The Population Structure and Mobile Genetic Elements Harboured by Hospital-Adapted Vancomycin-Resistant and Linezolid-Resistant *Enterococcus faecium* and *Enterococcus faecalis* in Ireland Investigated by Whole-Genome Sequencing

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Summary

Vancomycin-resistant enterococci (VRE) are a major cause of healthcare-associated infection worldwide. For over a decade, Ireland has consistently had some of the highest rates of invasive VRE infections in Europe. VRE harbouring the *vanA* operon encoding vancomycin resistance, which is often located on plasmids and associated with the Tn*1546* transposon, are the primary causative genotype. Linezolid is an antibiotic used to treat infections caused by multi-drug resistant (MDR) Gram-positive bacteria, such as VRE. Linezolid-resistant enterococci (LRE) have been reported with increasing frequency in recent years, with a recent rise in LRE harbouring transferable linezolid resistance, encoded by the *optrA*, *poxtA* and/or *cfr* genes. Little is known about the population structure of hospital-adapted VRE in Ireland or about the prevalence and genetic organisation of mobile genetic elements encoding linezolid resistance. The overall objectives of this study were to investigate the population structure of hospital-adapted *E. faecium* screening and bloodstream infection isolates (BSIs) from patients in a large acute hospital in Dublin, Ireland, and to investigate the prevalence and genetic organisation of transferable genetic elements encoding linezolid resistance genes in LRE from patients in multiple Irish hospitals. A combination of short-read (Illumina, The Netherlands) and long-read (Oxford Nanopore Technologies [ONT] Oxford, UK) whole-genome sequencing (WGS) technology was used as the principal approach to achieve these objectives.

The first principal aim of this project was to examine the population structure of hospital-adapted *E. faecium* in a large Dublin hospital using WGS. Between June 2017 and July 2019, 365 *E. faecium* isolates were collected at the clinical microbiology laboratory, including vancomycin-resistant *E. faecium* (VREfm) screening isolates (n=286) and all *E. faecium* BSI isolates (VREfm, n=45, vancomycin-susceptible *E. faecium* [VSEfm], n=34) recovered during the study period. All isolates underwent Illumina WGS and were typed using conventional multilocus sequence typing (MLST) and high-resolution core-genome (cg) MLST using the Ridom SeqSphere+ (version 7.0.4, Ridom GmbH, Germany) and BioNumerics (version 7.7, Applied Maths, Belgium) suite of software packages. A selection of isolates from representative sequence types (STs) underwent ONT long-read sequencing. The vast majority of isolates (360/365, 98.6%) belonged to the hospital-adapted clade A1, while five isolates (1.4%) (all VSEfm BSI) belonged to the community-adapted clade B. The majority (57.5%) of clade A1 *E. faecium* isolates belonged to ST80 (207/360) based on conventional MLST. However, cgMLST divided the 360 clade A1 isolates into 33 clusters and 63 singletons, with an inter-cluster allelic difference range of 25-1201. The entire isolate population was polyclonal, with highly related isolates (≤ 20 cgMLST allelic differences) identified in multiple hospital wards and persisting over extended periods of time (up to 21 months). Furthermore, the identification of highly related VREfm and VSEfm in the isolate collection indicated that VSEfm can readily acquire *vanA,* resulting in new VREfm. Persistence of isolates indicated that current hospital cleaning regimes are not effective at eradicating VREfm.

The *vanA* transposon regions of three selected VREfm isolates from representative STs, were resolved using hybrid assembly from isolates SJ10 (ST789, SJ10*vanA*; 11,210 bp), SJ11 (non-typeable, SJ11*vanA;* 13,252 bp) and BSI_SJ40 (ST80, BSI_SJ40*vanA*; 13,269 bp). In addition, two *vanA*-encoding plasmids were also resolved using hybrid assembly from SJ82 (ST203, pSJ82*vanA;* 48,934 bp) and SJ245 (ST117, pSJ245*vanA*; 40,559 bp). The *vanA* region in Irish isolates differed from the prototype Tn*1546 vanA* transposon (VREfm strain BM4147) by multiple insertions of IS*1216E*, differing orientations of the *vanA* operon genes, and in SJ11*vanA* and BSI_SJ40*vanA*, the insertion of a cadmium efflux accessory protein gene. The SJ10*vanA* sequence was used as a reference against which the corresponding *vanA* regions of all VREfm (n=331) isolates investigated were compared; 95.5% (316/331) of isolates harboured a *vanA* region with >90% sequence identity to the SJ10*vanA* reference. The repetitive insertion of IS*1216E* in the *vanA* operon observed in Irish VREfm is a likely source of instability within the operon. The *vanA* region of five isolates was successfully transferred to the recipient *E. faecium* 64/3 strain by conjugation including SJ11 (non-typeable), SJ82 (ST203), SJ245 (ST117), SJ267 (ST18) and BSI_SJ40 (ST80). In the resulting transconjugants (TCs), the *vanA* region from 4/5 donor strains transferred successfully and was identical to the *vanA* region in each respective donor strain. A discrepancy was noted with pSJ82*vanA* indicating only partial transfer from donor (SJ82) to TCs. Using hybrid assembly on the transconjugant SJ82:Efm 64/3 TC1, it appears that the *vanA* operon flanked on either side by IS*1216E* (9,826 bp) from pSJ82*vanA* (donor strain) likely moved via transposition to pSJ82_B, a 232,026 bp plasmid also present in the donor strain SJ82, and there was

loss of a 65,931 bp portion of pSJ82_B during/after conjugation into the *E. faecium* 64/3 recipient strain giving rise to a new hybrid plasmid pSJ82_TC (175,921 bp). It is evident that hospital-adapted *E. faecium* population dynamics are complicated by the efficient movement of the *vanA* transposon between clones.

The second principal aim of the project was to investigate the prevalence of transferable linezolid resistance genes in LRE recovered from patients in Irish hospitals. One-hundred and fifty-four LRE recovered from patients in 14 Irish hospitals between June 2016 and August 2019 were screened for the *optrA, poxtA* and *cfr* genes by PCR. All isolates harbouring at least one of these genes, and 20 without, underwent Illumina WGS. The *optrA* and/or *poxtA* genes were identified in 35/154 (22.7%) isolates, the highest prevalence of transferable linezolid resistance reported to date. Fifteen isolates with diverse STs harboured *optrA* only; one *E. faecium* isolate harboured *optrA* (chromosome) and *poxtA* (plasmid). Seven *E. faecalis* and one *E. faecium* harboured *optrA* on a 36,331 bp plasmid with 100% identity to the previously described *optrA*-encoding conjugative plasmid pE349. Variations around *optrA* were also observed, with *optrA* located on plasmids in five isolates and within the chromosome in three isolates. Nine *E. faecium* and 10 *E. faecalis* harboured *poxtA*, flanked by IS*1216E*, within an identical 4,001 bp region found on plasmids exhibiting 72.9%–100% sequence coverage to a 21,849 bp conjugative plasmid (pM16/0594) encoding *poxtA*. *Enterococcus faecalis* isolates primarily belonged to ST480, whereas *E. faecium* isolates belonged to diverse STs. Of the remaining 119 linezolid-resistant isolates without linezolid resistance genes, 20 representative isolates investigated all harboured the G2576T 23S RNA gene mutation associated with linezolid resistance. This high prevalence of *optrA* and *poxtA* in diverse enterococcal lineages found in Irish hospitals indicated the presence of significant selective pressure(s) for maintenance.

The third principal aim of the project was to investigate a hospital outbreak of linezolid-resistant VREfm (LVREfm) using WGS. The outbreak occurred in a second Dublin hospital in October 2019. Thirty-nine VREfm from patient screening (19 isolates, 17 patients) and environmental sites (20 isolates) were investigated. Isolates were screened for *optrA*, *poxtA* and *cfr* by PCR and underwent Illumina WGS. One LVREfm underwent hybrid assembly to resolve an *optrA*-encoding plasmid. Twenty isolates (51.3%) were LVREfm and *optrA*-positive, including the LVREfm from the index patient. A closely related cluster of 28 ST80 isolates was identified using cgMLST, including all 20 LVREfm and eight linezolid-susceptible VREfm (including a VREfm from the index patient recovered in October 2018 a year before the outbreak), with an average allelic difference of two (range 0-10), indicating an outbreak. Nineteen (95%) LVREfm harboured a 56,684-bp conjugative plasmid, termed pEfmO_03. The remaining LVREfm isolate exhibited 44.1% sequence coverage to pEfmO_03. Presence of pEfmO_03 in LVREfm and the close relatedness of the outbreak cluster isolates indicated the spread of a single clone. The inclusion of the October 2018 VREfm from the index patient, provided evidence this clone had persisted within the patient's gastrointestinal tract for at least one year prior to the acquisition of pEfmO_03 and the subsequent hospital outbreak. The outbreak was terminated by enhanced infection prevention and control (IPC) and environmental cleaning measures, ceasing ward admissions and ward-dedicated staff. WGS was central in confirming and understanding an outbreak of ST80 LVREfm.

VRE, and more recently LRE, have become a persistent and increasing problem in hospitals in Ireland. Urgent action is required to understand the true burden of MDR enterococci. Ireland is disadvantaged by its sub-optimal hospital infrastructure, especially a paucity of single rooms. Updating current IPC guidelines should be considered to reduce VRE/LRE rates. Implementing admission screening of all patients for VRE/LRE, increasing the frequency of and improving current cleaning regimes, improving hand hygiene compliance in healthcare and improved antimicrobial stewardship both in healthcare and agriculture would contribute significantly to managing this problem. The use of WGS can help to determine if IPC guidelines are effective in minimising/elimination of problematic clones. The main goal should be to halt the further spread of VRE/LRE in Ireland, with a particular focus on controlling these nosocomial pathogens within our already challenged healthcare environment. Additionally, national and international surveillance of VRE/LRE is critical for controlling their spread. WGS for typing has been vital in understanding VRE/LRE population structure along with the creation of a cgMLST scheme, that permits the comparison of clones across countries and continents. Finally, without action increasing levels of both VRE and LRE could lead to an increase in the transfer of antimicrobial resistance genes to other Gram-positive organisms, such as *S. aureus* and/or MRSA.

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Abbreviations

ABC	ATP-binding cassette
AMR	Antimicrobial resistance
BAPS	Bayesian Analysis of Population Structure
BED	Browser extensible data
bp	Base pair
BHIA	Brain Heart Infusion Agar
BHIB	Brain Heart Infusion Broth
BLT	Bead Linked Transposome
BURST	Based Upon Related Sequence Types
BSI	Bloodstream Infection
BWA	Burrows-Wheel Aligner
CA	Community-associated
CC	Clonal complex
CBA	Columbia Blood Agar
CDC	Centers for Disease Control and Prevention
cgMLST	Core genome MLST
cgSNPs	Core genome single nucleotide polymorphisms
cm	centimetre
CT	Complex type
DDD/100 BDU	Defined daily doses per 100 bed days used
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
EARS-Net	European Antimicrobial Resistance Surveillance Network
ECDC	European Centre for Disease Prevention and Control
ECOFFs	Epidemiological Cut-off Values
EDTA	Ethylenediaminetetraacetic acid
EEA	European Economic Area
e.g.	*Exempli graita*; for example
EPM	Enhanced PCR mix
et al.	*Et alia*; and others
etc.	*Et cetera*; and the rest
EU	European Union
EUCAST	European Committee of Antimicrobial Susceptibility Testing

g	Gravitational force
GC	Guanine-Cytosine
GFF	General feature format
GP	General medical practitioner
h	Hours
H	Hospital
HCA	Healthcare-associated
HCAIs	Healthcare-associated infections
HGT	Horizontal gene transfer
HPSC	Health Protection Surveillance Centre
HPV	Hydrogen peroxide vapour
HSE	Health Service Executive
HT1	Hybridization buffer
ICU	Intensive care unit
IPC	Infection prevention and control
IS	Insertion sequence
LRE	Linezolid-resistant enterococci
LREfm	Linezolid-resistant *Enterococcus faecium*
LREfs	Linezolid-resistant *Enterococcus faecalis*
LVREfm	Linezolid-resistant vancomycin-resistant *Enterococcus faecium*
M	Molar
MALDI-TOF-MS	Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry
MDR	Multidrug-resistant
MGE	Mobile genetic element
mg/ml	Milligram per millilitre
mg/L	Milligram per litre
MIC	Minimum inhibitory concentration
min	Minute
ml	Millilitre
MLST	Multilocus sequence type
MLT	Maximum-likelihood tree
Mm	Millimetre
MRSA	Methicillin-resistant *Staphylococcus aureus*
MST	Minimum-spanning tree

N/A	Not applicable or Not available
NAOH	Sodium hydroxide
NJT	Neighbour joining tree
nM	Nanometre
NMRSARL	National MRSA reference laboratory
NT	Non-typeable
ONT	Oxford Nanopore Technologies
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
pH	Potential Hydrogen
$PhLOPS_A$	Phenicol, lincosamide, oxazolidinone, pleuromutilin and streptogramin A resistance
POCT	Point of care testing
R	Resistant
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RSB	Resuspension Buffer
S	Susceptible
SBS	Sequencing by Synthesis
SLV	Single locus variant
SNV	Single nucleotide variation
SPBs	Sample Purification Beads
ST	Sequence type
TAT	Turn-around-time
TB1	Tagmentation Buffer
TBE	Tris-borate/EDTA
TC	Transconjugant
TSB	Tagment Stop Buffer
TWB	Tagment Wash Buffer
UK	United Kingdom
USA	United States of America
v	version
VRE	Vancomycin-resistant enterococci
VREfm	Vancomycin-resistant *E. faecium*
VREfs	Vancomycin-resistant *E. faecalis*

VSEfm	Vancomycin-susceptible *E. faecium*
v/v	Volume over volume
W	Wards
wgMLST	whole genome MLST
w/v	Weight over volume
WGS	Whole-genome sequencing
WHO	World Health Organisation
WT	Wild-type
ZAAPS	Zyvox® Annual Appraisal of Potency and Spectrum programme
µg	Microgram
µl	Microlitre
%	Percentage
ºC	Degrees Celsius
′	Prime
×	Times
>	Greater than
<	Less than
≥	Greater than or equal to
≤	Less than or equal to

Publications

Some of the original work presented in this thesis has been published in international peer-reviewed journals, as listed below. Offprints of the publications are included in the Appendix.

