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High-resolution genomic and molecular characterization of vancomycin-resistant *enterococci* from hospitalized patients in a tertiary care center in Riyadh, Saudi Arabia

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Vancomycin-resistant enterococci (VRE), particularly *Enterococcus faecium*, represent a significant nosocomial threat worldwide. In Saudi Arabia, limited genomic data exist to support phenotypic surveillance findings, impeding the understanding of resistance mechanisms, clonal diversity, and plasmid dynamics. To investigate the genomic and phenotypic characteristics of vancomycin-resistant *E. faecium* and *E. faecalis* clinical isolates from a tertiary care center in Riyadh, Saudi Arabia, and to assess antimicrobial resistance genes, virulence factors, sequence types, and plasmid replicons. Seventy-five VRE isolates were collected between 2017 and 2019 and subjected to antimicrobial susceptibility testing per CLSI guidelines. The whole genome sequencing (WGS) was performed using the Illumina MiSeq platform. Species identification, MLST/cgMLST typing, resistome, virulome, and plasmidome analyses were conducted using established bioinformatics pipelines (e.g., CARD, VFDB, PlasmidFinder, pyMLST). Among 75 isolates, 50 *E. faecium* and 6 *E. faecalis* passed WGS quality thresholds. *E. faecium* isolates showed high resistance to vancomycin (100%), ciprofloxacin (98%), and ampicillin (96%), while linezolid retained activity (98% susceptible). The *vanA* gene was detected in 93.9% of *E. faecium* isolates; other resistance determinants included *tet(M)*, *erm(B)*, and *liaR/liaS* mutations associated with daptomycin non-susceptibility. MLST revealed multiple STs, including ST136, ST102, and ST252, with no dominant clone, supporting polyclonality. Plasmid analysis identified 20 replicon types, predominantly rep11a, rep2, and repUS15, some co-associated with AMR genes. Virulence profiling showed enrichment of *bopD*, *acm*, and *cpsA/uppS* genes. *E. faecalis* isolates exhibited limited resistance and no clonal clustering. This is the most comprehensive genomic study of VRE from Saudi Arabia to date. Our findings reveal a diverse, polyclonal population of *E. faecium*

harboring high-risk resistance and virulence determinants disseminated via plasmids. These data underscore the need for routine genomic surveillance to guide infection control and antimicrobial stewardship.

Keywords Vancomycin-resistant *Enterococcus faecium*, Enterococcus faecalis, Whole-genome sequencing, Multilocus sequence typing, Daptomycin resistance, Saudi arabia

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Enterococci are Gram-positive bacteria that were once regarded as medically insignificant but have now emerged as important opportunistic pathogens[1]. They are ubiquitous in nature, found in water, soil, food products (such as dairy and meat), sewage, and are also natural colonizers of the gastrointestinal tract of humans and animals[2]. Among the genus, *Enterococcus faecalis* and *Enterococcus faecium* are the primary species responsible for clinical infections, particularly in healthcare settings, where they commonly cause endocarditis, urinary tract infections (UTIs), and bloodstream infections (BSIs)[3].

In the United States, *E. faecalis* and *E. faecium* together account for up to 30% of hospital-acquired infections, and globally they are recognized as the second most common cause of nosocomial infections[4]. In China, *E. faecium* was identified as the dominant species (74%) in BSI cases, followed by *E. faecalis* (20%), with an associated mortality rate of 24%[5]. The clinical challenge posed by enterococci is compounded by increasing antimicrobial resistance in both species. Although enterococci possess intrinsic resistance to aminoglycosides and β-lactams, their genomic plasticity facilitates the horizontal acquisition of resistance genes against a broader spectrum of antibiotics[6]. Since the 1980 s, multidrug-resistant enterococci have been increasingly implicated in hospital-acquired BSIs and UTIs[7]. Among these resistance mechanisms, glycopeptide resistance—particularly to vancomycin—is of significant concern. First identified in the 1980 s, vancomycin-resistant enterococci (VRE) are now a major cause of hospital-acquired infections, especially in intensive care units and among immunocompromised patients[8,9].

VRE have evolved into globally relevant multidrug-resistant organisms (MDROs), as recognized by the World Health Organization[10]. Invasive infections caused by VRE, such as BSIs, are associated with significantly higher mortality rates compared to those caused by vancomycin-susceptible strains[11]. In Europe, *E. faecium* accounts for over 93% of VRE isolates and several risk factors—including prior antibiotic use, immunosuppression, and advanced age—have been associated with VRE colonization and infection[12].

Glycopeptide resistance in enterococci is mediated by eight known gene clusters: *vanA*, *vanB*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM*, and *vanN*[13]. Data from the SENTRY Antimicrobial Surveillance Program showed that 8% of global enterococcal isolates were VRE, with most exhibiting the *VanA* phenotype. In this dataset, 67% of *E. faecium* isolates were vancomycin-resistant, compared to only 1.49% of *E. faecalis* isolates—a trend similarly observed in Europe[14]. Notably, the prevalence of VRE varies significantly by regions, with North America reporting the highest proportion (21.6%)[15].

In Saudi Arabia, the first VRE isolate was reported in 1993, and several studies since then have documented its prevalence across the country. However, many of these studies were limited by small sample sizes (typically fewer than 50 isolates) and a lack of molecular characterization[16–18]. Therefore, this study seeks to address existing knowledge gaps by undertaking an in-depth investigation of VRE isolates obtained from hospitalized patients in Riyadh, Saudi Arabia. The objectives are fourfold: (i) to systematically characterize the phenotypic antibiotic susceptibility patterns of these isolates using standardized methodologies; (ii) to elucidate the underlying genetic determinants of antimicrobial resistance, with a particular focus on glycopeptide resistance genes and other clinically significant resistance markers; (iii) to profile the distribution of key virulence factors that may contribute to pathogenicity and persistence in the hospital environment; and (iv) to delineate the population structure and clonal diversity of VRE by comprehensive sequence typing. By integrating phenotypic, genotypic, and epidemiological data, this study aims to provide a robust framework for understanding the local epidemiology and molecular landscape of VRE. Ultimately, the findings will inform regional infection control practices, support antimicrobial stewardship efforts, and contribute valuable baseline data for ongoing surveillance and future comparative studies within Saudi Arabia and beyond.

Materials and methods

Study design

This cross-sectional study was conducted between February 2017 and March 2019 in the Department of Clinical Microbiology at Prince Sultan Military Medical City, a 1,192-bed tertiary care hospital located in Riyadh, Saudi Arabia.

Bacterial isolates

A total of 75 vancomycin-resistant enterococci (VRE) isolates were included. These isolates were obtained as part of routine clinical diagnostic workflows from various clinical specimens, including blood, urine, rectal swabs, and aspirated fluids. Isolates were stored in brain heart infusion (BHI) broth supplemented with 15% glycerol at −80 °C for subsequent analysis. Duplicate isolates from the same patient were excluded. Relevant clinical and demographic data, including patient age, gender, hospital ward (medical, surgical, or ICU), and clinical diagnosis, were also collected.

