

Protracted transmission and persistence of ST80 vancomycin-resistant *Enterococcus faecium* clonal complex types CT2933, CT2932 and CT1916 in a large Irish hospital: a 39-month WGS study

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Running title: Transmission and persistence of ST80 VREfm

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Abbreviations: cgMLST, core-genome multilocus sequence typing; cgSNP, core genome single-nucleotide polymorphism; CT, complex type; H1, hospital 1 IPC, infection-prevention and control; MALDI-TOF-MS, Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass-Spectrometry; MLT, maximum likelihood tree; MLST, multi-locus sequence type; MST, minimum spanning tree; NJT, neighbour joining tree; PTC, potential transmission chain; ST, sequence type; SKA, split k-mer analysis (SKA); WGS, whole-genome sequencing; RIGs, related isolate groups, VREfm, vancomycin-resistant *E. faecium*; VSEfm, vancomycin-susceptible *E. faecium*.

ABSTRACT

Background: Vancomycin-resistant *Enterococcus faecium* (VREfm) are significant nosocomial pathogens. Sequence type (ST)80 *vanA*-encoding VREfm predominate in Irish hospitals, but their transmission is poorly understood.

Aims: To investigate transmission and persistence of predominant complex type (CT) VREfm in two wards of an Irish hospital (H1) using whole-genome sequencing, and their intra- and inter-hospital dissemination.

Methods: Rectal screening ($N=330$, September 2019-December 2022) and environmental ($N=48$, November 2022-December 2022) *E. faecium* were investigated. Isolate relatedness was assessed by core-genome multilocus sequence typing (cgMLST) and core-genome single nucleotide polymorphism (cgSNP) analysis. Likely transmission chains were identified using SeqTrack (<https://graphsnp.fordelab.com/graphsnp>) using cgSNP data and recovery location. Well-characterised *E. faecium* ($N=908$) from seven Irish hospitals including H1 (June 2017-July 2022) were also investigated.

Findings: Conventional MLST assigned isolates to nine STs (ST80, 82%). cgMLST identified three predominant ST80 CTs (CT2933, CT2932 and CT1916) (55% of isolates) of related isolates (≤ 20 allelic differences). cgSNP analysis differentiated these CTs into multiple distinct closely related genomic clusters (≤ 10 cgSNPs). Parisimonious network construction identified 55 likely inter- and intra-ward transmissions with epidemiological support between patients ≤ 30 days involving 73 isolates (≤ 10 cgSNPs) from seven genomic clusters. Numerous other likely transmissions over longer time periods without evident epidemiological links were identified, suggesting persistence and unidentified reservoirs contribute to dissemination. The three CTs predominated among *E. faecium* ($N=1,286$) in seven hospitals, highlighting inter-hospital spread without known epidemiological links.

Conclusion: This study revealed the long-term intra- and inter-hospital dominance of three major CT ST80 VREfm lineages, widespread transmission and persistence, implicating unidentified reservoirs.

Keywords: Vancomycin-resistant *Enterococcus faecium*, VREfm, whole-genome sequencing, cgMLST, cgSNP analysis, hospital transmission, environmental persistence.

Introduction

Enterococcus faecium has emerged as a significant nosocomial pathogen globally, associated with a wide range of infections and persistent environmental contamination[1]. Extensive studies have highlighted high-level intrinsic and acquired resistance amongst *E. faecium* hospital-adapted clones, limiting the effectiveness of important therapeutic options including vancomycin and linezolid[1-7]. Multidrug resistant vancomycin-resistant *E. faecium* (VREfm) constitute a substantial disease burden, particularly in immunocompromised or multimorbid patients. At-risk patient groups including critical care and haematology-oncology are disproportionately vulnerable to invasive infection, often following gastrointestinal colonisation, with high associated mortality and lengthy hospitalisation[1,5]. The World Health Organisation listed VREfm as a high-priority pathogen for which new antimicrobial therapies are urgently required[8].

The true prevalence of VREfm amongst individuals with frequent healthcare exposure in Ireland is unknown, with rates of 3-40% reported[9-13]. The Republic of Ireland has reported one of the highest rates of invasive VREfm infection in Europe for many years, ranging from 28.4%-45.8% (2006-2022)[14]. Widespread dissemination of VREfm in hospitals is partly attributable to environmental persistence despite rigorous infection-prevention and control (IPC) cleaning regimens, which have successfully curtailed dissemination of other significant nosocomial pathogens[15-17]. Additionally, current *E. faecium* screening practices in Irish hospitals are restricted to high-risk areas and it is likely that colonised asymptomatic patients constitute an important reservoir for transmission via environmental shedding[18]. Previous studies highlighted that VREfm environmental contamination, particularly in communal ward areas, may reach rates up to 50% in hyperendemic settings like Ireland, which can facilitate spread of epidemic clones by direct/ indirect patient transmission[19].

Recently, our laboratory described the polyclonal population structure and predominance of *vanA*-encoding ST80 VREfm in Irish hospitals using whole-genome sequencing (WGS)[20]. The present study aimed to undertake an in-depth retrospective investigation of the transmission and persistence of VREfm clonal lineages in two wards of a large Irish hospital using a genomic-based approach over 39-months. Patient screening and environmental *E. faecium* were investigated to identify predominant genomic clusters involved in transmission and persistence in the context of a protracted, silent outbreak. Predominant clonal lineages among isolates from six other Irish hospitals recovered over a five-year time period were also investigated.

Methods

Patient isolates

VREfm rectal screening isolates ($N=330$) from individual patients in two wards of a large acute Dublin hospital (H1) between September 2019-December 2022 were retrospectively investigated (Supplemental Table A1). Ward A (WA) is a 15-bed cardiothoracic unit, comprising three 4-bed bays with a shared bathroom and 3 single ensuite rooms. Ward B (WB) is a 35-bed oncology unit, comprising six 4-bed bays with a shared bathroom, a 2-bed bay and 9 single ensuite rooms. Infection prevention and control procedures, including hand hygiene and ward cleaning regimens were undertaken according to national guidelines (Supplemental Methods)[21,22,23].

Rectal screening swabs in WA and WB were taken routinely on admission with weekly universal screening thereafter as per national guidelines[18]. A total of 330/2885 screens from separate patients yielded VREfm following culture on chromID VRE chromogenic agar (bioMérieux, Marcy, L'Étoile, France). VREfm-positive isolates were stored at -80°C in Microbank cryovials (Pro-Lab Diagnostics, Toronto, Canada). Only the first positive VREfm rectal isolate per individual patient was included in the study. No VREfm outbreaks were identified on the wards during the isolate collection period. A well-characterised in-house collection of *E. faecium* isolates (VREfm $N=859$, VSEfm $N=49$) from seven Irish hospitals (June 2017-July 2022) were included as comparators, 648 of which were described previously[20] (Supplemental Table A1).

Environmental isolates

Forty-eight environmental *E. faecium* were recovered in H1 between November 2022 and December 2022 (Supplemental Table A1). Due to COVID-19 restrictions, environmental sampling was rudimentary and limited to two 'snap-shot' time-points at the end of the study period. Three single patient-occupied ensuite rooms (WA $N=1$, WB $N=2$) were chosen for surface-sampling of high-touch environmental sites ($N=30$, 10 sites per room) using *Enterococcus faecium* ChromoSelect agar (Sigma-Aldrich [Merck], Ireland) contact plates (25 cm^2 area). Areas sampled include bedside tables, bedframes, call bells, cardiac monitors, sats probes, toilet seats and shower drains. All suspected *E. faecium* colonies recovered on contact plates were picked for further analysis. A total of 13 ward handwash basin drain outlets (WA $N=10$, WB $N=3$) were sampled using nylon-flocked eSwabs (Copan Diagnostics, California, USA). On WA, the handwash basins on three 4-bed bays, the shared bathroom facilities, two single rooms and accompanying ensuites were sampled. On WB, the handwash basin in one

single room, accompanying ensuite and one shared patient bathroom were investigated. Detailed methodologies and metadata pertaining to environmental sampling are provided in Supplemental Methods.

Phenotypic and genotypic susceptibility testing

Linezolid, vancomycin and teicoplanin susceptibility was determined using the VITEK®2 system (bioMérieux, Marcy-l'Étoile, France) and EUCAST (v14.0) clinical breakpoints[24]. Antimicrobial-resistance and virulence-associated genes were detected *in silico* on short-read WGS assemblies using ABRicate v.0.9.8 (<https://github.com/tseemann/abricate>).

Whole-genome sequencing

Genomic DNA libraries were prepared for short-read WGS as described previously[20]. Libraries were scaled to exhibit $\geq 35\times$ coverage and underwent sequencing using the Illumina 600-cycle MiSeq paired-end Reagent Kit v3 and sequencer (Illumina, Eindhoven, The Netherlands). For long-read sequencing, genomic DNA was extracted using the Wizard HMW DNA extraction kit (Promega Corporation, Wisconsin, USA). Library preparation was performed using the Native Barcoding Kit 24 v14 (SQK-NBD114.24) and sequencing was performed on the MinION platform using a R10.4.1 Flow Cell with MinKNOW software v23.04.6 (Oxford Nanopore Technologies, United Kingdom).

Sequence analysis

Short-read sequences were quality assessed and trimmed using FastQC v0.11.7 and Trimmomatic v0.36 incorporated within Ridom SeqSphere+ v8.5.1 (Ridom GmbH, Münster, Germany). Sequence assembly was carried out under default settings using VELVET v1.1.04 (SeqSphere+ v8.5.1). Long-read sequencing files were base-called, trimmed and demultiplexed using Dorado v0.5.3 (Oxford Nanopore).

Hybrid assembly of paired-end short-read and long-read sequences was performed using Unicycler v0.5.0 (<https://github.com/rrwick/Unicycler>)[25]. Assembly quality was assessed using the graph connectivity and node size metrics in Bandage v0.8.1 (<https://github.com/rrwick/Bandage>)[26]. Assembled genomes were annotated using Prokka v1.14.5 (<https://github.com/tseemann/prokka>) and visualised using SnapGene v.7.0.4 (<https://www.snapgene.com>)[27]. VREfm and VSEfm sequence reads have been deposited in GenBank under BioProjects PRJNA734127 and PRJNA1074551.

Phylogenetic analysis

Phylogenetic neighbour-joining trees (NJTs) were constructed using gene-by-gene allele calling based on the 1,423 *E. faecium* core-genome multilocus-sequence typing (cgMLST) scheme in SeqSphere+[28]. Traditional MLST sequence types (STs) were extracted *in silico* (<https://pubmlst.org/organisms/enterococcus-faecium>).

Variant annotation and sequence alignment based on core genome single-nucleotide polymorphisms (cgSNPs) was performed by mapping to study-specific reference genomes using Snippy v4.6.0 (<https://github.com/tseemann/snippy>), removing recombinant SNPs using Gubbins v3.2.1 (<https://github.com/nickjcroucher/gubbins>)[29] and generating cgSNPs-based pairwise distance matrices using snp-dists v3 (<https://github.com/tseemann/snp-dists>). cgSNP maximum likelihood trees (MLTs) were constructed using IQ-TREE v2.2.2.6 (<http://www.iqtree.org>) with the extended ModelFinder and 1000 ultrafast bootstrap replicates [30]. Trees were visualised using Interactive Tree of Life v6.8.1 (<https://itol.embl.de/>).

Parsimonious transmission trees

Parsimonious transmission trees were constructed using cgSNP distance matrices and isolate metadata using the SeqTrack algorithm[31] and visualised using GraphSNP (<https://graphsnp.fordelab.com/graphsnp>) [32].

Ethics declaration

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

Results

Isolate description

In total, 378 *E. faecium* isolates recovered over 39 months from wards WA and WB of hospital H1 were investigated, including 330 patient rectal screening isolates (WA, 109 (33%); WB, 221 (67%)) (Supplementary Table A1). A total of 330/2885 individual patients screened (11.43%) yielded VREfm, 109/1232 (8.85%) from Ward A and 221/1653(13.37%) from Ward B. Twenty-eight environmental isolates were recovered from 3/13 handwash basin drains (19 from two WA drains and nine from one WB drain). Twenty environmental isolates were recovered from surface sampling of near-patient high-touch ward sites (Supplementary Table A1). All patient isolates were *vanA*-encoding VREfm. The majority (43/48, 89%) of

environmental isolates were vancomycin-susceptible *E. faecium* (VSEfm) and five were *vanA*-encoding VREfm. All isolates were linezolid susceptible.