Egan, S.A., Shore, A.C., O'Connell, B., Brennan, G.I., and Coleman, D.C. (2020). Linezolid resistance in *Enterococcus faecium* and *Enterococcus faecalis* from hospitalized patients in Ireland: high prevalence of the MDR genes *optrA* and *poxtA* in isolates with diverse genetic backgrounds. *J. Antimicrob. Chemother.* 75, 1704–1711. doi:10.1093/jac/dkaa075.

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Chapter 1

General Introduction

1.1 Enterococci and their clinical impact

Enterococci are facultative anaerobic Gram-positive cocci, which are capable of surviving and growing under harsh environmental conditions including extreme temperature (10ºC-45ºC), high salt concentrations and at variable pH (4.5-10) (Arias and Murray, 2012). Enterococci also have demonstrated a variable tolerance to chemical disinfectants and biocides used in healthcare settings, including alcohol and chlorhexidine (Bradley and Fraise, 1996; Guzmán Prieto *et al.*, 2017). Thus, enterococci have the ability to persist in the environment for prolonged periods of time, even in settings subjected to regular and rigorous cleaning and disinfection, such as in the hospital environment. Enterococci are commensal bacteria in the gastrointestinal tract of humans, farm and wild animals, birds and ubiquitous in the environment, soil, water, plants, and insects (Arias and Murray, 2012). It was not until the mid 1980s with advances in molecular technology that enterococci were moved from the genus *Streptococcus* into the genus *Enterococcus* (Patterson and Kelly, 1998). To date, 60 enterococcal species have been described (https://www.bacterio.net/genus/enterococcus), with this number increasing regularly with the identification of new species following the advent of high-throughput whole-genome sequencing (WGS) technology (Gilmore *et al.*, 2014; Parte, 2018). *Enterococcus faecium* and *Enterococcus faecalis* are the two species responsible for the overwhelming majority of human enterococcal infections (Gilmore *et al.*, 2013). These two species can cause a wide range of infections including endocarditis, bacteraemia, urinary tract, surgical wound, intra-abdominal and pelvic infections. Healthcare-associated infections (HCAIs) by *E. faecium* and *E. faecalis* are associated with significant morbidity and mortality, increased hospital stays and associated healthcare costs (Arias and Murray, 2012). In the 1970s, *E. faecalis* accounted for 90-95% of clinical enterococcal isolates (Arias and Murray, 2012). Over the past two decades this has changed and *E. faecium* has become the predominant nosocomial enterococcal pathogen worldwide. Surveillance data from Europe has shown a consistently increasing rate of *E. faecium* bacteraemia's with an annual increase of approximately 20% year on year from 2002 (n = 1118) to 2008 (n = 3128) (de Kraker *et al.*, 2013). Vancomycin-resistant *E. faecium* (VREfm) has also been listed as a "high" priority on the World Health Organisation's (WHO) global list of antibiotic-resistant bacteria (World Health Organisation, 2017).

1.2 Antimicrobial resistance mechanisms in enterococci

Antimicrobial resistance in enterococci can be intrinsic or acquired, with the latter being due to either genetic mutations or horizontal transfer of resistance genes from other enterococci or other bacterial species. Enterococci are intrinsically resistant to many classes of clinically relevant antimicrobial agents, including cephalosporins, beta-lactams, aminoglycosides, lincosamides, streptogramins and trimethoprim-sulfamethoxazole combinations (Hollenbeck and Rice, 2012). Acquired resistance to aminoglycosides, glycopeptides, streptogramins, oxazolidinones, daptomycin and tigecyclines has also been reported (Hollenbeck and Rice, 2012; Miller *et al.*, 2014). A multitude of intrinsic and acquired resistance mechanisms have been reported in *E. faecium* and *E. faecalis* (Table 1.1), which has contributed, at least in part, to their nosocomial success. *Enterococcus faecium* and *E. faecalis* can harbour a wide variety of antimicrobial resistance-encoding plasmids including pheromone-responsive plasmids, which can be swiftly disseminated among enterococcal species. These also include broad host range conjugative plasmids, which can be disseminated to and from other bacterial species via horizontal gene transfer (HGT) (Palmer *et al.*, 2010).

Table 1.1 Examples of mechanisms of antimicrobial resistance in *E. faecium* and *E. faecalis*[a]

Resistance to antibiotic classes	Mechanism of resistance	Associated genotype/enzyme	Phenotype	Intrinsic, sporadic or associated MGEs	Host range
Aminoglycosides	Low cell wall permeability	-	Low-level aminoglycoside resistance	Intrinsic	*E. faecalis*
	16S rRNA mutations	-	High-level aminoglycoside resistance with MIC > 128,000 mg/L	Sporadic	*E. faecalis*, *E. faecium*
	Modifying enzyme	AAC(6′)-Ii	Low-level tobramycin and kanamycin resistance	Intrinsic	*E. faecium*
	Modifying enzyme	APH(3′)-IIIa	Low-level kanamycin resistance	pJH1	*E. faecium*
	Modifying enzyme	ANT(4′)-Ia	Low-level resistance to kanamycin, tobramycin, amikacin and neomycin	plP810	*E. faecium*
	Modifying enzyme	APH(2′′)-Ia-AAC(6′)Ie	High-level gentamicin resistance	Tn*5281*	*E. faecalis*, *E. faecium*
	Modifying enzyme	ANT(6′)-Ia	High-level streptomycin resistance Tobramycin	Tn*1546*, Tn*5382,* Inc18 broad-host range plasmid	*E. faecalis*, *E. faecium*
	Ribosome-modifying methyltransferase	*efmM*	Tobramycin and kanamycin resistance	Intrinsic	*E. faecium*

Table 1.1 continued overleaf

Resistance to antibiotic classes	Mechanism of resistance	Associated genotype/enzyme	Phenotype	Intrinsic, sporadic or associated MGEs	Host range
β-lactams and cephalosporins	PBP4/5 production	-	Low-level penicillin resistance; moderate to high-level cephalosporin resistance	Intrinsic	*E. faecalis, E. faecium*
	PBP4/5 point mutation	-	High-level ampicillin and imipenem resistance	Sporadic	*E. faecalis* *E. faecium*
	Altered cell wall	L,D-transpeptidase	β-lactam resistance	Intrinsic	*E. faecium*
Glycopeptides	Terminal D-alanine residue in cell wall modified to D-lactate or D-serine	*vanA*, *vanB*, *vanH*/B, *vanY*/B, *vanX*/B, *vanR*/B, *vanS*/B, vanW	Resistance to vancomycin +/- teicoplanin depending on the phenotype	Tn*1546*, Inc18 broad-host range plasmid	*E. faecalis, E. faecium*
Macrolides, Streptogramins & Lincosamides	ABC-efflux pump	*msrC*	Low-level resistance to streptogramin B	Intrinsic	*E. faecium*
	Acetyltransferase	*vatH*	Streptogramin A resistance	Putative transposon	*E. faecium*
	Acetyltransferase	*vgbA*	Streptogramin B resistance	Unknown	*E. faecium*
	Altered ribosome by methylation	*ermA*	MLS_A phenotype	Tn*554*	*E. faecalis, E. faecium*
	Altered ribosome by methylation	*ermB*	MLS_B phenotype	Tn*917*, Tn*1545*	*E. faecalis, E. faecium*

Table 1.1 continued overleaf

Resistance to antibiotic classes	**Mechanism of resistance**	**Associated genotype/enzyme**	**Phenotype**	**Intrinsic, sporadic or associated MGEs**	**Host range**
Linezolid Oxazolidinones	rRNA point mutations	G2576T, G2505A, L3, L4[b]	Linezolid resistance	Sporadic	*E. faecalis* *E. faecium*
	Methylated rRNA	*cfr*, *cfr(B)* & other *cfr* variants[c]	PhLOPS$_A$ - resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A compounds[b]	pEF-01	*E. faecalis* *E. faecium*
	ABC transporter	*optrA*[d]	Oxazolidinones and phenicols resistance	pE349	*E. faecalis* *E. faecium*
	ABC transporter	*poxtA*[e]	Oxazolidinones, phenicols and tetracyclines resistance	Putative transposon	*E. faecium*
Daptomycin	Altered membrane-bound protein	*gdpD*	Daptomycin resistance, effect is amplified in combination with *liaF* mutation	Sporadic	*E. faecalis,* *E. faecium*
	Altered membrane-bound protein	*liaF*	Daptomycin resistance when combined with *gdpD* mutation	Sporadic	*E. faecalis,* *E. faecium*

[a] Table adapted from Hollenbeck and Rice, 2012 with up-to date information on oxazolidinone resistance from [c]Vester, 2018, [d]Wang *et al.*, 2015 and [e]Antonelli *et al.*, 2018.

[b] G2576T and G2505A common point mutations in the 23S rRNA binding site of the 50S subunit, L3 and L4 represent mutations in ribosomal proteins Abbreviations; MGE, mobile genetic element; PBP, penicillin binding protein; ABC, ATP-binding cassette; MLS$_A$, macrolides, lincosamides, streptogramin A; MLS$_B$, macrolides, lincosamides, streptogramin B.

1.2.1 Vancomycin-resistant enterococci (VRE)

Vancomycin is a glycopeptide antibiotic that is commonly used for the treatment of infections caused by multidrug resistant (MDR) bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA), penicillin-resistant *Streptococcus pneumoniae* and *Clostridium difficile* (Bruniera *et al.*, 2015). Vancomycin acts by binding the D-alanyl-D-alanine terminus of the peptidoglycan precursor to prevent cross-linking of peptidoglycan and thereby inhibiting cell wall synthesis. Vancomycin resistance is acquired in enterococci and is mediated by the production of a modified precursor where the terminal D-alanine is replaced by D-lactate or D-serine (Hollenbeck and Rice, 2012). Seven *van* genes (*vanS*, *vanR*, *vanH*, *vanA*, *vanX*, *vanY* & *vanZ*) encode the enzymes required to generate the modified precursor (Figure 1.1).

Worldwide, vancomycin resistance is more common among *E. faecium* than *E. faecalis*. According to the Centers for Disease Control and Prevention (CDC), 77% of all enterococcal HCAIs resistant to vancomycin in the USA in 2011 were caused by *E. faecium* and just 9% were due to *E. faecalis* (CDC, 2013). In the USA, the CDC reported a 41% decrease in VRE cases between 2013 and 2019 in their annual report on antibiotic resistant threats in the USA (CDC, 2019). In 2019 in Europe, vancomycin resistance among *E. faecium* from bloodstream infections (BSIs) ranged from 0-50% (n = 0-735) compared to 0-8.1% (n = 0-80) for *E. faecalis* (European Centre for Disease Prevention and Control, 2021). In contrast to what has been reported in the USA, Europe has reported an almost two-fold increase of VREfm invasive infections, increasing from 10.5% in 2015 to 18.3% in 2019 (EU/EEA population-weighted mean percentage) (European Centre for Disease Prevention and Control, 2019).

To date, nine vancomycin resistance genotypes (*vanA/B/C/D/E/G/L/M/N*) have been identified and five of these (*vanA/B/D/M/N*) have been reported in *E. faecium* (Werner *et al.*, 2008; Xu *et al.*, 2010; Lebreton *et al.*, 2011). The *vanA* and *vanB* genotypes are the most common vancomycin resistance genotypes associated with human infections and their prevalence varies based on geographical location (Werner *et al.*, 2008; Guzman Prieto *et al.*, 2016). In North America and Europe, *vanA* has predominated among vancomycin-resistant *E. faecium* (VREfm) since the 1980s, while in Australia *vanB* predominates (Bonten *et al.*, 2001; Coombs *et al.*, 2014). More recently, the *vanB* genotype has been reported with increasing frequency in Europe (Freitas *et al.*, 2016; Buetti *et al.*, 2019; Werner *et al.*, 2020; Fang *et al.*, 2021). As *vanA* remains the predominant genotype in Ireland, it will be the main focus of this study.