Bacterial identification and antibiotic susceptibility testing

Initial bacterial identification was performed using conventional biochemical methods and confirmed using the MicroScan WalkAway 96 Plus System (Beckman Coulter) with the Pos Combo 28 Panel Kit, following the manufacturer's instructions. Antimicrobial susceptibility testing was conducted using the same platform for the following agents: ampicillin, ciprofloxacin, gentamicin (high-level synergy), linezolid, rifampin, streptomycin (high-level synergy), Synercid (quinupristin/dalfopristin), tetracycline, teicoplanin, and vancomycin. Results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines, 2023[19]. Control strains used included *E. faecalis* ATCC 29,212 (vancomycin-susceptible) and ATCC 51,299 (vancomycin-resistant).

Whole genome sequencing

Genomic DNA was extracted from overnight bacterial cultures using the Qiagen bacterial DNA isolation kit. DNA concentration was measured with the Qubit dsDNA HS Assay Kit on the Qubit Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA purity was assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) by evaluating the A260/280 and A260/230 ratios. Libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina) along with the Nextera Index Kit (FC-131–1002). Libraries were normalized, pooled, and sequenced using 250-bp paired-end reads on the Illumina MiSeq platform with the MiSeq Reagent Kit v3.

Raw reads (FASTQ files) were assembled using Unicycler v0.48 with default parameters[20]. Assembly quality was assessed using QUAST v5.2.0[21] and only assemblies meeting the following thresholds were retained: Phred quality score ≥ 30, read length ≥ 50 bp after trimming, sequencing depth ≥ 30×, and genome coverage ≥ 90%. Antimicrobial resistance (AMR) genes were identified with RGI v6.0.3 against the CARD database v4.0.0, using default settings[22]. Plasmid incompatibility (Inc) types were detected using PlasmidFinder v2.10 with the Gram-positive database, a threshold identity of 60%, and default coverage settings. Virulence factors were identified using the VFDB online platform[23]. Species confirmation was performed using TYGS. For *E. faecium* isolates, multilocus sequence typing (MLST) and core genome MLST (cgMLST, 2023 scheme) were conducted using pyMLST v2.1.5 with default parameters[24], based on the cgMLST.org scheme (https://www.cgmlst.org/ncs). A k-mer-based analysis (k = 31) was conducted using Sourmash v4 on both in-house and publicly available isolates[25]. Phylogenetic clustering based on cgMLST was generated using GrapeTree v1.5.0 with the MSTreeV2 algorithm[26]. The resulting phylogenetic tree was visualized using iTOL (Interactive Tree Of Life)[27]. Public genomes and their metadata were obtained from two different studies and the ENA GenBank repository[28,29].

Ethical approval

This study was approved by the research ethics committee of Prince Sultan Military Medical City (Reg. #HAP01-R-015) project NO. 998; 16 Oct 2017). All methods were performed in accordance with the relevant guidelines and regulations. An informed consent was obtained from all subjects and/or their legal guardian(s) to participate in the study.

Results

Demographic and clinical characteristics of the patients

Table 1 summarizes the demographic and clinical characteristics of the 75 patients from whom VRE isolates were obtained. The majority of patients (62%) were aged ≥ 60 years, with a mean age of 56 years (range: 9 months to 98 years). Male patients constituted 57.3% (43/75) of the cohort. All individuals were hospitalized at the time of specimen collection, and 29.3% (22/75) were admitted to intensive care units (ICUs).

The most frequent co-morbidity conditions included liver and renal transplantation, renal failure, pneumonia, and orthopedic interventions such as knee amputation or replacement. Regarding specimen sources, VRE isolates were most frequently recovered from blood cultures and rectal swabs.

Antimicrobial susceptibility testing of VRE isolates

Antimicrobial susceptibility testing revealed that VREisolates, including both *E. faecium* and *E. faecalis*, exhibited high resistance rates to vancomycin, ciprofloxacin, and ampicillin. Among *E. faecium* isolates, resistance was also substantial for rifampin, teicoplanin, tetracycline, and streptomycin synergy, with linezolid remaining the most effective agent. For *E. faecalis*, resistance rates to vancomycin and ciprofloxacin were also high, but lower levels of resistance were observed for ampicillin and other agents compared to *E. faecium*. Overall, linezolid showed

Demographic characteristics	E. faecium *n* = 50 (100%)	E. faecalis *n* = 6 (100%)
Ages		
Elderly (60 and above)	28 (55.1%)	5 (83.3%)
Adult (18–59)	14 (28.5%)	1 (16.7%)
Child (0–12)	8 (16.3%)	0 (0%)
Adolescent (13–17)	0 (0%)	0 (0%)
Gender		
Male	32 (63.3%)	1 (16.7%)
Female	18 (36.7%)	5 (83.3%)
Wards		
Accident and emergency (A&E)	24 (48.9%)	1 (16.7%)
Intensive care unit	13 (26.5%)	3 (50%)
No information	3 (6.1%)	1 (16.7%)
Renal ward	2 (4.0%)	1 (16.7%)
Isolation ward	3 (6.1%)	0 (0%)
Surgical ward	2 (4.0%)	0 (0%)
Hepatology	1 (2.0%)	0 (0%)
Obstetrics and gynecology	0 (0%)	0 (0%)
Burns unit	1 (2.0%)	0 (0%)
Sample source		
Blood culture	21 (40.8%)	0 (0%)
Rectal swab	18 (34.7%)	3 (50%)
Midstream urine	6 (12.2%)	2 (33.3%)
Aspirate	1 (2.0%)	0 (0%)
Tissues	2 (4.0%)	1 (16.7%)
Stool	2 (4.0%)	0 (0%)
Urine from Catheter	1 (2.0%)	0 (0%)

Table 1. Demographic and clinical characteristics of patients with VRE in the period from 2017 until 2019.

Antibiotic susceptibility profile	*E. faeciumn* = 50 (100%)	E. faecalis *n* = 6 (100%)
	R	*R*
Ampicillin	48 (96%)	3 (50%)
Ciprofloxacin	50 (100%)	3 (50%)
Gentamycin synergy	18 (36.7%)	3 (50%)
Linezolid	1 (2%)	1 (16.7%)
Rifampin	49 (98%)	1 (16.7%)
Streptomycin synergy	28 (57.1%)	1 (16.7%)
Synercid	24 (47%)	1 (16.7%)
Tetracycline	30 (59.2%)	3 (50%)
Teicoplanin	45 (89.8%)	1 (16.7%)
Vancomycin	50 (100%)	6 (100%)

Table 2. Antibiotic susceptibility profile of the Vancomycin resistant isolates. R = resistance,.

the highest activity against both species, and gentamicin synergy retained moderate efficacy. Detailed resistance and susceptibility profiles for all tested antibiotics and both species are presented in Table 2.

