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Molecular insights into antimicrobial resistance and virulence in hospital-associated of vancomycin-resistant *Enterococcus faecium* isolates in a tertiary hospital in Bangkok Thailand

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Abstract

Vancomycin-resistant Enterococci (VRE) are major pathogens causing nosocomial infections globally. This study investigated the genetic characteristics of vancomycin-resistant *Enterococcus faecium* (VREfm) in Thailand between June and November 2022. Fifty-two clinical VREfm isolates from Bangkok hospitals were analyzed for antimicrobial susceptibility, resistance genes, virulence factors, and genotypes using multilocus sequence typing (MLST). Phylogenetic analysis and goeBURST assessed genetic relationships and population structure. The VRE detection rate was 14.5%, with 97.1% *E. faecium* and 2.9% *E. faecalis*, likely reflecting the impact of an active case-finding program. All isolates exhibited resistance to penicillin, ampicillin, vancomycin, levofloxacin, ciprofloxacin, and rifampin. Resistance to erythromycin, high-level streptomycin, teicoplanin, and tetracycline occurred in 98.1%, 53.8%, 51.9%, and 17.3% of isolates, respectively. Chloramphenicol, linezolid, and high-level gentamicin remained effective against all isolates. The *van*A gene was the sole resistance determinant detected. Virulence genes *esp* and *hyl* were present in 100% and 88.5% of isolates, respectively. MLST identified five sequence types (STs), with ST17 (86.5%) as the dominant lineage, followed by ST262 (7.7%), ST202, ST787, and ST80 (1.9% each). All isolates belonged to Clonal Complex 17. Genome analysis revealed various resistance genes (VanHAX, *aac*(6')-Ii, *aad*(6), *ant(6)-Ia*, *msr*C, and *tet*M) and virulence factors (*acm*, *bop*D, *cps*A/*upp*S, *cps*B/*cds*A, *ebp*A, *ebp*B, *ebp*C, *efa*A, *esp*, *sgr*A, and *srt*C). The *van*A gene primarily drives vancomycin resistance in Thailand's VREfm. Genome analysis reveals antibiotic resistance genes, virulence factors, and mobile genetic elements that may drive antimicrobial resistance,

JBKKKD000000000.1, JBKKKE000000000.1, JBKKKF000000000.1, JBKKKG000000000.1, and JBKKKH000000000.1 correspond to G239, G293, G1101, G1176, G384, and G1768, respectively.

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increase diversity, and support adaptation in hospital settings. Ongoing infection control and active surveillance are essential.

Introduction

Enterococci are facultative anaerobic Gram-positive cocci that naturally inhabit the gastrointestinal tract of humans and other animals. They can act as opportunistic pathogens under certain conditions, causing various infections, particularly in immunocompromised individuals and in hospital settings. The genus *Enterococcus* includes species such as *Enterococcus faecalis* and *E. faecium*, which are responsible for most human infections. Enterococci can cause urinary tract infections (UTIs), bacteremia, endocarditis, and intra-abdominal infections. They are a leading cause of hospital-acquired infections, including catheter-associated UTIs and surgical site infections [1,2].

Enterococcus has high pathogenic potential because of the presence of virulence factors that facilitate colonization, persistence, and immune evasion. The key virulence genes in *Enterococcus* include *asa* (aggregation substance), *gel* (gelatinase), *cyt* (cytolysin), *esp* (enterococcal surface protein), *hyl* (hyaluronidase), *cpd* (sex pheromone determinant), and e*bp* (endocarditis and biofilm-associated pilus subunit A) [3–5]. Compared with *E. faecalis*, *E. faecium* can develop multidrug resistance and is tolerant to various antibiotics, including aminoglycoside, ampicillin, and vancomycin [6,7]. The synergistic interaction between antimicrobial resistance and virulence is central to the clinical impact of vancomycin-resistant *Enterococcus faecium* (VREfm). Strains that harbor both multidrug resistance determinants and major virulence factors pose a substantially greater public health threat than resistant strains with limited virulence, because they are simultaneously difficult to treat and highly capable of colonization, transmission, and invasion [8,9]. In VREfm, the coexistence of resistance genes such as *van*A, aminoglycoside- and tetracycline-resistance determinants with key virulence factors including *esp* and *hyl*, as well as multiple adhesins (e.g., *acm*, *efa*A) and biofilm-associated loci (*ebp* operon), enhances persistence in the hospital environment and promotes host colonization [10].

The emergence of vancomycin-resistant *Enterococcus* (VRE) is particularly concerning because of *van* genes such as *vanA*, *vanB*, and *vanC*. These genes encode enzymes that modify d-alanyl-d-alanine to d-alanyl-d-lactate or d-alanyl-d-serine, significantly reducing vancomycin binding affinity [11–13]. VRE have spread worldwide because of their multidrug resistance. The World Health Organization has specified VRE as a priority for developing new drugs [14]. In many countries, the prevalence of VRE has significantly increased. In Germany, the percentage of vancomycin-resistant *Enterococcus faecium* (VREfm) has increased from <5% in 2001 to 14.5% in 2013 [15]. The European Antimicrobial Resistance Surveillance Network reported an increase in the VRE rate from 10.4% in 2014 to 17.3% in 2018 [16]. According to Thailand's National Antimicrobial Resistance Surveillance Center, the frequency of VRE increased from 2.8% in 2015 to 8.1% in 2023. (Thailand National Antimicrobial Resistance Surveillance Center, 2025) [17]

VRE have significant implications for infection control in healthcare settings because of their association with outbreaks and their ability to transfer resistance genes to other pathogens. Understanding the virulence factors for the circulation of VRE strains within hospitals provides insights into the pathogenic potential and severity of infections caused by these strains, thereby enabling better assessment of the ongoing outbreak trends in the area. In Thailand, studies on the molecular characteristics of *E. faecium* are limited, and data on the prevalence of virulence factors are lacking. Therefore, this study aimed to provide a detailed molecular characterization of hospital-associated VRE *E. faecium* isolates from a tertiary hospital in Bangkok, Thailand. Specifically, we sought to: (i) determine the phenotypic antimicrobial susceptibility profiles of the isolates; (ii) identify the genetic determinants of vancomycin resistance (e.g., *van*A and *van*B genes); and (iii) evaluate the prevalence of key virulence markers associated with clinical severity and biofilm formation.

Materials & methods

Study setting

This study was conducted at Vajira Hospital, a public tertiary-care teaching hospital in Bangkok, Thailand, with approximately 740 inpatient beds. As a major referral center for complex medical and surgical cases, the hospital includes high-risk units such as internal medicine, surgery, and intensive care, where high antibiotic pressure and vulnerable patient populations facilitate the emergence and transmission of VREfm. The hospital has implemented active case-finding surveillance for VRE, routinely screening high-risk patients to identify colonization and prevent nosocomial spread. This practice increases the detection of VRE, including both infected and colonized patients. Although routine culture and phenotypic susceptibility testing are well established, systematic molecular characterization of VRE has been limited and circulating clones may be underrecognized. Therefore, this study was specifically designed to perform molecular characterization of VREfm isolates detected through active surveillance and clinical cultures, providing essential baseline genomic data to support future antimicrobial resistance surveillance, infection control, and antimicrobial stewardship efforts.

Study design

A cross-sectional study with data collected retrospectively was conducted at a tertiary care hospital in Bangkok, Thailand. Clinical isolates were consecutively collected from patients admitted to Vajira Hospital between 13/06/2022 and 30/11/2022. Bacterial collection was obtained from leftover cultures generated during routine diagnostic laboratory work. Data were accessed for research purposes on 18/02/2023. The Institutional Review Board committee approved this study, and patient consent was waived due to its use of leftover specimens and deidentified samples. This study was approved by The Institutional Review Board of Faculty of Medicine of Vajira Hospital, Navamindradhiraj University (COA 010/2566).

