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Co-carriage of diverse vancomycin-resistant *Enterococcus faecium* ST80-lineages by 70% of patients in an Irish hospital

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Background: Vancomycin-resistant *Enterococcus faecium* (VREfm) are significant nosocomial pathogens. Irish VREfm comprise diverse *vanA*-encoding ST80-complex type (CT) lineages. Recent studies indicate that within-patient VREfm diversity could confound surveillance. This study investigated the intra-host VREfm genetic diversity among colonized Irish hospital patients.

Methods: Rectal VREfm ($n=150$) from 10 patients (15 isolates each) were investigated by WGS, core-genome MLST and split *k-mer* (SKA)-SNP analysis. Plasmids and *vanA*-transposons from 39 VREfm representative of CTs identified were resolved by hybrid assembly of short-read (Illumina) and long-read (Oxford Nanopore Technologies) sequences. Plasmid relatedness was assessed based on Mash distances. Thirty vancomycin-susceptible *E. faecium* (VSEfm) from four VREfm-positive patients were also investigated.

Results: All isolates were clade A1 and most were ST80 (VREfm, 147/150; VSEfm, 25/30). Seventy-percent of patients (7/10) harboured either two ($n=4$), three ($n=2$) or four ($n=1$) VREfm CTs. Individual patient isolate pairs from different CTs differed significantly (median SKA-SNPs 2933), but differences were minimal between isolate pairs of the same CT (median SKA-SNPs 0). In total, 193 plasmids were identified in 39 VREfm investigated. Near-identical plasmids (≥99.5% average nucleotide identity) were identified in divergent CTs from multiple patients. Most VREfm (28/39, 72%) harboured *vanA* on closely related transferable, linear plasmids. Divergent CTs within individual patients harboured either indistinguishable *vanA*-transposons or *vanA*-transposons with distinct organizational iterations. Four VSEfm from different CTs investigated harboured similar plasmids to VREfm.

Conclusion: VREfm within-host diversity is highly prevalent in Irish hospital patients, which complicates surveillance. Linear plasmids play an important role in the emergence of Irish VREfm.

Introduction

Vancomycin-resistant *Enterococcus faecium* (VREfm) are major nosocomial pathogens, associated with a wide-range of infections and persistent environmental contamination. The Republic of Ireland reported one of the highest European rates (28.4%–45.8%) of invasive VREfm infection between 2006 and 2022.[1,2] Irish VREfm are polyclonal with a predominance of *vanA*-encoding ST80 encompassing diverse MLST complex types (CTs) differentiated by significant genomic variation.[3,4]

High-throughput WGS has revolutionized surveillance and phylogenetic analysis of nosocomial pathogens including VREfm. VREfm gastrointestinal colonization is an important cofounder for subsequent infection.[5,6] Current approaches for investigating nosocomial pathogens rely on the assumption of single-strain carriage/monostrain infection and selection of

a single isolate for investigation.[7] However, heterogeneous colonizing/infecting populations could significantly complicate surveillance and outbreak investigations.[8–10]

Previous *E. faecium* investigations identified a well-defined hospital population, termed clade A1, distinct from community and animal isolates.[11–13] Clade A1 tend to be enriched with mobile genetic elements, insertion sequences and pathogenicity islands.[12,14] Many studies highlighted the highly recombinogenic nature of clade A1, with the possibility of new clones emerging *in situ* mediated by horizontal gene transfer (HGT) and recombination.[15,16] The dynamic environment of the gut microbiome and hospital antibiotic selective pressures may promote multi-VREfm strain colonization in patients with frequent hospital exposure.[12,17–19] Additionally, patients can disseminate VREfm into the environment, which can persist and act as a reservoir of infection.[6] This may result in novel VREfm acquisitions for colonized patients and contribute to VREfm gut diversity. Only a few studies investigated the genomic properties and frequency of multi-strain VREfm gastrointestinal carriage in hospitalized patients.[20–25]

This study aimed to investigate the population dynamics, intra-host genetic diversity and plasmidome characteristics of clade A1 VREfm amongst asymptomatic carriers in a large Irish hospital.

Materials and methods

Ethics

The School of Dental Science and Dublin Dental University Hospital Research Ethics Committee approved this study (Reference DSREC2020-01-02).

Patient isolates

VREfm-positive routine admission rectal screening swabs (October 2022–January 2023) from 10 patients (P1-P10) on seven wards (W) (W1 $n=1$, W2 $n=1$, W3 $n=4$, W9 $n=1$, W18 $n=1$, W20 $n=1$, W21 $n=1$) in a large Irish hospital (H1) were inoculated on CHROMID® VRE agar (bioMérieux, Marcy-l'Étoile, France). Following aerobic incubation for 48 h at 37°C, 15 presumptive VREfm colonies per swab ($n=150$) were randomly selected based on previous within-patient heterogeneity studies.[25] Isolates were identified as *E. faecium* by MALDI-TOF-MS (Bruker Daltonics, Bremen, Germany) and stored at −80°C in Microbank cryovials (Pro-Lab Diagnostics, Toronto, Canada). Four VREfm-positive swabs (P1, P3, P5 and P8) were randomly selected to provide a snapshot of the co-carriage of vancomycin-susceptible *E. faecium* (VSEfm). Swabs were inoculated on *E. faecium* ChromoSelect agar (Merck, Ireland) and incubated aerobically for 48 h at 37°C. Multiple, separate green colonies, indicative of *E. faecium*, were subcultured on ChromoSelect and CHROMID® VRE agars. Colonies that failed to grow on CHROMID®VRE agar were identified as VSEfm by MALDI-TOF-MS and susceptibility testing.