Molecular characterization of VRE isolates via whole genome sequencing

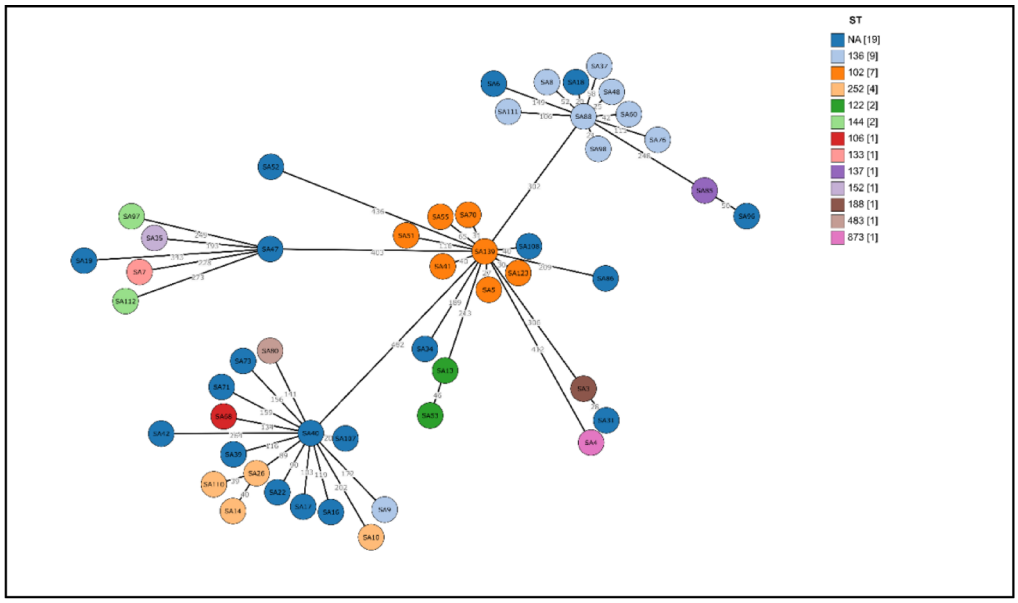
The whole genome sequencing (WGS) identified 62 isolates as *E. faecium*, 6 as *E. faecalis*, and 7 as other *Enterococcus* species, which were excluded from further analysis. Among the *E. faecium* isolates, 50 passed quality control thresholds and were included in downstream analysis, while all six *E. faecalis* isolates met quality standards and were retained.

Of the 50 *E. faecium* isolates, 55% were from patients aged ≥ 60 years, and 63% were from male patients. The predominant specimen source was blood cultures (40%), followed by rectal swabs (34%). Additionally, 50% of isolates originated from patients admitted to the Accidents and Emergency Department.

Clonality of the *Enterococcus faecium* isolates

Whole genome sequencing (WGS) analysis of *E. faecium* isolates revealed multiple distinct sequence types (STs) based on Bezdíček scheme (eight loci: *copA*, *dnaE*, *HP*, *mdlA*, *narB*, *pbp2b*, *rpoD*, *urvA*) (Fig. 1 A). Isolates with unknown sequence types represented 38.7% ($n = 19$). The most prevalent known sequence type was ST136, which accounted for 18.3% of the isolates ($n = 9$), followed by ST102 ($n = 7$, 14.2%), ST252 ($n = 4$, 8.1%), ST122,

(A)



(B)

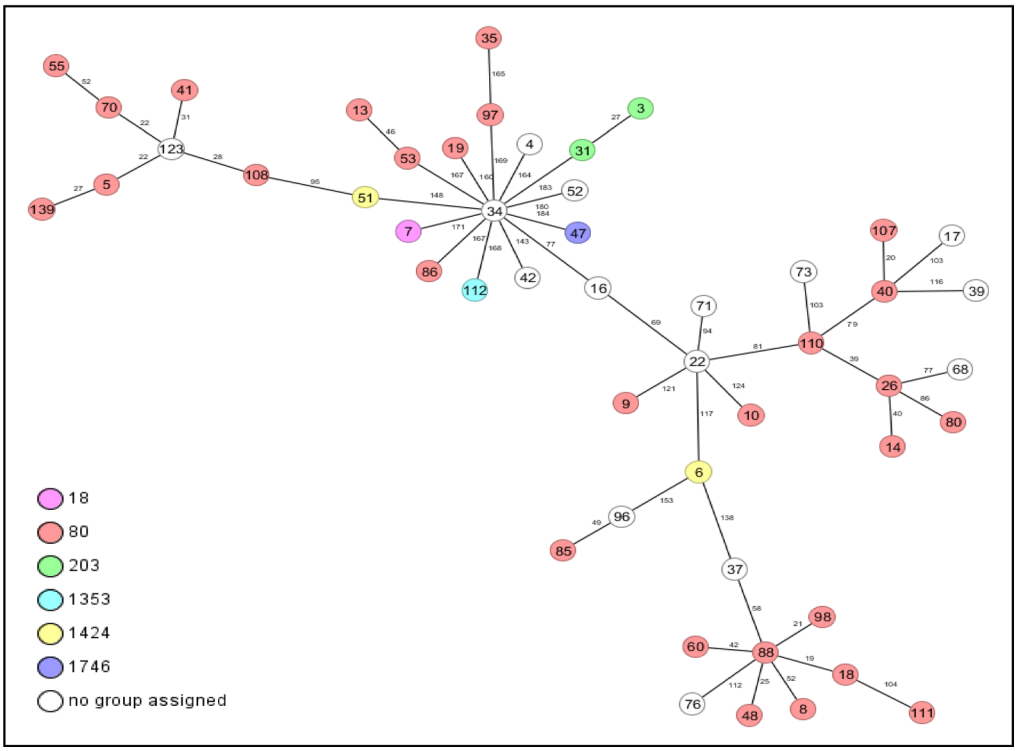


Fig. 1. Minimum spanning tree (MST) illustrating the clonal relatedness among vancomycin-resistant *E. faecium* (VREfm) isolates based on core genome multilocus sequence typing (cgMLST) profiles. Each node represents a single isolate, and the numbers on the connecting lines indicate allelic differences between them. Isolates are color-coded according to their respective sequence types (STs). (**A**) STs assigned according to the Bezdíček scheme, providing a higher-resolution and updated classification framework for *E. faecium* population structure. (**B**) STs assigned according to the original *E. faecium* MLST scheme based on seven housekeeping genes, allowing for historical comparison with legacy datasets.

ST144 (each $n=2$, 4.08%) and ST106, ST133, ST137, ST152, ST188, ST483 and ST873, each represented by a single isolate (2%). The ST136 isolates were distributed across several wards, with 44.0% originating from ICU, 33.3% from the Accidents and Emergency Department, and the remaining isolates from hepatology and isolation wards. Isolates with unknown STs were also distributed across various wards, with 73.5% being from the Accidents and Emergency Department, and ICU. The ST102 isolates were sourced from the ICU, Accidents and Emergency Department and the isolation ward, while the ST252 isolate was identified from the Accidents and Emergency Department and the isolation ward. Finally, the ST122 and ST144 isolates were traced to the Accidents and Emergency Department and ICU. However, when the isolates were analyzed using the original MLST scheme (based on 7 housekeeping genes (*adk*, *atpA*, *ddl*, *gyd*, *gdh*, *purK*, *pstS*)), (Fig. 1 B) a more consolidated population structure emerged. ST80 was identified as the dominant lineage, accounting for 30 of 50 isolates (~60%). An additional 32% of isolates could not be assigned to a known ST, indicating the presence of novel or highly divergent lineages. ST203 was detected in two isolates, while ST18, ST1353, and ST1746 were each represented by a single isolate. In both schemes, core genome MLST (cgMLST) analysis did not reveal any clustering patterns, supporting a polyclonal structure (Fig. 1 A, B).