VREfm screening and identification

Bacterial isolates were collected and identified according to the routine clinical bacteriology guidelines. The isolates originated from clinical specimens, including urine, blood, pus, and stool, collected from both outpatients and inpatients. All enterococcal isolates were incubated overnight on sheep blood agar at 37°C and were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker Microflex, Bremen, Germany). VREfm was preliminarily screened using the antibiotic disk diffusion technique for seven antibiotics including ampicillin (10 μg), erythromycin (15 μg), linezolid (30 μg), teicoplanin (30 μg), vancomycin (30 μg), tetracycline (30 μg), and gentamicin (120 μg). The VREfm isolates were selected for further microbroth dilution to test antimicrobial susceptibility.

Antimicrobial susceptibility testing

All VREfm isolates (n = 52) were tested for antimicrobial susceptibility using the Sensititre® microbroth dilution system according to the manufacturer's instructions. Overnight cultures were transferred to sterile water to achieve a 0.5 McFarland standard. Then, 30 μL of each suspension was transferred to sterile cation-adjusted Mueller–Hinton broth, and 50 μL

of the broth solution was dispensed into Sensititre™ THAPF (Trek Diagnostic Systems). Minimal inhibitory concentration (MIC) plates that included the following antibiotics (range of concentrations in μg/mL) were used: vancomycin (0.25–32 μg/mL), teicoplanin (4–32 μg/mL), penicillin (0.06–8 μg/mL), rifampin (1–4 μg/mL), ampicillin (0.12–8 μg/mL), erythromycin (0.25–4 μg/mL), levofloxacin (0.25–8 μg/mL), daptomycin (0.5–4 μg/mL), linezolid (0.5–8 μg/mL), chloramphenicol (8–16 μg/mL), tetracycline (2–16 μg/mL), gentamicin (500 μg/mL), ciprofloxacin (0.5–4 μg/mL), and streptomycin (1,000 μg/mL). *E. faecalis* ATCC 29212, *E. faecalis* ATCC 51299, and *S. aureus* ATCC 29213 were used as quality control strains. The MICs were recorded as the lowest concentration of an antimicrobial that completely inhibited bacterial growth (M100 33rd CLSI,2023). Resistance breakpoints published by the Clinical and Laboratory Standards Institute were used (M100 33rd CLSI, 2023). Multidrug resistance was defined as resistance to two or more classes of antibiotics. The breakpoints for vancomycin susceptibility were ≤4 μg/mL for "susceptible," 8–16 μg/mL for "intermediate resistance," and ≥32 μg/mL for "resistant." Strains with vancomycin MICs of ≥32 μg/mL were regarded as VRE. This study was limited by the upper concentration of vancomycin tested in the Sensititre™ THAPF panel, which capped MIC determination at 32 μg/mL. The exact MIC values beyond this threshold could not be determined.

DNA extraction

Genomic DNA was extracted using 10% Chelex solution in 0.1% Triton X-100 and 10-mM Tris buffer (pH 8.0). Then, 200 μL of 10% Chelex solution was added to each bacterial cell pellet. The mixture was vortexed for 10 s, heated at 95°C for 20 min, and centrifuged at 10,000 × g for 20 s. The supernatant was used as the substrate for polymerase chain reaction (PCR).

Detection of antimicrobial resistance and virulence genes

Eight antimicrobial resistance genes (i.e., *van*A, *van*B, *aac*, *aph(3')-IIIa*, *ant(6)-Ia*', *tet*L, *tet*O, and *tet*M) and eight virulence genes (i.e., *asa*, *gel*, *esp*, *hyl*, *cpd*, *ebp*, *pai*, and *spr*E) were detected using PCR using the primers and amplification conditions detailed in S1 Table. PCR was performed using FIREPol Master Mix Ready to Load (Solis Biodyne) following the manufacturer's instructions. PCR was performed in a reaction volume of 20 μL PCR reaction mixture containing 0.8 μM of each primer and 20 μL of 5 × FIREPol Master Mix Ready to Load (Solis Biodyne) containing buffer, nucleotides, and Taq polymerase. All reactions were run on a T100 Thermal Cycler (Bio-Rad Laboratories Inc, Hercules, CA, USA). *E. faecalis* ATCC 51299 was used as a positive control for *van*B, *asa*, *gel*, *esp*, *cpd*, *ebp*, *pai*, *spr*E, *aph(3')-IIIa*, and *ant(6)-Ia*. The expression of virulence and resistance genes was confirmed by DNA sequencing.

Genotyping and phylogenetic tree construction

All VREfm isolates were genotyped using MLST as previously published for *E. faecium*. Seven gene fragments were used for typing: *atp*A, *ddl*, *gdh*, *pur*K, *gyd*, *pst*S, and *adk*. PCR was performed using Kit PCR 5 × FIREPol Master Mix Ready to Load (Solis Biodyne) according to the manufacturer's instructions. S1 Table lists the primers used for the amplification of fragment genes. All DNA fragments were sequenced (U2Bio Sequencing Service, Korea) in both directions using amplification primers. DNASTAR Lasergene was used to align and edit the DNA sequences. The consensus sequences of the seven loci were determined for all isolates. The consensus sequences of each locus of all isolates were submitted to the MLST database to define the allele profile and sequence type (ST). The population structures were analyzed using the goeBURST algorithm with Phyloviz.

Each sequence of the seven genes was trimmed to the correct length with its start and end nucleotides. Sequences were concatenated in the order of *atp*A, *ddl*, *gdh*, *pur*K, *gyd*, *pst*S, and *adk*. The best evolutionary model for concatenated datasets was selected using the Bayesian Information Criterion (BIC) in MEGA-X. The model with the lowest BIC score was chosen as the basis for constructing. Initial tree for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood

(MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The phylogenetic trees was constructed using the maximum likelihood method. Bootstrap analysis was conducted using 1,000 replicates.

Whole-genome sequencing (WGS) and data analysis

Six representative isolates with different STs were selected for WGS. The genomic DNA was isolated using phenol-chloroform extraction. In brief, a loop of bacterial colonies was transferred to a 1.5 ml microcentrifuge tube and frozen at −20°C for 1 hour. Five hundred microliter of lysis buffer containing 0.5 g sodium dodecyl sulfate, 1.4 g NaCl, 0.73 g EDTA, and 20 mL of 1 M Tris–HCl per 100 mL lysis buffer was added. Then, add 5 μL of 2-mercaptoethanol. The suspension was vortexed and incubated at 65°C for one hour. The lysate was extracted with 500 μL of phenol–chloroform–isoamyl alcohol (25:24:1, v:v:v) and mixed by pipetting. The tubes were centrifuged for 5 min at 12,500 rpm at 4°C. The aqueous phase was transferred to a fresh tube, and DNA was precipitated with 500 μL of isopropanol and incubated at 4°C for 1 hour. The mixture was centrifuged at 12,500 rpm for 5 minutes at 4°C to pellet the DNA. The DNA pellet was washed with 70% ethanol, centrifuged for 5 minutes at 12,500 rpm, and air dried. The DNA was resuspended in 100 μL sterile deionized water and kept at −20°C.