Major cgMLST clusters of E. faecium

Isolates were assigned to nine STs by conventional MLST, with ST80 predominating (310/378, 82%), followed by ST203 ($N=32$), ST612 ($N=14$), ST18 ($N=10$), ST17 ($N=3$), ST117 ($N=3$), ST787 ($N=3$), ST789 ($N=1$), ST1395 ($N=1$) and one novel isolate submitted to pubMLST.org (Figure 1). cgMLST grouped isolates into 27 related isolate clusters using the previously recommended threshold of ≤ 20 allelic differences[28] with an average cluster size of 13 (median: seven; range: 2-96) and 21 singletons (Figure 1). Three major ST80 complex types (CTs) were evident in an cgMLST-based NJT (CT2933, CT2932 and CT1916), comprising 55% (208/378) of isolates (Figure 1).

CT2933 (termed CI), contained 96 isolates (62 screening, 34 environmental) recovered over 39 months with an average allelic difference of eight (median: seven; range: 0-21). In total, 54% (52/96) of CI isolates were from WB, with the remainder from WA (Supplementary Table A2). Six CI environmental isolates recovered from areas including toilet seats, cardiac monitors and shower drains were VSEfm and differed from their closest neighbouring screening VREfm by an average of two allelic differences (range: 2-12). Twenty-eight additional CI environmental VSEfm from three washbasin drains on WA and WB differed from their closest neighbouring screening VREfm by an average of eight allelic differences (range: 6-21). The majority of isolates in CT2932 (termed CII) ($N=58$) were recovered in WB (37/58; 64%) over 39 months with an average allelic difference of six (median: six, range 0-18). CII-specific environmental isolates ($N=six$) were predominantly VSEfm (4/6) and differed by an average of five allelic differences to their closest neighbouring screening VREfm (range: 1-17). CT1916 (termed CIII) comprised 52 screening VREfm recovered over 38 months with an average allelic difference of 10 (median: 11, range: 0-27). These findings indicated that isolates within CI-CIII, respectively, were closely related, suggesting potential widespread transmission.

cgSNP analysis identifies closely-related E. faecium subclusters

Diversity within CI-CIII was further investigated by cgSNP analysis. Isolates were categorised into related isolate groups (RIGs) based on a median pairwise cgSNP difference of ≤ 15 to identify CT-specific subclusters. This threshold was based on reported annual *E. faecium* diversification estimates of 6-122 SNPs[33-36] and the 39-month study duration. For clarity, only RIGs with $\geq$ four isolates are described.

CI (CT2933) isolates exhibited a pairwise median SNP distance of 25 (average 26, range: 0-84) (Supplementary Table S2). Two major subclusters were evident in an cgSNP MLT based on 366 SNPs using the CI-specific reference JH005 (earliest CI isolate), termed subcluster CIA (35 isolates (28 environmental and seven screening), median SNP difference: 26, range: 0-53) and subcluster CIB (61 isolates (six environmental and 55 screening), median SNP difference: 14, range: 0-84) (Figure 2). CIA segregated into three RIGs; CIA1 (nine environmental VSEfm, median SNP difference: 0), CIA2 (19 environmental VSEfm, median SNP difference: 0) and CIA3 (four screening VREfm, median SNP difference: three) (Supplemental Tables A3a-c). Subcluster CIB consisted of one major RIG, CIB1 (40 screening VREfm and six environmental VSEfm, median SNP difference: 11) and 15 intra-RIG outlier isolates (median pairwise SNP difference between isolates >15) (Figure 2, Supplemental Table A3d).

CII (CT2932) isolates exhibited a pairwise median SNP distance of 17 (average: 21, range: 0-56) based on 220 SNPs. Two major subclusters were evident in a cgSNP-based MLT using the CII-specific JH004 reference (earliest CII isolate), termed sub-clusters CIIA and CIIB (Supplemental Figure A1). Subcluster CIIA isolates ($N=13$) (seven screening VREfm, four environmental VREfm and two environmental VSEfm) exhibited a median SNP distance of 15. Subcluster CIIB isolates (45 screening VREfm) exhibited a median SNP distance of nine (Supplemental Figure A1). Subcluster CIIA comprised two major RIGs; CIIA1 (four environmental VSEfm, median SNP distance: one) and CIIA2 (seven screening VREfm and two environmental VREfm, median SNP distance: nine) (Supplemental Table A3e-f). Subcluster CIIB comprised one major RIG (CIIB1) (39 screening VREfm, median SNP distance: seven) (Supplemental Table A3g).

CIII (CT1916) isolates ($N=52$) exhibited a pairwise median SNP distance of 30 (range 0-68), using the CIII-specific JH026 reference (earliest CIII isolate) based on 341 SNPs. CIII isolates were more clonal, with isolate JH424 forming an out-group (CIIA) and the rest forming one major subcluster (CIIB) (median SNP distance: 28) (Supplemental Figure A2). CIIB comprised three RIGs; CIIB1 (four screening VREfm, median SNP difference: 3.5), CIIB2 (four screening VREfm, median SNP difference: 11) and CIIB3 (27 screening VREfm, median SNP difference: eight) (Supplemental Tables A3h-j). CIIB3 comprised over 52% (27/52) of CIII isolates and contained six intra-RIG outliers (median SNP difference >15).

VSEfm harbouring vanA remnants

In silico analysis of VSEfm identified four subcluster CIIA1 environmental isolates harbouring *van* operon remnants. These isolates harboured the *vanZ* and *vanY* genes, but lacked

the *vanHAX* and *vanRS* genes. One isolate (JHE024) was fully closed by hybrid assembly, which identified the *vanZ* and *vanY* genes on a 88,110 bp circular plasmid. The organization of the *van* remnant and adjacent sequences were similar to *vanA* operon regions on closed plasmids in three Irish clinical VREfm isolates representing the three main CT groups (CT2933, CT2932 and CT1916) (Supplemental Figure A3).

VRE transmission and persistence

To investigate isolate transmission, a more stringent cgSNP threshold of ≤ 10 SNPs was adopted to define closely related isolates based on previous surveillance studies, mutational rates, and intra-patient isolate diversity[19,36-38]. Isolates attributed to the same cgSNP subclusters and RIGs were investigated according to their likely epidemiological linkage based on recovery date and ward. The mean patient length of stay during the study was 11.3 days for WA and 12.3 days for WB. During the study period, the mean glycopeptide use per quarter in defined daily doses (DDDs)/100 bed days was 7.35 for Ward A and 5.38 for Ward B. Isolates recovered on the wards within ≤ 30 days were deemed to be epidemiologically linked, whereas isolates recovered >30 days were deemed to have no evident epidemiological linkage.

CI ($N=96$) comprised 4,608 potential isolate pairs with 667 exhibiting ≤ 10 cgSNPs. To gain insights into the directionality of potential transmissions, a parsimonious transmission tree based on the SeqTrack algorithm was constructed (Figure 3), which determines the most plausible genealogy for isolate sets based on recovery date and ward location[31,32]. In total, 46.8% (29/62) of CI patient isolates were likely involved in 23 transmissions with epidemiological support (blue branches in Figure 3), while CI-specific environmental isolates ($N=34$) could not be linked to any patient isolate within the 30-day timeframe.

CI RIGs CIA1 and CIA2 (Figure 2, Supplemental Table A3a-b) contained 28 VSEfm from three washbasin drains and differed from their closest neighbouring patient isolate by 24 cgSNPs (range: 24-79). This indicates that the sampled handwash basin drain outlets were not involved in likely transmission events (Figure 3). Additionally, the close relatedness of isolates within CIA1 and CIA2 reflect multiple colony picks from individual drain outlets ($N=3$) and represents within-drain diversity. CI RIG CIA3 contained four WB patient VREfm with a median pairwise SNP difference of three (range: 0-5). The earliest isolate, JHW125, was recovered 112 days before the other three with no evident epidemiological linkage. However, these three isolates were recovered ≤ 7 days suggesting transmission (Figure 3, Supplemental Table A3c).

Subcluster CIB was more complex and comprised 61 isolates from 19 WA patients, four WA environmental sources, 36 WB patients and two WB environmental sources over 37

months (average pairwise cgSNP difference of 14, range 0-84). Of the 1,861 potential CIB isolate pairs, 454 exhibited pairwise cgSNPs of ≤ 10 (Figure 2, Supplemental Table A3d). Three presumptive index VREfm were identified (JH031, JH099 and JH0131; marked with an $\times$ in Figure 3) with epidemiological links recovered from one WA patient and two WB patients over 20 days (median cgSNP difference: 4, range: 0-10), which were likely involved in three potential transmission chains (PTCs) (Figure 3).

PTC1-PTC3 comprised 22, 21 and 16 isolates, respectively. A significant proportion of likely transmissions with epidemiological support across both wards within all three PTCs could be attributed to the index isolates (i.e., 11/24 (46%) patient isolates connected to index isolates by immediate network branches). However, intermediary isolates (e.g., PTC1 isolates JHW157, JHW159 and JHW209; median SNP difference: 0) also provide evidence of spread between patients on the same wards on coinciding dates.

Likely indirect transmission was evident amongst patients occupying the same ward months apart (e.g., PTC1 WB patient isolates JHW242 and JHW61, SNP difference: 0), which is indicative of a long-term ward-specific outbreak. Additionally, the results indicated dissemination from a potential common source(s), with closely related isolates recovered on different wards with no evident epidemiological link (e.g., PTC1 patient isolates JHW9 (WB) and JHW20 (WA), SNP difference: one, time between isolates: 40 days) (Figure 3). This is further emphasised by the CI-specific environmental isolates, with four PTC3 environmental VSEfm (JHE017, JHE018, JHE026 and JHE027) recovered from a cardiac monitor and shower drain outlets on WA 29 months after the PTC3 JH131 index isolate with a median pairwise cgSNP difference of six suggesting environmental persistence (Figure 3).

The next two largest CT isolate groups, CII ($N=58$) and CIII ($N=52$) comprised a total of 1,682 and 1,352 potential isolate pairs, respectively, with 1,318 and 662 exhibiting ≤ 10 cgSNPs. Similar findings of likely patient-to-patient transmission with apparent epidemiological links and indirect transfer from an unsampled carrier and/or communal source were observed with CII and CIII isolates (Supplemental Figures A4 and A5), with 15 and 17 transmissions with epidemiological support evident, respectively.

Inter-hospital VRE transmission

We previously investigated the population structure of *E. faecium* ($N=648$) from five Irish hospitals including H1, with ST80 predominance and most isolates assigned to CT1916, CT2933 and CT1598[20]. To investigate the impact of additional isolates on the predominant CTs on a national level, all previously described isolates, an additional 260 well-characterised

isolates from seven Irish hospitals (June 2017-July 2022), and WA and WB isolates were investigated by cgMLST (Supplemental Figure A6) ($N=1,286$).

Conventional MLST assigned isolates to 32 STs with ST80 predominating (944/1286, 73%). Isolates segregated into 71 cgMLST clusters with an average size of 17 (median: 4; range: 2-215) and 67 singletons. The three major CT clusters (CI-CIII) identified in WA and WB (Figure 1) accounted for over 34% (440/1,286) of the larger collection consisting of 215, 88 and 137 isolates, respectively.

An additional 119 ST80 CT2933 (CI) isolates were identified in the national collection from multiple H1 wards and five other Irish hospitals (H2-H6) in distinct geographical locations between October 2018-December 2022. An MLT based on cgSNP analysis of 215 CI isolates (WA and WB $N=96$, national $N=119$) based on 731 cgSNPs identified 4,698 closely related isolate pairs (≤ 10 SNPs) from four Dublin (H1-H4) and two regional hospitals (H5 and H6) (Supplemental Figure A7, Supplemental Table A3k-p). In the larger isolate collection, RIG CIB1 comprised 114 isolates from six hospitals (H1 $N=87$, H2 $N=6$, H3 $N=1$, H4 $N=16$, H5 $N=1$ and H6 $N=3$) recovered over 45 months with a median pairwise cgSNP difference of nine (range: 0-23) (Supplemental Table A3p). Within CIB1, many examples of closely related isolates (≤ 10 SNPs) recovered in geographically distant hospitals within ≤ 7 days were evident (e.g., isolates JH239 from H1 and RC-19-037 from H6, SNP difference: nine, recovered on the same day, >100 km distance; isolates BS307 from H4 and RC-19-044 from H5, SNP difference: 5, recovered within 2 days, >100 km distance) (Supplemental Figure A7, Supplemental Table A3p). Additionally, closely related VREfm were identified from 39 H1 wards providing further evidence for widespread dissemination of highly related isolate groups that may reflect potential communal or environmental sources with no evident epidemiological links. For instance, RIG CIA5 comprised eight screening VREfm from seven H1 wards and one ward within H4 over a 16-month period with a median cgSNP difference of 10.5 (range: 0-22) (Supplemental Table A3o). Similar findings were evident within CII and CIII (Supplemental Figure A8 and A9, Supplemental Table A3q-y).