The *vanA* gene cluster is located on a transposon originally described as Tn*1546*. In addition to the *van* genes, Tn*1546* harbours two transfer-related genes, a transposase (*orf1*) and a resolvase (*orf2*) (Arthur *et al.*, 1993; Hollenbeck and Rice, 2012). Multiple variants of Tn*1546* have been reported among clinical isolates, with five Tn*1546*-like transposons being defined from a large study of VREfm in Denmark (Figure 1.2) (Wardal *et al.*, 2014; Pinholt *et al.*, 2017). Commonly the *vanA* gene complex is located on a plasmid and is acquired by HGT (Hollenbeck and Rice, 2012; Wardal *et al.*, 2014). In contrast, the *vanB* gene complex is commonly integrated in the chromosome within a transposon (Tn*1549*) (Howden *et al.*, 2013).

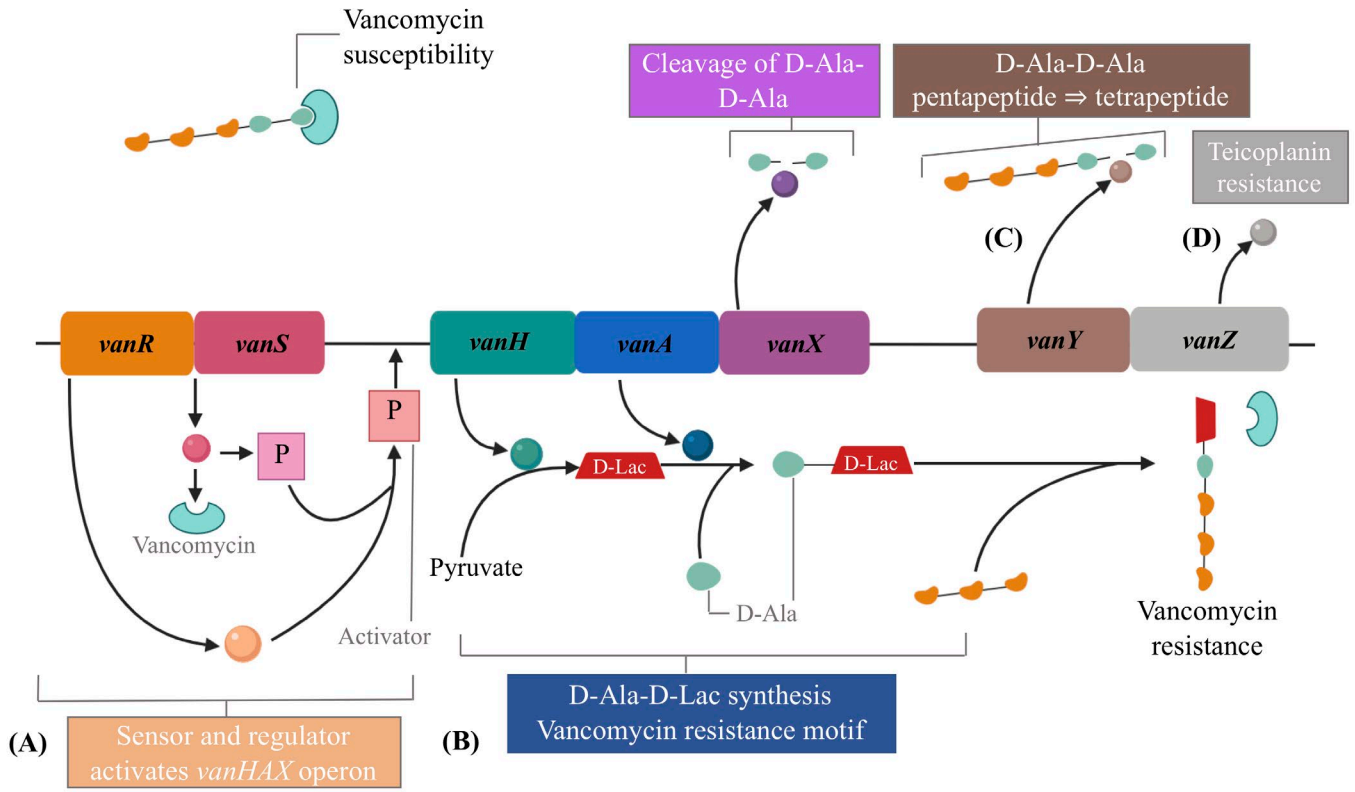


Figure 1.1 Schematic overview of the mechanism of vancomycin resistance mediated by the *vanA* operon. (A) The expression of vancomycin resistance is regulated by a two-component regulatory system; *vanS* encodes a sensor (of vancomycin) that controls the level of phosphorylation of the *vanR* gene, which encodes a transcriptional activator of the operon. (B) The *vanH*, *vanA* and *vanX* genes encode the synthesis of D-Ala-D-Lac i.e. the *vanH* gene encodes a dehydrogenase that reduces pyruvate to D-Lac, *vanA* encodes a ligase that catalyses the formation of an ester bond between D-Ala and D-Lac and *vanX* encodes a dipeptidase that hydrolyses the normal peptidoglycan component D-Ala-D-Ala. (C) The *vanY* gene encodes a D,D-carboxypeptidase that hydrolyses the terminal D-Ala residue of late peptidoglycan precursors that are produced if elimination of D-Ala-D-Ala by *vanX* is not complete. Ultimately, D-Ala-D-Lac replaces the normal dipeptide D-Ala-D-Ala during peptidoglycan synthesis resulting in vancomycin resistance. (D) The *vanZ* gene confers resistance to teicoplanin by an unknown mechanism. Adapted from Hughes, 2003.

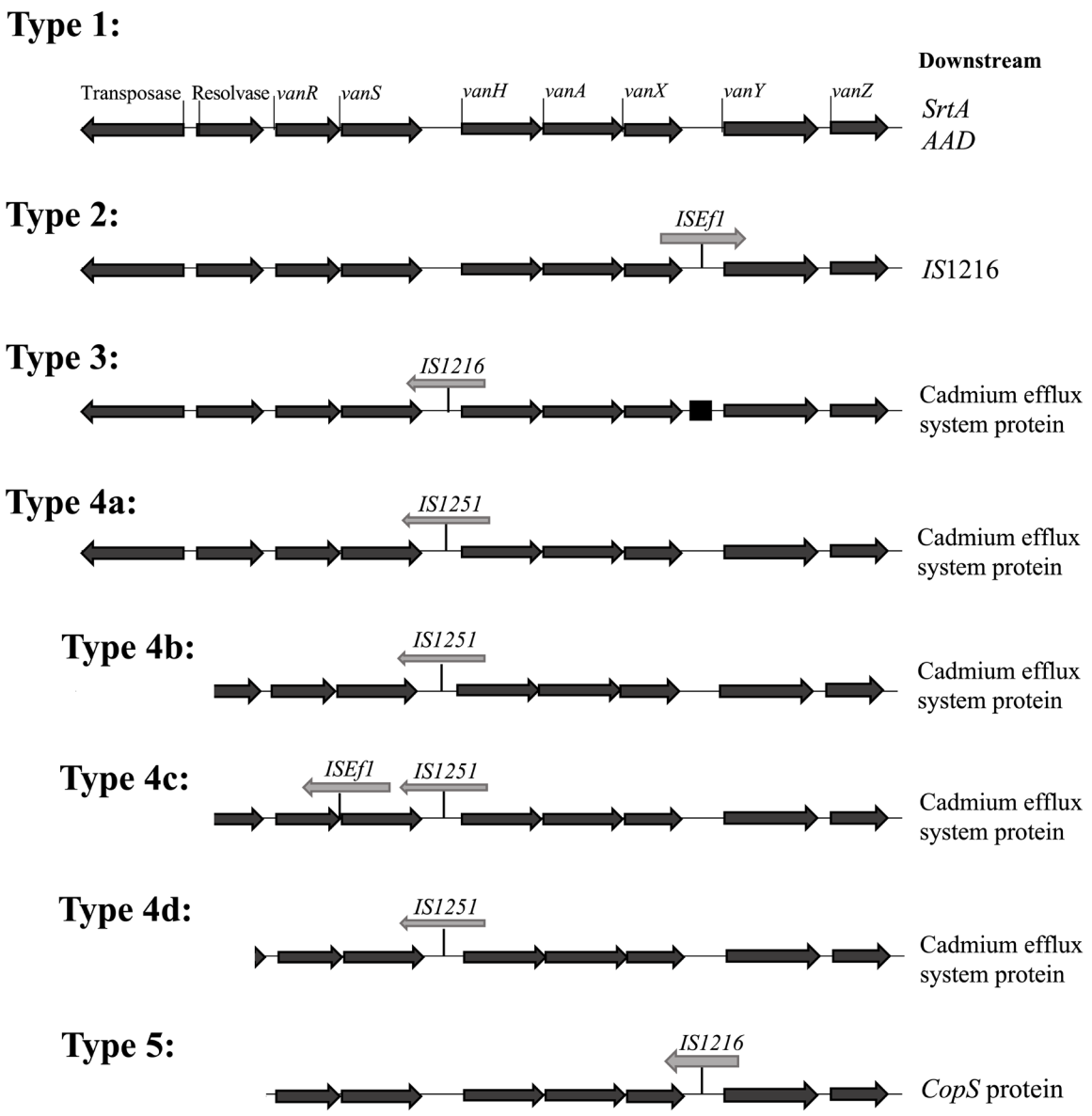


Figure 1.2 Tn*1546*-like transposon types identified among 493 *vanA*-positive vancomycin-resistant *E. faecium* isolates recovered from patients in hospitals in Copenhagen, Denmark between 2012 and 2014. Type 1 was identical to the Tn*1546* prototype characterised previously by Arthur *et al.* (1993). The positions of the transposase (*orf1*), resolvase (*orf2*) and *van* genes and their direction of transcription are depicted using black arrows. Grey arrows represent IS elements and their direction of transcription. The filled black box in Type 3 represents a sequence in the transposon that is not identical to the prototype. Adapted from Pinholt *et al.*, 2017.

1.2.2 Linezolid-resistant enterococci (LRE)

Linezolid is an oxazolidinone antibiotic first approved for clinical use in 2000 and is considered a drug of "last resort" for the treatment of infections caused by MDR Gram-positive bacteria, including VRE. Resistance to linezolid was first reported during initial clinical trials and later in a hospital-acquired VREfm isolate in Greece in 2004, just four years after its approval for clinical use (Gonzales *et al.*, 2001; Bersos *et al.*, 2004; Zahedi Bialvaei *et al.*, 2017). Linezolid inhibits bacterial protein synthesis by binding within the peptidyl transferase centre in the V domain of the 23S rRNA component of the 50S ribosomal subunit and inhibits transfer of the aminoacyl-tRNA to the A site. This prevents formation of the initiation complex and consequently inhibits protein synthesis (Swaney *et al.*, 1998). Linezolid partially shares its binding site with phenicols, lincosamides, pleuromutilins and streptogramin A compounds, all of which also inhibit protein synthesis (Long and Vester, 2012). Linezolid resistance in enterococci is primarily encoded by (i) mutations in the 23S rRNA binding site of the 50S subunit or mutations in the ribosomal proteins L3 and/or L4 and/or (ii) acquisition of the commonly plasmid-located and multidrug resistance-encoding methyltransferase gene *cfr,* an ATP-binding cassette (ABC) transporter gene *optrA* and/or the novel ABC-F transporter gene *poxtA* (Table 1.1) (Wang *et al.*, 2015; Antonelli *et al.*, 2018; Bi *et al.*, 2018). The most frequently reported mechanism of linezolid resistance in enterococci involves mutations of the 23S rRNA gene, most commonly a G2576T transition, which is often reported following linezolid exposure (Bender *et al.*, 2018a; Bi *et al.*, 2018). Although the numbers of *E. faecium* and *E. faecalis* isolates reported to carry the mobile linezolid resistance genes *cfr, optrA* and/or *poxtA* is low, recently *optrA* in particular, has been reported with increased frequency (Mendes *et al.,* 2016, Mendes *et al.*, 2018). Transmission of linezolid-resistance by HGT is a concern, particularly its transmission to VREfm, which would give rise to, new linezolid- and vancomycin-resistant *E. faecium* (LVREfm) derivatives, for which treatment options would be very limited.

