resistance factor, daptomycin resistance	Ernst and Peschel (2011)	*rny*	RNA metabolism protein	Salze *et al*. (2020)
yidD	Novobiocin resistance	Nasaj *et al*. (2020)	*sufBCD*	Sulfur assimilation	Riboldi *et al*. (2009)

Table 5: Antimicrobial resistance genes and virulence factors common in *E. faecium* C33a and *E. faecium* S98b strains according to whole genome sequencing analysis

Antimicrobial resistance genes	Function	Reference	Virulence factors	Function	Reference
vanH and *vanY*	Vancomycin resistance	Leclercq (1997)	*acm*	Adhesin from collagen	Nallapareddy *et al.* (2008)
msr(C)	Quinupristin, erythromycin, azithromycin, tylosin, telithromycin, virginiamycin S resistance	Cattoir and Leclercq (2017)	*spxB*	Regulator in oxidative stress tolerance and virulence	Kajfasz *et al.* (2012)
efmA	Macrolide and fluoroquinolone resistance	Fatoba *et al.* (2022)			
liaS	Daptomycin resistance	Tran *et al.* (2015)			
aac(6')	Aminoglycoside resistance	Peykov *et al.* (2022)			
pbp5	Ampicillin resistance	Freitas *et al.* (2022)			
rpoN	Rifampicin resistance	Taniguchi *et al.* (1996)			
eat(A)	Enterococcus ABC transporter and also involved in pleuromutilin resistance	Li *et al.* (2022)			

Table 6: Antimicrobial resistance genes and virulence factors detected in *E. faecalis* C32 strains according to whole genome sequencing analysis

Antimicrobial resistance genes	Function	Reference	Virulence factors	Function	Reference
lsa(A)	Clindamycin, lincomycin, quinupristin-dalfopristin, pristinamycin iia, virginiamycin M resistance	Singh *et al.* (2002)	*glsA*	Bile resistance and are crucial for adaptation to the intestinal environment	Bhatty *et al.* (2017)
nfsA	Nitrofurantoin resistance	Wan *et al.* (2021)	*prgU*	Suppressor of sex pheromone toxicity	Nunez *et al.* (2018)
dfr	Sulfamethoxazole-trimethoprim resistance	Scholtzek *et al.* (2020)	*elrABCDR*	Enterococcal leucine-rich proteins	Segawa *et al.* (2021)
			opp1ABCDF and *opp2ABCDF*	Peptide transport	Gião *et al.* (2022)

predominant species in other studies, respectively (Gião *et al*., 2022; Samad *et al*., 2022). Interestingly, when multidrug-resistant *E. faecium* isolates were examined phylogenetically, it was determined that the emergence of hospital-adapted strains was due to human antibiotic use, whereas strains of animal origin made up the majority of bacterial populations (Lebreton *et al*., 2013). Data supporting this information are provided by Yan *et al*. (2022) reported 42 of 46 (91.3%) VRE strains as *E. faecium*.

Resistance to glycopeptides in enterococci has been generally associated with the *vanA* and *vanB* genes, *vanA*/*vanB* is mostly reported as the predominant resistance genes in VRE infections (Ahmed and Baptiste, 2018; Yan *et al*., 2022). A co-occurrence of *vanA* and *vanM* among VRE has also been reported previously (Sun *et al*., 2019; Zhou *et al*., 2020; Yan *et al*., 2022). However, to our knowledge, the co-occurrence of *vanB* and *vanM* genes in VRE isolates has not been reported to date. The fact that all isolates with the *vanB* gene can also carry the *vanM* gene, stands out from our data. On the other hand, similar to the *van* genes profile in our study, *vanM*-type VRE was also reported to be more common than *vanA*-type VRE in China (Chen *et al*., 2015; Sun *et al*., 2019). The *vanM* genotype has been reported with a high incidence (4.36%) in clinical enterococci, mostly with the silent-resistant phenotype, and the clinical isolations of *vanM*-type VRE reported from China have been increased (Sun *et al*., 2019). The term 'silent resistance' is used for isolates that have the *vanM* resistance gene, such as the isolates from our study but do not express vancomycin resistance phenol-typically.

As in our study, the absence of CRISPR-Cas loci in food-producing animal-origin *E. faecalis* and *E. faecium* isolates has been previously shown (Palmer and Gilmore, 2010). It has been reported that CRISPR-Cas loci, which protect bacterial DNA, are not found especially in *E. faecium*, so *E. faecium* has high recombination rates (Palmer and Gilmore, 2010). On the other hand, CRISPR-Cas loci have been reported in *E. faecalis* and *E. faecium* isolates analyzed from hospital wastewater by Alduhaidhawi *et al*. (2022). They highlighted in their study that CRISPR-Cas loci are less common in MDR isolates compared to non-MDR enterococci. In our study, it was also observed that the MIC values of *E. faecium* isolates against antibiotics were generally higher than those of *E. faecalis* isolates (Table 3). Similarly, the lack of CRISPR-Cas genes was explained by the higher rates of antibiotic resistance in *E. faecalis* and *E. faecium* isolates from hospital wastewater (Alduhaidhawi *et al*., 2022).