Discussion

A WGS-based approach was used to gain insights into transmission and persistence of VREfm in two hospital wards over 39 months. Twenty-seven *E. faecium* CT clusters were identified, with three predominant ST80 lineages (CT2933, CT2932 and CT1916) comprising 55% of isolates (Figure 1). These three CTs appeared highly clonal by cgMLST and were considered closely related (≤ 20 allelic differences)[28], however cgSNP analysis revealed greater diversity and discriminated isolates into more distinct genomic clusters. This supports

recent studies that emphasised the higher discriminatory value of pairwise genome comparison approaches including cgSNP and split k-mer analysis (SKA), during outbreak scenarios in hyperendemic regions[38,39]. These findings indicate multiple hospital introductions of VREfm and subsequent genomic specialisation resulting in the establishment of major endemic groups. This phenomenon has been extensively reported in clade A1 *E. faecium*, where multidrug resistant hospital-adapted subpopulations emerge, evolve, and eventually predominate[40,41].

Probable transmissions were defined as follows: (i) likely direct/indirect patient transmission with epidemiological support (i.e., involving patients present in wards within ≤ 30 days) and (ii) direct/indirect transmission with no epidemiological support (i.e., involving patients present in wards > 30 days). SeqTrack algorithm-generated parsimonious trees indicated a total of 55 potential transmissions with epidemiological support involving the three CTs. Importantly, inferred transmissions based on SeqTrack parsimonious trees do not consider patient movement data, spatial location, and other key factors, which were not available for this study, that may complicate transmission inference[31,32]. Nonetheless, our findings provide convincing evidence for extensive transmissions, even with the implementation of routine IPC interventions. Additionally, our findings strongly suggest that multiple overlapping outbreaks caused by genomically distinct isolates within the same CT occurred both within and across multiple H1 wards.

The complexity and duration of the transmission networks identified suggest the influence of multiple factors with the potential involvement of unidentified reservoirs contributing to dissemination. The highly dynamic pattern of spread of VREfm between patients and the hospital environment and environmental persistence is well documented[2,42,43]. A recent UK study reported that 48% of environmental swabs from two haematology wards over six months yielded VREfm and 20% of bedroom/bathroom areas remained positive following cleaning after patient discharge[19]. We identified four genetically indistinguishable environmental isolates (median cgSNP difference: 0) from cardiac monitors and shower drains on WA 29-months after the recovery of patient screening isolate JH131, which differed by a median of six cgSNPs (Figure 3). The ability of *E. faecium* to persist, resist decontamination and the repeated reintroduction of endemic clones from patients with frequent healthcare exposure likely contributes to ongoing transmissions. The selection of ≤ 30 days as the cut-off for epidemiological support was conservative as VREfm can persist for considerable periods. The use of a longer threshold (e.g., ≤ 60 days) would have identified a significantly higher number of potential transmissions.

Most environmental isolates (89%, 43/48) recovered here were VSEfm, yet four harboured a *van* remnant (*vanY* and *vanZ*), suggesting they arose from VREfm. The ability of *E. faecium* to readily acquire resistance elements via horizontal transfer is well documented[1,44,45] and the loss of resistance genes is to be expected. The high prevalence of *IS1216E* in Irish VREfm in the absence of a functional *vanA* transposase gene may facilitate the gain/loss of segments of, or the entirety of the *vanA* transposon in an environmental setting[20]. These findings indicate that VSEfm should also be included in surveillance.

Analysis of *E. faecium* from seven Irish hospitals over five years support the circulation of prevalent ST80 lineages, with a predominance of the three major CTs identified in H1 reflected nation-wide in the absence of known communal sources or epidemiological links. Even if transmission seems epidemiologically unlikely, genomic results should not be immediately disregarded. Long-term cross-institutional CT-specific dominance with limited genetic diversity (<10 SNPs in cases) has been reported in other European studies[36,37,38,46]. Patient and staff movement across hospital networks may contribute to this. Alternatively, it may reflect the carriage of common VSEfm lineages by patients that acquire resistance elements following healthcare exposure. There is also potential for unidentified community sources. Unfortunately, data regarding community prevalence of VREfm in Ireland is limited with ranges of 0.98%-3% reported[9,11,13,47], whereas community reservoirs have been reported in Europe[48-53].

Limitations

Environmental screening was restricted due to limited access during the COVID-19 pandemic. Potential transmissions without evident epidemiological support could have been due to colonisation by unsampled patients or hospital staff.

Conclusions

This study identified the protracted intra-hospital dissemination of highly related isolates from endemic CTs across multiple wards ($N=39$) including areas not subject to routine VREfm screening. Inter-hospital dissemination of the same clones was evident. Current screening practices in Ireland are limited to ‘at-risk’ groups. Admission-based screening may identify hidden reservoirs to help curtail hospital transmission. Finally, investigation of VSEfm should be considered in surveillance.

Acknowledgements

The authors acknowledge the support of the staff of the Irish National MRSA Reference Laboratory (NMRSARL) and the staff of the referring hospitals who submitted isolates for

investigation. We thank Maebh Kelleher, Antimicrobial Stewardship Pharmacist at St. James's Hospital for collating the glycopeptide usage data for the study period.

Conflict of interest statement

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

Funding source

This work was supported by Irish Health Research Board grant number ILP-POR-2019-010.

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Figure legends

Figure 1. Neighbour joining tree (NJT) based on core-genome multilocus sequence type (cgMLST) analysis of 1,423 target genes for 378 clade A1 *Enterococcus faecium* isolates from two wards (WA and WB) of a large Dublin acute hospital recovered between September 2019 and December 2022. Isolates segregated into 27 clusters and 21 singletons. Three predominant clusters (CI, CII, CIII) of closely related isolates (≤ 20 allelic differences) were identified, which are shaded in grey with the corresponding complex type (CT) indicated; [CI] CT2933 (96 isolates): average allelic difference 7.9, range 0-21; [CII] CT2932 (58 isolates): average allelic difference 6.2, range 0-18; [CIII] CT1916 (52 isolates): average allelic difference 10.0, range 0-27. Detailed metadata pertaining to sequence type (ST), isolate source, ward location and vancomycin susceptibility phenotype are indicated in the legends. A tree scale bar is shown at the top left.

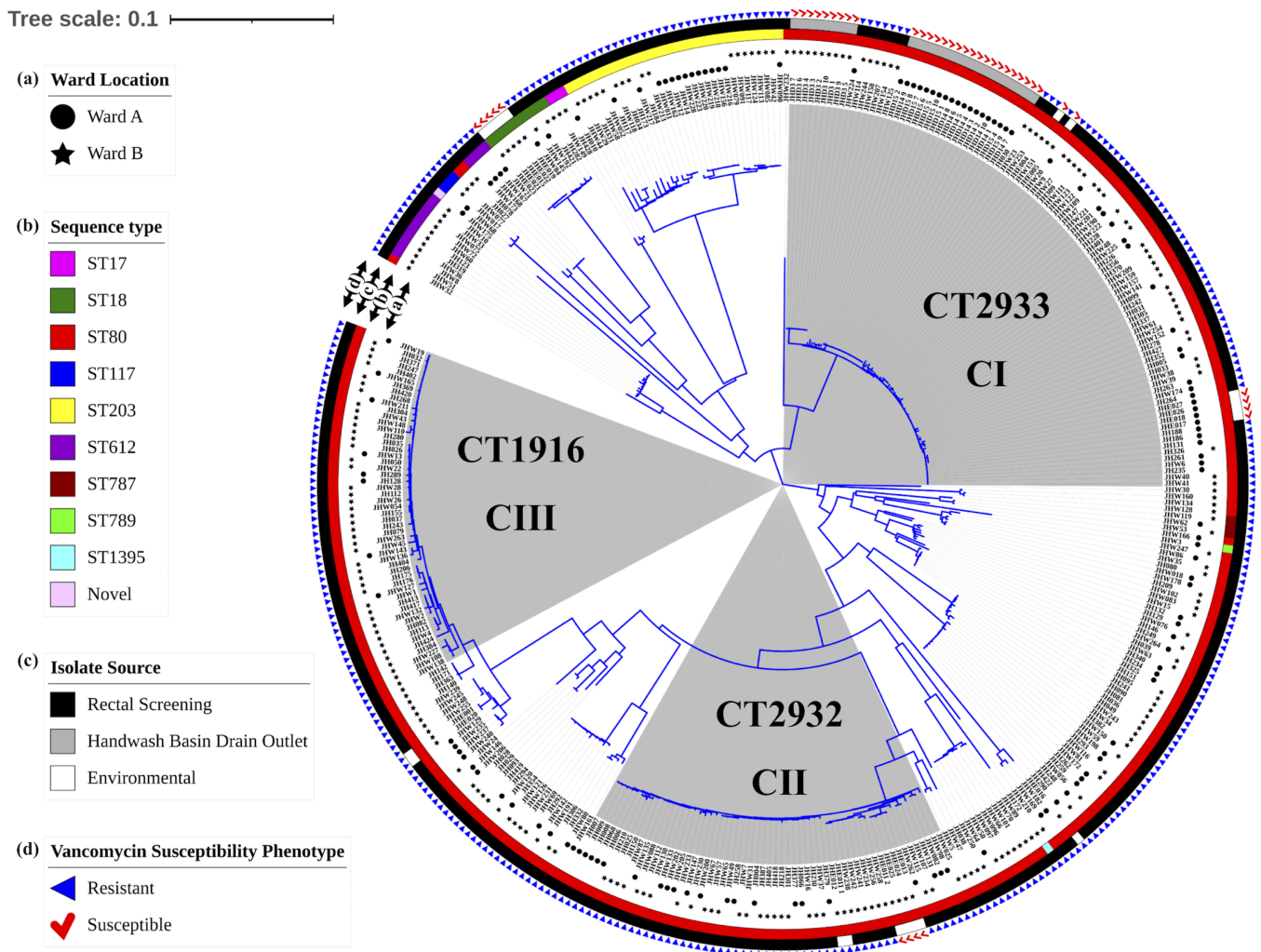
Figure 2. Maximum likelihood tree (MLT) based on phylogenetic analysis of 366 core-genome single nucleotide polymorphisms (cgSNPs) for the 96 clade A1 *Enterococcus faecium* isolates belonging to clonal complex type (CT)2933 identified by core-genome multilocus sequence typing (cgMLST) of isolates from wards WA and WB. Two major subclusters were evident within CT2933 in the MLT: sub-cluster A (CIA), represented by blue branches and inner blue ring and subcluster B (CIB), represented by green branches and inner green ring. Isolates recovered over the three-year study period that exhibited a median pairwise cgSNP difference of ≤ 15 were considered highly related and were further segregated into related isolate groups (RIGs). Four distinct RIGs (≥ 4 isolates) were identified within CI (CIA1, CIA2, CIA3 and CIB1) which are shaded in dark and light grey with the corresponding labels indicated; CIA1 (9 isolates): median cgSNP distance 0, range 0, time between earliest and latest isolate 0 days; CIA2 (19 isolates) median cgSNP distance 0, range 0-3, time between earliest and latest isolate 0 days; CIA3 (4 isolates) median cgSNP distance 3, range 0-5, time between earliest and latest isolate 119 days; CIB1 (46 isolates) median cgSNP distance 11, range 0-19, time between earliest and latest isolate 1143 days. Metadata pertaining to isolate source, ward location and vancomycin susceptibility phenotype are indicated in the legend. A tree scale bar is shown at the top left. The tree is mid-point rooted. The corresponding cgSNP distance matrix for all isolates is provided in Supplementary Table S3.

Figure 3. Parsimonious transmission tree based on cgSNP distance and collection date of 96 clade A1 *Enterococcus faecium* isolates from the ST80/CT2933 lineage (referred to as

CI) recovered from wards WA and WB between September 2019 and December 2022.