Resistome analysis of the *Enterococcus faecium* isolates

Whole genome sequencing (WGS) revealed a high prevalence of vancomycin resistance genes among the *E. faecium* isolates (Fig. 2). The *vanA* gene was identified in 94% of isolates, while *vanY* (part of the *vanB* operon) was found in 98%. Only one isolate (2.04%) carried the *vanB* gene. Additional components of the *vanA* operon, including *vanH*, were detected in 10.20% of isolates. No isolates carried vanC-type resistance genes. Tetracycline resistance genes were common, with *tet(M)* detected in 46.94% of isolates and *tet(U)* in 42.86%. Other tetracycline resistance determinants, including *tet(L)* and *tet(S)*, were observed at lower frequencies. Aminoglycoside resistance was also prominent. The *aac(6')-Ii* gene was present in 65.31% of isolates, and *aad(6)* in 61.22%. For macrolide resistance, e*rmB* was detected in 65.31% of isolates, whereas *ermA* was identified in only one isolate (2.04%). Daptomycin resistance-associated mutations in the liaFSR regulatory system were identified, with *liaR* and *liaS* mutations detected in 38.78% and 34.69% of isolates, respectively. In addition, one isolate (2.04%) harbored the β-lactamase gene $bla_{\text{TEM-207}}$, and another carried the *E. faecalis* chloramphenicol acetyltransferase gene. All major sequence types (STs) harbored the *vanA* gene, with the exception of ST152 and ST188, which lacked this key vancomycin resistance determinant. Daptomycin resistance-associated mutations in both *liaR* and *liaS* were found across multiple STs, including ST106, ST133, ST144, ST152, ST252, ST136, and isolates with unknown STs.

Virulence gene profiling of *Enterococcus faecium* isolates

A total of 30 virulence genes spanning 13 functional categories were identified among the *E. faecium* isolates (Fig. 3). The most frequently detected gene was *bopD*, associated with biofilm formation, present in 48 isolates. This was followed by the capsule-associated gene *cpsA/uppS* (anti-phagocytosis), identified in 45 isolates. Other highly prevalent genes included the serine protease *htrA/degP* ($n=42$), the iron uptake gene *vctC* ($n=40$), and the lipoprotein anchoring gene *lgt* ($n=39$). Adherence-related genes—such as *acm*, *ebpA*, *ebpB*, *ebpC*, *efaA*, and *sgrA*—were widely distributed, with *acm* present in nearly all isolates. Genes involved in capsule biosynthesis (*cpsB/cdsA*), adherence and invasion (*stp*, *EF-Tu*), and immune evasion (*cps2K*, *rpgG*) showed variable presence. In contrast, genes related to serum resistance (*wbtE*, *wbtP*) and magnesium uptake (*mgtB*) were less frequently observed across the isolate set.

Plasmidome analysis

Comprehensive analysis of the plasmidome across the studied *Enterococcus faecium* isolates revealed a diverse set of 20 distinct plasmid replicon types. The overall plasmid landscape was characterized by a small number of dominant replicons coexisting with a broader array of low-frequency elements (Fig. 4 A). The most prevalent replicon was rep11a, detected in 48 isolates, followed by rep2 (38 isolates) and repUS15 (31 isolates), indicating these replicons may serve as major plasmid backbones for the dissemination of mobile genetic elements, including antimicrobial resistance determinants. Moderately abundant replicons included rep17, rep18b, and rep14a, which were present in 16–26 isolates. In contrast, several low-frequency replicons—such as rep7a, rep6, rep29, and rep14b—were detected in only one to three isolates, forming a long-tail distribution. The number of replicons per isolate ranged from 1 to 7, reflecting substantial inter-strain variability in plasmid content. A subset of isolates exhibited high replicon diversity, suggestive of increased plasmid acquisition capacity or the maintenance of multiple compatible replicons. Conversely, isolates harboring only one or two replicons may represent genomically streamlined lineages or strains inhabiting more stable ecological niches with limited selective pressure for horizontal gene transfer. Further integration of plasmid replicon data with resistance gene profiles revealed distinct associations between specific incompatibility (Inc) types and antimicrobial resistance determinants (Fig. 4B). The most prominent linkage was observed for rep14a, which was present in 10 isolates and consistently co-harbored the *tet(U)* gene. Additionally, repUS43 was frequently associated with *tet(M)*, identified in four isolates. Other Inc types such as rep22 and repUS15 were each associated with unique resistance profiles.

Phylogenetic analysis

The comparative circular genome visualization of vancomycin-resistant *Enterococcus faecium* (VREfm) isolates, including those from this study (highlighted in green fluorescence), demonstrates the distribution of antimicrobial resistance (AMR) genes, plasmid content, and associated metadata. The AMR genes are categorized as aminoglycoside-modifying enzyme (AME) genes (red), vancomycin resistance genes (blue), and other resistance genes (green). Plasmid content is marked in light blue, with plasmid-encoded AMR genes shown in yellow (Fig. 5). The isolates from this study are distributed across several distinct clades and genomic lineages,