Contextual collection

Study isolates were compared with well-characterized collections of clade A1 *E. faecium* ($n=1286$) from seven Irish hospitals including H1 (June 2017–July 2022) and international isolates from 30 countries (1986–2016) (Table S1, available as Supplementary data at *JAC-AMR* Online).[3,4,16,26]

Susceptibility testing

Susceptibility to linezolid, vancomycin and teicoplanin was determined using the VITEK®2 system (bioMérieux) and EUCAST (v14.0) clinical breakpoints.[27] Plasmid-encoded antimicrobial-resistance genes were detected *in silico* on WGS hybrid assemblies ($n=39$) using ABRicate v.0.9.8 (https://github.com/tseemann/abricate).

PFGE and S1 nuclease analysis

PFGE was performed as previously described using a Bio-Rad CHEF DRIII system (Bio-Rad, Richmond, CA, USA) with 0.8% (w/v) agarose gels and switching from 1 to 17 s for 17 h (120-degree angle, 6 V/cm).[28] Plasmid linearity was investigated by S1 nuclease (Promega Corporation, Madison, WI, USA) treatment of agarose plug slices as described previously.[29]

Plasmid transfer

Plasmid transfer was undertaken by filter mating as described previously using the plasmid-free rifampicin- and fusidic acid-resistant recipient *E. faecium* 64/3.[3,30]

Whole-genome sequencing

Short-read and long-read WGS and sequence analysis were performed as described previously.[31,32] Details are provided in Supplemental Methods. VREfm and VSEfm sequence reads are available in GenBank under BioProjects PRJNA734127 and PRJNA1207810.

MLST and core-genome MLST

Traditional MLST STs were extracted *in silico*. Gene-by-gene allele calling and CT assignment was performed using the standardized *E. faecium* core-genome MLST (cgMLST) scheme in Ridom SeqSphere+v8.5.1 (Ridom GmbH, Münster, Germany) based on 1423 genes.[33] cgMLST clusters were determined using single-linkage clustering and a previously recommended pairwise allelic difference threshold of ≤20.[33]

Split k-mer analysis

Target free pairwise whole-genome comparisons were conducted using the split *k*-mer analysis (SKA) software package v1.0 (https://github.com/simonrharris/SKA).[34] The fastq command with default settings was used to generate *k*-mer files ($k=15$) for each isolate from short-read data. Pairwise distances and single-linkage clustering was performed using SKA distance (identity cut-off of ≥0.9) using a previously recommended seven SKA-SNPs threshold.[35] Reference-free alignment of split *k*-mer files was undertaken using the SKA align command under default settings.

Phylogenetic analysis

Phylogenetic neighbour-joining trees were constructed using gene-by-gene allele calling for 1423 core genes in the SeqSphere+*E. faecium* cgMLST scheme.[33] SKA-generated maximum likelihood trees (MLTs) were generated using IQ-TREE v2.2.2.6 (http://www.iqtree.org) with the extended ModelFinder with tree inference (-m MFP) and 1000 ultrafast bootstrap replicates.[36] MLTs were mid-point rooted and visualized using Interactive Tree of Life v6.8.1 (https://itol.embl.de/).[37]

Plasmid analysis

Plasmid sequences from polished hybrid assemblies were visualized using SnapGene v.7.0.4. (https://www.snapgene.com) and characterized using the STAMAR pipeline (https://github.com/phac-nml/staramr). MOB Subfamilies were assigned using the MOB-Typer tool as part of the MOB-suite (https://github.com/phac-nml/mob-suite).[38] Pairwise plasmid genomic distance estimation was calculated using Mash v2.3 (https://github.com/marbl/Mash) using default *k*-mer and sketch sizes.[39,40] Clustering was performed by the complete-linkage method based on