The tree was generated using the SeqTrack algorithm with GraphSNP which determines the most plausible genealogy for isolate sets based on recovery date and ward location[28,29]. Isolates are represented by shaded circular nodes with inner node shading indicating ward location as shown in the legend. Node outer ring colour refers to isolate recovery date range as shown in the legend. Isolate names are shown outside the nodes. Isolate nodes connected by blue network branches share over-lapping recovery dates of ≤ 30 days. Transmission links for isolates with a cgSNP distance of >10 are hidden and are indicated with a dashed outer node ring. Numbers on the network branches show cgSNP distances. The earliest CI isolate (JH005) used as a reference for cgSNP analysis is indicated by the black star within the node. Three central presumptive index isolates (JH031, JH099 and JH0131) within CIB (indicated by a $\times$ symbol within the nodes) with epidemiological links recovered from one WA patient and two WB patients over 20 days (median cgSNP difference: 4, range: 0-10), were likely involved in three potential transmission chains (PTCs), which are highlighted by background shading. A significant proportion of likely isolate transmissions with epidemiological support was evident within all three PTCs. Vancomycin-susceptible environmental isolates are indicated by a black triangle within the nodes. Branches not to scale. None of the VSEfm comprising CIA1 and CIA2 were involved in likely transmission events. The nine and 19 VSEfm in CIA1 and CIA2, respectively, were multiple isolates from the same samples from one and two handwash basin drains, respectively.

Figure 1



Tree scale: 0.001

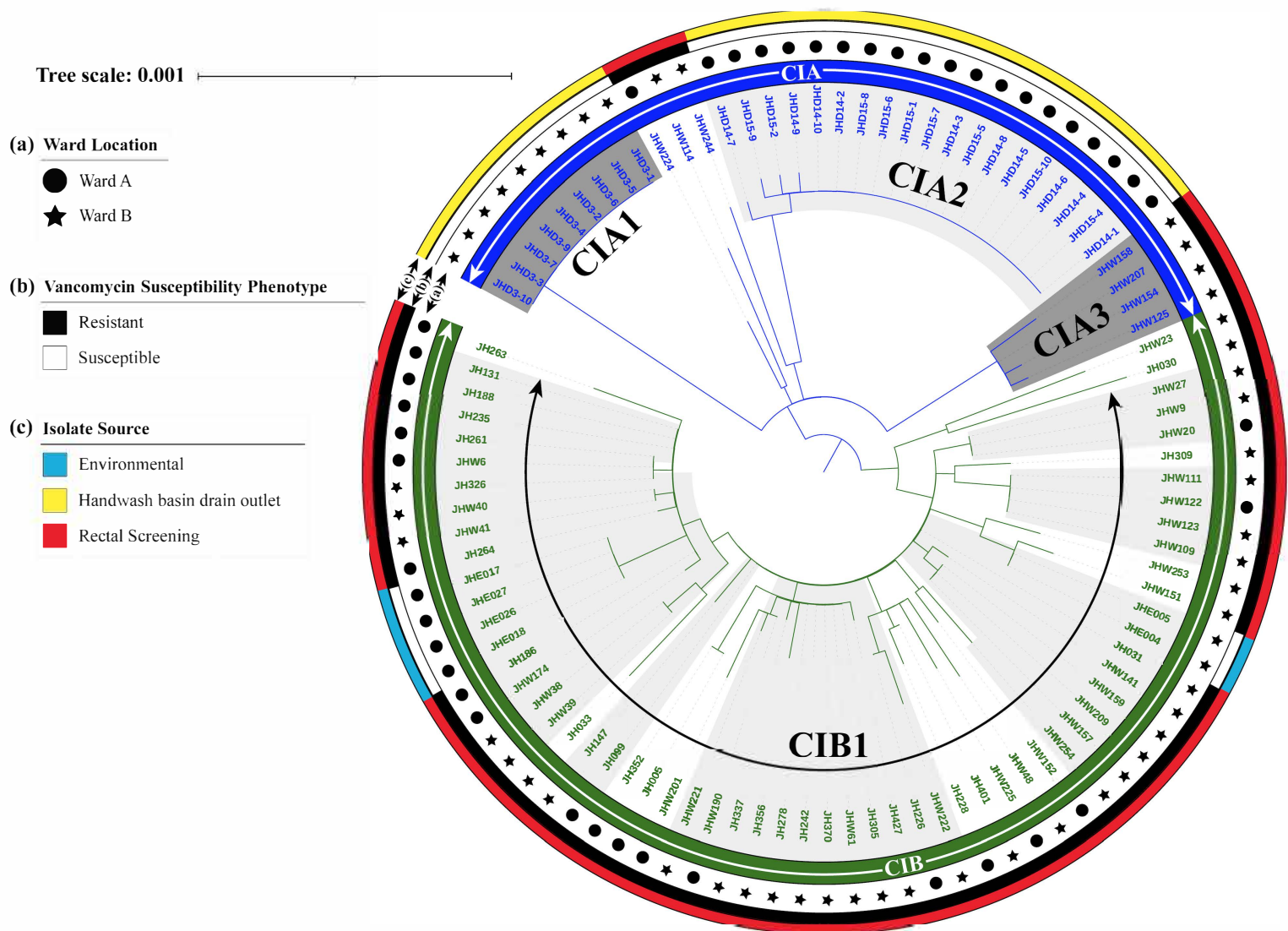
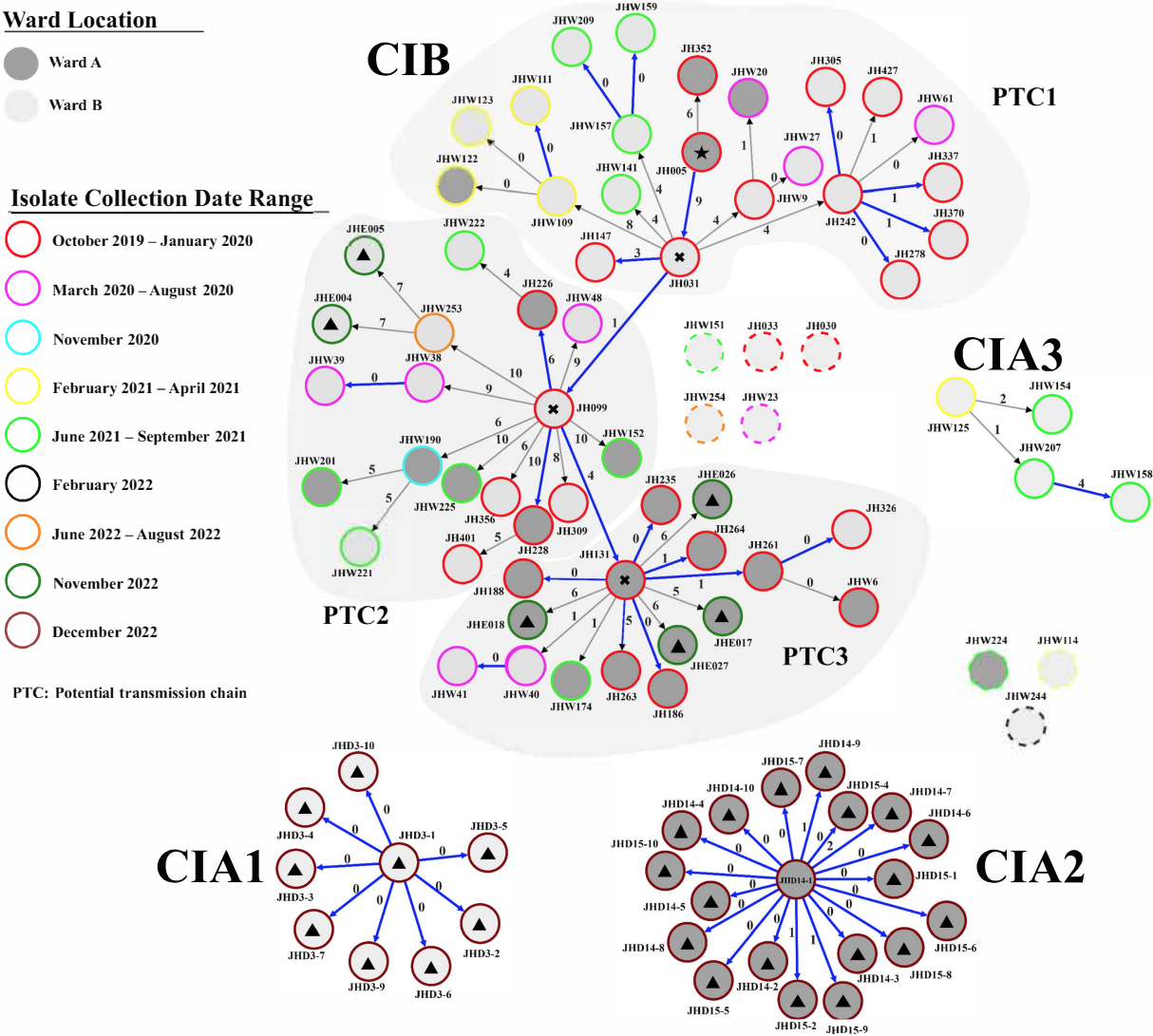


Figure 3



Supplementary Information

Protracted transmission and persistence of ST80 vancomycin-resistant *Enterococcus faecium* belonging to clonal complex types CT2933, CT2932 and CT1916 in a large Irish hospital: a 39-month WGS study.

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This file includes:

- Supplementary Methods
- Supplementary Table A2
- Supplementary Figure A1
- Supplementary Figure A2
- Supplementary Figure A3
- Supplementary Figure A4
- Supplementary Figure A5
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- Supplementary Figure A8
- Supplementary Figure A9

Supplemental Methods

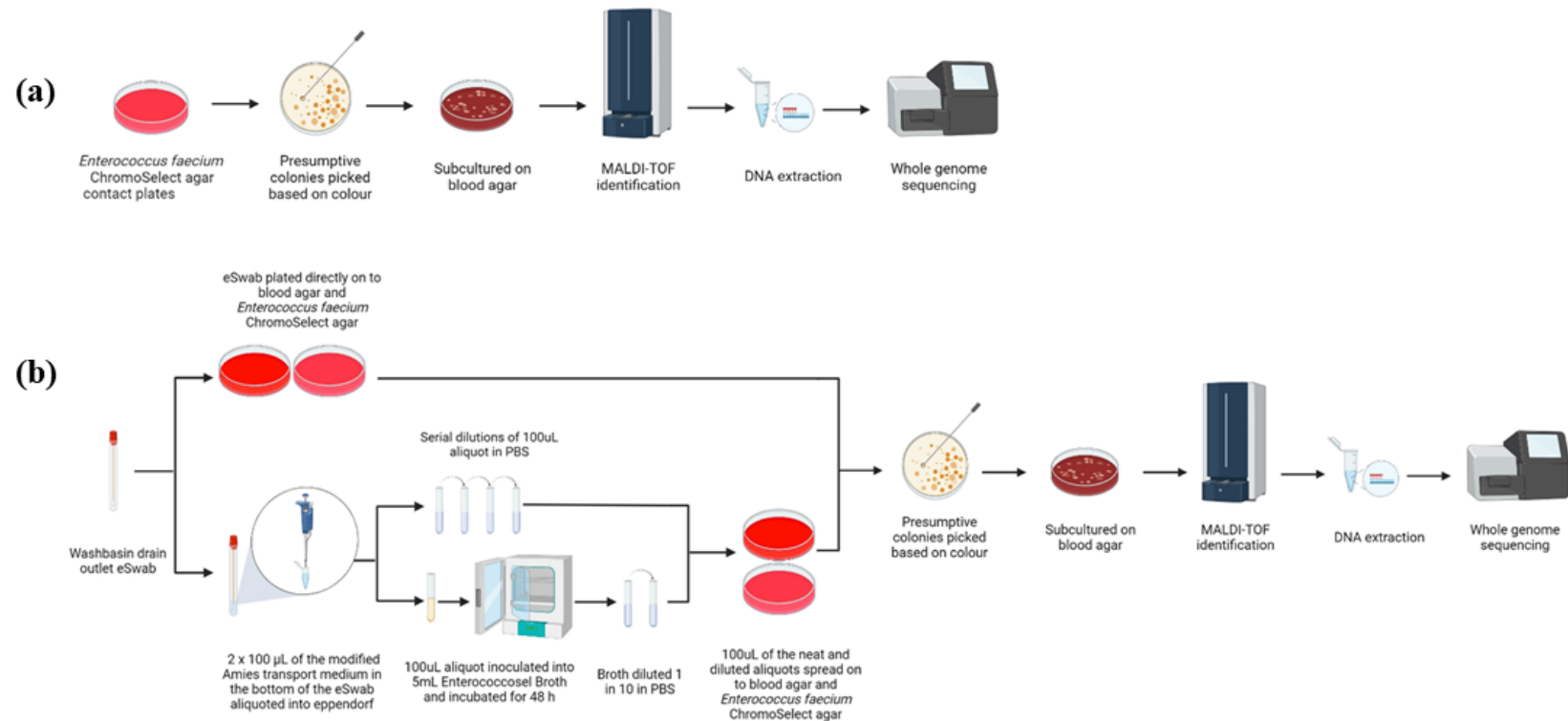
Infection control procedures and antimicrobial stewardship