WGS was conducted using a short-read, paired-end sequencing strategy on the Illumina Nextseq550 platform at CELEMICS (Celemics, Inc., Seoul, Korea), in collaboration with U2Bio (Thailand) Co., Ltd. (Bangkok, Thailand). The sequencing reads were trimmed and filtered using AfterQC (version 0.9.6). *De novo* genome assembly was performed using Unicycler (version 0.5.0), and the quality of the assembled genomes was assessed using QUAST (version 5.0.2). Genome completeness and contamination were estimated using CheckM (version 1.1). The assembled genomes were annotated using Prokka (version 1.14.6). Taxonomy based on the genomes was determined using the Type (Strain) Genome Server (https://tygs.dsmz.de/). The sequencing reads were trimmed and filtered using AfterQC (version 0.9.6). cgMLST were carried out using established typing schemes in MBioSEQ Ridom Typer. version 11.1. Antibiotic resistance genes were identified based on the Comprehensive Antibiotic Resistance Database [18] and ResFinder4.1 [19]. Plasmid replicons and mobile genetic elements were analyzed using PlasmidFinder2.1 [20] and MobileElementFinder [21]. Virulence genes were predicted using VirulenceFinder2.0 [22] and the virulence factor database (http://www.mgc.ac.cn/VFs/).

Statistical analysis

Descriptive statistical analyses were performed using IBM SPSS Statistics 30.0 for Windows (IBM Corp., Armonk, NY, USA). Frequencies and percentages were calculated to describe the distribution of virulence genes, antimicrobial resistance genes, and genotypes, including sequence types and cgMLST complex types, among the isolates.

Results

Species distribution of *Enterococcus* spp.

From the clinical isolates collected from patients treated at Vajira Hospital between 13th June 2022 and 30th November 2022, a total of 8,187 patients underwent laboratory testing. *Enterococcus* spp. were identified in 470 cases, yielding an overall detection rate of 5.74%. The most commonly isolated *Enterococcus* species was *E. faecalis* (271, 57.7%), followed by *E. faecium* (180, 38.3%), *E. raffinosus* (8, 1.7%), *E. gallinarum* (5, 1.1%), *E. hirae* (3, 0.6%), *E. casseliflavus* (2, 0.43%), and *E. cecorum* (1, 0.2%). Phenotypic testing revealed vancomycin-resistant *Enterococcus* (VRE) in 68 of the 470 isolates, corresponding to an overall VRE detection of 14.5%. Among these, 97.1% (66/68) were identified as *E. faecium* (VREfm), and 2.9% (2/68) as *E. faecalis*. Accordingly, vancomycin resistance was identified in 36.7% (66/180) of *E. faecium* isolates and only 0.7% (2/271) of *E. faecalis* isolates. In this study, 52 VREfm isolates were recovered for further analysis; 75% (39/52) were recovered from urine samples, followed by fecal specimens (15.4%, 8/52), wound exudates (5.8%, 3/52), and one isolate each (1.9%) from blood and bile.

Antimicrobial susceptibility pattern of VREfm

The antibiotic susceptibility of all 52 VREfm clinical isolates was analyzed (Table 1). All isolates exhibited dose-dependent susceptibility to daptomycin; resistance to penicillin, ampicillin, vancomycin, ciprofloxacin, levofloxacin, and rifampin; and sensitivity to chloramphenicol, linezolid, and high-level gentamicin (HLG) (MIC > 500 μg/mL). Resistance to erythromycin, teicoplanin, and tetracycline was observed in 51 (98.1%), 27 (51.9%), and 9 (17.3%) isolates, respectively. High-level streptomycin resistance (HLSR) with MIC > 1,000 μg/mL was observed in 28 (53.8%) isolates. Table 2 presents the antibiotic resistance patterns of the VREfm isolates; 11 antibiotic resistance patterns were recorded. All VREfm isolates were resistant to at least four categories of antibiotics, with 51 (98.1%) isolates being resistant to ≥5 antibiotics (Table 2).

Antibiotic-resistant gene identification

All VREfm strains (100%) harbored the *van*A gene, whereas the *van*B gene was not detected in any isolate. The prevalence of tetracycline-resistant genes in the VREfm isolates was as follows: *tet*M was detected in 28 of the 52 isolates, exhibiting the highest rate of occurrence among *tet* genes at 53.9% (28/52). The frequency of *tet*L and *tet*O was 13.5% (7/52) and 3.9% (2/52), respectively. Among the nine phenotypically tetracycline-resistant VREfm isolates, 77.8% (7/9), 55.6% (5/9), and 22.2% (2/9) contained *tet*M, *tet*L, and *tet*O, respectively. *tet*M was detected simultaneously along with *tet*L and *tet*O in 55.6% (5/9) and 11.1% (1/9) of the isolates, respectively. High-level aminoglycoside-resistant genes were observed in 53.9% (28/52) of the isolates for *ant(6)-Ia*, 53.9% (28/52) for *aph(3')-IIIa*, and 11.5% (6/52) for *aac(6')-Ie-aph(2")-Ia*. Among the 28 phenotypically HLSR VREfm isolates, 89.3% (25/28) had *ant(6)-Ia*, 82.1% (23/28) had *aph(3')-IIIa*, and 7.1% (7/28) had *aac(6')-Ie-aph(2")-Ia*. Concurrent *ant(6)-Ia* and *aph(3')-IIIa* were observed in 82.1% (23/28) of the isolates. The simultaneous presence of *ant(6)-Ia* and *aac(6')-Ie-aph(2")-Ia* was detected in 7.1% (2/28) of the isolates (Table 3 and Fig 1).

A correlation between sequence types and antibiotic resistance genes revealed ST-specific patterns, with ST17 isolates most commonly carrying *aph(3')-IIIa, ant(6)-Ia'*, and *tet*M. ST262 and ST787 exhibited broader resistance genes, including

Table 1. Antimicrobial susceptibility test results of the 52 VREfm isolates.

Antibiotics	Antibiotic class	Susceptibility rate No. (%)			
		% R	% I	% SDD	% S
Penicillin	β-lactams	52 (100)	ND	ND	0 (0)
Ampicillin		52 (100)	ND	ND	0 (0)
Vancomycin	glycopeptides	52 (100)	0 (0)	ND	0 (0)
Teicoplanin		27 (51.9)	22 (42.3)	ND	3 (5.8)
Daptomycin	lipopeptide	ND	ND	52 (100)	0 (0)
Erythromycin	macrolide	51 (98.1)	0 (0)	ND	1 (1.9)
Tetracycline	tetracycline	9 (17.3)	0 (0)	ND	43 (82.7)
Ciprofloxacin	fluoroquinolones	52 (100)	0 (0)	ND	0 (0)
Levofloxacin		52 (100)	0 (0)	ND	0 (0)
Rifampin	ansamycin	52 (100)	0 (0)	ND	0 (0)
Chloramphenicol	phenol	0 (0)	0 (0)	ND	52 (100)
Linezolid	oxazolidinone	0 (0)	0 (0)	ND	52 (100)
Gentamicin HLAR	aminoglycoside	0 (0)	0 (0)	ND	52 (100)
Streptomycin HLAR		28 (53.8)	0 (0)	ND	24 (46.2)

Abbreviations R: Resistant, I: Intermediate, S: Susceptible, SDD: Susceptible-dose dependent, ND: Not determined, HLAR: High-level aminoglycoside resistance.

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Table 2. Phenotypic resistance patterns of the 52 clinical VREfm isolates.

Antimicrobial susceptibility pattern	Resistant	
	n	%
VAN-TEI-RIF-PEN-AMP-ERY-LEV-CIP-HLS	17	32.7
VAN-RIF-PEN-AMP-ERY-LEV-CIP	14	26.9
VAN-RIF-PEN-AMP-ERY-LEV-CIP-HLS	6	11.5
VAN-TEI-RIF-PEN-AMP-ERY-LEV-CIP-TET	4	7.7
VAN-TEI-RIF-PEN-AMP-ERY-LEV-CIP-TET-HLS	3	5.8
VAN-TEI-RIF-PEN-AMP-ERY-LEV-CIP	3	5.8
VAN-RIF-PEN-AMP-ERY-LEV-CIP-TET-HLS	1	1.9
VAN-RIF-PEN-AMP-ERY-LEV-CIP	1	1.9
VAN-RIF-PEN-AMP-ERY-LEV-CIP-TET	1	1.9
VAN-RIF-PEN-AMP-LEV-CIP	1	1.9
VAN-RIF-PEN-AMP-ERY-LEV-CIP-HLS	1	1.9
Total	52	100

Abbreviations PEN: Penicillin, AMP: Ampicillin, TEI: Teicoplanin, ERY: Erythromycin, TET: Tetracycline, CIP: Ciprofloxacin, LEV: Levofloxacin, RIF: Rifampin, HLS: High-level streptomycin.