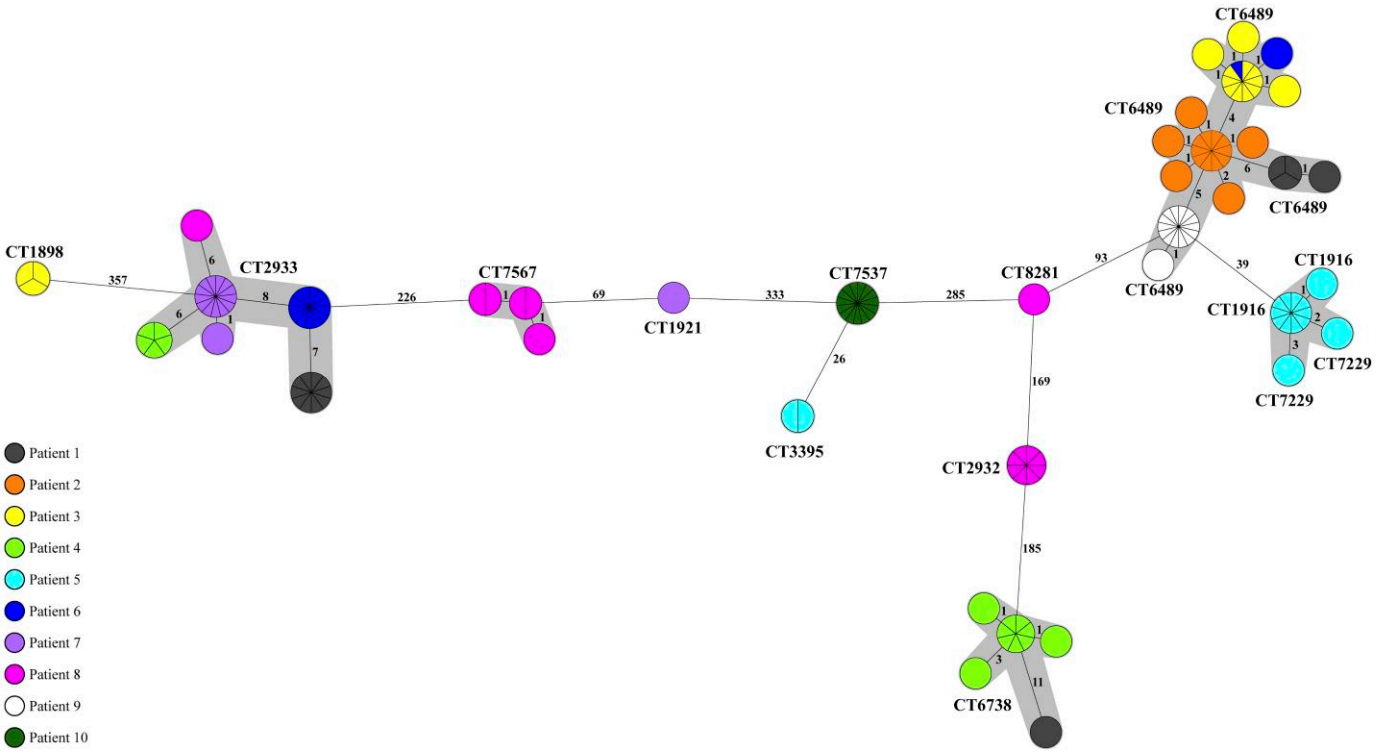

Figure 1. Minimum spanning tree (MST) based on core genome multi-locus sequence typing (cgMLST) analysis of 1423 loci for 150 clade A1 VREfm isolates recovered from a single rectal screening swab from each of 10 separate patients. Node colours represent the patient number (P1-P10) from which the isolate was recovered as indicated in the legend. Partitions within the nodes represent the presence of ≥2 isolates. Branch numbers indicate cgMLST allelic differences between neighbouring isolates. cgMLST segregated isolates into 11 clusters and two singletons using the previously recommended threshold of ≤20 allelic differences.[33]

the Mash-calculated distance matrix using the hclust command in RStudio v2024.04.01 with a threshold of 0.06.[39]

Results

Isolates

All 150 VREfm from rectal screening swabs from 10 patients (15 VREfm per swab) were sequenced, harboured *vanA* exclusively and were linezolid susceptible (Table S2). Thirty VSEfm sequenced from four VREfm-positive patient swabs lacked *vanA*.

Heterogeneity of within-patient VREfm

Most VREfm (147/150, 98%) were ST80. The remaining three ST80 double-locus variants were submitted to pubMLST.org for ST assignment. Recent studies of within-patient VREfm heterogeneity primarily utilized STs to differentiate between strains.[20,24,25,41,42] As Irish VREfm are polyclonal, with a predominance of ST80 comprising genetically diverse CTs, ST assignment could not be used to investigate within-patient diversity.[3,4] Therefore, VREfm belonging to distinct CTs were considered separate strains.

VREfm grouped into nine CT clusters (average size 16, median: 11; range: 2–48) (Figure 1) and two singletons using a previously recommended cgMLST relatedness threshold (≤20 allelic differences).[33] A core-genome based MST comprising study ($n=150$), national ($n=1286$) and global ($n=1325$) clade A1 *E. faecium* assigned all study VREfm to clade A1 (Figure S1).[3,4,16,26] Most (7/10) patients harboured diverse populations, comprising two ($n=4$), three ($n=2$) or four ($n=1$) VREfm-CTs. Three patients each yielded one VREfm-CT (Figure 1; Table S2). The frequency of different VREfm-CTs varied in all patients with multi-strain carriage, with high-frequency, moderate-frequency and low-frequency strains identified in each patient (Figure 1; Table S2).

Isolate pairs from different CTs within individual patients exhibited significant variation. For example, P8 yielded four CTs with a median inter-CT pairwise cgMLST allelic difference of 428 (range: 169–429) (Figure 1; Table S2) and a median pairwise SKA-SNP difference of 2469 (range: 0–3232) (Figure S2 and Tables S2 and S3h). Similar findings were observed for the remaining six patients harbouring multiple VREfm-CTs (Tables S3a and S3c-h). Genetic diversity was minimal among isolate pairs of the same CT from the same patient, with median pairwise cgMLST allelic and SKA-SNP differences of 0 (range: 0–4) and 0 (range: 0–34), respectively, (Tables S2 and S3b, S3i and S3j). The median pairwise SKA-SNP difference between isolates in each of the three patients with one VREfm-CT was 0 (average: 1, range 0–8) (Tables S3b, S3i and S3j).