Internal infection prevention and control (IPC) procedures were followed according to national guidelines [1,2,3]. Ward-specific antimicrobial stewardship policies were developed in line with the recommendations of the Strategy for the Control of Antimicrobial Resistance in Ireland (SARI) Hospital Antibiotic Stewardship Working group [4]. The isolation strategy for patients with clinical infection and/or colonisation with a transmissible multi-drug resistant (MDR) organism involved contact isolation in a single room with adjoining toilet facilities if available. Due to the limited number of ensuite rooms on ward A and ward B, organisms such as methicillin resistant *Staphylococcus aureus* (MRSA) and carbapenemase-produce Enterobacterales (CPE) were prioritised in patient cohorts for isolation. As a result, patients with a clinical or screening specimen positive for vancomycin resistant Enterococci (VRE) were not isolated and instead enteric contact precautions were observed. Additionally, a flag of 'VRE positive' was added to the patient's electronic health record. Compliance to hand hygiene on each ward was monitored using regular audits based on Health Protection Surveillance Centre (HPSC)-developed standard operating procedures for hand hygiene observation [5].

Environmental sampling

Due to the COVID-19 pandemic, IPC restrictions and staff shortages, access to the hospital and ward environments was limited. As a result, the two wards were screened for the presence of environmental *E. faecium* on two separate time-points between November 2022 and December 2022 to provide a rudimentary 'snap-shot' of the strains in circulation. On the first occasion, high-touch environmental sites in patient-occupied single ensuite rooms were investigated by surface-sampling using *Enterococcus faecium* ChromoSelect agar (Sigma-Aldrich [Merck]) contact plates (67 mm diameter, 25 cm² area). This ensured that the presence of both VREfm and VSEfm isolates were investigated. In total, ten environmental sites were chosen per room to encompass the patient bed space, vital-monitoring devices and ensuite bathroom facilities. The high-touch areas sampled included bedframes, both bedside tables, patient call bells, cardiac monitors, SATs probes, toilet seats and shower drains. Contact plates were incubated aerobically at 37°C for 48 h. All colonies from each sampling site that turned green, indicating the growth of *E. faecium*, were picked for subculture on Columbia blood agar (Fannin Ltd.) and subjected to identification, susceptibility testing and whole genome sequencing as described in Materials and Methods section.

On the second occasion, handwash basin wastewater drain outlets ($N=13$) from both wards were sampled using nylon-flocked eSwabs (Copan Diagnostics) and then placed in a tube of modified Amies transport medium. The handwash basins on three 4-bed bays and accompanying shared bathroom facilities ($N=6$) and two single patient rooms and accompanying ensuite bathrooms ($N=4$) in ward A were sampled. For ward B, the handwash basin in a single ensuite room and accompanying bathroom ($N=2$) and a shared bathroom for 4-bed bay ($N=1$) were investigated. eSwabs were directly plated on *Enterococcus faecium* ChromoSelect agar and Columbia blood agar (Fannin Ltd.) and incubated aerobically at 37°C for 48 h. A 100 µL aliquot of transport medium was collected from each eSwab and serial ten-fold dilutions prepared in sterile phosphate buffered saline (PBS). 100 µL of each dilution was spread in duplicate on *Enterococcus faecium* ChromoSelect agar and CBA agar and incubated aerobically at 37°C for 48 h. Simultaneously, an additional 100 µL was collected from each eSwab transport medium and incubated aerobically in 5 mL of Enterococcus Selective Broth (CondaLab) at 37°C for 48 h. Each broth was then diluted 10-fold in PBS. 100 µL of each neat and diluted sample was incubated in duplicate as described above. Presumptive *E. faecium* isolates ($N=28$) were selected based on colony colour, purified by subculture aerobically on CBA at 37°C for 24 h and identified by MALDI-TOF-MS (Bruker Daltonics). An overview of both protocols is available in Supplemental Methods Figure A1 below



Supplemental Methods Figure A1. Schematic diagram pertaining to the laboratory processing of environmental isolates ($N=48$) from two critical care wards of a large Dublin acute hospital. (a) High-touch environmental sites ($N=30$) were sampled in the two wards using *Enterococcus faecium* ChromoSelect agar (Sigma-Aldrich [Merck]) contact plates (67 mm diameter, 25 cm² area) and incubated aerobically at 37°C for 48 h. (b) Handwash basin wastewater drain outlets ($N=15$) from both wards were sampled using nylon-flocked eSwabs (Copan Diagnostics) and then placed in a tube of modified Amies transport medium. Swabs were directly plated on *Enterococcus faecium* ChromoSelect agar and CBA (Fannin Ltd.) and incubated aerobically at 37°C

for 48 h. A 100 µL aliquot of transport medium was collected from each eSwab and serial ten-fold dilutions prepared in sterile phosphate buffered saline (PBS). 100 µL of each dilution was spread in duplicate on *Enterococcus faecium* ChromoSelect agar and CBA agar and incubated aerobically at 37°C for 48 h. Simultaneously, an additional 100 µL was collected from each eSwab transport medium and incubated aerobically in 5 mL of Enterococcus Selective Broth (CondaLab) at 37°C for 48 h. Each broth was then diluted 10-fold in PBS. 100 µL of each neat and diluted sample was incubated in duplicate as described above. Presumptive *E. faecium* isolates ($N=28$) were selected based on colony colour, purified by subculture aerobically on CBA at 37°C for 24 h and identified by MALDI-TOF-MS (Bruker Daltonics).

Supplemental Method Table A1. Detailed metadata on positive environmental sites from WA and WB

Surface sampling of high-touch environmental sites			
	Room 1	Room 2	Room 3
Ward Location	Ward A	Ward B	Ward B
Total Number of Positive Sites (%) ¹	2 (20%)	6 (60%)	1 (10%)
Positive Site Location (<i>N</i> of colonies recovered from positive site)	Cardiac Monitor (2) Shower Drain (2)	Bed Frame (1) Bedside Table (9) Call Bell (1) Shower Drain (2) Sats Probe (1)	Toilet Seat (2)
Flocked eSwab sampling of handwash basin drain outlets			
	Room 2	Room 4	
Ward Location	Ward A	Ward B	
Total Number of Positive Drain Sites (%) ²	2 (20%)	1 (33%)	
Positive Site Location (<i>N</i> of colonies recovered from positive site)	Handwash basin in patient bedroom (9) Handwash basin in patient ensuite bathroom (10)	Handwash basin in patient bathroom (9)	

¹ Percentage calculated on the basis of a total 10 sampling sites per single patient room in both Ward A and Ward B.

² Percentage calculated on the basis of a total 10 sampling drain sites in Ward A and 3 in Ward B.

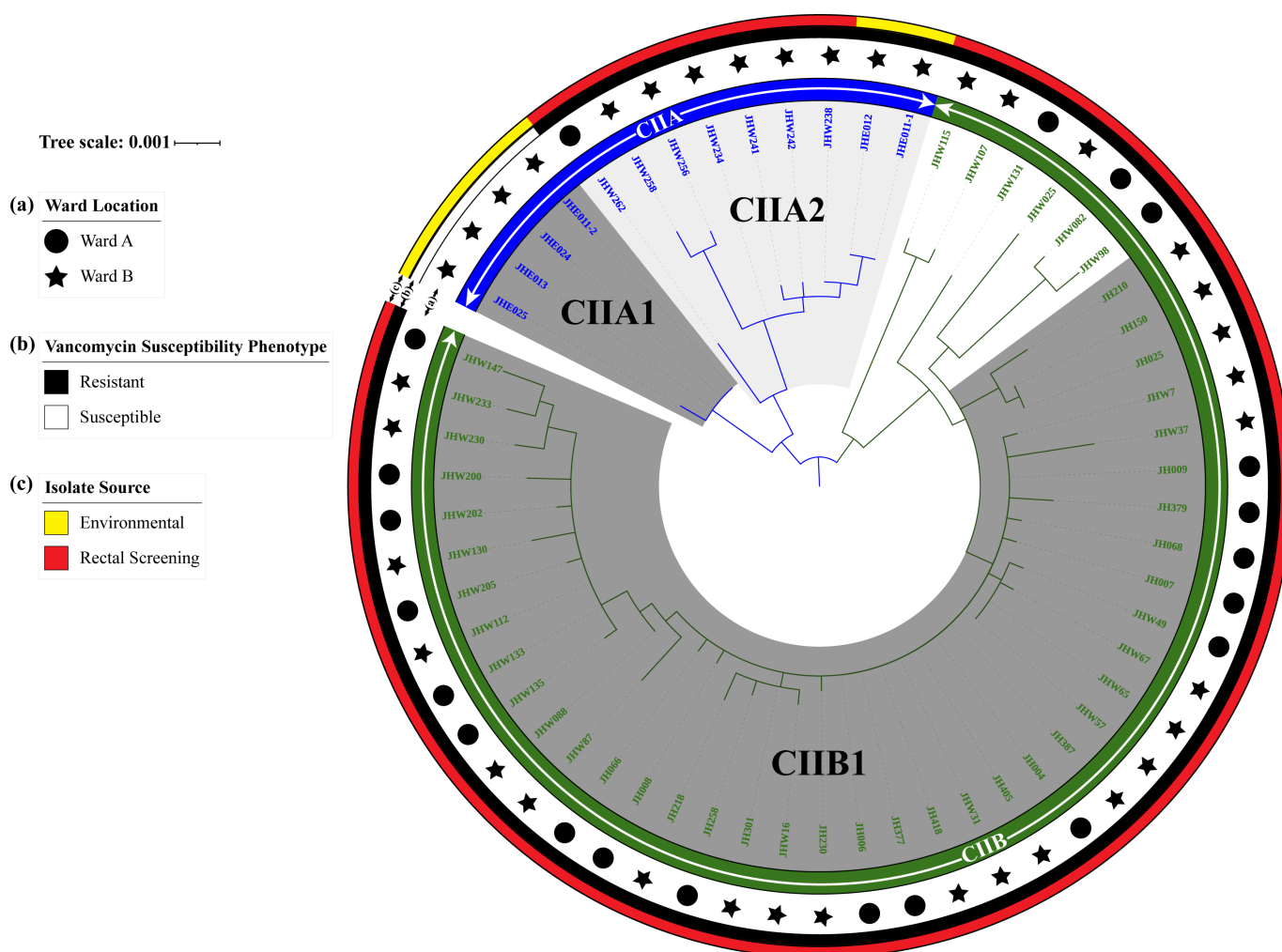
Supplemental Table A2. Detailed descriptions of three major complex types (CI, CII, CIII), subclusters and their related isolate groups (RIGs) identified using cgMLST and cgSNP analysis of 378 clade A1 *Enterococcus faecium*^I isolates from two wards (WA and WB) of a large Dublin acute hospital recovered between September 2019 and December 2022.