1.2.2.1 Resistance to oxazolidinones and phenicols by acquisition of optrA

The *optrA* gene encodes an ABC-F protein and mediates resistance through target protection (Sharkey and O'Neill, 2018). First detected in an *E. faecalis* of human origin, it has since been also reported in *E. faecium*, along with *S. aureus*, *Staphylococcus sciuri* and *Streptococcus suis* (Cai *et al.*, 2015; Wang *et al.*, 2015; Huang *et al.*, 2017; Guo *et al.*, 2018). Surveillance studies from China have also shown that *optrA* is detected more frequently in enterococci from food-producing animals, such as pigs, than in humans

(Wang *et al.*, 2015). Since its discovery, *optrA* has been noted with increased frequency in clinical isolates. In 2014 the Zyvox® Annual Appraisal of Potency and Spectrum (ZAAPS) programme, which monitors the *in-vitro* activity of linezolid in Gram-positive clinical infection isolates from across the globe, reported that 3/9 (33.3%) of linezolid non-susceptible isolates with a linezolid minimum inhibitory concentration (MIC) ≥ 4 mg/L, were *optrA*-positive *E. faecalis* isolates; two of the three *optrA*-positive isolates were from Ireland. The prevalence of linezolid-resistant Gram-positive organisms increased to 17 isolates according to ZAAPS in 2016. Of which 9 (52%) were *optrA*-positive isolates, 8 of these were *optrA*-positive *E. faecalis* (Mendes *et al.,* 2016, Mendes *et al.*, 2018). A large reference centre in Germany retrospectively screened 698 linezolid-resistant enterococci recovered between 2007 and 2017 for *optrA* and found 43 *optrA*-positive isolates (first *optrA*-positive isolate identified in 2007), including 25 *E. faecalis* and 18 *E. faecium* isolates and also demonstrated the conjugative transfer of *optrA* encoding plasmids between enterococci of the same and different species (Bender *et al.*, 2018b).

1.2.2.2 Phenicol–oxazolidinone–tetracycline resistance by acquisition of poxtA

The novel phenicol–oxazolidinone–tetracycline resistance gene *poxtA* was first identified in a MRSA isolate in Italy in 2015 and was subsequently found in an *E. faecium* isolate from porcine faeces, from an Italian farm which previously used florfenicol (Antonelli *et al.*, 2018; Brenciani *et al.*, 2019). Similar to *oprtA*, *poxtA* has also been found in *E. faecium* and *E. faecalis* isolates recovered from both food-producing animals (pig faeces and cow's milk) and from isolates of human origin (urine and rectal screening). The *poxtA* gene is often encoded on conjugative plasmids (Elghaieb *et al.*, 2019; Hao *et al.*, 2019; Papagiannitsis *et al.*, 2019; Freitas *et al.*, 2020). A recent unique finding in a *poxtA*-positive *E. faecium* isolated from pig faeces, demonstrated its ability to form small translocatable elements (TEs) when flanked on either side by IS*1216E* (Shan *et al.*, 2020). This arrangement is advantageous for the transmission of *poxtA* as forming this TE allows the movement of *poxtA* around the genome, permitting the translocation of *poxtA* to a conjugative plasmid(s) to aid its dissemination to new strains.

1.2.2.3 Acquisition of cfr and its variants

The resistance phenotype mediated by the Cfr methyltransferase is commonly referred to as PhLOPS$_A$, an acronym for resistance to the phenicol, lincosamide, oxazolidinone, pleuromutilin and streptogramin A antimicrobials (Table 1.1) (Long *et al.*, 2006). Variants

of the *cfr* gene, termed *cfr*-like variants, have been described and it has been suggested that a >20% amino acid sequence identity difference to the original *cfr* gene would signify a new variant, denoted by a single letter alphabetic extension e.g. *cfr(B), cfr(C)* etc. (Vester, 2018). As of January 2021, *cfr* variants reported include *cfr(B), cfr(C), cfr(D)* and *cfr(E)* (Vester, 2018; Guerin *et al.*, 2020; Stojković *et al.*, 2020). To date *cfr, cfr(B) and cfr(D)* have been reported in *E. faecium* (Bender *et al.*, 2016; Lazaris *et al.*, 2017; Guerin *et al.*, 2020), whereas only *cfr* has been reported in *E. faecalis* (Diaz *et al.*, 2012). The *cfr* gene is more commonly reported in staphylococci than enterococci, but it is important to note that the transfer of plasmid-borne *cfr* has been demonstrated to occur even across bacterial species and genera (Bender *et al.*, 2016; Lazaris *et al.*, 2017). In this regard enterococci may act as a reservoir for the spread of linezolid resistance across species, most concerningly to staphylococci, and especially to MRSA.

1.3 The enterococcal genome

The genomes of the clinical enterococcal species of interest in this study *E. faecium* and *E. faecalis* were examined *in silico* for genome size, %GC content and estimated protein encoding sequences. *Enterococcus faecium* is reported to have a median genome size of 2.92 Mb, exhibits approximately 37.8% GC content and includes a median of 2724 protein-encoding sequences (https://www.ncbi.nlm.nih.gov/genome/?term=Enterococcus +faecium). Similarly, *E. faecalis* exhibits a median genome size of 2.97 Mb, has a GC content of ~37.4% and includes a median of 2762 protein-encoding sequences (https://www.ncbi.nlm.nih.gov/genome/?term=enterococcus+faecalis).

1.3.1 Recombination

Enterococci (in particular *E. faecium* and *E. faecalis*) are characterised by their malleable genomes and high recombination rate. It has been reported that up to 44% of the *E. faecium* genome is affected by recombination, primarily due to acquisition of foreign DNA, while around 25% of the *E. faecalis* genome consists of mobile and exogenously acquired DNA (de Been *et al.*, 2013; Howden *et al.*, 2013; Ramos *et al.*, 2020). Recombination is defined as the transfer of DNA from one bacterium to another and includes both the acquisition of new genes as well as the replacement of existing genes by different allelic variants of the same gene (homologous recombination) (Martin and Beiko, 2010). The core genome can be defined as essential genes encoding housekeeping functions vital for survival and is less affected by recombination compared to accessory genes encoding non-essential functions (Martin and Beiko, 2010). Recombination which

occurs in the core genome commonly occurs in "hotspots" where some regions exhibit frequent changes, whereas other regions show little to no recombination events (Martin and Beiko, 2010; de Been *et al.*, 2013; Howden *et al.*, 2013). Thus *E. faecium* and *E. faecalis* have a primarily stable core genome and a highly flexible accessory genome, the latter of which enables the organisms to adapt readily to new environmental stresses and challenges.

1.3.2 Mobile genetic elements and horizontal gene transfer

Dissemination of drug resistant enterococci and resistance genes they harbour occurs by clonal expansion and by HGT involving mobile genetic elements (MGEs) (Werner *et al.*, 2008; Hegstad *et al.*, 2010; Huddleston, 2014). Studies have shown the human GI tract serves as a significant reservoir for MGEs encoding resistance genes, with evidence in VREs that *vanB* can be acquired via HGT from anaerobes in the GI tract (Howden *et al.*, 2013; Huddleston, 2014). The GI tract environment is an ideal place for exchange of resistance genes, due to the abundance of other microbes and MGEs, which provides opportunities for acquisition or exchange of resistance genes between enterococci and other species (Huddleston, 2014). The predominant MGEs include plasmids, transposons, insertion sequence (IS) elements and bacteriophages, which provide vehicles for transfer of DNA within the bacterial cell and from one bacterium to another (Werner *et al.*, 2008; Hegstad *et al.*, 2010; Huddleston, 2014). These MGEs encode genes involved in their own mobilization. HGT via MGEs plays a significant role in the ability of a bacterium to adapt to host and environmental challenges, as genes carried on MGEs are mainly antibiotic resistance genes and virulence-associated genes (Martin and Beiko, 2010).

The main MGEs of interest in the present study are plasmids, transposons and IS elements. Plasmids are extrachromosomal, circular double stranded DNA molecules capable of controlling their own replication (Norman *et al.*, 2009). The size of plasmids described in *E. faecium* and *E. faecalis* ranges from a few kb up to around 250 kb (Hegstad *et al.*, 2010; Palmer *et al.*, 2010; Sadowy, 2018). Some plasmids are conjugative and encode genes for transfer machinery, allowing the formation of a mating bridge between donor and recipient bacterial cells, which facilitates plasmid transfer horizontally (Figure 1.3). Some non-conjugative plasmids encode mobilization genes, but have to use the conjugation machinery encoded on conjugative plasmids to facilitate plasmid transfer (Norman *et al.*, 2009). Transposons or transposable elements are sequences of DNA that can change their location within a genome or insert copies of themselves into another part of the genome. Along with genes responsible for mobilization, most transposons encode

accessory genes such as antibiotic resistance genes (Werner *et al.*, 2008; Martin and Beiko, 2010). The *vanA* transposon Tn*1546* belongs to the Tn*3* transposon family (Arthur *et al.*, 1993). Tn*3* family transposons mobilize DNA within and between the chromosome and plasmids within a single bacterial cell (Werner *et al.*, 2008). Lastly, IS elements are small pieces of DNA between 700-2500 bp that only contain genes involved in the mobilization of the element (Martin and Beiko, 2010). There is evidence that identical IS elements flanking an antimicrobial resistance gene can loop out and form small TEs, which can move location within a single bacterial cell (Shan *et al.*, 2020).

Horizontal gene transfer occurs by three predominant mechanisms; (i) natural transformation which involves the direct uptake of foreign or exogenous DNA by a bacterial cell from the environment and integration into the bacterial genome (Figure 1.3 (A)) (Martin and Beiko, 2010; Huddleston, 2014), (ii) phage transduction, which involves the transfer of DNA via a bacteriophage (Figure 1.3(B)) and (iii) conjugation, which is the transfer of plasmids or conjugative transposons from on bacterial cell to another by direct cell-to-cell contact via a conjugative pilus or mating bridge (Figure 1.3(C)). Non-conjugative plasmids can also be transferred to other bacterial cells in the presence of a conjugative plasmid if a bridge is formed by a conjugative pilus (Martin and Beiko, 2010; Huddleston, 2014).

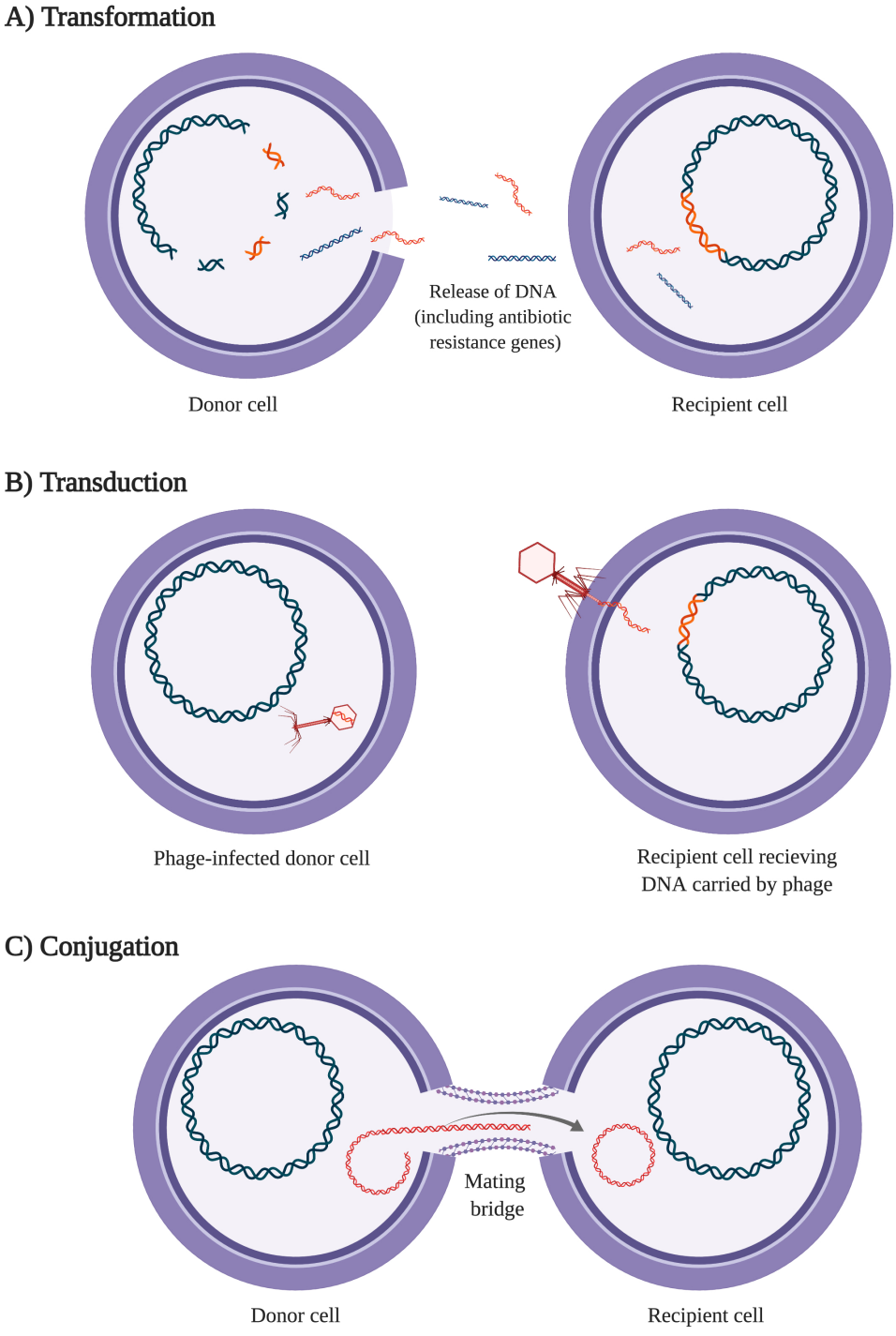


Figure 1.3 Horizontal gene transfer between bacteria. (A) Transformation occurs when naked DNA is released into the environment and picked up by another competent bacterial cell. (B) Transduction occurs when fragments of DNA are transferred between cells via bacteriophage. (C) During conjugation, bacterial cells make direct contact and a mating bridge is formed through which DNA is exchanged. Modified from Furuya and Lowy, 2006.