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Table 3. Characteristics of isolated VREfm—genotypic profile.

Genotypic profile	N	%
Sequence type (ST); Clonal complex (CC)		
ST17; CC17	45	86.5
ST80; CC17	1	1.9
ST202; CC17	1	1.9
ST787; CC17	1	1.9
ST262; CC17	4	7.7
Vancomycin resistant genes		
*van*A	52	100
*van*B	0	0
Aminoglycoside resistance genes		
aac(6')-Ie-aph(2")-Ia	6	11.5
aph(3')-IIIa	28	53.9
ant(6')-Ia	28	53.9
Tetracycline resistance genes		
*tet*L	7	13.5
*tet*O	2	3.9
*tet*M	28	53.9
Virulence genes		
asa	0	0
gel	0	0
esp	52	100
hyl	46	88.5
cpd	0	0
ebp	0	0
pai	0	0
sprE	0	0

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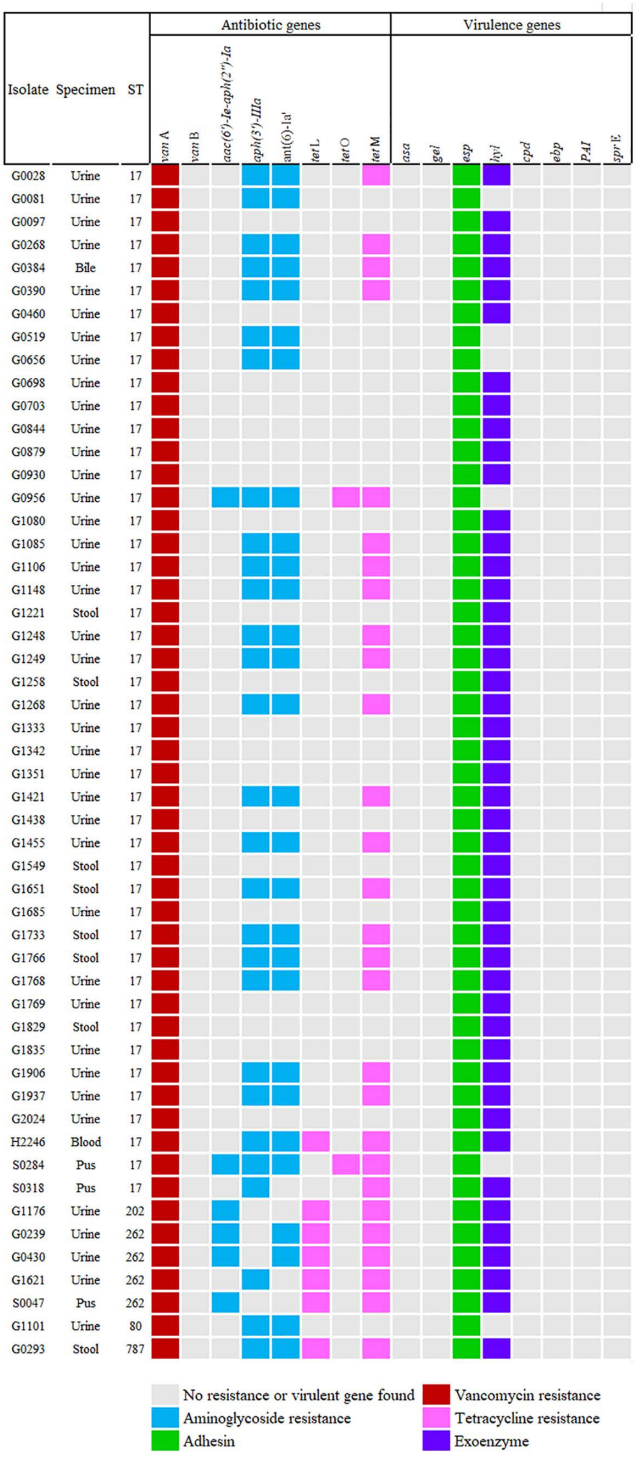


Fig 1. The heatmap of the antibiotic resistance and virulence genes across the 52 VREfm isolates. Colored blocks represent the presence of genes, and gray blocks represent the absence of genes. The rows represent isolates, and the columns represent genes involved in antimicrobial resistance and virulence factors.

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aac(6')-Ie-aph(2")-Ia, *aph(3')-IIIa*, ant(6)-Ia', *tet*L, and *tet*M. The presence of ST-specific resistance gene patterns, with ST262 and ST787 representing a potential for multidrug resistance and therapeutic failure. ST202 harbored *aac(6')-Ie-aph(2")-Ia*, *tet*L, and *tet*M, but lacked *aph(3')-IIIa*, *ant(6)-Ia'*. ST80 carried *aph(3')-IIIa*, and *ant(6)-Ia'*. (Fig 1).

Resistance gene and specimen Correlation; urine isolates represented the majority of samples and exhibited the greatest variability in antibiotic resistance genes, likely due to the larger sample size. Among the 39 urine isolates, *aph(3')-IIIa* was detected in 20 isolates (51.3%), *ant(6)-Ia* in 21 (53.8%), and *tet*M in 19 (48.7%). The *tet*L gene was found exclusively in four ST262 isolates (10.3%). The *tet*O gene was detected in only one ST17 isolate. Notably, four urine isolates (10.3%) carried the high-level aminoglycoside resistance gene *aac(6')-Ie-aph(2")-Ia*, including two ST262 isolates, one ST17, and one ST202. Stool isolates (n = 8) generally harbored a narrower resistance profile, with *aph(3')-IIIa* and *ant(6)-Ia* each detected in four isolates (50%), *tet*M in four (50%), and *tet*L in one (12.5%). Neither *aac(6')-Ie-aph(2")-Ia* nor *tet*O was detected in stool isolates. Pus isolates demonstrated broader resistance profiles, with detection of 66.67% *aac(6')-Ie-aph(2")-Ia*, 66.67% *aph(3')-IIIa*, 33.33% *ant(6)-Ia*, 33.33% *tet*L, 100% *tet*M, and 33.33% *tet*O. The single blood isolate harbored all major resistance genes, including *aph(3')-IIIa, ant(6)-Ia*, tetL, and *tet*M. The bile isolate exhibited a limited profile, carrying *aph(3')-IIIa*, *ant(6)-Ia*, and *tet*M.

Virulence gene identification

Eight virulence genes—*asa*1, *gel*E, *esp*, *hyl*, *cpd, ebp*, *pain*, and *spr*E—were tested to determine which enterococcal virulence factor was present in the clinical VREfm isolates. *asa*1, *gel*E, *cpd, esp, pain,* and *spr*E were absent in all isolates, whereas *esp* was present in all isolates, and *hyl* was detected in 88% (46/52) of the isolates. The percentage of VREfm isolates carrying the *esp*+ and *hyl*+ genes (88%, 46/52) was higher than that of VREfm isolates carrying the *esp*+ gene alone (13.5%) (Table 3 and Fig 1).