Previous studies have shown that antibiotic resistance is inversely related to the presence of full CRISPR loci, and the members of recently emerged high-risk enterococcal strains lacked full CRISPR loci (Palmer and Gilmore, 2010; Rodrigues *et al*., 2019). The lack of full CRISPR1-Cas, CRISPR2, and CRISPR3-Cas loci in our isolates was in agreement with the previous studies. Supporting this data, one CRISPR-Cas element (CRISPR Id/Cas type: NZ_CP038997_1_consensus_1) and feefteen different CRISPR-Cas elements (CRISPR Id/Cas type: NZ_KB944666_1_consensus_1 to 15) were found in the S98b and C32 isolate by using the CRISPRCasFinder tool (Grissa *et al*., 2007). These findings indicate that isolates containing more CRISPR-Cas elements have fewer antimicrobial resistance genes. As can be seen in Tables 5 and 6, antimicrobial resistance genes of the C32 *E. faecalis* isolate containing 15 different CRISPR-Cas elements were found to be

more limited than those of S98b. *In-vivo* and *in-vitro* studies have also shown that CRISPR-Cas elements prevent in the uptake of antibiotic resistance genes by *E. faecalis* isolates, and the presence of CRISPR-Cas elements in the murine intestine reduces the formation of antibiotic-resistant *E. faecalis* (Hullahalli *et al*., 2018; Rodrigues *et al*., 2019).

MIC values against 13 antibiotics of *E. faecalis* and *E. faecium* isolates are shown in Table 3. The antibiotics selected in the study were preferred according to their widespread use in livestock, but meropenem, erythromycin, and nitrofurantoin are not widely used. Conspicuously, the highest phenotypic resistance was observed for nitrofurantoin (80%), the most preferred in human urinary tract infections (Gardiner *et al*., 2019), with MIC ≥64 µg/ml, making a significant contribution to the literature. Therefore, all *vanM*-positive isolates in the study were found to be resistant to nitrofurantoin but surprisingly, according to our sequencing results, the *nfsA* gene encoding nitrofurantoin resistance was found only in *E. faecalis* C32 strain. While, MIC values of meropenem, erythromycin, fosfomycin, chloramphenicol, tobramycin, trimethoprim-sulfamethoxazole, and tetracycline for Enterococci are not mentioned in the EUCAST guidelines, CLSI guidelines recommended erythromycin, fosfomycin, chloramphenicol, and tetracycline breakpoints for Enterococci (CLSI, 2023). According to EUCAST, the epidemiological cut-off value of trimethoprim-sulfamethoxazole is 1 mg/L for both *E. faecium* and *E. faecalis* (EUCAST, 2022b). In light of this information, we can mention that *E. faecalis* and *E. faecium* isolates are mostly (n=14/20, 60%) resistant to trimethoprim-sulfamethoxazole. Surprisingly, the *dfr* gene encoding trimethoprim-sulfamethoxazole resistance was found again only in *E. faecalis* C32 strain among *vanB* and *vanM* positive isolates, but the MIC value in this isolate was observed to be considerably lower than the others. All isolates, and thus all *vanM* positive isolates, have a tobramycin MIC value of ≥4 mg/L and the *aac(6')* gene found in strains C33 and S98b. In this study, it was determined that although the isolates were phenotypically resistant, they did not carry the main genes encoding resistance according to general knowledge about resistance-encoding genes. In this context, it should be considered that it would be important in the future to analyze the resistance mechanisms to antibiotics, which are especially important for public health, in a more comprehensive way. Likewise, *rplA-X* genes related to linezolid resistance were found in C32, C33a, and S98b strains but were not phenotypically resistant to linezolid. This is most likely due to the absence of the plasmid-borne *cfr*, *cfr(B)*, *poxtA* genes, and the ATP binding cassette (ABC) transporter gene *optrA* in isolates (Ma *et al*., 2021). It is noteworthy that C33a and S98b strains have both *vanM* and *pbp5* genes, and both isolates with erythromycin resistance according to the latest CLSI guideline (CLSI, 2023). Since these strains are phenotypically sensitive to both ampicillin and vancomycin, it is thought that they may show variable resistance to vancomycin and ampicillin.