1.4 Typing of E. faecium and E. faecalis

The first widely used molecular typing method employed to investigate the relatedness of VRE isolates recovered during outbreaks was macrorestriction digestion of genomic DNA with restriction endonucleases that cleave DNA infrequently followed by pulsed-field gel electrophoresis (PFGE) of the resulting DNA fragments in the early 1990s (Patterson and Kelly, 1998). Although PFGE was considered to be the gold standard for typing *E. faecium* (particularly in outbreak situations), it is a very time-consuming procedure, involves the analysis of undefined genetic variation and data interpretation is subjective and lacks standardisation. In addition, the extent of genome plasticity in the enterococci, which results in a high degree of DNA banding pattern polymorphisms among strains, complicates the interpretation of isolate banding patterns, particularly with *E. faecium* isolates from hospital-associated outbreaks (Pinholt *et al.*, 2015).

During the 2000s, multilocus sequence typing (MLST) schemes were introduced for *E. faecium* and *E. faecalis* (Homan *et al.*, 2002; Ruiz-Garbajosa *et al.*, 2006a), which involve DNA sequencing of PCR-amplified internal fragments of seven housekeeping genes of each species. The different sequences of each housekeeping gene are assigned distinct allele numbers and the alleles at each of the loci define the allelic profile or sequence type (ST). Clonal complexes (CC) are defined as clusters of related STs differing from at least one other ST within the CC in no more than two of the seven loci and are considered to be descended from a recent common ancestor (Ruiz-Garbajosa *et al.*, 2006a). The main advantages of MLST compared to PFGE are that it is a DNA-sequencing based method with standardised methods and data interpretation. Although MLST has been used for both short-term/outbreak and long-term/global VREfm and vancomycin-resistant *E. faecalis* (VREfs) epidemiological investigations, it is more suited to the latter due to the stability of housekeeping genes and lacks the discrimination required for short-term/outbreak investigations. (Homan *et al.*, 2002; Pinholt *et al.*, 2015). Deletions of one of the MLST housekeeping genes in *E. faecium*, *pstS,* has also been reported, rendering some isolates non-typeable by this method (Carter *et al.*, 2016; van Hal *et al.*, 2018). More recent WGS studies have highlighted the poor resolution powers of both PFGE and MLST for typing of VRE due to high levels of recombination associated with these organisms (de Been *et al.*, 2015; Guzman Prieto *et al.*, 2016; Raven *et al.*, 2016; Pinholt *et al.*, 2017).

1.4.1 Whole-genome sequencing for typing of *E. faecium* and *E. faecalis*

Since the release of the first high-throughput sequencing platform by Illumina in the mid-2000s, the technology has continued to evolve, with increasing capacity and also has become more accessible and affordable. Second generation WGS, or short-read sequencing approaches, fall under two broad categories i.e. sequencing by ligation and sequencing by synthesis (SBS). One of the most widely used platforms, and one which has been widely used in bacterial typing studies, is the Illumina MiSeq platform, which is based on SBS technology (Figure 1.4). Illumina's platform works by using solid-phase bridge amplification where fragmented DNA is ligated to adapter sequences and bound to a primer immobilised on a patterned flow cell. The free end of the strand can interact with other primers nearby, forming the "bridge" structure. PCR is used to create a second strand from this immobilised fragment (Figure 1.4(A)). After several rounds of amplification 100-200 million clonal clusters are formed and the complimentary reverse strands are washed away. To sequence these clusters, a mixture of all four individually labelled and 3´-blocked deoxyribonucleotide triphosphates (dNTPs) are added. After the incorporation of a single dNTP to each elongating complementary strand, unbound dNTPs are removed, and the surface is imaged to identify which dNTP was incorporated at each cluster (Figure 1.4(B)) (Goodwin *et al.*, 2016; Illumina, 2018). Following an Illumina sequencing run, two (paired end reads) FASTQ files are generated for each sample included in the library. These files contain all sequence read and quality data associated with the sample (Quainoo *et al.*, 2017).

WGS has transformed our understanding of the evolution and molecular epidemiology of microorganisms, particularly for organisms with highly variable genomes, such as enterococci. This is because it allows mapping of genome-wide variation, thereby providing the optimal resolution to infer phylogenetic relatedness and to identify possible, probable, or unlikely cases of epidemiological linkage of isolates.

1.4.1.1 Core-genome MLST and further typing methods

The development of a core-genome MLST (cgMLST) scheme for *E. faecium,* which involves the comparative sequence analysis of 1,423 stable single-copy coding genes/alleles found on the chromosome of multiple *E. faecium* isolates, has allowed for rapid and discriminative interpretation of WGS typing data of VREfm from hospital outbreaks and has been shown to be effective at tracking trends in predominant VREfm clones over time (de Been *et al.*, 2015). Using cgMLST, it was deemed that isolates with ≤20 allele differences are likely to be epidemiologically linked (de Been *et al.*, 2015;

Hammerum *et al.*, 2017), although this threshold has to be considered together with available epidemiological data, and higher allelic differences (21-40 alleles) should also be considered (de Been *et al*., 2015).

In addition to cgMLST, whole-genome MLST (wgMLST) and single nucleotide variation (SNV) analysis can be used to analyse and interpret WGS data. For wgMLST allelic differences within the stable genes of the core genome and stable genes in the accessory genome are examined, whereas SNV analysis generally involves the analysis of genome wide changes at a single nucleotide base level, excluding indels (genome insertions and deletions). At the beginning of this study, no cgMLST scheme existed for *E. faecalis*, but a wgMLST scheme was released in 2018 (BioNumerics, 2018). At the end of 2019, a cgMLST scheme for *E. faecalis* consisting of 1,972 gene targets was released (Neumann *et al.*, 2019). Due to the highly recombining nature of the enterococcal genome, SNV analysis alone is not sufficient for investigating an outbreak. However cgMLST or wgMLST has been shown to give the same results as core-genome SNVs (both outbreak and over time), but require less computational infrastructure (de Been *et al.*, 2015; Raven *et al.*, 2016).

Further discrimination of VREfm and VREfs isolates can be achieved by comparative sequence analysis of MGEs present, in particular those harbouring antimicrobial resistance genes, as the same transposon or plasmid can spread among multiple strains (Zhou *et al.*, 2018). For example, in Denmark, it was found by using WGS data obtained from the Illumina platform, that 82% of VREfm (495 isolates) harboured the same *vanA* plasmid (Pinholt *et al.*, 2017). This plasmid was highly similar to a plasmid originally reported in the USA indicating the possibility that this predominant *vanA* plasmid had spread between two continents (McKenney *et al.*, 2016). This provided evidence that the successful nosocomial transmission of VREfm is due to the spread of a promiscuous and dominant plasmid/transposon throughout a polyclonal *E. faecium* population followed by clonal expansion and spread of the resulting VREfm.

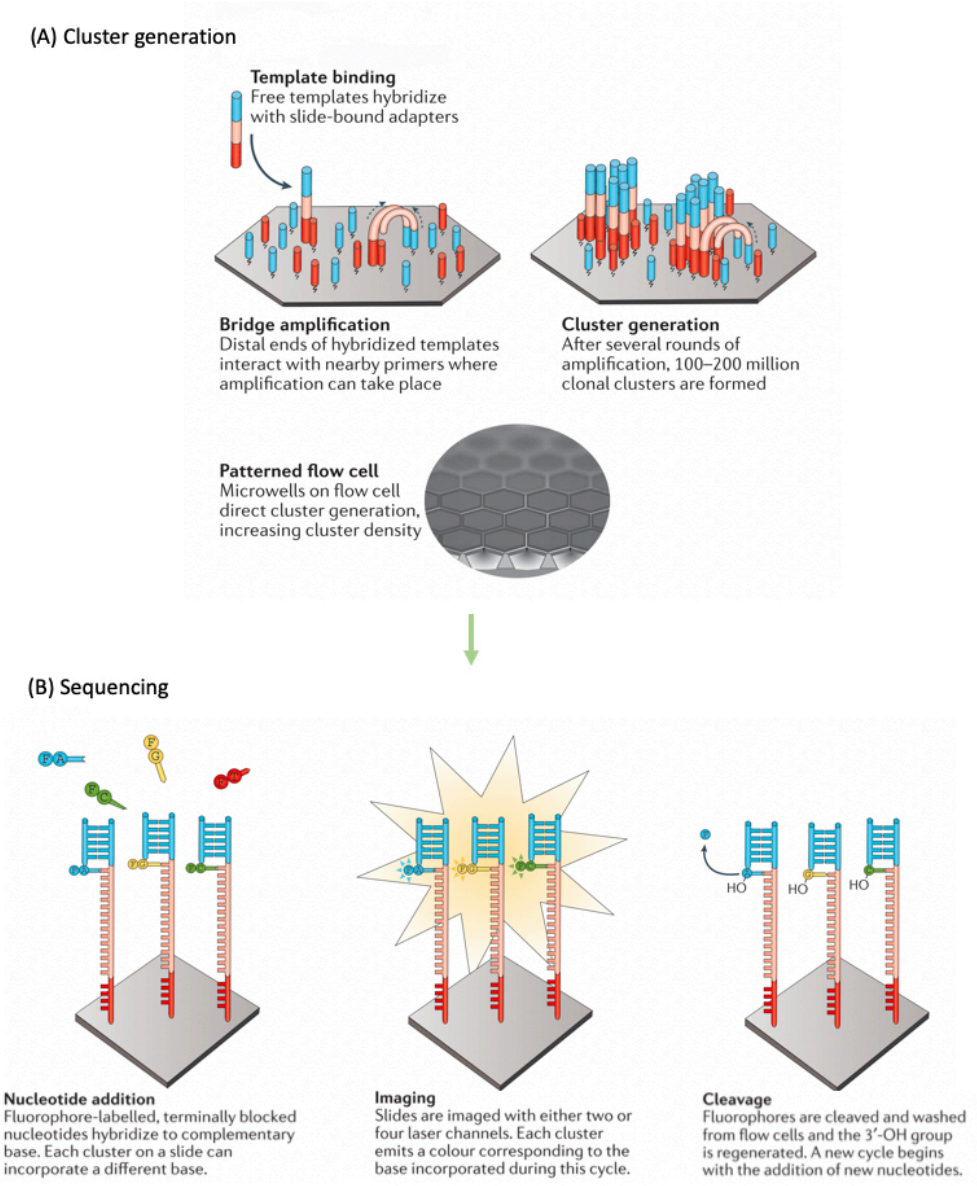


Figure 1.4 Overview of sequencing by synthesis technology used by Illumina sequencing platforms. (A) Cluster generation occurs by solid-phase bridge amplification, fragmented DNA is ligated to adapter sequences and bound to a primer immobilised on a solid support i.e. Illumina's patterned flow cell. The free end can interact with other nearby primers, forming a bridge structure. PCR is used to create a second strand from the immobilized primers, and unbound DNA is removed. (B) Sequencing begins after completion of cluster generation, a mixture of primers, DNA polymerase and modified nucleotides are added to the flow cell. Each nucleotide is blocked by a 3′-O-azidomethyl group and is labelled with a base-specific, cleavable fluorophore. During each cycle, fragments in each cluster will incorporate just one nucleotide as the blocked 3′ group prevents additional incorporations. After base incorporation, unincorporated bases are washed away, the slide is imaged, and the colour identifies which base was incorporated in each cluster. The dye is then cleaved and the 3′-OH is regenerated and the cycle of nucleotide addition, elongation and cleavage can then begin again. Adapted from Goodwin, McPherson and McCombie, 2016.

1.4.2 Third generation sequencing (MinION)

Third-generation sequencing, also known as long-read sequencing, works by base calling at a single nucleotide level from a single long strand of DNA. In contrast to second generation methods, such as Illumina's SBS, third generation sequencing reads nucleotides at the single molecule level and does not require the fragmentation of DNA. (Oxford Nanopore Technology, 2015; Goodwin *et al.*, 2016). The two main third generation sequencing technologies are Pacific Biosciences (PacBio), which is expensive and not readily accessible, and Oxford Nanopore Technology (ONT) which is more accessible and cost efficient (Goodwin *et al.*, 2016). The ONT technology works via a device which passes an ionic current through nanopores (a nano-scale hole) which is located on a flowcell. The device measures the changes in current as biological molecules pass through the nanopore or near it. The information about the change in current can be used to identify that molecule i.e a single nucleotide base (Oxford Nanopore Technology, 2015). Currently third-generation sequencing such as the ONT is error prone in comparison to Illumina's MiSeq platform, with error rates of ~30% per base call and 0.1% in >75% of base calls, respectively (Quainoo *et al.*, 2017). The quality of ONT sequencing is improving year-on-year.