Genotypic classification of VRE strains using MLST

Among the 52 VREfm isolates, five STs were identified: ST17, ST80, ST202, ST262, and ST787. The predominant strain identified within the hospital under study was ST17, accounting for 86.54% (45/52) of the isolates, followed by ST262 (7.69%, 4/52), ST787 (1.92%, 1/52), ST80 (1.92%, 1/52), and ST202 (1.92%, 1/52) (Fig 1). All isolates belonged to the same ancestral lineage and were classified under Clonal Complex (CC) 17. Fig 2 presents the goeBURST network diagram of CC17, illustrating the relationships between the STs using Phyloviz 2.0. From the analysis, ST17 was identified as the primary founder because of its highest frequency. ST202 was the closest relative to ST17, with a single-locus variant, followed by ST80, which exhibits a double-locus variant. In contrast, ST262 and ST787 exhibited a triple-locus variant. Notably, ST787 was closely connected to ST80 within the network (Fig 2). Evolutionary analyses of seven concatenated sequences were conducted using MEGA-X. Hasegawa-Kishino-Yano model. The BIC score was 11110.06999. Then, a phylogenetic tree was constructed using the maximum likelihood method based on the HKY model with 1,000 replicates in bootstrap analysis. The tree with the highest log likelihood (−4919.79) is presented. This analysis involved 52 nucleotide sequences. The first, second, third, and non-coding codon positions were included. The final dataset included 3,458 positions.; S1Fig shows the resulting phylogenetic tree. The relationships observed were as follows: ST17 was most closely related to ST202, followed by ST262, ST80, and ST787.

Genome characteristics

To characterize the genome of VREfm circulating in our hospital, six VREfm representative isolates were selected for WGS based on different STs, including G239 (ST262), G293 (ST787), G384 (ST17), G1101(ST80), G1176 (ST202), and G1768 (ST17). S2 Table presents an overview of the genome and assembly characteristics of the VREfm sequences. The total assembled genome size ranged from 2.8 to 3.0 MB, the GC content ranged from 37.48 to 37.75. S2 Table also presents the total number of contigs.

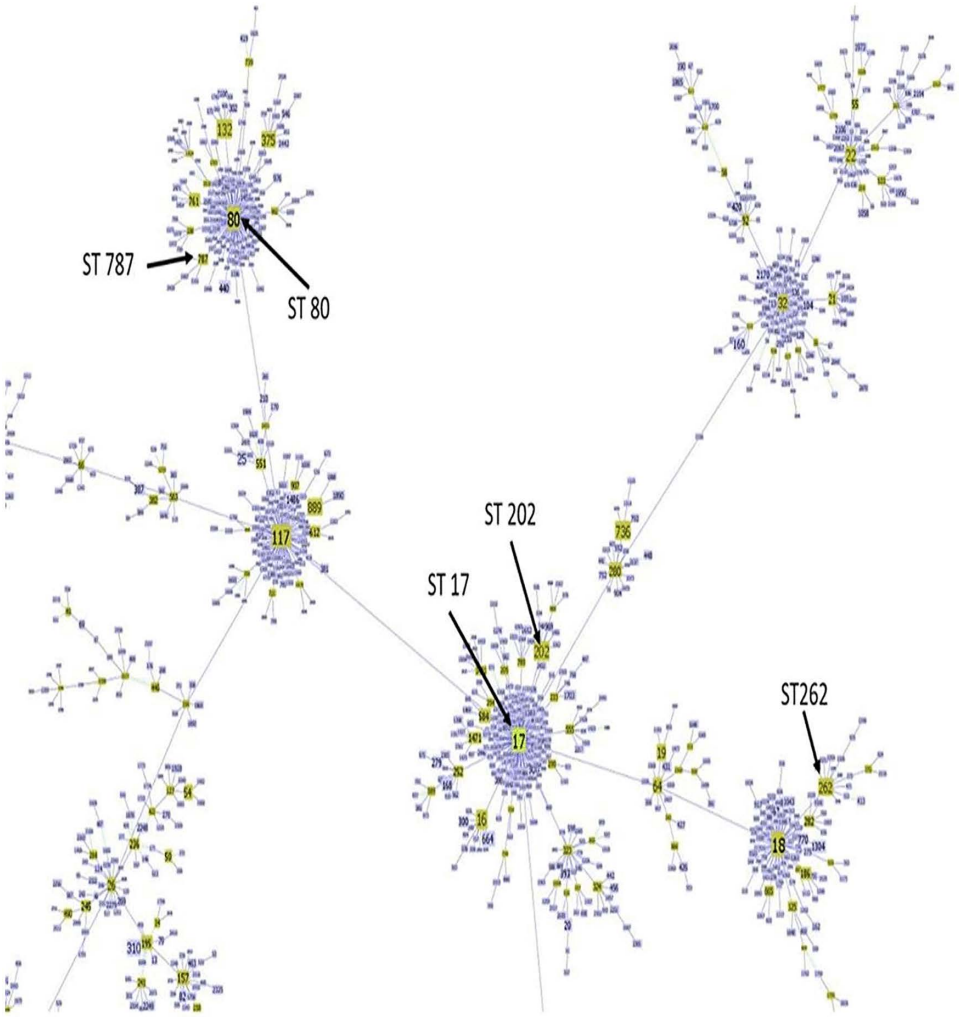


Fig 2. Population snapshot of *Enterococcus faecium* isolates based on multilocus sequence typing (MLST), generated using goeBURST analysis. The diagram illustrates the genetic relationships among *E. faecium* sequence types (STs) identified through MLST. Each node (numbered box) represents a unique ST, and the size of each box is proportional to the number of isolates corresponding to that ST in the global MLST database. Solid lines connect STs that are single-locus variants (SLVs), indicating close genetic relatedness. STs identified in this study are indicated with arrows. A total of 52 vancomycin-resistant *E. faecium* (VREfm) isolates were analyzed, and five distinct STs were detected. These were categorized into three groups based on their genetic clustering: Group 1: ST17 and ST202, with ST17 serving as the central (founder) ST within the group, suggesting it may be the ancestral type. Group 2: ST80 and ST787, which clustered closely together and formed a distinct subgroup. Group 3: ST262, which did not group with other STs and thus constituted a separate lineage.

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Core genome multilocus sequence typing (cgMLST) was applied to six representative vancomycin-resistant *Enterococcus faecium* (VREfm) genomes belonging to five different sequence types (STs), including ST262 (G239), ST787 (G293), ST80 (G1101), ST202 (G1176), and ST17 (G384 and G1768). cgMLST analysis further assigned the six isolates to distinct complex types (CTs): CT7004-ST262 (G239), CT10028-ST787 (G293), CT10025-ST80 (G1101), CT10026-ST202 (G1176), CT10029-ST17 (G384), and CT10027-ST17 (G1768). Among these, one previously described CT (CT7004) and five novel CTs were identified. Two isolates (G384 and G1768) belonged to ST17; cgMLST distinguished them into two separate CTs (CT10029 and CT10027, respectively), demonstrating the higher discriminatory power of cgMLST compared with conventional MLST for differentiating closely related VREfm strains.

Using VFanalyzer and Virulence Finder to predict virulence-associated genes, 14 virulence genes were detected (Table 4). The virulence genes present in the VREfm genome included adhesin (*acm, ebp*A, *ebp*B, *ebp*C, *srt*C, *ecb*A, *efa*A, *esp, scm, sgr*A), capsule-associated antiphagocytosis (*cps*A/*upp*S, *cps*B/*cds*A), biofilm formation (*bop*D), and hyaluronidase enzyme *(hyl*E) (Table 4).