Related VREfm in different patients

The cgMLST-based MST revealed three CTs (CT2933, CT6489 and CT6738) containing related (<20 allelic differences) VREfm from

multiple patients (Figure 1). The largest cluster comprised 48 CT6489-VREfm from five patients (P1 $n=4$, P2 $n=15$, P3 $n=12$, P6 $n=2$ and P9 $n=15$) with a median pairwise allelic difference of five (range: 0–11). Whole-genome comparison of CT6489-VREfm revealed greater diversity with a median pairwise SKA-SNP difference of 66 (range: 0–140) (Table S4e). Similar findings were recorded for CT2933 and CT6738 (Tables S4d and S4f). Using a previously recommended threshold of ≤7 SKA-SNPs to deem isolates closely related,[35] ward W3 patients P3 and P6 harboured closely related CT6489-VREfm (recovered 104-days apart, median pairwise SKA-SNP difference: three, range: 1–6) with no apparent epidemiological links.

Study isolates ($n=150$) were placed in a wider genomic context by comparison with 622 previously described clade A1 clinical and screening *E. faecium* (June 2017–December 2022) from patients and environmental sites from the same seven H1 wards (VREfm $n=579$, VSEfm $n=43$) (Table S1).[3,4] The circulation and persistence of highly related low-frequency strains across multiple wards was evidenced by a small number of examples (see Supplemental Results and Tables S5a-i).

Plasmidome of VREfm from patients harbouring multiple strains

Plasmid sequences from 39 VREfm from the 10 patients (P1 $n=6$, P2 $n=2$, P3 $n=3$, P4 $n=4$, P5 $n=5$, P6 $n=4$, P7 $n=4$, P8 $n=7$, P9 $n=2$ and P10 $n=2$), representative of CTs, were resolved by hybrid assembly of short- and long-read sequences (Table S6). In total, 193 fully resolved plasmids (size range: 2947–289 861 bp) were identified with isolates harbouring a median of 5 plasmids (range: 3–8). Ten additional plasmid sequence assemblies comprising multiple contigs, were excluded from further analysis. Plasmids were grouped by associated replication genes and nine distinct replicon types (REP) were identified, with rep11a ($n=28$) and repUS15 ($n=39$) predominating (Table S6). Twelve-percent (23/193) of plasmids were multi-replicon, encoding up to three REPs and 42% of plasmids (82/193) exhibited no known REP. The predicted transferability of plasmids was assessed using Mob and mate-pair formation (MPF) protein typing.[38] In total, 56.5% (109/193) of plasmids were deemed non-mobilizable, 30% (58/193) as mobilizable and 13.5% as conjugative (26/193). Most plasmids (81/84) characterized as transferable contained MOBP relaxase gene family types (Table S6).

Mash distance-based plasmid clustering

Plasmid ($n=193$) relatedness was assessed using a reference-free approach based on Mash distances using a previously recommended Mash distance threshold of 0.06.[39] Plasmids grouped into 16 clusters (C1-C16) and two singletons (average cluster size 12, median: eight, range: 2–39) (Figure 2; Tables S7a-o). The phylogenetic clustering of plasmid sequences was mainly independent from the core genome phylogeny. Plasmid clusters were predominantly restricted to single replicon types, with 3/16 clusters exhibiting combinations of multiple plasmid family types (Tables S7a-o).

A recently described stringent Mash distance threshold of ≤0.0001,[43] equivalent to ≥99.5% average nucleotide identity, was adopted after investigating several thresholds for differentiating plasmids into nested secondary clusters. Near-identical plasmids were observed in genetically divergent VREfm CTs indicative of widespread dissemination (Tables S7a-o). A more extensive evaluation of the plasmidome from the 39 VREfm investigated is provided in Supplementary Results.

Linear plasmids as vehicles of vanA *dissemination*

Among the 39 VREfm investigated for plasmids, 72% (28/39) harboured *vanA* on linear topology plasmids, most (25/28) of which were deemed non-transferable by conventional MOB typing and lacked known mobilization or conjugation genes (Table S6). However, two linear plasmids investigated from this study (see below) were transferable by filter mating.[3] Linear topology plasmids resided within genomic cluster C4 in the Mash-based dendrogram, with a median total pairwise Mash distance of 0.00797 (range: 0–0.02980) (Figure 2; Table S7d). Of the remaining 11 VREfm, three P8 ST80/CT7567-VREfm harboured *vanA* on 40 922 bp non-transferable circular plasmids, while eight harboured *vanA* on plasmids not fully closed by hybrid assembly. PFGE was performed with S1 nuclease treatment on four representative VREfm (JHM1-15 (ST80/CT2933, P1), JHM2-15 (ST80/CT6489, P2), JHM5-6 (ST80/CT1916, P5) and JHM8-1 (ST80/CT2932, P8) harbouring *vanA*-encoding linear plasmids. The electrophoretic mobility of these plasmids during PFGE was comparable with or without S1 nuclease treatment, which nicks supercoiled circular plasmid DNA, thus confirming plasmid linearity (Figure S4). Transfer of *vanA*-encoding linear plasmids from two donor isolates JHM5-6 (ST80/CT1916, P5) and JHM5-13 (ST80/CT3395, P5) to the plasmid-free *E. faecium* 64/3 (ST21) recipient yielded *vanA*-positive transconjugants phenotypically resistant to vancomycin and teicoplanin, which harboured linear plasmids with near-complete homology to the donor plasmids (average nucleotide identity >99.9%, Figure S5).