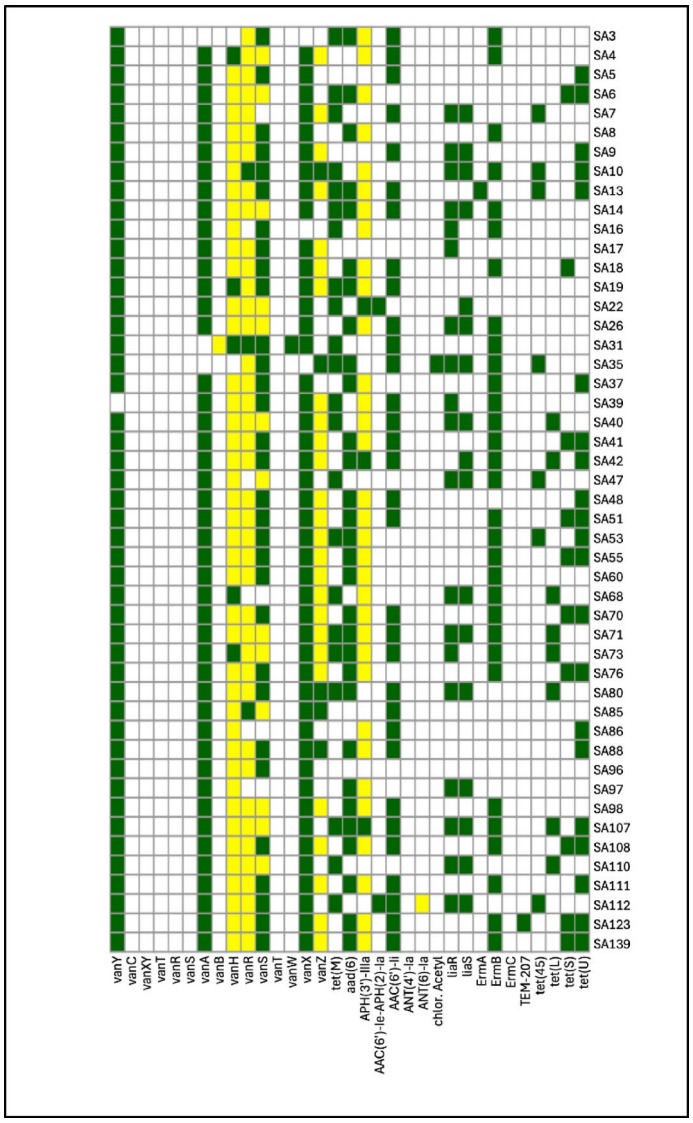


Fig. 2. Heatmap illustrating the presence and identity of antimicrobial resistance (AMR) genes among vancomycin-resistant *E. faecium* (VREfm) isolates. Each row represents an individual isolate, and each column corresponds to a specific AMR gene. Colored cells indicate gene presence, with green denoting 100% nucleotide identity and yellow indicating 98–99% identity, based on reference sequences. White cells represent gene absence. The panel includes resistance determinants for glycopeptides (*vanA*, *vanB*, *vanS*, etc.), aminoglycosides (*aac(6')-Ii*, *aph(3')-IIIa*, *ant(6)-Ia*), macrolides (*ermB*, *lnuB*), tetracyclines (*tet(M)*, *tet(L)*), and other classes.

indicating polyclonal origins. These isolates harbor a dense accumulation of AMR genes, including *vanA*, *aac(6')-Ii*, *erm(B)*, and *tet(M)*, which are frequently co-localized with plasmid-associated regions. The yellow bars denoting plasmid-encoded resistance suggest that horizontal gene transfer via plasmids plays a central role in the dissemination of resistance within these strains. Most of the study isolates cluster with global genomes from both human and animal hosts, indicating potential inter-host transmission. Notably, these isolates share high genomic similarity with international strains from Europe, Asia, and North America, reflecting the global spread of dominant VREfm lineages such as ST136 and ST102. The isolates also span multiple years of collection, primarily between 2017 and 2019, with several exhibiting similar resistance and plasmid profiles to historical isolates from earlier years, suggesting long-term circulation and persistence.

Genomic and phenotypic characterization of *Enterococcus faecalis* isolates

For the *Enterococcus faecalis* isolates ($n=6$), the majority of patients were elderly, with 83% aged 60 years or above. Most of the isolates were obtained from female patients (83%). Half of the isolates (50%) were sourced from patients admitted to the intensive care unit (ICU), and rectal swabs accounted for 50% of the isolate sources. Whole genome sequencing (WGS) analysis of the *E. faecal*is isolates identified that 3 out of the 6 isolates corresponded to known sequence types (STs): ST6, ST179, and ST394. The remaining 3 isolates were of unknown

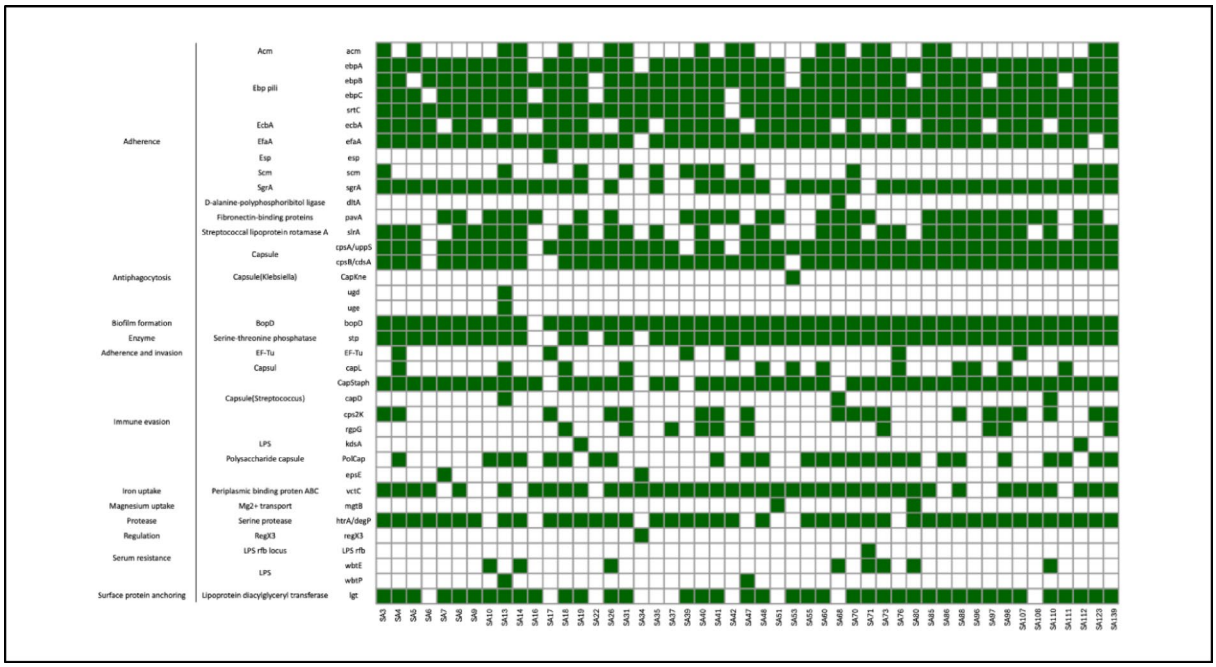


Fig. 3. Heatmap showing the distribution of virulence genes among vancomycin-resistant *E. faecium* (VREfm) isolates. Each row represents a virulence gene, grouped by functional categories (e.g., adherence, antiphagocytosis, biofilm formation, immune evasion, iron uptake, and others), while each column corresponds to an individual isolate. The presence of a gene is indicated by a green cell, and its absence by a white cell.

sequence types. Isolates belonging to ST6 and ST179 were collected from the ICU, while the ST394 isolate was sourced from the renal ward. cgMLST analysis did not identify any clustering among the isolates. In contrast, the *E. faecalis* isolates exhibited a more limited resistome profile compared to *E. faecium*. The *vanA* gene was detected in only one isolate, specifically associated with ST6, indicating a restricted presence of vancomycin resistance within this species. Both ST6 and ST179 harbored genes conferring resistance to macrolides, aminoglycosides, and tetracyclines. The remaining isolates did not carry any known resistance genes, highlighting the lower overall prevalence and diversity of resistance mechanisms in *E. faecalis*.

Discussion

This study provides genomic and phenotypic characterization of vancomycin-resistant *Enterococcus faecium* (VREfm) and *Enterococcus faecalis* (VREfs) isolates from a tertiary care center in Riyadh, Saudi Arabia. Our findings reveal a high burden of multidrug resistance in *E. faecium*, characterized by widespread *vanA* carriage, extensive resistance to first-line antibiotics, and the presence of plasmid-mediated resistance genes. The polyclonal nature of the isolates, with predominant STs such as ST136 and ST102, aligns with global patterns of high-risk *E. faecium* clones. In contrast, *E. faecalis* isolates demonstrated limited resistance mechanisms, with only one isolate harboring *vanA*, and no detectable clonal clustering, suggestive of a limitedrole in VRE burden.