Antimicrobial resistance genes conferring resistance to six antimicrobial classes were detected among six representative VREfm genomes. Genes encoding resistance to tetracyclines [*tet*M and *tet*L], macrolides [*erm*B, *erm*T, *msr*C, and *efm*A], aminoglycosides [*aac(6')-Ii, aph(3')-III, ant(6)-Ia, aac(6')-aph(2''), aph(2'')-Ia, aad(6),* and *SAT-4*], phosphonic acid (*FosXCC* and *FosI*), trimethoprim (*dfr*G and *dfr*F), and glycopeptide (*Van*HAX) were detected among the six isolates. Tetracycline resistance was mediated mainly by the *tet*M gene in all TET-resistant isolates (i.e., G239, G293, G384, G1176, and G1768) (Table 4).

Table 4. Comparative genomic features of six VREfm sequence types and a vancomycin-sensitive *E. faecium* (VSE) strain.

Isolate	cgMLST	Antibiotype	AMR genes	Virulence factors	Plasmid replicon	MEG
G239 (ST262)	CT7004	VAN-TEI-RIF-PEN-AMP-ERY-LEV-CIP-TET-HLS	*aac(6')-Ii*, *ant(6)-Ia*, *aac(6')-aph(2'')*, *aph(2'')-Ia*, *aad(6)*, *msr*C, *erm*T, *dfr*G, *Van*HAX, *Fos*I, *SAT*-4, *FosXCC*, *tet*M	*acm*, *ebp*A, *ebp*B, ebpC, *srt*C, *ecb*A, *efa*A, *esp*, *sgr*A, *cps*A/*upp*S, *cps*B/*cds*A, *bop*D, *hyl*Efm	rep11a rep18b rep22 repUS15 repUS43	ISEfa5 IS16
G293 (ST787)	CT10028	VAN-TEI-RIF-PEN-AMP-ERY-LEV-CIP-TET-HLS	*aac(6')-Ii, ant(6)-Ia, aac(6')-aph(2''), aph(2'')-Ia, aph(3')-III, aad(6), msr*C, *efm*A, *erm*B, *Van*HAX, *tet*M*, *tet*L*	*acm*, *ebp*A, *ebp*B, *ebp*C, *srt*C, *efa*A, *scm*, *sgr*A, *cps*A/*upp*S, *cps*B/*cds*A, *bop*D, *hyl*E	rep1 rep11a rep2 rep17 repUS15 repUS43*	ISLgar5 IS16
G1101 (ST80)	CT10025	VAN-RIF-PEN-AMP-ERY-LEV-CIP-HLS	*aac(6')-Ii, ant(6)-Ia, aph(3')-III, aad(6), msr*C, *efm*A, *erm*B, *Van*HAX, *SAT-4*	*acm*, *ebpA*, *ebp*B, *ebp*C, *srt*C, *ecb*A, *efa*A, *esp*, *scm*, *sgr*A, *cps*A/*upp*S, *cps*B/*cds*A, *bop*D	rep11a rep14a rep17 rep2 rep22 repUS15 repUS7	ISEfa11 IS16 IS256
G1176 (ST202)	CT10026	VAN-TEI-RIF-PEN-AMP-ERY-LEV-CIP-TET	*aac(6')-Ii, aac(6')-aph(2''), aph(2'')-Ia, msr*C*, *erm*T, *dfr*G, *Van*HAX, *FosI*, *tet*M	*acm*, *ebp*A, *ebp*B, *ebp*C, *srt*C, *ecb*A, *efa*A, *esp*, *scm*, *sgr*A, *cps*A/*upp*S, *cpsB*/*cdsA*, *bopD*, *hylE*	rep11a rep18b rep22 repUS15 repUS43 repUS7	ISEfm2* ISEfa5 IS16
G384 (ST17)	CT10029	VAN-RIF-PEN-AMP-ERY-LEV-CIP-TET-HLS	*aac(6')-Ii, ant(6)-Ia, aad(6), msr*C, *efm*A, *dfr*G, *Van*HAX, *tet*M	*acm*, *ebp*A, *ebp*B, *ebp*C, *srt*C, *ecb*A, *efa*A, *esp*, *sgr*A, *cps*A/*upp*S, *cps*B/*cds*A, *bop*D, *hyl*Efm	rep11a rep18b rep17 rep2 repUS15	IS16
G1768 (ST17)	CT10027	VAN-TEI-RIF-PEN-AMP-ERY-LEV-CIP-HLS	*aac(6')-Ii, ant(6)-Ia, aac(6')-aph(2'')*, *aad(6)*, *msr*(C), *efm*A, *dfr*F, *dfr*G, *Van*HAX, *FosXCC*, *tet*M	*acm*, *ebp*A, *ebp*B, *ebp*C, *srt*C, *ecb*A, *efa*A, *esp*, *sgr*A, *cps*A/*upp*S, *cps*B/*cds*A, *bop*D, *hyl*Efm	rep11a rep18b rep17 rep2 repUS15	IS256 IS16
E. faecium VSE1036 (JAAJ01000001.1)		NA	*aac(6')-Ii, ant(6)-Ia, aph(2'')-Ib, aac(6')-Im, aph(3')-III, msr*C, *lsa*E, *lnu*B, *erm*B, *tet*L, *tet*M	*acm*, *ebp*B, *ebp*C, *srt*C, *ecb*A, *efa*A, *sgr*A, *cps*A/*upp*S, *cps*B/*cds*A, *bop*D, *hyl*Efm	rep11a, repUS12, rep2, rep14b, rep17, repUS15	IS16, ISEfa4, ISS1N

*Indicates a plasmid replicon or mobile genetic element (MGE) found alongside antimicrobial resistance (AMR) genes within the same contig.

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Plasmid sequence analysis using PlasmidFinder revealed ten plasmid-associated replication genes (i.e., *rep11a, rep18b, rep22, repUS15, repUS43, rep1, rep2, rep17, repUS7,* and *rep14a*). All six isolates had at least five plasmid replicons in their genome. *rep11a* and *repUS15* were the most common replicon types in all six isolates, followed by *rep18b, rep17*, and *rep2* in four isolates*; rep22* in three isolates; *repUS43*, *rep1*, and *repUS7* in two isolates; and *rep14a, rep11a, repUS15, rep2*, and *rep17* in one isolate, which occurred in the same four isolates. *repUS43* coexisted with *tet*M and *tet*L on the same contig on the G293 (ST787) genome (Table 3).

Using the Mobile Element Finder tool, six insertion sequence (IS) elements were identified. Among them, IS16 was the most prevalent IS element, appearing in all six genomes, followed by ISEfa5 and IS256 appearing in two isolates each and ISLgar5, ISEfa11, and ISEfm2 appearing in one isolate each. ISEfm2 was found alongside the *msr*C gene in the same contig in the G1176 (ST202) genome (Table 4).