Most linear plasmids (82%, 23/28) encoded one resistance determinant (*vanA*). Core gene analysis of the 28 linear plasmids (i.e. genes present in ≥95%) using Roary v13.3.046,[44] with a 95% BLASTP sequence identity threshold identified 87 coding sequences, with 77/87 encoding hypothetical proteins. Seven of the genes with a known function were *vanA* operon components. The 28 linear plasmids from multiple VREfm lineages had near-identical backbones (Mash threshold ≤0.0001) (Figure 2; Table S7d).

VREfm diversity and vanA *transposon organization*

The genetic organization of the Tn*1546*-like *vanA* regions in the 39 VREfm investigated for plasmids were resolved by hybrid assembly (Table S6, Figures 3 and S6-S14). Structural analysis of the *vanA* region from multiple VREfm from individual patients (e.g. P1, P4 and P7) revealed identical *vanA* regions (≥99.9% average nucleotide identity) in divergent CTs (Figures S6, S9 and S12). There were also many examples of distinct iterations of the *vanA* transposon in different CTs in individual patients (e.g. P5, P6 and P8; Figures 3 and S10-S11). A more detailed description of clonal diversity and *vanA*-transposon structural organization is provided in Supplementary Results.

VSEfm carriage

Thirty VSEfm from four VREfm-positive patients (P1, P3, P5 and P8) were clade A1 with 25/30 identified as ST80 and 5/30 as

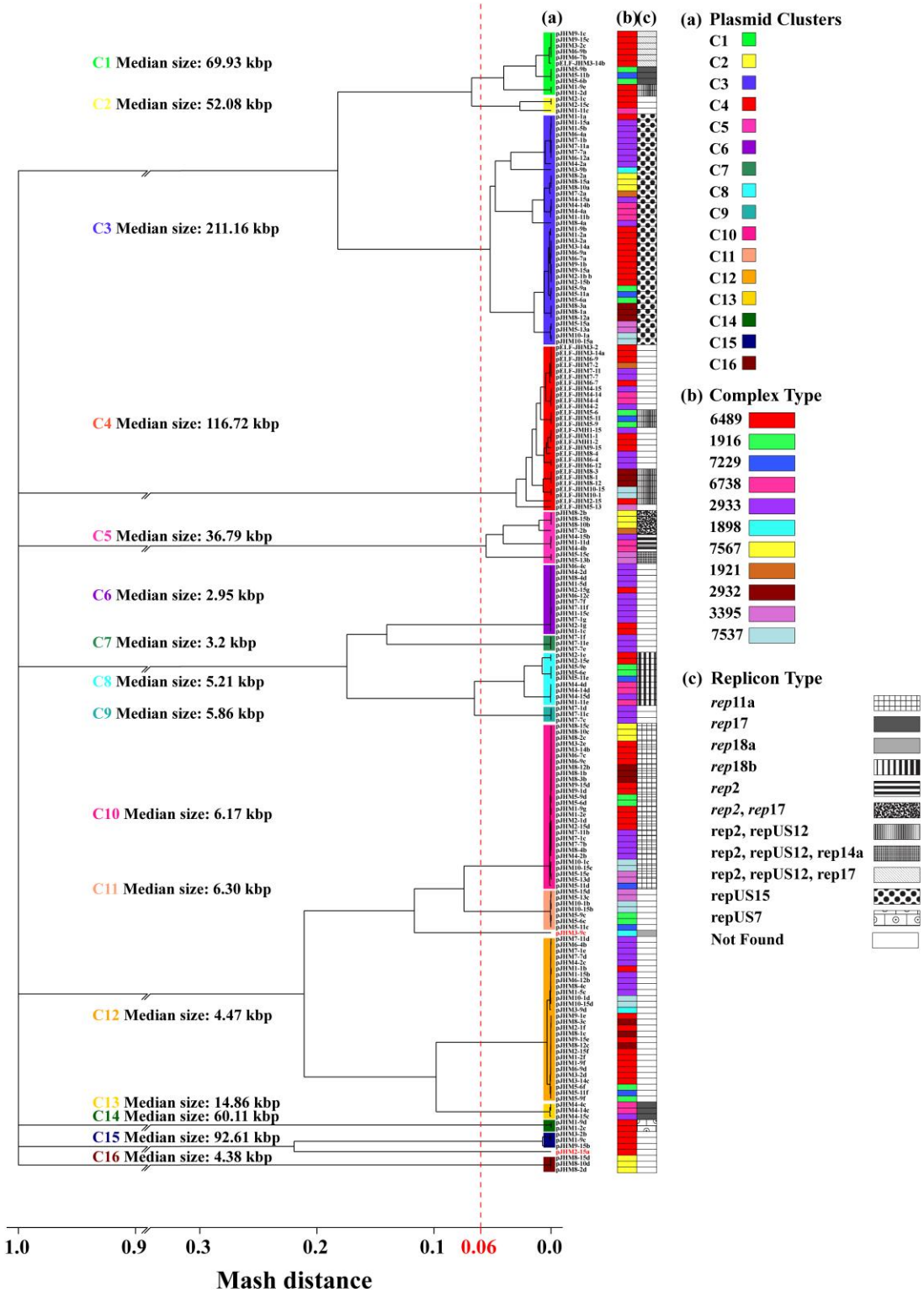

Figure 2. Pairwise Mash distance-based clustering of plasmids ($N = 193$) from 39 representative VREfm investigated from 10 patient rectal screening samples. Plasmids segregated into 16 distinct clusters (C1-C16) and 2 singletons with an average cluster size of 12 (median: 8, range: 2–39) using a Mash distance of 0.06.[39] Metadata pertaining to the plasmid cluster, CT of the isolate harbouring the plasmid and replicon type are indicated in the legends. The median plasmid size of each cluster is indicated on the branches. The two singleton plasmid labels are coloured red.