Clusters						
CI			CII		CIII	
ST/CT	ST80/CT2933		ST80/CT2932		ST80/CT1916	
No. isolates	96		58		52	
Source (N)	Rectal screening (62) Wash-hand basin drain outlet (28) Toilet seat (2) Cardiac monitor (2) Shower drain (2)		Rectal screening (52) Bedside table (6)		Rectal screening (52)	
Median pairwise cgSNP difference (range)	25.5 (0-84)		17 (0-56)		30 (0-68)	
Time period (days)	1172		1145		1174	
Sub-clusters						
CIA		CIB	CIIA	CIIB	CIIIA	CIIBB
No. isolates	35	61	13	45	1	51
Source (N)	Rectal screening (7) Wash-hand basin drain outlet (28)	Rectal screening (55) Toilet seat (2) Cardiac monitor (2) Shower drain (2)	Rectal screening (7) Bedside table (6)	Rectal screening (45)	Rectal screening (1)	Rectal screening (51)
Median pairwise cgSNP difference (range)	26 (0-53)	14 (0-84)	15 (0-35)	9 (0-41)	-	28 (0-68)
Time period (days)	651	1146	46	779	-	1174
RIGs						
	No. isolates	Isolate Source (N)	Median pairwise cgSNP difference (range)	Time period (days)	Vancomycin susceptibility phenotype (N)	Ward Distribution % (N)
CIA1	9	Washhand basin drain outlet (9)	0	0	VSEfm (9)	WB: 100% (9)
CIA2	19	Washhand basin drain outlet (19)	0 (0-3)	0	VSEfm (19)	WA: 100% (19)
CIA3	4	Rectal screening (4)	3 (0-5)	112	VREfm (4)	WB: 100% (4)

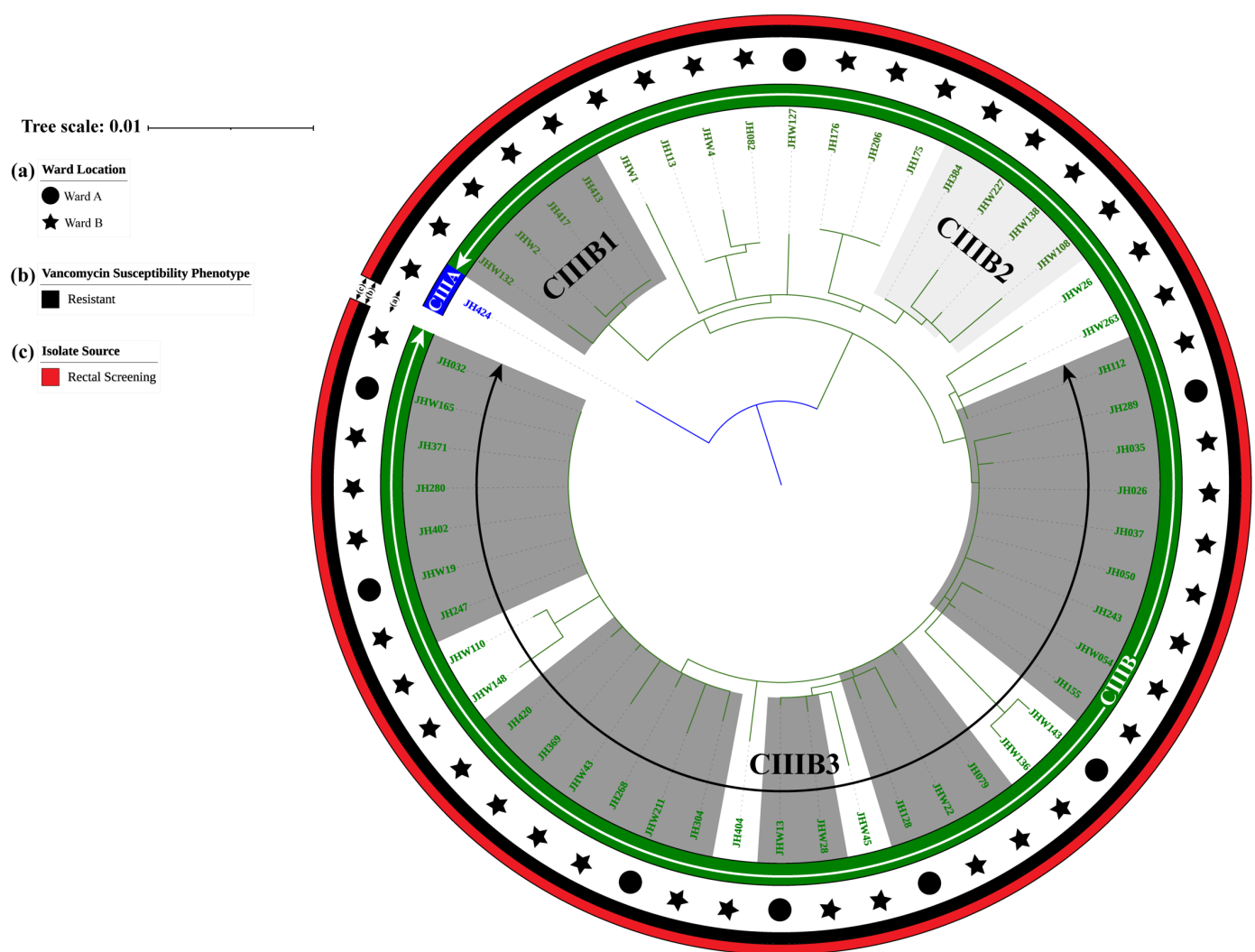
RIGs						
	No. isolates	Isolate Source (N)	Median pairwise cgSNP difference (range)	Time period (days)	Vancomycin susceptibility phenotype (N)	Ward Distribution % (N)
CIB1	46	Rectal screening (40) Toilet seat (2) Cardiac monitor (2) Shower drain (2)	11 (0-19)	1143	VREfm (40) VSEfm (6)	WA: 37% (17) WB: 63% (29)
CHIA1	4	Bedside table (4)	1 (0-2)	0	VSEfm (6)	WB: 100% (4)
CHIA2	9	Rectal screening (7) Bedside table (2)	9 (0-19)	363	VREfm (9)	WB: 100% (9)
CHIB1	39	Rectal screening (39)	7 (0-24)	1144	VREfm (39)	WA: 44% (17) WB: 56% (22)
CHIB1	4	Rectal screening (4)	3.5 (0-7)	505	VREfm (4)	WB: 100% (4)
CHIB2	4	Rectal screening (4)	11 (0-18)	656	VREfm (4)	WB: 100% (4)
CHIB3	27	Rectal screening (27)	8 (0-20)	687	VREfm (27)	WA: 22% (6) WB: 78% (21)

¹All 330 rectal screening isolates were VREfm and 43/48 environmental isolates were VSEfm.

Abbreviations: ST, sequence type; CT, complex type; cgSNP, core-genome single nucleotide polymorphism; RIGs, related isolate groups; VSEfm, vancomycin susceptible *E. faecium*; VREfm, vancomycin resistant *E. faecium*; WA, Ward A; WB, Ward B



Supplemental Figure A1. Maximum likelihood tree (MLT) based on phylogenetic analysis of 220 core-genome single nucleotide polymorphisms (cgSNPs) for the 58 clade A1 *Enterococcus faecium* isolates from clonal complex (CT)2932 (referred to as CII) identified by core-genome multilocus sequence typing (cgMLST) of isolates from two wards of a large Dublin acute hospital between September 2019 and December 2022. Two major subclusters were evident within CII in the MLT: subcluster A (CIIA), represented by blue branches and inner blue ring and subcluster B (CIIB), represented by green branches and inner green ring. Isolates recovered over the three year study period which exhibited a median pairwise cgSNP difference of ≤ 15 were considered closely related and were further segregated into related isolate groups (RIGs). Three distinct RIGs (≥ 4 isolates) were identified within CII (CIIA1, CIIA2 and CIIB1) which are shaded in dark and light grey with the corresponding labels indicated accordingly; CIIB (4 isolates): median cgSNP distance 1, range 0-2, time between earliest and latest isolate 0 days; CIIA2 (9 isolates) median cgSNP distance 9, range 0-19, time between earliest and latest isolate 363 days; CIIB1 (39 isolates) median cgSNP distance 8, range 0-24, time between earliest and latest isolate 779 days. Detailed metadata pertaining to isolate source, ward location and vancomycin susceptibility phenotype are indicated in the legend. A tree scale bar is shown at the top left. The tree is mid-point rooted. The corresponding cgSNP distance matrix for all the isolates is provided in Supplemental Table A3.



Supplemental Figure A2. MLT based on phylogenetic analysis of 341 cgSNPs for the 52 clade A1 *Enterococcus faecium* isolates from clonal complex CT1916 (referred to as CIII) identified by cgMLST of isolates from two wards of a large Dublin acute hospital between September 2019 and December 2022. One isolate formed an out-group (JH424) termed CIIIA and is represented by the blue branch and inner blue ring labelled CIIIA. The remaining isolates belonged to one major subcluster termed CIIIB, represented by green branches and the inner green ring labelled CIIIB. Isolates recovered over the three year study period that exhibited a median pairwise cgSNP difference of ≤ 15 were considered closely related and were further segregated into related isolate groups (RIGs). Three distinct RIGs (≥ 4 isolates) were identified within CIII (CIIB1, CIIB2 and CIIB3) which are shaded in dark and light grey with the corresponding labels indicated accordingly; CIIB1 (4 isolates): median cgSNP distance 3.5, range 0-7, time between earliest and latest isolate 505 days; CIIB2 (4 isolates) median cgSNP distance 11, range 0-18, time between earliest and latest isolate 656 days; CIIB3 (27 isolates) median cgSNP distance 8, range 0-20, time between earliest and latest isolate 687 days. Isolates within CIIB3 ($N=6$) which are not shaded in grey and exhibited a median pairwise cgSNP of >15 were excluded from the RIG and considered to be intra-RIG outliers. Detailed metadata pertaining to isolate source, ward location and vancomycin susceptibility phenotype are indicated in the legend. A tree scale bar is shown at the top left. The tree is mid-point rooted. The corresponding cgSNP distance matrix for all the isolates is provided in Supplemental Table A3.

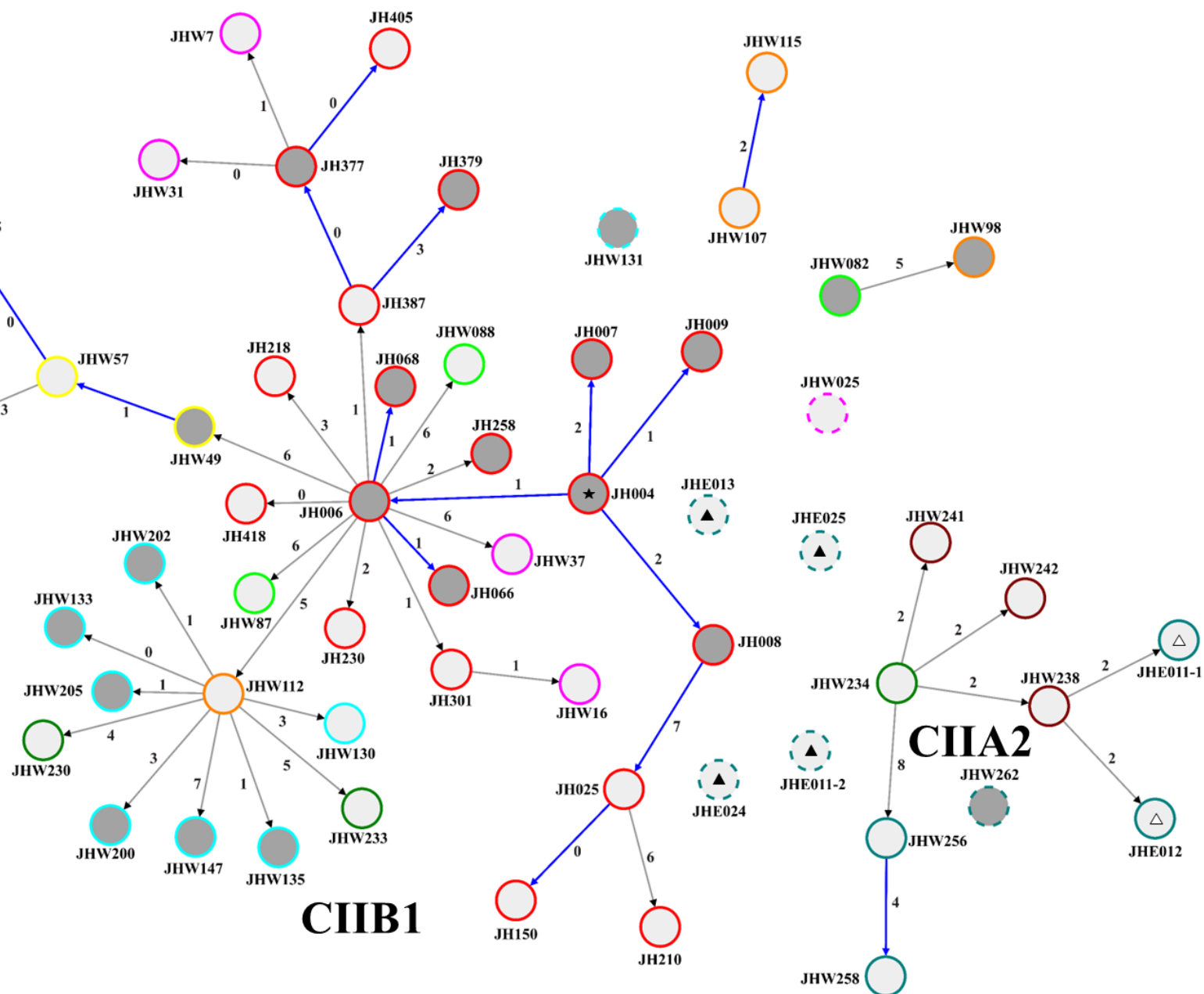
Genomic map of the *vanA* locus on the 100,000 bp chromosome of *E. coli* O157:H7 strain 26869. The map shows genes as arrows and IS elements as boxes. Genes include *dinB*, *ISEfa4*, *ISEfa4*, *ISEfa4*, *ISEfa4*, *ISEfa4*, *tagU*, *IS1216E*, *hisB*, *vanY*, *vanA*, *tnpA*, *vanS*, and *relG*. IS elements include *IS1216E*, *IS1216E*, and *IS1216E*. The map is divided into segments of 25,000, 50,000, 75,000, and 100,000 bp.