Discussion

Enterococcus is a significant challenge in hospital-acquired infections, particularly in intensive care units. The bacteria can spread through person-to-person contact or contaminated medical equipment. Therefore, effective infection control measures to monitor and prevent the dissemination of *Enterococcus* within hospital settings are critical. In this study, *E. faecalis* was the most commonly isolated species (57.7%), followed by *E. faecium* (38.3%). However, vancomycin resistance was observed more frequently in *E. faecium* than in *E. faecalis*, consistent with the results of previous studies [23,24]. The *van* gene, a genetic determinant of vancomycin resistance, can be horizontally transferred among *Enterococcus* spp. [25]. In this study, all VREfm isolates carried the *van*A gene, whereas no isolates contained the *vanB* gene, which is consistent with the results of previous studies in Thailand [26,27]. Urine samples accounted for most VRE isolates (75%), consistent with the findings from Songklanagarind Hospital, which reported the highest detection of VRE in urinary specimens [26]. This is unsurprising because *Enterococcus* is the second leading cause of UTIs after *Escherichia coli*. The overall detection rate of vancomycin-resistant *Enterococcus* (VRE) was 14.5% among *Enterococcus* spp., while 36.7% of *E. faecium* isolates (VREfm) were found to be vancomycin-resistant. These rates are substantially higher than those reported by the Health Policy and Systems Research on Antimicrobial Resistance (HPSR-AMR) Network, which documented an increase in vancomycin resistance among *Enterococcus* spp. in Thailand from 6.0% in 2019 to 6.9% in 2023, and in *E. faecium* specifically from 7.6% to 15.7% during the same period (https://www.thaiamrwatch.net) [28]. Moreover, a study conducted in a hospitals in Thailand has reported lower and more variable VRE detection rates among *Enterococcus* spp., ranging from 0.2% to 4.3% [26]. The high VRE and VREfm detection rate observed in our hospital is likely attributable to the implementation of active surveillance programs. These programs systematically screen patients, particularly those in high-risk wards, for VRE colonization to prevent the transmission of nosocomial infections. Consequently, the VRE detection rate reported in this study does not accurately reflect the actual infection rate among hospitalized patients, but rather includes all VRE-positive findings from specimens submitted to the laboratory, encompassing both clinical infections and asymptomatic colonization (carriers). When compared to other countries in Asia, our observed VREfm rate (36.7%) also exceeds regional averages. A meta-analysis study reported a pooled vancomycin resistance rate of 8.1% among *Enterococcus* spp. Across Asia, with resistance in *E. faecium* reaching 22.4% [29]. Country-specific reports have documented VRE rates of 3.1% in East Asia (China), 7.7% in South Asia (India and Pakistan), and 11.4% in Western Asia (Iran, Saudi Arabia, Jordan, and Kuwait) [29]. In this context, the 36.7% VREfm detection rate in our study is significantly higher than both regional and global estimates. Overall, these findings highlight the critical need for ongoing surveillance of vancomycin-resistant *Enterococcus* (VRE), especially in healthcare settings that implement active screening measures. Although active surveillance facilitates prompt identification and helps reduce hospital-acquired transmission, it may also lead to an overestimation of prevalence by detecting both clinical infections and asymptomatic colonization. Therefore, it is essential to interpret surveillance data with caution, differentiating between actual infections and colonization, to support informed decisions regarding antimicrobial stewardship and infection prevention efforts.

VRE often exhibits multidrug resistance, limiting therapeutic options [30,31]. Antimicrobial susceptibility testing revealed that all isolates were resistant to β-lactams (ampicillin and penicillin), glycopeptides (vancomycin), fluoroquinolones (levofloxacin and ciprofloxacin), and ansamycin (rifampin). However, these isolates exhibited susceptibility to phenol (chloramphenicol), oxazolidinone (linezolid), and lipopeptide (daptomycin), suggesting that these three antibiotics are available for treating VRE-related infections. Linezolid remains the treatment of choice for VRE infections. In the literature, chloramphenicol effectively treats VRE bacteremia [32]. Resistance to the tetracycline class in *Enterococcus* is mediated by efflux pumps [*tet*K and *tet*L] and ribosomal protection proteins [*tet*M*, tet*O*, tet*S*, tet*T*,* and *tet*W] [33]. In this study, phenotypically tetracycline-resistant VREfm was detected in 17.3% of the isolated strains, and the genotypically tetracycline-resistant (*tet*) gene was observed in 53.9% of the isolated strains, including *tet*M in 53.9%, *tet*L) in 13.5%, and *tet*O in 3.9%. Fifty-five percent of the phenotypically tetracycline-resistant isolates concurrently harbored the *tet*L and *tet*M genes. The presence of both the *tet*L efflux pump and the *tet*M protection factor was found to be associated with resistance in clinical isolates of *E. faecium* [34]. The *tet*M gene, which is carried by conjugative transposons and mediates the ribosomal protection mechanism to prevent 23S rRNA binding, was mainly associated with tetracycline resistance in this study. A recent study found that the expression of two tetracycline resistance determinants, *tet*L and *tet*M, can confer tigecycline resistance in enterococcal clinical isolates [35]. *Enterococcus* intrinsically exhibit low-level aminoglycoside resistance, mostly because of the presence of the *aac(6′)-Ii* gene; however, high-level aminoglycoside resistance is mediated by the acquisition of aminoglycoside resistance genes, including *aac(6′)-Ie–aph(2′′)-Ia, aph(3′)-IIIa,* and *ant(6)-Ia* [36]. In this study, 53.8% of the VREfm isolates exhibited phenotypic HLSR. Furthermore, the VREfm isolates carried a gene associated with high-level aminoglycoside resistance, specifically *aph(3′)-IIIa* (53.9%), *ant(6)-Ia* (53.9%), and *aac(6′)-Ie–aph(2′′)-Ia* (11.5%). Eighty-two percent of the phenotypic HLSR isolates carried both *aph(3′)-Ia* and *ant(6)-Ia*. The correlation between specimen type and the distribution of antibiotic resistance genes in VREfm isolates demonstrates urine samples, which constituted the majority, showing the highest diversity of resistance genes, likely due to the larger sample size and selective antibiotic pressure in urinary tract infections. In contrast, stool isolates exhibited a more limited resistance profile. Pus and blood isolates carried broader resistance determinants, including genes associated with high-level aminoglycoside and tetracycline resistance, reflecting their clinical severity. These findings highlight the importance of specimen-specific surveillance and continuous genomic monitoring, alongside prudent antimicrobial stewardship, to effectively manage the spread and clinical impact of multidrug-resistant *E. faecium* in healthcare settings.

Pathogenicity-associated gene analysis revealed that 100% of the isolates carried the *esp* gene, and 88.5% carried the *hyl* gene. The high prevalence of *esp* is consistent with the findings of previous studies, demonstrating its common occurrence in uropathogenic *E. faecium* [26,37]. The 88.5% prevalence of *hyl* observed in this study is notably higher than the 5.6% reported in previous studies within the same country (16). These findings suggest that *esp* and *hyl* are major virulence factors in the strains under study. The *esp* gene contributes to bacterial adherence and biofilm formation in the urinary tract, whereas the *hyl* gene, which encodes hyaluronidase, facilitates colonization and tissue invasion.

The MLST analysis identified five STs associated with the hospital setting: ST17, ST80, ST202, ST262, and ST787. Among them, ST17 was the most frequently observed strain. Numerous studies have reported outbreaks of *E. faecium* ST17 and ST80 in various regions, including Germany [38], France [39], Australia [40], Thailand [27,41], and China [42]. ST262 has been linked to infections in South Korea [43], Europe [44], Cuba [45], and Russia [46]. ST202 has been associated with hospital-acquired infections in Norway [47], Germany [48], and China [49]. Furthermore, ST787 has been reported in South Korea, Sweden, Pakistan, Denmark, and Ireland (www.pubmlst.org), indicating that the identified STs, particularly ST17, ST80, ST202, ST262, and ST787, are globally distributed and associated with hospital-acquired infections. Notably, this study is the first to report the presence of VREfm ST202, and ST787 in Thailand, contributing new insights into the molecular epidemiology of VREfm in the region. The correlation between sequence types (STs) and antibiotic resistance genes revealed distinct ST-specific patterns of resistance. ST17, the most common lineage, predominantly carried *aph(3')-IIIa*, *ant(6)-Ia*, and *tet*M, reflecting a more conserved resistance profile. In contrast, ST262 and

ST787 harbored a broader array of resistance genes, including *aac(6')-Ie-aph(2")-Ia*, indicating a higher potential for multi-drug resistance and treatment failure. ST202 showed a unique resistance profile, lacking some aminoglycoside-modifying enzymes, while ST80 carried only *aph(3')-IIIa* and *ant(6)-Ia*. These findings highlight the value of ST-based resistance profiling in informing surveillance, antimicrobial stewardship, and infection control efforts. However, this ST-based analysis has limitations, as conventional MLST may lack sufficient resolution to distinguish closely related strains within the same lineage in a hospital setting. Therefore, small genetic differences and potential transmission relationships may not be fully identified without higher-resolution approaches, such as core genome multilocus sequence typing (cgMLST).