Phenotypic antimicrobial susceptibility testing of VREfm isolates revealed alarming levels of multidrug resistance, with high resistance rates to ciprofloxacin, tetracycline, gentamicin, and streptomycin, reflecting a severely limited therapeutic arsenal. However, linezolid retained high efficacy (97.3% susceptibility). These findings are consistent with previous reports, including Hota et al. (2025), who describe widespread high-level resistance in VREfm to vancomycin, teicoplanin, aminoglycosides, and fluoroquinolones, mediated by *vanA* operons and target site mutations[30]. Similar susceptibility trends were also observed in Japan, where over 90% of *E. faecium* isolates exhibited resistance to β-lactams and fluoroquinolones, while linezolid retained full efficacy[31].

Our findings are consistent with global reports of VREfm resistance genotypes, particularly the frequent co-occurrence of *vanA*, *tet(M)*, and *erm(B)*, a pattern widely recognized as indicative of high-level multidrug resistance. Similar ARG combinations were reported by Toc et al. during the COVID-19 pandemic in Romania, where the *vanA-tet(M)-erm(B)* genotype was the most common among ICU-derived *E. faecium* isolates[32]. Importantly, this association was also occasionally accompanied by *tet(L)*, as seen in our cohort, which has been implicated in reduced susceptibility to tigecycline. The convergence of glycopeptide, tetracycline, and macrolide resistance determinants particularly in isolates harboring *vanA-tet(M)-tet(L)-erm(B)* raises concerns about treatment-limiting genotypes emerging under antibiotic pressure. These findings reinforce the need for continuous genomic monitoring of AMR gene combinations to anticipate phenotypic resistance and guide therapeutic decisions.

Our detection of *liaR* and *liaS* mutations in over one-third of VREfm isolates is clinically significant, given their established role in daptomycin resistance through modulation of cell envelope stress responses[33] (REF).

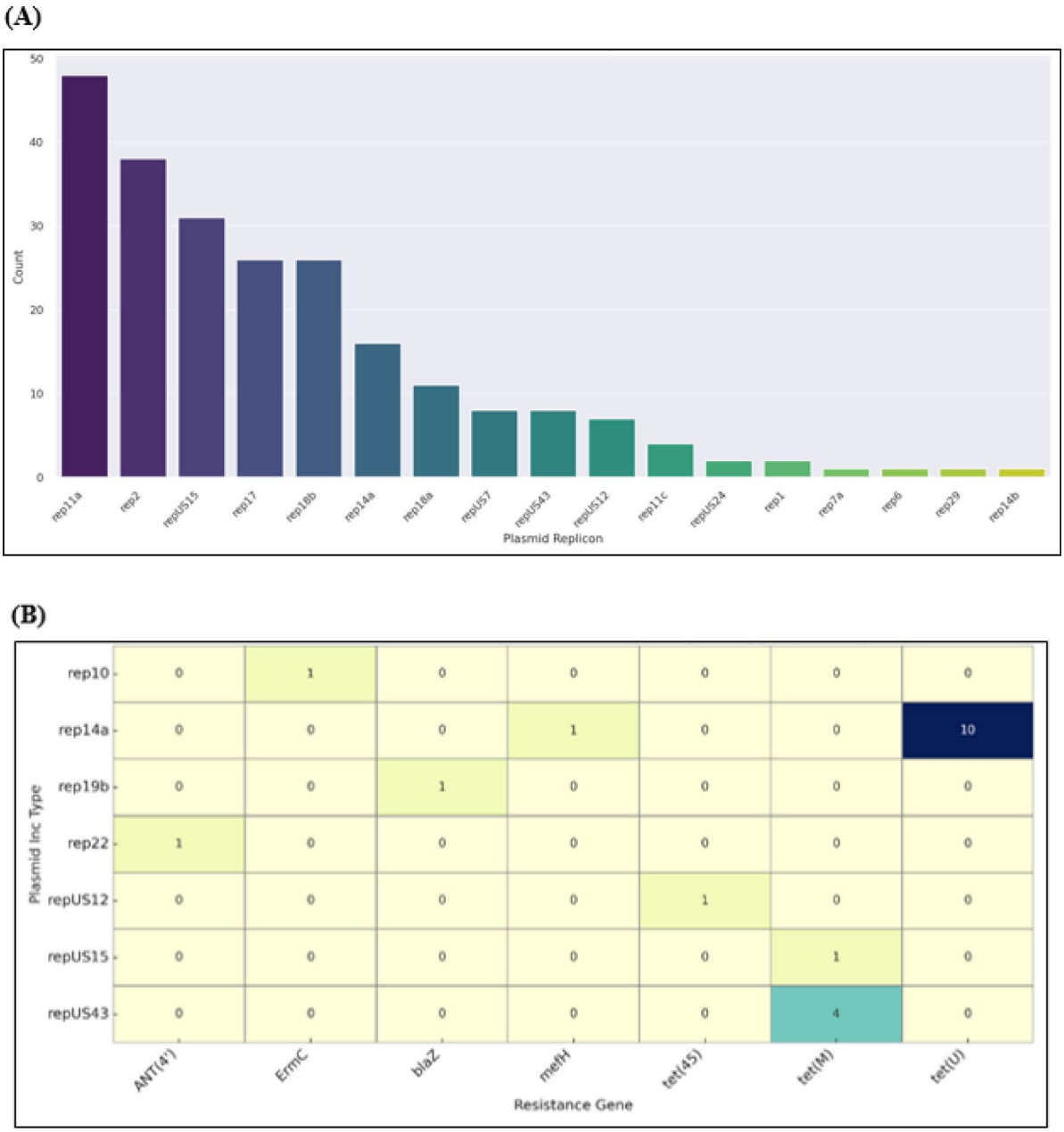


Fig. 4. Plasmid replicon distribution and associated resistance gene profiles among *E. faecium* isolates. **(A)** Bar chart showing the frequency of plasmid replicon types identified among the sequenced *E. faecium* isolates. The x-axis lists individual replicon types, while the y-axis indicates the number of isolates harboring each replicon. **(B)** Heatmap illustrating the co-occurrence of plasmid incompatibility (Inc) types and specific antimicrobial resistance genes across isolates. The y-axis represents Inc types, and the x-axis lists resistance genes detected through WGS-based annotation. Numerical values within each cell indicate the number of isolates carrying both the Inc type and the corresponding resistance gene.

These mutations have been associated with reduced daptomycin binding and increased MICs, contributing to treatment failure in VRE bloodstream infections[33]. In a large prospective study of 661 patients treated with high dose daptomycin, doses ≥ 11 mg/kg were independently associated with improved survival, particularly among isolates with MIC ≥ 2 mg/L. These findings emphasize the importance of both genomic resistance markers and MIC data in guiding therapy. The prevalence of *liaFSR*-associated mutations in our isolates suggests that routine genomic surveillance may be critical to optimize daptomycin dosing and improve outcomes in VREfm infections, especially in critically ill patients. Nonetheless, the potential toxicity at higher doses (e.g., elevated CK) should be considered when balancing efficacy and safety.