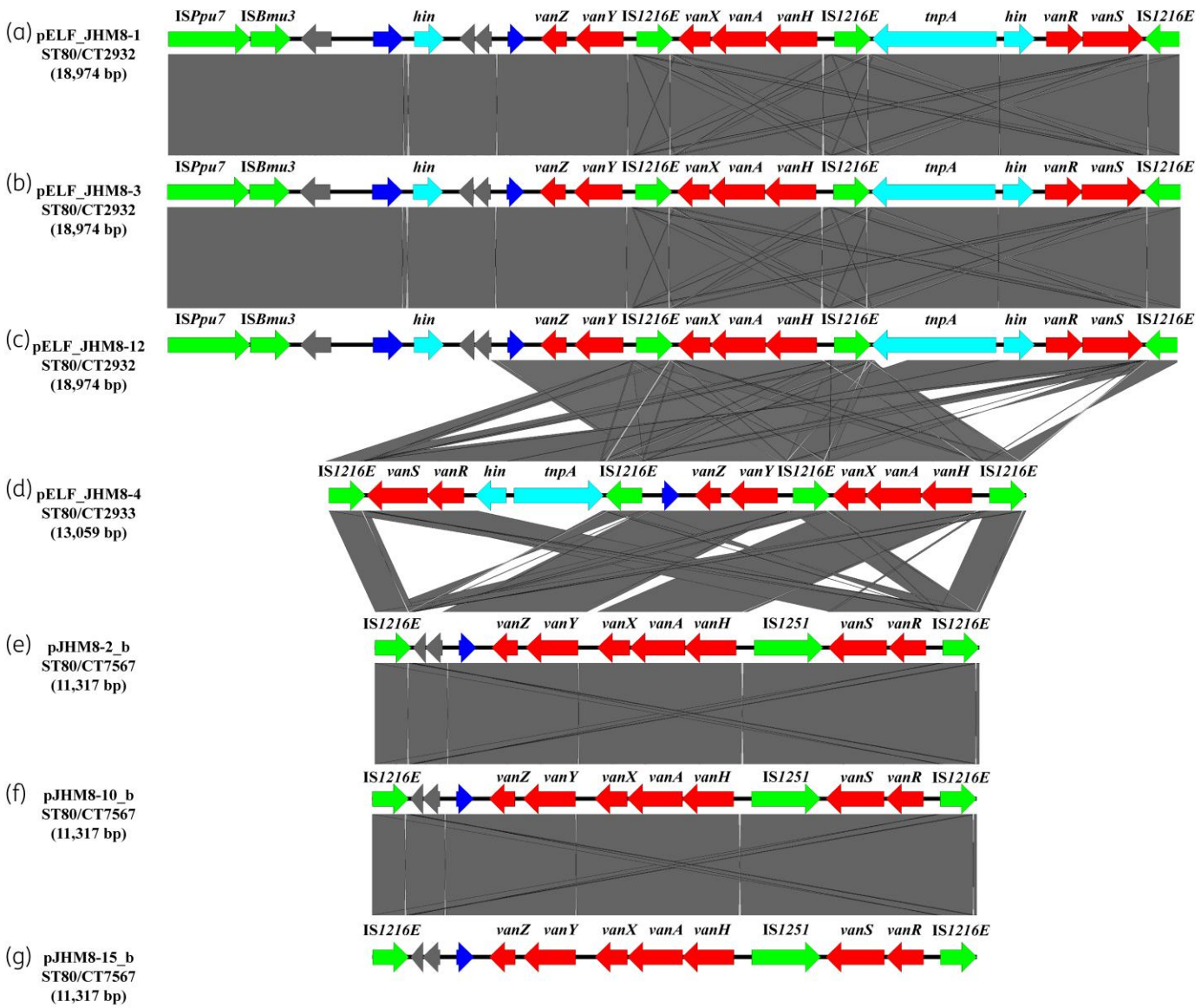

Figure 3. Comparative genetic organization of *vanA* transposon regions closed by hybrid assembly of seven VREfm isolates from patient P8 belonging to three CTs. The figure shows an alignment of the *vanA* region encoded by seven plasmids from separate VREfm isolates as follows: (a) isolate JHM8-1 (ST80/CT2932), *vanA* region 18 974 bp encoded on pELF_JHM8-1 (linear plasmid 146 603 bp), (b) isolate JHM8-3 (ST80/CT2932), *vanA* region 18 974 bp encoded on pELF_JHM8-3 (linear plasmid 146 604 bp), (c) isolate JHM8-2 (ST80/CT7567), *vanA* region 18 974 bp encoded on pELF_JHM8-12 (linear plasmid 194 497 bp), (d) isolate JHM8-4 (ST80/CT2933), *vanA* region 13 059 bp encoded on pELF_JHM8-4 (linear plasmid 167 557 bp), (e) isolate JHM8-2_b (ST80/CT7567), *vanA* region 11 317 bp encoded on pJHM8-2_b (circular plasmid 40 922 bp), (f) isolate JHM8-10 (ST80/CT7567), *vanA* region 11 317 bp encoded on pJHM8-10_b (circular plasmid, 40 922 bp) and (g) isolate JHM8-15 (ST80/CT7576), van region, 11 317 bp encoded on pJHM8-15_b (circular plasmid, 40 922 bp). Images were generated using Easyfig.[45] Coding sequences of interest and their orientation are represented by arrows as follows: red, vancomycin resistance genes; green, insertion sequences; blue, proteins with a known coding function; cyan, transposase and invertase genes; grey, hypothetical proteins. Full details of plasmid profiles and SKA-SNP matrices are provided in Supplemental Tables A3h, A6 and A7. Regions between alignments with shared homology are represented by grey connecting blocks and/or lines with the colour gradient indicating percentage nucleotide identity (82%–100%). A scale bar is included beneath the figure.

ST203 (Table S8). Phylogenetic analysis using 1423 cgMLST loci identified five CTs including CT2932 ($n=7$), CT2950 ($n=5$), CT2940 ($n=12$), CT3395 ($n=1$) and CT2933 ($n=5$) (Table S8). A cgMLST based MST of the 150 VREfm and 30 VSEfm using the ≤20 allelic differences relatedness threshold revealed 11 clusters and two singletons (average cluster size 16, median: 12, range: 3–48) (Figure S17). CT2933 and CT3395 comprised mixtures of VSEfm and VREfm from individual patients (Figure S17 and Table S8). The largest cluster, CT2933, comprised 48 isolates (five VSEfm and 43 VREfm) from five patients (P1 $n=10$, P4 $n=5$, P6 $n=13$, P7 $n=14$ and P8 $n=5$) with a median cgMLST allelic difference of eight (range: 0–11). Patient P8 CT2933-VSEfm and CT2933-VREfm and Patient P5 CT3395-VSEfm and CT3395-VREfm both exhibited a median allelic difference of one, indicating close relatedness (Figure S17). SKA-SNP analysis supported these findings (median