Supplemental Figure A3. Schematic diagram showing the comparative structural organisation of *vanA*- and *van* remnant-encoding plasmids closed by hybrid assembly from clinical and environmental *Enterococcus faecium* isolates from Irish hospitals. (a) pELF_BS256 (ST80/CT2933 111,849 bp linear plasmid from VREfm rectal screen); (b) pELF_RC_19_045 (ST80/CT2932, 153,351 bp linear plasmid from VREfm bloodstream infection); (c) pELF_RC_19_023 (ST80/CT1916, 109,124 bp linear plasmid from VREfm rectal screen); (d) pJHE024 (ST80/CT2932, 88,110 bp circular plasmid from VSEfm isolate recovered from patient bedside table). Isolates were recovered from H4 (a), H5 (b-c) and H1 (d). Genes and their orientation are denoted by directional arrows and labelled with corresponding gene names. A reference size scale bar is shown above each plasmid map. Genes encoding hypothetical proteins are hidden. The plasmid map shown in (c) encoding an intact *vanA* operon was previously described by Egan et al [3]. Plasmid pJHE024 (d) was identified in VSEfm environmental isolate JHE024 from the present study and harbours a *van* remnant encoding the *vanY* and *vanZ* genes.

Ward Location



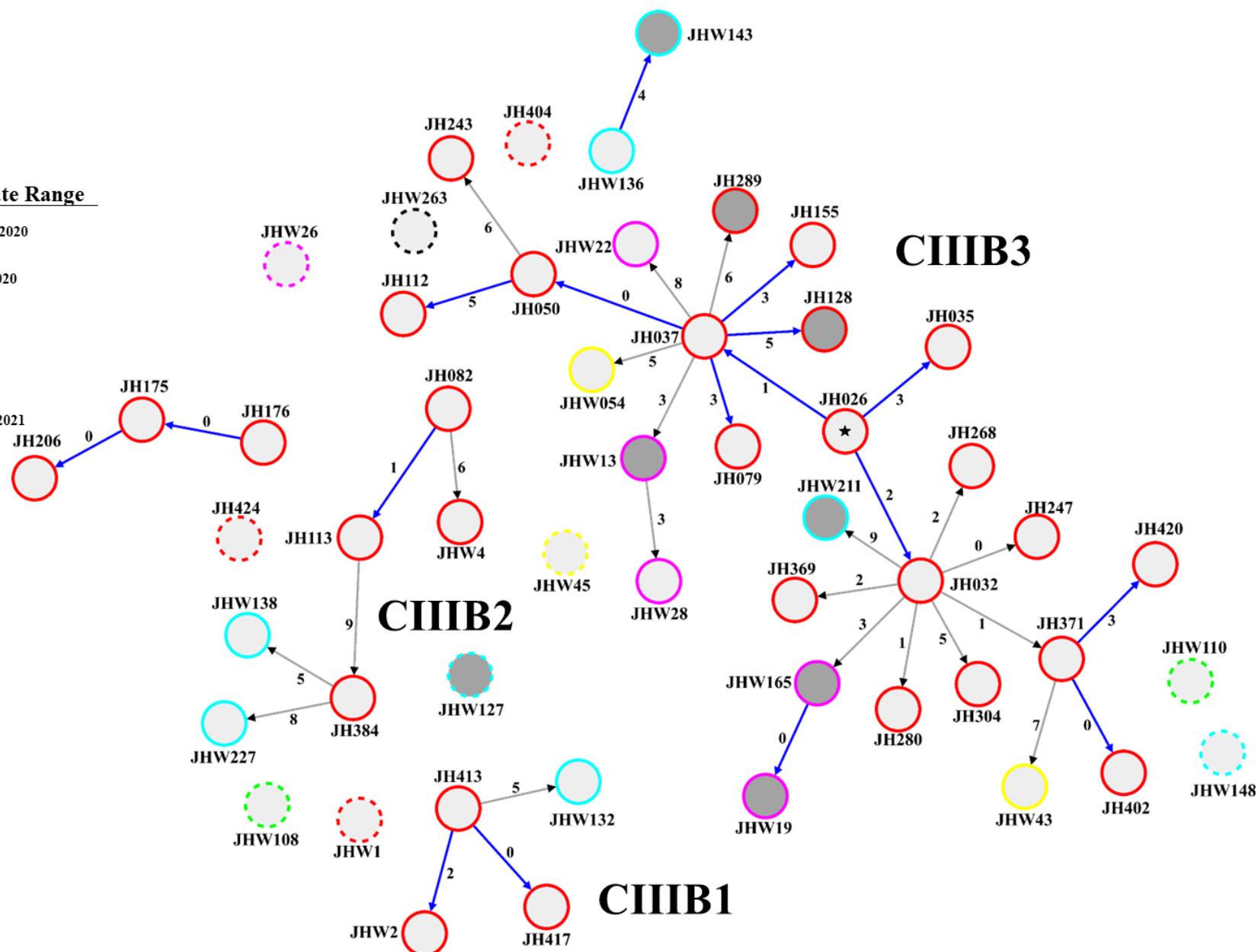
Isolate Collection Date Range



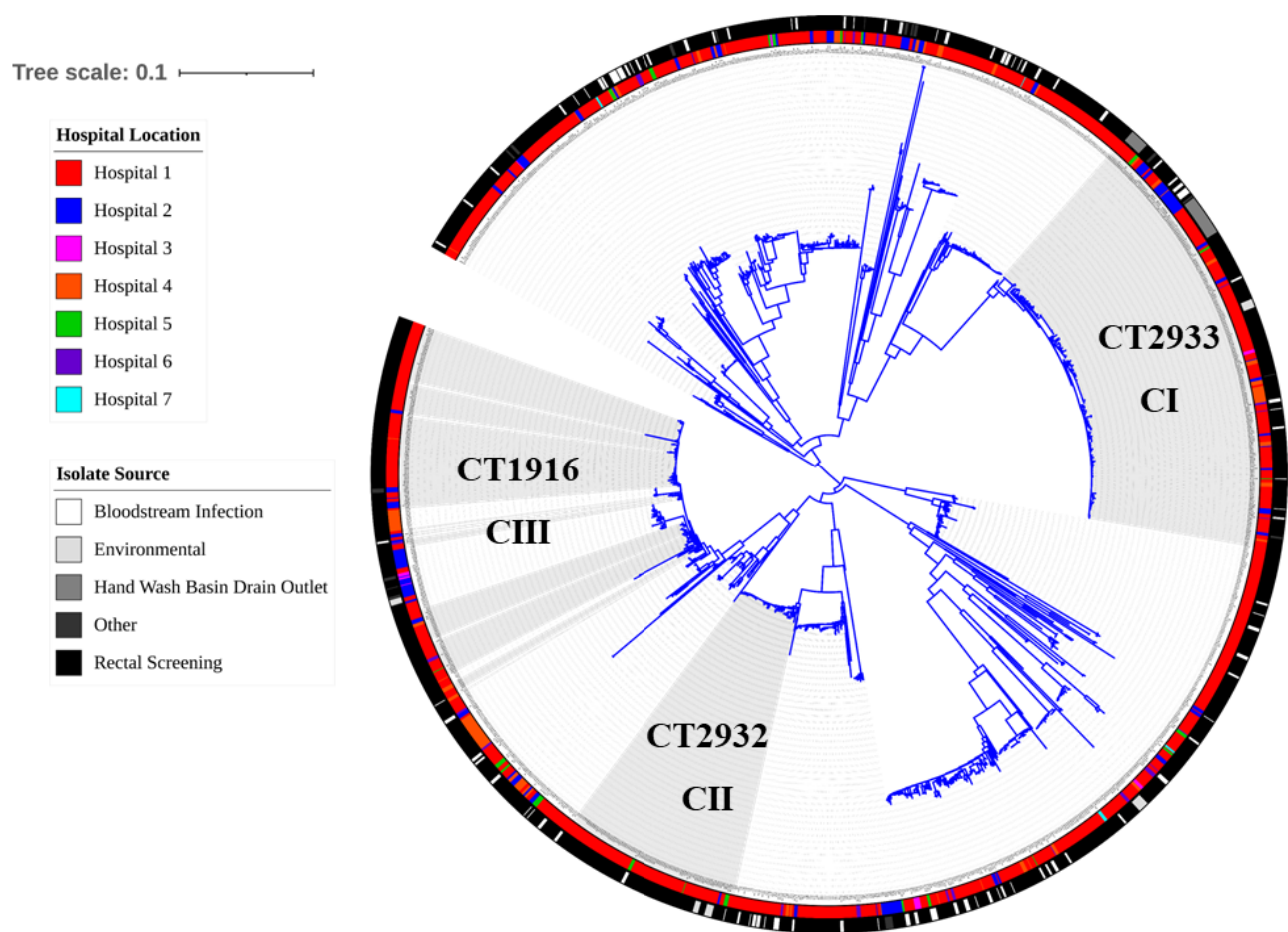
Supplemental Figure A4. Parsimonious transmission tree based on cgSNP distance and collection time of 58 clade A1 *Enterococcus faecium* isolates from the ST80/CT2932 lineage (referred to as CII) recovered from Wards A and B between September 2019 and December 2022 generated using the SeqTrack algorithm with GraphSNP [6,7]. Isolates are represented by shaded circular nodes with inner node shading indicating ward location as shown in the legend. Node outer ring colour refers to isolate recovery date range as shown in the legend. Isolate names are shown outside the nodes. Isolates connected by blue network branches share over-lapping recovery dates of ≤ 30 days. Transmission links for isolates with a cgSNP distance of >10 are hidden and are indicated with a dashed outer ring. Numbers on the network branches show cgSNP distances. The earliest CII isolate (JH004) used as a reference for cgSNP analysis is indicated by the black star within the node. Environmental isolates ($N=6$) are indicated by a triangle within the node: black triangle, vancomycin susceptible isolate; white triangle, vancomycin resistant isolate. An MLT based on phylogenetic analysis of 220 cgSNPs for the 58 CII isolates is shown in Supplemental Figure A1. The four VSEfm environmental isolates JHE011-2, JHE013, JHE024 and JHE05 that formed RIG CIIA1 (Supplemental Figure A1) were multiple colony isolates recovered from a contact plate sample from a patient bedside table, none of which was involved in likely transmission events.