The polyclonal structure of *E. faecium* observed in our study reflected by multiple sequence types (STs) such as ST136, ST252 and ST80, ST18, and ST203 is consistent with the known global diversity of hospital-adapted lineages. These STs are well-recognized members of the hospital-associated clade A1, which has emerged

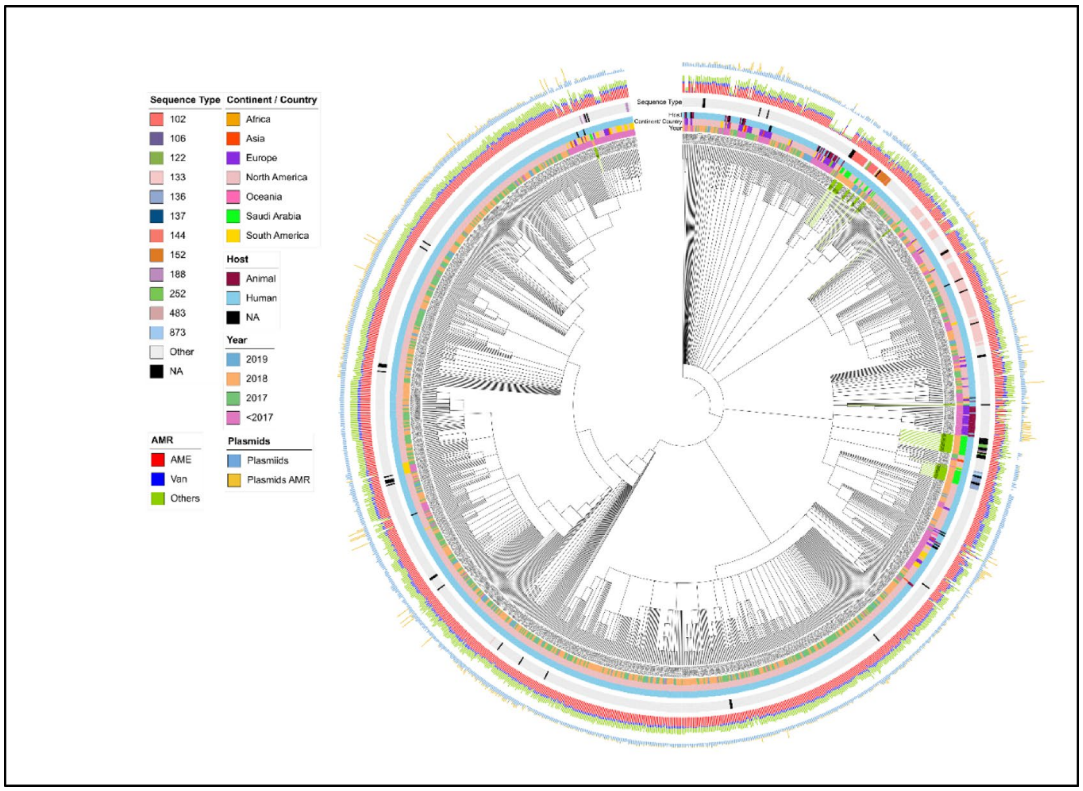


Fig. 5. Comparative genome map of vancomycin-resistant *E. faecium* (VREfm) isolates showing distribution of antimicrobial resistance (AMR) genes and plasmid content. The circular plot displays genomic data from multiple isolates aligned against a reference genome. From outermost to innermost rings: the presence of AMR genes (green = other AMR genes, red = aminoglycoside-modifying enzymes [AME], blue = vancomycin resistance genes), plasmid regions (light blue), and plasmid-associated AMR genes (yellow). Metadata rings annotate each isolate by sequence type (ST), continent/country of origin, host (human/animal), year of isolation, and unclassified categories (NA).

through genomic exchange and selective adaptation to the nosocomial environment[34]. While traditional MLST has been instrumental in identifying high-risk clones, its discriminatory power is limited by recombination events affecting housekeeping genes. In contrast, core genome MLST (cgMLST) provides higher resolution by assessing thousands of conserved loci. Our cgMLST findings revealed no tight clustering among isolates, suggesting that the burden of VREfm in our setting is not due to clonal spread but reflects ongoing importation and horizontal gene transfer of resistance determinants across diverse backgrounds. These findings reinforce recent genomic evidence that endemic VREfm populations exhibit blurred clade boundaries due to frequent recombination and plasmid-mediated gene dissemination, necessitating the use of high-resolution typing tools like cgMLST for effective surveillance and outbreak differentiation[34].

Comparable findings have been reported by Fujii et al. (2024), who conducted a genomic and antimicrobial susceptibility analysis of *Enterococcus* species isolated from hospitalized patients in a Japanese tertiary center without any apparent VRE outbreak. Despite the absence of documented transmission events, their study identified significant antimicrobial resistance among *E. faecium* isolates, particularly to β-lactams and fluoroquinolones, with genotypic resistance mechanisms such as *pbp5* and *gyrA/parC* mutations being prevalent[31]. These results highlight that substantial resistance burdens can persist even outside of outbreak scenarios, emphasizing the endemic nature of VRE in hospital environments. Their use of MLST and whole-genome analysis revealed diverse sequence types, including high-risk ST17, and no close genomic relatedness between strains, reinforcing our own observation of polyclonality and genetic heterogeneity among clinical VREfm isolates. Together, these findings support the growing consensus that robust genomic surveillance is essential—not only for outbreak detection—but also for understanding the silent persistence and evolution of resistant *Enterococcus* populations in healthcare settings.

Plasmidome analysis revealed extensive diversity, with 20 distinct replicons identified. Among them, rep11a, rep2 and repUS15 were the most frequently observed, suggesting their potential role as conserved plasmid backbones involved in AMR gene dissemination. Our findings are consistent with previous genomic studies that highlight the critical role of plasmids in the dissemination of resistance determinants among *Enterococcus* spp. Hourigan et al. (2024) reported that hospitalized *E. faecium* isolates harbor a larger number and size of plasmids compared to community isolates, with over 300 replication and mobilization proteins identified across the plasmidome, contributing significantly to genome size and diversity[34]. Similarly, Founou et al. (2024) identified multiple replicon types in clinical *E. faecium* (e.g., rep1, repUS15, repE) and *E. faecalis* (e.g., repA2, rep(pUB110)),

many of which were co-located with resistance genes such as *vanA*, *erm(B)*, and *tet(M)*, supporting their role in the horizontal transfer of AMR elements[35].

Virulence profiling showed a high prevalence of *bopD*, *cpsA/uppS*, *acm*, and *htrA/degP* genes, indicating that these VREfm strains possess both virulence and resistance traits. Our analysis mirrors findings from prior studies emphasizing distinct virulence profiles between *E. faecalis* and *E. faecium*. Hourigan et al. (2024) identified key surface-anchored adhesins (e.g., *esp*, *acm*) and enzymes such as *gelE* and *hyl* that facilitate biofilm formation and host tissue invasion, particularly in hospital-adapted *E. faecium* lineages like CC17. In contrast, Founou et al. (2024) reported a markedly broader virulence repertoire in *E. faecalis*, with at least 14 genes per strain, including *fsrB*, *ebpA/B/C*, and *ace*, which are involved in adhesion, biofilm development, and immune evasion. These differences suggest species-specific adaptation strategies, where *E. faecalis* may rely more on virulence mechanisms while *E. faecium* exhibits stronger resistance traits, both converging to enhance persistence in healthcare environments[34,35].