pairwise SKA-SNP difference of one, range: 1–2). Hybrid assembly of a representative Patient P8 CT3395-VSEfm (JHMS8-4) identified five plasmids (size range 2947–167 561 bp). Comparison of the plasmidome of JHMS8-4 with a genetically indistinguishable P8 CT3395-VREfm (JHM8-4) also identified five plasmids, including four near-identical plasmids (average nucleotide identity ≥99.99%) (Figures S17-S18). VREfm isolate JHM8-4 harboured an additional *vanA*-encoding linear plasmid (110 419 bp) that was absent in JHMS8-4 (VSEfm), which harboured a 40 337 bp circular plasmid instead. No *vanA* genes were identified in the VSEfm isolate. Screening of the short-read derived FASTA contigs from the remaining four P8 CT2933-VSEfm using ABRicate revealed similar findings. See Supplementary Results for a detailed description of VSEfm carriage.

Discussion

This study provides insights into within-host genetic diversity of clade A1 VREfm in Irish hospital patients. Seventy-percent of patients carried diverse VREfm populations (≥2 CTs), significantly higher than previously reported (27–51% of patients with 2–4 variants).[20,24,25,41,42] Discrepancies between reported rates likely reflect differences in the number of VREfm colonies investigated per patient (5–14).[20,24,25,41,42]

Significant genomic variation was observed between individual patient isolate pairs from different CTs. For example, median pairwise cgMLST allelic and SKA-SNP differences of 427 and 2933, respectively, were recorded between different VREfm CTs within the same patient (Tables S2 and S3), possibly reflecting independent strain acquisitions. Longitudinal studies on genetic changes between VREfm collected sequentially from patients support this conclusion, with within-patient mutation rates of 6–122 SNPs/genome/year reported.[11,20,22,23] Intra-CT variation within individual patients was also observed. For example, 13 patient P6 CT2933-VREFm exhibited a pairwise SKA-SNP difference range of 0–10, highlighting micro-evolution (Table S4d). The occurrence of CT2933 and CT6489 in 5/10 patients in each case likely reflects the institutional dominance of specific CTs widely reported in clade A1 *E. faecium,* where hospital-adapted subpopulations emerge, evolve, and subsequently predominate.[11,19,35] We recently reported the intra- and inter-hospital dominance of three major ST80 CTs including CT2933 in Irish hospitals.[4]

Most plasmid types identified (179/193, 93%) in the 39 VREfm investigated were distributed across diverse CTs (Figure 2). This indicates a reservoir of similar plasmid backbones circulating within Irish ST80-VREfm irrespective of lineage, consistent with previous studies.[4,25,46]