1.4.2.1 Hybrid assembly

Assembly using short reads (Illumina MiSeq) into contiguous sequences, known as contigs is often performed. Use of short reads alone can produce a genome across many contigs, as there are difficulty resolving repetitive genomic regions, such as insertion sequences. Hybrid assembly using a combination of both short reads and long reads (ONT) can be used to overcome this and resolve a genome sequence or close gaps remaining in a MGE of interest following *de novo* assembly of short read sequences. Unicycler, a command line hybrid assembly tool, first assembles the short reads into an accurate and connected assembly graph using the SPAdes *de novo* assembler software (Bankevich *et al.*, 2012) (Figure 1.5(A)). The SPAdes algorithm looks for pairs of overlapping short reads and combines the corresponding sequence to create a longer contiguous sequence, often referred to as a contig. Unicycler then uses long reads to find the best solution to bridge the gaps present in the assembly (Figure 1.5(B)). By following a short-read-first approach, it effectively uses low quantities of long reads, leading to lower misassembly rates and lower error rates. Unicycler finishes by using Bowtie2 and Pilon software to correct errors in the assembly or "polish" by using short-read alignments, reducing the rate of small errors (Figure 1.4(C)) (Langmead and Salzberg, 2012; Walker *et al.*, 2014; Wick *et al.*, 2017).

Hybrid assembly has permitted more effective closure of genome gaps left following second generation sequencing, and this approach has been shown to be particularly useful for accurately determining the genetic organisation of MGEs including VRE transposons (Zhou *et al.*, 2018).

1.4.2.2 Data analysis following hybrid assembly

Third generation sequencing data needs to be processed from raw reads to assembly and analysed using command line bioinformatic tools. As third generation sequencing is more expensive (cost per isolate) than second generation sequencing, a selection of isolates can be chosen to create representative hybrid assemblies to be used as references for downstream analysis. Analysis can then be upscaled by the use of a number of software tools e.g. Burrows-Wheel Aligner (BWA), SAMTools and bedtools. The BWA tool (Li and Durbin, 2009) is a suite of command line tools which can be used to efficiently align short sequencing reads against the hybrid reference sequences created, allowing mismatches and gaps to be identified. It writes the output in sequence alignment map (SAM) format which is a text-based format for storing biological sequences aligned to a reference and is compatible with many other tools. SAMTools is another suite of command line tools that can be used to separate the alignment step from further downstream analyses, such as SNV calling. (Li *et al.*, 2009; Li, 2011). Following this, SNV calling can be performed on specific regions e.g. a plasmid or transposon, to examine evolutionary changes. Bedtools (Quinlan and Hall, 2010) is a suite of utilities for common operations on genomic features, such as comparison, manipulation and annotation in browser extensible data (BED) and general feature format (GFF) format. The coverageBed tool within Bedtools summarises the depth and breadth of coverage of features in one BED file relative to another and this is useful in screening large numbers of isolates for a particular plasmid or transposon and outputs a percentage similarity of this element to the chosen hybrid reference. Bedtools also allows for a more detailed look at the coverage by providing a GFF annotation file from which the depth and breadth of coverage of each gene can be calculated (Quinlan and Hall, 2010).

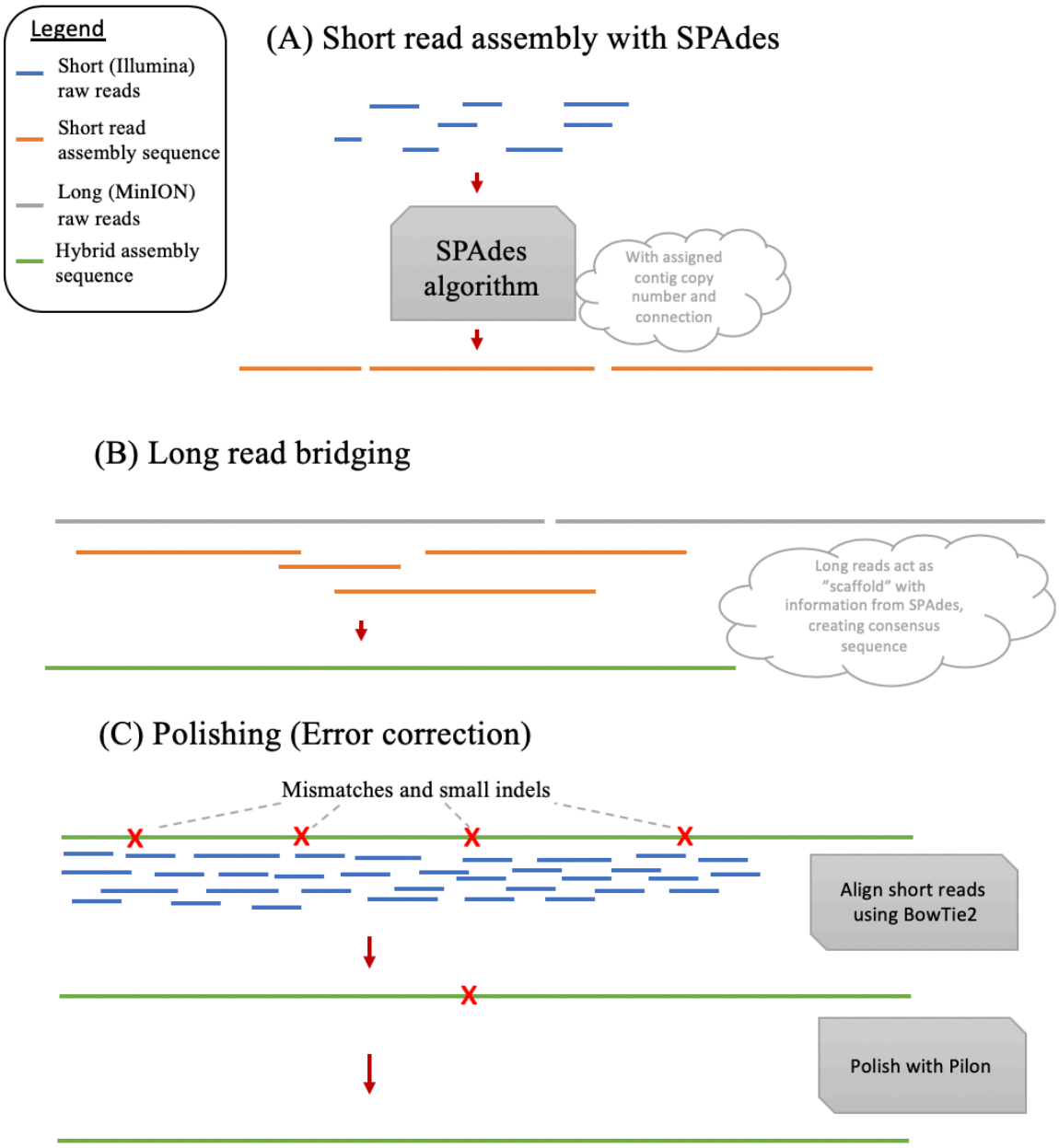


Figure 1.5 Basic overview of key steps in the Unicycler hybrid assembly process. (A) Short reads are first assembled using SPAdes *de novo* assembler (Bankevich *et al.*, 2012) and the value of repeating contigs is then determined for each contig and a feasible assembly graph is created. (B) Using the information from the SPAdes assembly, long reads that align to multiple single-copy contigs can be used for further bridging. Quality scores are then applied to bridges based on many factors including the number of reads confirming this bridge structure. (C) Contigs are merged as consensus sequence and are corrected for mismatched and small indels using BowTie2 and Pilon (Langmead and Salzberg, 2012; Walker *et al.*, 2014). Adapted from Wick *et al.*, 2017.

1.5 Evolution and population structure of hospital-associated enterococci

1.5.1 Separation of clades noted in *E. faecium*

Early studies of the population structure of *E. faecium* were based on MLST and the Based Upon Related Sequence Types (BURST) algorithm and revealed a distinct subpopulation of *E. faecium* designated CC17, where ST17 was the predicted founder. Isolates of CC17 are characterised by ampicillin and quinolone resistance and the presence of a putative pathogenicity island harbouring the enterococcal surface protein gene (*esp)*, a virulence factor that plays a vital role in biofilm formation and is present in the majority of isolates. Isolates belonging to CC17 were found to be globally dispersed and the vast majority of healthcare-associated (HCA) *E. faecium* isolates were associated with this clonal complex (Willems *et al.*, 2005; Top *et al.*, 2008). Later it was reported that BURST- and MLST-based typing were inaccurate in species with high levels of recombination such as *E. faecium* (Willems and van Schaik, 2009; Galloway-Peña *et al.*, 2012; Pinholt *et al.*, 2015).

The use of Bayesian analysis of the *E. faecium* population structure (BAPS) using software on publicly available MLST whole-genome sequence data revealed a division between HCA, and community-associated (CA) *E. faecium* isolates termed clade A and clade B, respectively (Galloway-Peña *et al.*, 2012; Willems *et al.*, 2012; Lebreton *et al.*, 2013). It has been estimated that the two clades diverged from each other more than 300,000 years ago (Galloway-Peña *et al.*, 2012). Further WGS analysis using cgSNVs showed that *E. faecium* belonging to clade A underwent a second evolutionary split more recently (approximately 75 years ago) with the newly termed clade A1 consisting mainly of clinical isolates and clade A2 mostly containing animal-derived isolates (Lebreton *et al.*, 2013) (Figure 1.6(A)). Confirmation of this divide was further demonstrated using WGS data and a combination of MLST, BAPS and SNV analysis on patient bloodstream infection isolates and isolates obtained from livestock from the United Kingdom (Gouliouris *et al.*, 2018). Multiple studies have also shown that the prevalence of MGEs (plasmids, transposons and IS elements) is significantly higher in isolates from clade A1 in comparison to isolates from clade B, indicating the MGEs and accessory genes play a vital role in the adaptation of *E. faecium* to the hospital environment (Figure 1.6(B)) (Leavis *et al.*, 2006; Lebreton *et al.*, 2013; Wurster *et al.*, 2016).

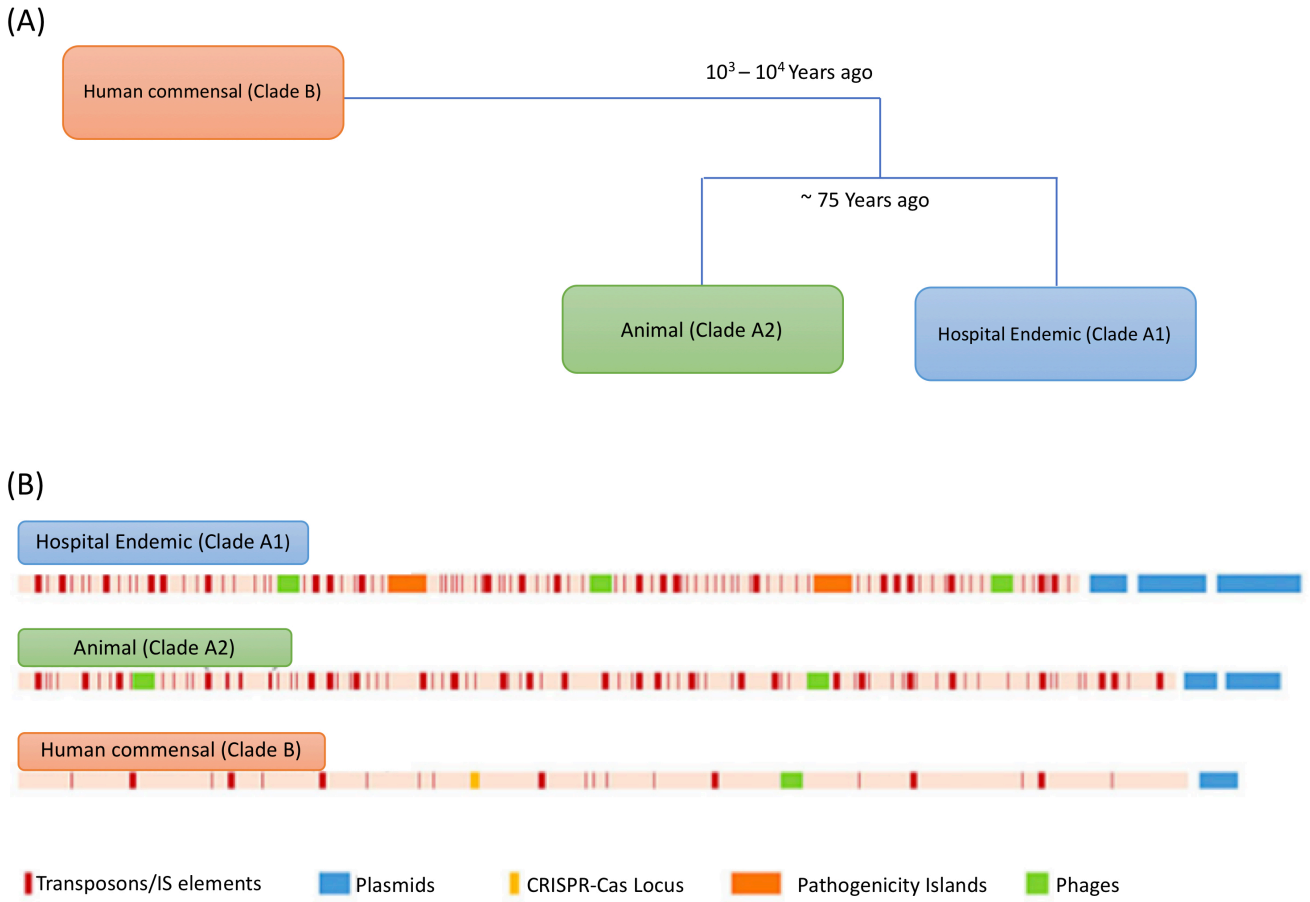


Figure 1.6 Population structure of *E. faecium.* (A) Phylogenetic tree of *E. faecium* isolates demonstrating the timeline of the split between clade A1 (hospital associated), clade A2 (animal associated) and clade B (human commensal isolates). (B) A graphical representation of a genome belonging to each of the clades (A1, A2 and B) highlighting the increased presence of mobile genetic elements (MGEs) in clade A1 hospital isolates in comparison to clade A2, with clade B showing significantly lower levels of MGEs. Adapted from Wurster *et al.*, 2016.