In contrast, *E. faecalis* isolates (n = 6) displayed a more limited resistance and virulence profile. Only one isolate harbored *vanA* (ST6), while most showed susceptibility to linezolid and other antibiotics. WGS-based MLST assigned three isolates to ST6, ST179, and ST394, all of which have been previously associated with nosocomial colonization but not large-scale outbreaks[14]. No cgMLST clustering was observed, indicating sporadic acquisition rather than clonal expansion. Moreover, the resistome in *E. faecalis* was less complex; only ST6 and ST179 carried resistance genes to macrolides, aminoglycosides, and tetracyclines, while the remaining isolates harbored no known acquired AMR genes. This distinction suggests that *E. faecalis*, in this cohort, plays a lesser role in the propagation of resistance compared to *E. faecium*.

These observations are consistent with previous genomic studies, which have shown that *E. faecalis* generally exhibits a less complex resistome and is less frequently implicated in large-scale hospital outbreaks than *E. faecium*. Founou et al. (2024) demonstrated that while *E. faecalis* isolates can carry a broad array of virulence genes, including *efaAfs*, *ace*, and components of the fsr quorum-sensing system, their resistance gene content is often limited to specific lineages such as ST6 and ST563, particularly when compared to multidrug-resistant *E. faecium*[35]. Similarly, Hourigan et al. (2024) highlighted the species' tendency to maintain functional CRISPR-Cas systems, which may reduce horizontal acquisition of resistance plasmids and contribute to a comparatively stable genomic architecture[34]. The lack of cgMLST clustering in our cohort further supports sporadic acquisition rather than clonal dissemination, reinforcing the notion that, in contrast to *E. faecium*, *E. faecalis* may play a more passive role in the spread of antimicrobial resistance within healthcare settings.

Comparison of the original MLST scheme with the new typing scheme revealed a marked improvement in discriminatory resolution. The original scheme predominantly identified ST80 among the isolates; however, this lineage was subdivided into at least eight distinct sequence types (e.g., ST102, ST122, ST136, ST137, ST144, ST152, and ST252) by the new scheme, reflecting enhanced phylogenetic granularity. Several isolates that were untypeable using the original method (n = 17) were successfully assigned new sequence types, including ST102 and ST106, underscoring the expanded typing capability of the updated scheme. Conversely, a subset of ST80-typed isolates (n = 12) remained unresolved under the new system, potentially due to incomplete allelic coverage or limitations in reference allele representation. The identification of unique sequence types such as ST873 suggests that the new scheme may also detect sporadic or emerging lineages not captured by earlier methods. These findings are in agreement with a recent study by Karino et al., which demonstrated that the new Bezdíček MLST scheme outperforms the original scheme and shows comparable discriminatory power to PFGE, facilitating the resolution of sequential VREfm outbreaks that would otherwise remain undifferentiated[36]. Collectively, our results further support the utility of the new MLST scheme in refining epidemiological investigations, particularly in resolving clonal diversity within prevalent hospital-associated *E. faecium* lineages.

Molecular epidemiological studies in Saudi Arabia have predominantly reported *Enterococcus faecium* ST17 and ST80 among clinical VRE isolates, reflecting their widespread distribution across multiple healthcare settings[18]. In line with these previous observations, our study also identified ST80 as the dominant lineage when applying the original MLST scheme. However, with the use of the updated Bezdíček MLST scheme, we detected a far greater diversity of sequence types, with ST136, ST102, and ST252 emerging as prevalent lineages in our cohort, alongside a substantial proportion of untypeable isolates. This expanded ST diversity suggests that the current dissemination of VRE in our setting involves multiple clones, some of which were not captured by earlier studies using less discriminatory methods. With respect to *E. faecalis*, although our sample size was limited, our findings align with prior reports in Saudi Arabia, identifying ST179 and ST16 as the predominant sequence types[18–37].

Despite multiple epidemiological investigations of VRE across Saudi Arabia, our study represents the most comprehensive genomic and phenotypic characterization of *Enterococcus faecium* and *Enterococcus faecalis* conducted to date. Nationwide surveillance over 10 years period documented extensive *vanA*-mediated resistance in *E. faecium* and high phenotypic resistance to ciprofloxacin, ampicillin, and rifampicin, while linezolid and daptomycin retained clinical utility[37]. Although Farman et al. (2019) performed whole-genome sequencing on *E. faecalis* isolates from the western region, their focus was limited to 44 isolates of a single species, without a broader exploration of clonal spread, resistance mechanisms, or plasmid content in *E. faecium*[18]. In contrast, our study spans both species, encompasses around 50 VREfm isolates, and integrates detailed analyses of sequence types, cgMLST, resistomes, plasmid replicons, and virulence profiles. To our knowledge, this constitutes the largest and most detailed genomic investigation of VRE in Saudi Arabia, offering new insights into the polyclonal structure, resistance dissemination, and clinical implications of these hospital-adapted pathogens. By bridging the gap between phenotypic surveillance and genomic epidemiology, our findings underscore the urgent need for nationwide genomic surveillance strategies to preempt the emergence and spread of high-risk VRE lineage.

Conclusion

This study provides the most comprehensive genomic insight to date into vancomycin-resistant *Enterococcus faecium* (VREfm) and *Enterococcus faecalis* (VREfs) circulating in a tertiary care hospital in Riyadh, Saudi Arabia. The predominance of *E. faecium* harboring *vanA*, along with extensive resistance to multiple antibiotic classes and the presence of diverse plasmid replicons, underscores the organism's adaptability and the central role of mobile genetic elements in resistance dissemination. The polyclonal structure revealed by MLST and cgMLST analysis indicates ongoing horizontal gene transfer rather than clonal expansion. In contrast, *E. faecalis* isolates exhibited limited resistance profiles and no evidence of clonal spread, suggesting a less prominent role in the regional VRE burden. Together, these findings highlight the urgent need for integrating genomic surveillance into national AMR monitoring frameworks to detect emerging high-risk clones, guide therapeutic decision-making, and inform infection control policies in healthcare settings.

Data availability

The datasets generated and analyzed during the current study are available in publicly accessible repositories. Whole-genome sequences of the isolates have been deposited in NCBI under BioProject accession number [PRJEB90586].

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Author contributions

Author Contributions: L.D.A., M.M.A. and M.K. conceptualized the study. A.G., N.S., and A.M. contributed to methodology, genomic analysis, and data visualization. L.D.A., H.H.A., D.M., M.S.A. and M.M.A. provided the clinical data and bacterial isolates. A.G., N.S., and L.D.A. drafted the original manuscript. A.G., N.S., L.D.A., M.M.A., A.M., M.K., A.S., H.H.A., and M.S.A. contributed to writing—review and editing of the final version. All authors read and approved the final manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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