The genomic characterization of the six selected VREfm isolates allowed us to identify more details in the resistance and virulence genes of each representative ST strain. All six representative genomes contained resistance genes associated with three antimicrobial classes (aminoglycosides, glycopeptides, and macrolides). All VREfm isolates carried genes associated with vancomycin resistance, specifically the *van*A gene cluster (i.e., *van*R, *van*S, *van*H, *van*X, *van*Y, and *van*Z). All selected VREfm genomes carried the *aac(6')-Ii* gene, which was previously reported to be associated with intrinsic resistance to low-level aminoglycosides in *E. faecium* [50]. The presence of the *ant(6)-Ia* gene, which is responsible for HLSR, was mainly associated with phenotypically identified high-level resistance. In terms of macrolide and lincosamide resistance determinants, in addition to *msr*C, which was intrinsically present in all selected VREfm isolates, other genes such as *efm*A, *erm*B, and/or *erm*A were discovered in phenotypically erythromycin-resistant strains. The VREfm isolates harbored *tet*M and/or *tet*L, which are commonly associated with tetracycline-resistant *Enterococcus*. Fosfomycin has shown good *in vitro* action against *Enterococci*, including VRE strains, and is used to treat uncomplicated UTIs [51–53]. In our study, the *fos*XCC and *fos*I genes associated with fosfomycin resistance were detected in isolates G239-ST262-CT7004, G1176-ST202-CT10026, and G1768-ST17-CT10027. Consistent with these genotypic findings, fosfomycin disk-diffusion testing showed resistance in G239-ST262-CT7004 and G1768-ST17-CT10027, while G1176-ST202-CT10026 exhibited intermediate susceptibility. Overall, these results demonstrate a clear genotype–phenotype correlation, as the presence of *fos*XCC/*fos*I was associated with resistant or reduced susceptibility to fosfomycin. A study from China [54] reported increasing fosfomycin resistance among VREfm, frequently associated with fos genes, consistent with our observation of fosXCC/fosI in multiple isolates.Three VREfm genomes (G239-ST262-CT7004, G384-ST17-CT10029, and G1768-ST17-CT10027) carried genes associated with trimethoprim resistance (*dfr*F and *dfr*G). Linezolid is currently FDA-approved for VRE infection treatment [34]. Linezolid resistance is a significant concern. Fortunately, linezolid-resistant genes (i.e., *cfr, optr*A, and *poxt*A) were not detected in this study, supporting the linezolid phenotypic antimicrobial susceptibility results. Numerous virulence genes, such as those involved in biofilm formation, adhesion, and host cell invasion, can be expressed by *Enterococci* [55]. The genomic profiles of VREfm isolates in this study demonstrate the association between sequence types and resistance gene patterns. ST262 and ST787 stood out with the highest resistance gene diversity. These sequence types may pose a greater risk of therapeutic failure due to their potential for multidrug resistance. In contrast, ST17 remains the dominant strain with a stable resistance profile, reflecting endemic hospital adaptation.

Genome analysis from six representative strains identified several virulence genes with adhesin protein (i.e., *acm, ebp*A, *ebp*B, *ebp*C, *srt*C, *ecb*A, *efm*A, *esp, scm,* and *sgr*A), capsule (*cps*A/*upp*S and *cps*B/*cds*A), biofilm formation (*bop*D), and hyaluronidase enzyme (*hyl*E). A previous report stated that the *cps*A and *cps*B genes are common in *E. faecium*, and the *esp* and *hyl* genes are exclusively present in HA clade strains, including most strains responsible for human infections [56]. The *hyl*E gene responsible for intestinal colonization has been detected in ampicillin-resistant *E. faecium* ST17 and VREfm strains in hospitals worldwide [57]. Characterizing plasmids and mobile genetic elements is essential for understanding phenotypic traits such as the ability to colonize hosts, enhance bacterial virulence, and acquire antimicrobial resistance. These elements play a critical role in the diversification of *Enterococci* and appear to contribute to their adaptation in hospital environments [58]. Eleven types of plasmid replicons were identified in the analyzed genomes, with rep11a and repUS15 being the most common replicons in representative VREfm genomes, and some contained rep14a,

rep17, rep1, rep2, repUS43, rep22, rep17, repUS7, and rep18b. One isolate from this study (G293-ST787) exhibited the repUS43 plasmid carrying the *tet*M and *tet*L genes. The findings of a previous study indicated that the *tet*M gene is often associated with the chromosomally integrated repUS43 locus [59]. Six IS families were identified in the analyzed genomes. All six representative VREfm genomes contained IS16, a specific marker for hospital-associated strains of *E. faecium* [60], whereas some genomes also included ISEfa5, ISLgar5, ISEfa11, IS256, and ISEfm2. These mobile genetic elements may play roles in the antibiotic resistance, adaptation, and genome plasticity of VRE. The genomic characterization of vancomycin-resistant *Enterococcus* (VRE) provides valuable insights into the genetic features, resistance mechanisms, and clonal lineages of VRE strains circulating in our hospital, thereby supporting local surveillance and infection control strategies. However, a limitation of this comparative analysis is the uneven distribution of sequence types among the whole-genome sequenced isolates. While the majority of isolates belonged to ST17, only a single representative was available for ST262, ST202, ST80, and ST787. This limited sample size for non-ST17 sequence types may restrict the generalizability of resistance and virulence gene comparisons across different lineages and may not fully capture the genetic diversity within those STs.

This study has several key strengths. First, it provides comprehensive characterization of VREfm by combining phenotypic antimicrobial susceptibility testing with molecular analyses, including MLST, resistance gene profiling, and virulence gene detection, offering an in-depth view of circulating strains. Second, the work is based on clinical isolates from a tertiary-care referral hospital, making the findings directly applicable to real-world patient care, infection control, and antimicrobial stewardship. Third, the study identifies the dominant clonal lineage (CC17/ST17) and describes the population structure and associated resistance/virulence profiles, contributing important baseline molecular data for Thailand. Finally, the study contributes valuable local genomic data for VREfm, supporting infection control and antimicrobial stewardship in tertiary-care hospital settings.

Conclusions

This study revealed that the *van*A gene primarily mediates vancomycin resistance in the study strains. In terms of virulence, the strains carried at least one key virulence gene, *esp*, which encodes an adhesin protein associated with enhanced colonization and persistence. Notably, *esp* was frequently co-harbored with the *hyl* gene, which encodes hyaluronidase—an enzyme that facilitates tissue invasion. These findings indicate a potential link between virulence factors and antimicrobial resistance mechanisms. Effective infection control practices and active surveillance measures should be continuously implemented. Molecular techniques can successfully identify antibiotic-resistant genes, thereby enabling better monitoring and management of VRE infections in hospitals. Furthermore, this study provides an overview of the genomic characteristics of six representative VREfm isolates, examining various antibiotic resistance genes, virulence genes, and mobile genetic elements that may contribute to the spread of antimicrobial resistance. These factors enhance genetic diversity and improve adaptation in hospital environments.

Supporting information

S1 Table. List of primers and PCR conditions used in this study.
(DOCX)

S1 Fig. The phylogenetic tree illustrates the relationships of the nucleotide sequences of seven concatenated housekeeping genes (i.e., *atpA*, *ddl*, *gdh*, *purK*, *gyd*, *pstS*, and *adk*) from 52 VREfm isolates. These isolates were classified based on the following STs: ST17 (•), ST202 (•), ST262 (•), ST80 (•), and ST787(•).
(TIFF)

S2 Table. Genome characteristics of six representative VREfm strains.
(PDF)

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