 Ward A
 Ward B

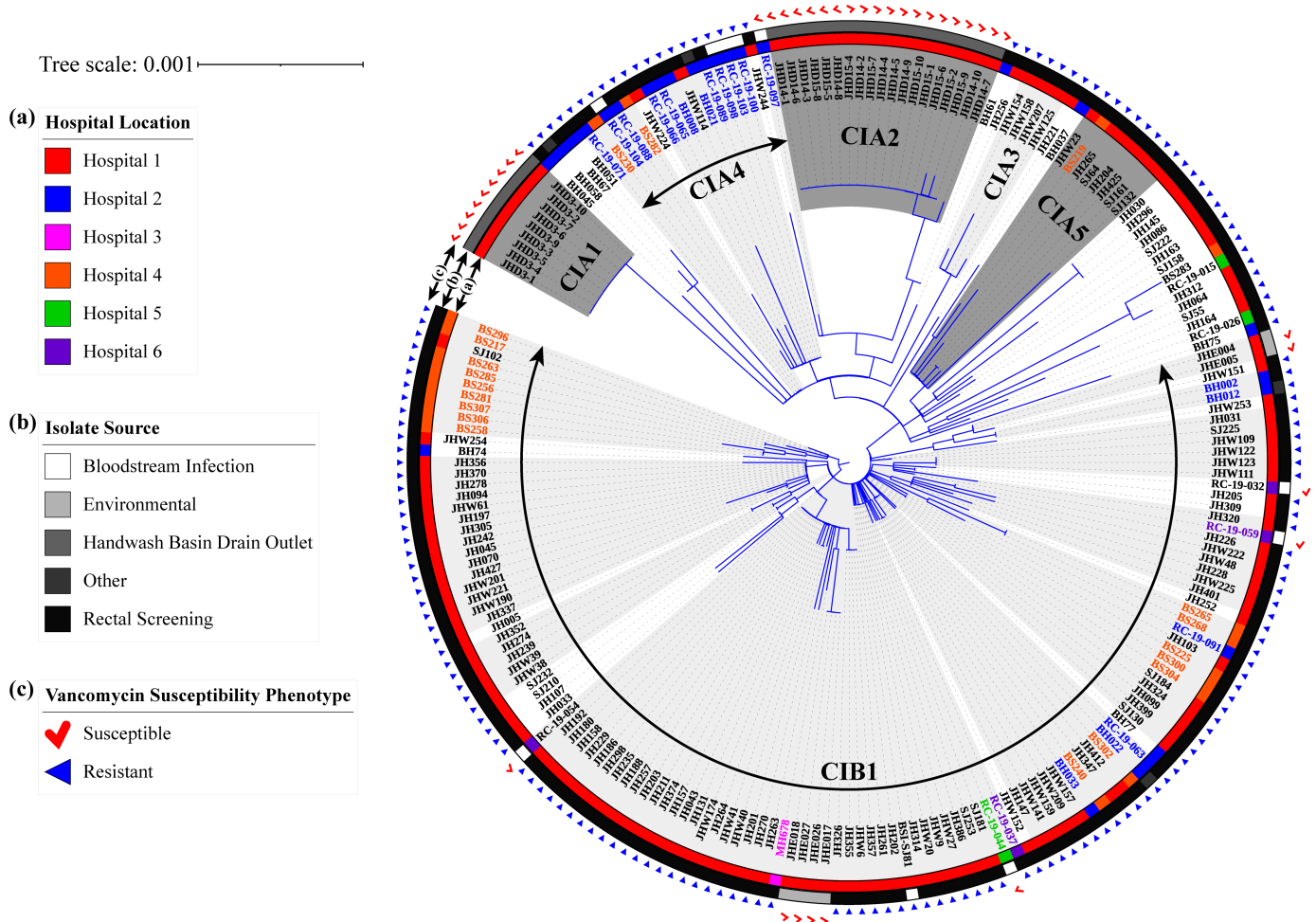
- October 2019 – January 2020
- February 2020 – April 2020
- June – July 2020
- February 2021
- April 2021 – September 2021
- December 2022



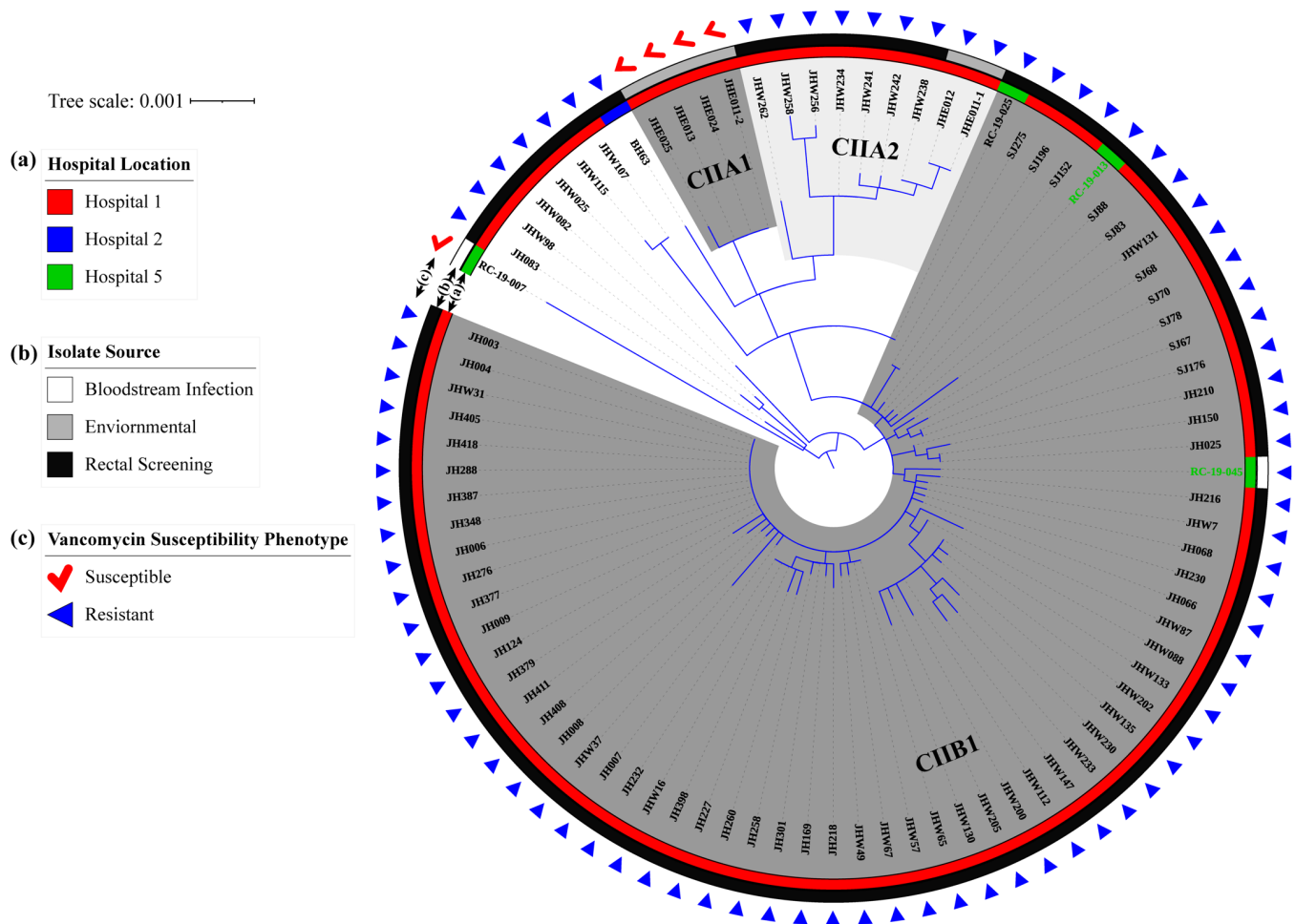
Supplemental Figure A5. Parsimonious transmission tree based on cgSNP distance and collection time of 52 clade A1 *Enterococcus faecium* isolates from the ST80/CT1916 lineage (referred to as CIII) recovered from Wards A & B between September 2019 and December 2022 generated using the SeqTrack algorithm with GraphSNP [6,7]. Isolates are represented by shaded circular nodes with inner node shading indicating ward location as shown in the legend. Node outer ring colour refers to isolate recovery date range as shown in the legend. Isolate names are shown outside the nodes. Isolates connected by blue network branches share over-lapping recovery dates of ≤ 30 days. Transmission links for isolates with a cgSNP distance of >10 are hidden and are indicated with a dashed outer ring. Numbers on the network branches show cgSNP distances. The earliest CIII isolate (JH026) used as a reference for cgSNP analysis is indicated by the black star within the node. An MLT based on phylogenetic analysis of 341 cgSNPs for the 52 CIII isolates is shown in Supplemental Figure A2.



Supplemental Figure A6. Neighbour joining tree (NJT) based on core-genome multi locus sequence type (cgMLST) analysis of 1,423 target genes for 1,286 clade A1 *Enterococcus faecium* isolates recovered from seven Irish hospitals between June 2017 and December 2022. The NJT comprises 648 isolates from five Irish hospitals including H1 reported in a previous study from this laboratory [8], an additional 260 well-characterised isolates recovered from seven Irish hospitals (June 2017-July 2022), and WA and WB isolates from this study ($N=378$). Isolates segregated into 71 clusters and 67 singletons. Three distinct clusters (CI, CII, CIII) of closely related isolates (≤ 20 allelic differences) are highlighted, which are shaded in grey with the corresponding complex type (CT) indicated as follows: [CI] CT2933 (215 isolates): average allelic difference 7, range 0-28; [CII] CT2932 (88 isolates): average allelic difference 5, range 0-26; [CIII] CT1916 (137 isolates): average allelic difference 9, range 0-28. Metadata pertaining to hospitals and isolate source are indicated in the legends. A tree scale bar is shown at the top left.



Supplemental Figure A7. MLT based on phylogenetic analysis of 731 cgSNPs for 215 clade A1 *Enterococcus faecium* belonging to CT2933 (referred to as CI) identified by cgMLST of isolates from six Irish hospitals between October 2018 and December 2022. Isolates recovered over the four-year period which exhibited a median pairwise cgSNP difference of ≤ 15 were considered highly related and were further segregated into RIGs. Six distinct RIGs (≥ 4 isolates) were identified within CI (CIA1, CIA2, CIA3, CIA4, CIA5 and CIB1) which are shaded in dark and light grey with the corresponding labels indicated accordingly; CIA1 (9 isolates): median cgSNP distance 0, time between earliest and latest isolate 0 days; CIA2 (19 isolates) median cgSNP distance 0, range 0-3, time between earliest and latest isolate 363 days; CIA3 (4 isolates) median cgSNP distance 3, range 0-5, time between earliest and latest isolate 112 days; CIA4 (15 isolates) median cgSNP distance 9, range 0-18, time between earliest and latest isolate 537 days; CIA3 (4 isolates) median cgSNP distance 3, range 0-5, time between earliest and latest isolate 112 days; CIA5 (8 isolates) median cgSNP distance 10.5, range 0-22, time between earliest and latest isolate 433 days; CIB1 (114 isolates) median cgSNP distance: 9, range 0-23, time between earliest and latest isolate 1381 days. Isolates within CIA4 ($N=2$) and CIB1 ($N=15$) which are not shaded in grey and exhibited a median pairwise cgSNP of >15 were excluded from the RIG and are considered intra-RIG outliers. Metadata pertaining to isolate source, ward location and vancomycin susceptibility phenotype are indicated in the legends. A tree scale bar is shown at the top left. The tree is mid-point rooted. The corresponding cgSNP distance matrix for all the isolates is provided in Supplementary Table A3.



Supplemental Figure A8. MLT based on phylogenetic analysis of 315 cgSNPs for the 88 clade A1 *Enterococcus faecium* isolates CT2932 (referred to as CII) identified by cgMLST of isolates from three Irish hospitals between October 2018 and December 2022. Isolates recovered over the four-year period which exhibited a median pairwise cgSNP difference of ≤ 15 were considered highly related and were further segregated into RIGs. Three distinct RIGs (≥ 4 isolates) were identified within CII (CIIA1, CIIA2 and CIIB1) which are shaded in dark and light grey with the corresponding labels indicated accordingly; CIIA1 (4 isolates): median cg SNP distance 1, range 0-2, time between earliest and latest isolate 0 days; CIIA2 (9 isolates) median cgSNP distance 9, range 0-19, time between earliest and latest isolate 363 days; CIIB1 (114 isolates) median cgSNP distance 8, range 0-24, time between earliest and latest isolate 1125 days. Metadata pertaining to isolate source, ward location and vancomycin susceptibility phenotype are indicated in the legends. A tree scale bar is shown at the top left. The tree is mid-point rooted. The corresponding cgSNP distance matrix for all the isolates is provided in Supplementary Table A3.

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