The division of clade A1 and clade A2 is still under debate, as this distinction is not always corroborated when larger collections have been used (Raven *et al.*, 2016; Arredondo-Alonso *et al.*, 2020; Rios *et al.*, 2020; Freitas *et al.*, 2021). It is evident that healthcare-associated *E. faecium* emerged from an ancestor lineage linked to animals which diverged prior to clonal expansion of human clinical clades, with the hospital-associated populations evolving faster than animal clades (Rios *et al.*, 2020). A key factor in the success and adaptability of these healthcare-associated *E. faecium* is the plasmidome (Arredondo-Alonso *et al.*, 2020). Clinical *E. faecium* present a plasmidome highly dissimilar to that of other bacteria, with several plasmids per cell, larger size plasmids and unique plasmid chimeras (Freitas *et al.*, 2016; Arredondo-Alonso *et al.*, 2020). Studies to date have described clinical VREfm as a polyclonal population, with widespread dissemination of similar clones, as well as genetically indistinguishable clinical VREfm and VSEfm isolates at the core genome level, providing evidence that new VREfm are arising from VSEfm following acquisition of a vancomycin resistance gene i.e. *vanA* by HGT (Raven *et al.*, 2016; Gouliouris *et al.*, 2018; Pinholt *et al.*, 2019).

1.5.2 Unclear boundaries within *E. faecalis* populations

Less is known about the population structure of *E. faecalis*, which may be due to the fact it poses less of a clinical burden than that of hospital-associated *E. faecium*. In *E. faecalis* it seems there is a lack of such a clear clade structure, like that seen in *E. faecium*. It has been suggested that this is because *E. faecalis* occupies a larger variety of ecological microniches, and therefore is exposed to a more heterogeneous spectrum of alleles than *E. faecium* (Ruiz-Garbajosa *et al.*, 2006b; Tedim *et al.*, 2015). As a result, no clear genotypic differences have been observed between hospital and community isolates of *E. faecalis* (Ruiz-Garbajosa *et al.*, 2006b; Howden *et al.*, 2013; Tedim *et al.*, 2015). A very recent study suggests that hospital-adapted *E. faecalis* strains existed in the pre-antibiotic era (isolates ranging from 1936 up to 2018), the population is cohesively connected through homologous recombination, metabolic flexibility and a stable large core genome, suggesting adaptations seen in these "hospital-adapted" strains likely occurred in alternative niches (Pöntinen *et al.*, 2021).

1.7 Enterococci in Ireland

1.7.1 The prevalence and molecular epidemiology of VREfm in Ireland

In 2019, it was reported that 38.4% of *E. faecium* BSI isolates in Ireland were vancomycin-resistant compared to only 0.3% of *E. faecalis* isolates (European Centre for Disease Prevention and Control, 2021). Only a few studies in Ireland have investigated VRE or specifically VREfm, of which the majority have used traditional and mostly unreliable typing methods such as PFGE. One study in a large university hospital in Cork in 2014 revealed that 19.1% (67/350) of patient faecal samples were positive for VREfm and identified a heterogeneous CC17 population, albeit using random amplified polymorphic DNA (RAPD) fingerprinting, which has poor discriminatory power (Whelton *et al.*, 2016). Another study investigated patients and their immediate environment between 2012 and 2014 for VRE in an intensive-care unit (ICU) in a large north Dublin tertiary referral hospital and found that 19% of patients and 6.5% of environmental sites were positive for VREfm. High-touch ICU sites such as drip stands, bed control panels, and chart holders accounted for 61% of contaminated environmental sites (McDermott *et al.*, 2018). The study revealed a greater heterogenicity among patient isolates compared to environmental isolates, albeit using PFGE, which has been shown to be unreliable for typing of VREfm (Pinholt *et al.*, 2015; Lytsy *et al.*, 2017).

A study in another Dublin tertiary referral hospital investigated the epidemiology and molecular types of VRE recovered from BSIs between 2009 and 2012 and found that 95% were VREfm and compared these to other nearby referral hospitals. A combination of PFGE and MLST suggested the spread of individual VREfm clones within and between hospitals with four CC17 STs dominating (ST17, ST18, ST78 and ST203), although once again the unreliability of these methods for typing *E. faecium* is a major limitation of that study (Ryan *et al.*, 2015).

The only WGS-based study that has included Irish VREfm isolates was a study of 495 *E. faecium* (VREfm and VSEfm) BSI isolates recovered over a decade (between 2001 and 2011), predominantly from the UK (n = 474) but also from Ireland (n = 21) (Raven *et al.*, 2016). The study revealed a polyclonal VREfm population structure using WGS and core-genome SNV analysis, but with two clusters that were geographically restricted to Ireland/Northern Ireland and evidence of transmission of VREfm between different hospitals. A unique *vanA* transposon region was also identified in the Irish isolates with deletions in the transposase, resolvase and *vanZ* genes, suggesting a local source and evolution of *vanA* among Irish isolates VREfm (Raven *et al.*, 2016).

Because of a scarcity of studies that have used accurate and highly discriminatory typing methods to investigate the molecular epidemiology of VREfm in Ireland, coupled with the high rate of VREfm BSIs among patients in Irish hospitals, it is clear that detailed WGS-based studies are required to provide insights into the emergence, evolution and spread of VREfm in Ireland and to ultimately provide an evidence base to inform infection, prevention and control (IPC) procedures. In addition, in light of recent studies highlighting the emergence and clonal spread of VREfm from VSEfm, the role of VSEfm in the evolution and spread of VREfm here requires investigation. Lastly, although rectal and faecal screening of high-risk patients for VRE is widely used as part of IPC procedures in an attempt to curtail the spread of VRE (Health Protection Surveillance Centre, 2014), little is known about the relatedness of VREfm carriage and infection isolates in Ireland.

1.7.2 Linezolid-resistant enterococci in Ireland

The first report of an outbreak of linezolid-resistant VREfm in Ireland was in 2014, involving 15 patients and the spread of a single clone was confirmed by PFGE. All isolates harboured the G2576T mutation in the gene encoding 23S ribosomal RNA, however investigation of mobile linezolid resistance genes was not undertaken (O'Driscoll *et al.*, 2015). In 2014, the ZAAPS programme reported the first two isolates from Ireland harbouring the mobile linezolid resistant gene *optrA,* one each from Dublin and Galway (Mendes *et al.*, 2016). Following this, in 2016 the Health Service Executive (HSE) in Ireland requested that all linezolid-resistant enterococci should to be sent to the National MRSA Reference Laboratory (NMRSARL) to be screened for the presence of the transferable resistance genes i.e. *cfr* and *optrA* and, more recently, *poxtA*. In 2017, a VREfm isolate harbouring a plasmid harbouring both *cfr* and *optrA,* was reported from a patient in an Irish hospital (Lazaris *et al.*, 2017). However, to date little is known about the prevalence and spread of mobile linezolid resistance genes among linezolid-resistant enterococci in Ireland.

1.8 Aims of the study

The main aims of this project were to use WGS to investigate the population structure of vancomycin-resistant *E. faecium* (VREfm) screening and bloodstream isolates from patients in a large acute hospital in Dublin, Ireland and to investigate the prevalence of mobile linezolid resistance genes in linezolid-resistant enterococci recovered from patients in Irish hospitals.

- The first research chapter (Chapter 3) aimed to examine the population structure of VREfm in a large teaching hospital in Ireland and to gain insights into the predominant clone or clones in circulation. Extending from this was the aim to examine the structural organisation of *vanA* in Irish VREfm isolates to determine whether particular features were unique to Irish isolates and to examine the ability of *vanA* to spread (via transposons or conjugative plasmids) among Irish VREfm isolates.

- The second research chapter (Chapter 4) aimed to use WGS to examine the population structure of linezolid resistant *E. faecium* and *E. faecalis* isolates recovered from patients in Irish hospitals that harboured mobile linezolid resistance genes (*optrA, poxtA* and/or *cfr)*. The variability of MGEs harbouring these resistance genes, their presence and transmissibility throughout different genetic lineages and their presence in hospitals in different geographical locations within Ireland was also investigated.

- The final research chapter (Chapter 5) aimed to use insights learned from Chapter 3 & 4 to assist in the investigation an outbreak of LVREfm in real-time in a north Dublin hospital. This section of the study used WGS to examine the initiation and expansion of the outbreak (whether clonal or spread of MGEs) and the role of the environment as a reservoir for spread of LVREfm during the time period of the outbreak.

Chapter 2

General Materials and Methods

2.1 Bacterial isolates

Three main study subgroups consisting of a total of 459 enterococcal isolates were investigated in detail, as detailed in Table 2.1. These included VREfm (n=350), VSEfm (n=34), LREfm (n=30), LREfs (n=25) and LVREfm (n=20), recovered from a variety of hospital locations across Ireland.

2.1.1 Ethical approval

Ethical approval for this project was granted by the Trinity College Dublin Dental School Research Ethics Committee (DSREC), under the research ethics committee (REC) reference number: DSREC2020-01-02.

2.2 General microbiological methods

2.2.1 Bacterial culture and isolate storage

Isolates were stored in Microbank^{TM} cryogenic bead vials (Pro-Lab Diagnostics, Cheshire, UK) at -80°C. Unless otherwise stated, isolates were cultured on Columbia Blood agar (CBA; Fannin Ltd., Dublin, Ireland). Isolates were cultured by removing a single bead from the storage vial using sterile forceps, inoculating the media plate using a sterile inoculating loop, and incubating the plate overnight (18-24 h) in a static incubator (Gallenkamp, Leicester, UK) at 37^{o}C. For liquid culture, enterococcal species were grown overnight at 37^{o}C in Brain Heart Infusion broth (BHIB) (Merck Ireland Ltd., Wicklow, Ireland) in an orbital incubator (Gallenkamp) set at 200 rpm.

2.2.2 Chemicals, water, buffers and solutions

All chemicals used were of analytical grade or molecular biology grade and were purchased from Merck Ireland Ltd., unless otherwise stated. Ultra-purified water generated using the Milli Q Biocel system (Millipore Ireland, Cork, Ireland) was used for the preparation of buffers and other chemical solutions. Tris-borate/EDTA (TBE) electrophoresis buffer was prepared at 10× concentration, and consisted of 0.45 M Trizma base, 0.45 M boric acid and 0.01 M EDTA, pH 8. This buffer was diluted to 0.5× concentration in ultra-pure water before use. The TBE buffer was used for preparing agarose gels and acted as the running buffer for gel electrophoresis (Section 2.4.2.2). Unless otherwise stated, Molecular Biology Grade Water (Merck) was used in all PCR reactions, and DNA dilutions and elution's.