As a striking detail in terms of macrolide resistance genes in WGS analysis, although *msrA* and *msrB* genes were found in all C32, C33a, and S98b strains, the MIC value in the C32 strain without *msr(C)* gene was found to be quite lower than the others. This shows that the effects of *msr(C)* and *efmA* genes, which are found only in C33 and S98b strains, on erythromycin resistance are pretty high. It has been noted that 7/12 (58.3%) of the isolates with meropenem MIC ≥8 mg/L, 3/5 (60%) of them with chloramphenicol MIC ≥8 mg/L, and 7/19 (36.8%) of them with fosfomycin MIC ≥32 mg/L were *vanM* positive isolates. Although all C32, C33a, and S98b strains contain *murABCDGQ* genes encoding fosfomycin resistance, the S98b strain has a very low MIC value than the others, maybe due to the lack of *fos* genes. The high MDR profile of enterococci may be due to horizontal transfer and/or physical exposure to antibiotics.

All of the isolates were found susceptible to teicoplanin and vancomycin, similar to the study conducted by Gião *et al*. (2022). Sun *et al*. (2019) also reported no resistance to teicoplanin despite the coexistence of *vanA* and *vanM* genes in their isolates. The absence of vancomycin resistance in isolates may be associated with the lack of the *vanA* gene in the study. Seven different peptides have been reported to be associated with the expression of *vanA*, these peptides have been studied in three different groups: those responsible for the regulation of glycopeptide resistance genes (*vanR*/S), those that induce resistance to glycopeptides by the production of the altered target (*vanH*/*A*/*X*), and non-essential accessory genes (*vanY*/*Z*) for the expression of glycopeptide resistance (Leclercq, 1997). In recent years, researchers have focused on the *vanH/A/X* gene cluster analysis (Anahtar *et al*., 2022; Jozefíková *et al*., 2022). Similarly, Lee *et al*. (2022), while identifying *van* operons, they also identified *vanR*, *vanS*, *vanH*, *vanA*/*vanB*, and *vanX* genes together with *vanA* and *vanB* operons. *E. faecium* isolates containing the silent *vanA* gene without *vanRS* were identified as vancomycin-variable *E. faecium* (VVEfm). Because these isolates can acquire vancomycin resistance during treatment and cause failure in treatment (Anahtar *et al*., 2022). This is consistent with that 83.3% of sequenced *E. faecium* isolates from our study were matched to the VVEswe-R chromosome with ≥84.21 identities in BLAST (Table 2).

Whole genome sequence analysis of enterococci isolates showed that strains harbored numerous chromosomal antibiotic and virulence genes. It is noteworthy that *E. faecalis* and *E. faecium* isolates have species-specific resistance, and virulence genes, as the *efmA* gene was found only in *E. faecium* isolates. Also, the *opp1ABCDF* and *opp2ABCDF* operons were found only in *E. faecalis* isolate. Similar studies also support that these genes are mostly species-specific (Nishioka *et al*., 2009; Segawa *et al*., 2021).

Although *vanA*, *vanX*, *vanR*, and *vanS* genes were not detected in the isolates obtained in our study, *vanB*, *vanM*, *vanH*, and *vanY* genes were present in the isolates.

E. faecium isolates with the silent *vanM* gene without *vanR* and *vanS* genes are reported as VVEfm in this study. Hospital outbreaks of VVE also emphasize that VVE can spread within the community and healthcare facilities (Hammerum *et al*., 2019). Therefore, to identify VVE and VRE isolates prospectively, vancomycin determination may be modified to detect *vanA* and *vanB* genes as well as other genes of the *van* operon (*vanX/Y/H/R/S*) and to indicate the presence of *vanH/A/X* (Anahtar *et al*., 2022). These data highlight the importance of whole genome sequencing-based approaches to identify potential strains of clinical importance, apart from the analysis methods routinely used today. More research is needed to determine an effective method for VVE detection by evaluating the sensitivity of various selective agars with or without vancomycin, and the cost efficiency of molecular assays.

The first *vanM*-positive VVEfm isolates from food-producing animals were identified, and the molecular characteristics of these isolates were determined using Sanger and whole genome sequencing. Although several studies have reported the occurrence of *vanA* and *vanB* genotype VRE, only certain countries have recognized the prevalence of the *vanM*-type. Determination of *vanM*-resistance genotype in enterococci isolates with rapid screening tests will prevent vancomycin resistance from being overlooked and will provide clinically appropriate treatment. Based on this information, we think that it is important to conduct new studies from animal, food, human, and environmental sources in order to better understand the distribution and characteristics of VVE isolates. Therefore, new studies are needed to facilitate the identification of *vanM*-resistant enterococci and VVE strains.

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Conflict of interest

The authors declare no conflict of interest.

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