The high prevalence of within-patient VREfm heterogeneity likely drives exchange of near-identical plasmids between diverse lineages within the gut with onward dissemination to other patients. VREfm harboured a median of five plasmids (range: 3–8), in agreement with previous studies, and frequently carried antimicrobial-resistance genes (39%, 73/193, (Table S6).[46] Interestingly, most plasmid-encoded resistance genes were co-localized on a single plasmid type. A recent investigation of within-host diversity of *Pseudomonas aeruginosa* suggested that mixed strain populations may significantly accelerate the evolution of within-host antibiotic resistance.[47]

The significantly higher prevalence of transferable linear plasmids in VREfm than previously reported (72%, 28/39 isolates)[48–50] suggests *vanA* acquisition is an important driver of linear plasmid dissemination/evolution in Irish VREfm. These results support the high-prevalence of transferable *vanA*-encoding linear plasmids identified in VREfm from several Irish hospitals previously reported.[3] Linear plasmids were enriched with IS*1216E* elements, potentially contributing to translocation of the *vanA* transposon to these plasmids.[3] IS*1216E*-mediated instability was previously shown to be a contributory factor in the restructuring and mobility of Tn*1546*-like *vanA* in Irish VREfm and in vancomycin-resistant enterococci from other countries.[3,51,52] The presence of distinct *vanA*-transposon iterations on both circular and linear plasmids in different VREfm CTs suggests multiple, separate *vanA* acquisition events by VSEfm (Figures 3 and S10 and S11). These findings indicate extensive recombination within VREfm *vanA* transposon regions, likely mediated by IS*1216E*, as reported previously.[53]

Most (25/28) linear plasmids were deemed non-transferable by MOB-suite tools due to the absence of known relaxase and mate-pair formation type genes. However, we demonstrated the conjugative transfer of VREfm linear plasmids under selective pressure here and previously.[3] Current databases do not list all mobilization genes and the high proportion of 'hypothetical proteins' encoded by linear plasmids may include undescribed genes involved in plasmid transfer.

The implications of VREfm multi-strain carriage for surveillance are significant as conventional surveillance approaches rely on single strain sequencing strategies. While this study lacks epidemiological context due to the unavailability of patient movement data, examples of potential acquisition/transmission of low-frequency strains were evident. For example, P3 and P6 harboured closely related CT6489-VREfm (≤6 SKA-SNPs) recovered within 101 days on ward W3 (Table S4e). In P6, CT6489-VREfm represented a minor frequency strain that could be overlooked using conventional surveillance (Table S2). Evaluation of study isolates with a comparator dataset of clade A *E. faecium* from H1 recovered over 5 years[3,4] revealed that divergent lineages were circulating within H1 study wards and some low-frequency strains identified here persisted for protracted periods (>40 months) within and between H1 wards (Tables S1, S2 and S5a-i).

Routine sequencing of multiple VREfm per sample for surveillance would entail significant costs, more complex bioinformatics and increased turnaround-time. Identifying an optimal number of colonies for sequencing is crucial to accurately

reflect within-patient diversity, while remaining justifiable financially. For example, McHugh *et al*. demonstrated that analysing <14 VREfm colonies per sample identified fewer transmission links and lower phylogenetic network clustering scores.[25] However, the authors concluded that single-colony picks are suitable in many settings, such as in Scotland, where 73% of cases showing limited within-patient diversity.[25] However, because of the extensive within-patient VREfm diversity identified in Ireland (70%), investigating multiple colonies would significantly improve the resolving power of WGS in surveillance.

Multiple colony sequencing may be challenging for hospital surveillance. A potential alternative could be a 'one plus pool' approach, which involves sequencing a single colony per specimen to represent the 'dominant' variant in conjunction with pooling and sequencing all remaining colonies to identify within-patient diversity.[54] Alternatively, a strain-specific 'plate sweep' metagenomics approach could be used, where a mixture of strains are harvested from enrichment cultures, sequenced and bioinformatic pipelines are employed to detect individual strains.[55–57]

All 30 VSEfm investigated from four VREfm-positive patients were clade A1. Highly similar plasmidomes were evident within VREfm and VSEfm, which is unsurprising since VREfm and VSEfm were predominantly ST80. However, there were instances of genetically indistinguishable VSEfm and VREfm from the same patient with plasmidome differences. For example, P8 yielded six CT2933 isolates (five VSEfm and one VREfm), which exhibited a median SKA-SNP difference of one. Comparison of the accessory genomes of a representative CT2933-VSEfm and CT2933-VREfm revealed near-identical plasmidomes (four plasmids in both isolates sharing ≥99.99% nucleotide identity) (Figure S18). A notable difference was the absence of a linear plasmid in the CT2933-VSEfm. It is likely that new VREfm arise in hospital patients colonized with VREfm and VSEfm by *in vivo* plasmid transfer. Patients would only have to acquire a VREfm strain once and then subsequently exhibit within-patient diversity. However, it is likely that separate VREfm acquisition events and plasmid acquisition by VSEfm contribute to within-patient diversity.

Conclusions

VREfm within Irish hospital patients exhibit significant intra-host diversity with 70% of patients harbouring multiple strains. The presence of near-identical plasmids in Irish VREfm/VSEfm facilitates HGT across diverse lineages, with linear plasmids constituting a significant vehicle for *vanA* dissemination.

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Transparency declarations

None of the authors have any conflicts of interest to declare.

Supplementary data

Figures S1–S18 and Tables S1–S8 are available as Supplementary data at *JAC-AMR* Online.

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