Table 2.1 Summary of enterococcal isolates investigated in the present study

Chapter	Title of substudy	Isolate source	Number of isolates		Sample types	Period of isolation
3	The problem of healthcare-associated *Enterococcus faecium* in Ireland; spread of vancomycin-resistance via IS*1216E*-mediated transposition and conjugative plasmid transfer of *vanA* throughout diverse genetic lineages	South Dublin large acute teaching hospital	VREfm 331	VSEfm 34	Rectal screening & bloodstream infections	June 2017 – July 2019
4	Linezolid resistance in *Enterococcus faecium* and *Enterococcus faecalis* from hospitalized patients in Ireland: high prevalence of the multidrug resistance genes *optrA* and *poxtA* in isolates with diverse genetic backgrounds	14 Irish hospital sites	LREfm 30	LREfs 25	Rectal screening & a range of infections	June 2016 -August 2019
5	A hospital outbreak of linezolid-resistant and vancomycin-resistant ST80 *Enterococcus faecium* harbouring an *optrA*-encoding conjugative plasmid investigated by whole-genome sequencing	North Dublin teaching hospital	LVREfm 20	VREfm 19	Rectal screening & hospital environment sources	October/November 2019

Abbreviations: VREfm, vancomycin-resistant *E. faecium*; VSEfm, vancomycin-susceptible *E. faecium*; MDR, multi-drug resistance; LREfm, linezolid-resistant *E. faecium;* LSEfs, linezolid-susceptible *E. faecalis*; LVREfm, linezolid-resistant and vancomycin-resistant *E. faecium.*

2.3 Isolate identification and antimicrobial susceptibility testing

2.3.1 Identification of vancomycin-resistant *E. faecium*

All VREfm screening and bloodstream infection isolates for population analysis investigations were initially identified as VREfm in the respective hospital's clinical microbiology laboratory, as part of routine work. This was performed by inoculating screening swabs onto Brilliance VRE agar (Oxoid Ltd., Hampshire, UK). This chromogenic media can be used to presumptively identify VREfm based on colony colour; i.e. on Brilliance VRE agar VREfm grow as indigo to purple colonies. The identity of presumptive colonies were then definitively confirmed using the Vitek MS Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF-MS) system (Vitek, bioMérieux Marcy l'Etoile, France) according to the manufacturer's instructions. For BSIs all presumptive *E. faecium* from CBA were also identified using the MALDI-TOF-MS system. Isolates underwent antimicrobial susceptibility testing as outlined in Section 2.3.3. All confirmed VREfm isolates (screening and from BSIs) and all VSEfm (BSI only), from the hospital microbiology laboratory were cultured on CBA and incubated at 37ºC for 24 h prior to transfer to the Irish National MRSA Reference Laboratory (NMRSARL) for inclusion in the present study. The bacterial species to which isolates belonged were further confirmed by in-house multiplex PCRs, as were the presence/absence of the *vanA/vanB* genes encoding vancomycin resistance as detailed in Section 2.4.2.3.

2.3.2 Identification of linezolid-resistant enterococci

Unless otherwise stated, all linezolid resistant enterococci (LRE) investigated in the present study were identified in the hospital of origin during routine clinical microbiology laboratory work. Since 2016, all LRE recovered in Irish hospitals are sent to the NMRSARL for further investigation. All isolates were screened for the linezolid resistance genes *optrA*, *poxtA* and *cfr* by multiplex PCR as detailed in Section 2.4.2.2. The bacterial species to which isolates belonged was confirmed using MALDI-TOF-MS (Vitek). When isolates were transferred from the NMRSARL to the laboratory in the Dublin Dental University hospital, the species and presence of linezolid-resistance genes was confirmed again using multiplex PCRs, as detailed in Section 2.4.2.3.

2.3.3 Antimicrobial susceptibility testing

For the VREfm isolates investigated in Chapter 3, antibiotic susceptibility testing (AST) was performed using the ST203 card on the VITEK-2 system (BioMérieux). Susceptibility results for vancomycin and teicoplanin were recorded using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) interpretive criteria (European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2019). Breakpoints were defined as vancomycin (Resistant (R) > 4 mg/L) and teicoplanin (R > 2 mg/L)

For all isolates investigated in Chapters 4 and 5, initial AST was performed at the origin hospital before transfer to the NMRSARL. Linezolid, vancomycin, chloramphenicol and tetracycline MICs were determined using the VITEK-2 system or by using E-test strips (both BioMérieux) at the NMRSARL and susceptibility interpretations were based on EUCAST guidelines (EUCAST, 2019). As there are no clinical breakpoints for enterococcus species for chloramphenicol and tetracycline, the most up-to-date epidemiological cut-off values (ECOFFs) were used (EUCAST, 2017). Breakpoints were defined as linezolid (R > 4 mg/L), vancomycin (R > 4 mg/L), chloramphenicol (R > 32 mg/L) and tetracycline (R > 4 mg/L).

2.4 Conventional molecular methods

2.4.1 DNA extraction

2.4.1.1 Qiagen DNeasy method

Unless otherwise stated, DNA was extracted from isolates using broth culture and the Qiagen DNeasy kit (Qiagen, Crawley, UK). Briefly, samples were reactivated from cryogenic storage beads (-80°C) onto CBA and incubated overnight at 37°C. A single colony was selected and inoculated into 4 ml BHIB and was incubated in a shaking incubator (Gallenkamp) set at 200 rpm, overnight at 37°C. The overnight culture was centrifuged at 2,376 × g for 5 min. The supernatant was removed and the resulting pellet was resuspended in 200 µl of lysis buffer A1 (Alere Technologies GmbH, Jena, Germany) and transferred to a fresh tube containing the lyophilised lysis enhancer A2 (Alere Technologies) and incubated for 2-3 h at 450 rpm at 37°C. Following lysis, the Qiagen DNeasy Blood and Tissue kit was used as per the manufacturer's instructions to complete DNA extraction. DNA was eluted in the final step in 50 µl of molecular grade water (Sigma-Aldrich [Merck KgaA], Wicklow, Ireland), the quality (260/280 between 1.8-2.0 and 260/230 between 2.0-2.2) and concentration of DNA was checked using the NanoDrop

2000 spectrophotometer (Thermo Fischer Scientific, Waltham, Massachusetts, USA). Extracted DNA was stored at 4°C or -20°C.

2.4.1.2 Qiagen HMW MagAttract method

Template DNA for third generation sequencing in Chapters 3 and 5, (Section 2.5.2) was extracted using the Qiagen High-Molecular Weight MagAttract kit (Qiagen). The following modifications were made, samples were reactivated from cryogenic storage beads (-80ºC) onto CBA and incubated overnight at 37ºC. A single colony was selected and inoculated into 4 ml BHIB and was incubated in a shaking incubator overnight at 37ºC. The overnight culture was then centrifuged at 2,376 × *g* for 5 min and the resulting pellet was resuspended in 200 µl of lysis buffer A1 and transferred to a fresh tube containing the lyophilised lysis enhancer A2 (Alere Technologies), and incubated for 2-3 h at 450 rpm at 37ºC. Following lysis, the Qiagen HMW MagAttract kit was used as per the manufacturer's instructions to complete DNA extraction. DNA was eluted in the final step in 100 µl of Sigma-Aldrich molecular grade water and the quality and concentration of DNA was checked using the NanoDrop 2000 spectrophotometer. Extracted DNA was stored at 4ºC or -20ºC.

2.4.1.3 Agencourt Genfind v3 method

Template DNA for third generation sequencing in Chapter 4, (Section 2.5.2) was extracted using a Agencourt Genfind v3 kit (Beckman Coulter, Brea, California, USA) with the following modifications to prevent DNA shearing. Samples were reactivated from cryogenic storage beads (-80ºC) onto CBA and incubated overnight at 37ºC. A single colony was selected and inoculated into 4 ml BHIB and was incubated in a shaking incubator overnight at 37ºC. The overnight culture was then centrifuged at 2,376 × *g* for 5 min and the resulting pellet was resuspend in 509 µl of lysis mix; consisting of 400 µl lysis buffer, 9 µl proteinase K (Beckman Coulter), 100 µl 16% (w/v) SDS/17% (w/v) Triton X-100 (Merck) and was then transferred to a 1.5 ml Safelock microfuge tube (Eppendorf, Hamburg, Germany) and incubated for 30 min at 37ºC. Following cell lysis the 1.5 ml tube was centrifuged at 17,968 × *g* for 2 min, the resulting supernatant transferred to a fresh 1.5 ml tube and 1 µl of 100 mg/ml RNase A (Sigma-Aldrich [Merck]) was added and incubated at room temperature for 5 min. The protocol was followed as per manufacturers guidelines following the addition of 300 µl of the Binding buffer (Beckman Coulter) supplied with the kit to complete DNA extraction. DNA was eluted in the final step in 60

µl of Sigma-Aldrich molecular grade water and the quality and concentration of DNA was determined using the NanoDrop 2000. Extracted DNA was stored at 4ºC or -20ºC.

2.4.1.4 Boiling method

Template DNA for PCRs for screening putative transconjugant derivatives (Section 2.9.1) for *optrA* and/or *poxtA*, for the confirmation of isolates as *E. faecium* or *E. faecalis* and for the detection of *vanA* and *vanB* was extracted from isolates using a quick boiling method. One colony from a 37ºC overnight culture on CBA was inoculated into 5 µl of Sigma-Aldrich molecular biology grade water in a 0.2 ml PCR tube (Molecular BioProducts, Fisher Scientific, New Hampshire, USA) and incubated on a SimpliAmp thermal cycler (Applied Biosystems, Foster City, California, USA) at 95ºC for 5 min. PCRs were then performed as described in Section 2.4.2.3.

2.4.2 Polymerase-chain reaction

2.4.2.1 Oligonucleotides, enzymes and chemicals

All oligonucleotide primers were custom synthesised by Merck Sigma-Aldrich. GoTaq G2 Flexi DNA Polymerase with buffers and dNTPs were purchased from the Promega Corporation (Madison, Wiconsin, USA). Oligonucleotide primers and dNTPs were stored as stock solutions of 10 mM at -20ºC.

2.4.2.2 Agarose gel electrophoresis

Agarose was prepared for gel electrophoresis at concentrations of 1 or 2% (w/v) by dissolution of agarose powder in TBE buffer (Section 2.2.2), GelRed™ (Biotium, Fremont, California, USA) was added to the agarose gel at a final concentration of 1% (v/v). Agarose gels were cast in trays (10 x 8 x 3 cm) with 10-well sample combs (1.5 mm depth) (Genesee Scientific, San Diego, California, USA). Agarose gel electrophoresis was carried out using a Consort nv model EV222 power pack (Parklaan, Turnhout, Belgium) set at 120 V and 80 mA, in Galileo Bioscience (Cambridge, Massachusetts, USA) gel. Agarose gel electrophoresis was carried out for 2-3 h.

Following electrophoresis, DNA in gels was visualised under ultraviolet light at a wavelength of 312 nm in an Alpha Innotech transilluminator (Protein Simple, San Jose, California, USA) model AVT26U and the AlphaImager mini software (Protein Simple). Captured images were printed using a Mitsubishi (Sant del Vallés, Barcelona, Spain) printer model P93DW.

2.4.2.3 Multiplex PCRs

Multiplex PCRs were performed to confirm enterococcal species identity and to detect the presence or absence of the vancomycin resistance genes *vanA* and *vanB* in all isolates. In Chapters 4 and 5, multiplex PCRs were used to confirm the presence of specific linezolid resistance associated genes prior to WGS (Table 2.2). A previously described multiplex PCR was used for enterococcal species and *van* gene detection (Dutka-Malen *et al.*, 1995a), although this was modified to include primers for *E. faecium*, *E. faecalis*, *vanA* and *vanB,* with an alternative reverse primer being designed in-house for *E. faecium* due to an error in the original manuscript, which was highlighted by the later publication of a correction (Dutka-Malen *et al.*, 1995b). An in-house multiplex PCR was designed to confirm the presence of *optrA* and *poxtA*. Primer details and PCR conditions used are shown in Table 2.2. All PCRs were performed using GoTaq G2 Flexi DNA Polymerase and buffers (Promega) according to the manufacturer's instructions on a SimpliAmp thermal cycler. Amplimers were visualised by conventional agarose gel electrophoresis, as described in Section 2.4.2.2.

Table 2.2 Primers used in the present study

Primer purpose	Gene amplified	Primer pair	Nucleotide Sequence (5'-3')	Product size (bp)	Nucleotide coordinates	PCR conditions	Reference
Multiplex PCR to confirm enterococcal species and *van* gene type	*ddl*$_{E.\ faecium}$	Efm-1 Efm-2	TAGAGACATTGAATATGCC ACCTAACATCGTGTAAGCT[a]	529	210949-210967[b] 211460-211478[b]	94°C for 2 min. 30 cycles of 94°C for 1 min, 54°C for 1 min, 72°C for 1 min. Final elongation step of 10 min at 72°C	Dutka-Malen *et al.*, 1995a
	ddl$_{E.\ faecalis}$	Efs-1 Efs-2	ATCAAGTACAGTTAGTCT ACGATTCAAAGCTAACTG	941	802443-802460[c] 803366-803383[c]		
	vanA	*VanA*-1 *VanA*-2	GGGAAAACGACAATTGC GTACAATGCGGCCGTTA	732	10540-10556[d] 9825-9841[d]		
	vanB	*VanB*-1 *VanB*-2	ATGGGAAGCCGATAGTC GATTTCGTTCCTCGACC	635	2213806-2213822[b] 2213188-2213204[b]		
Multiplex PCR for detection of linezolid-resistance genes	*optrA*	*optrA*-F *optrA*-R	GAAGAAGGAACTGGTGAAAGTGAG GTGTCATTTAGCTCAGGGTATTCG	1103	217-240[e] 1296-1319[e]	94°C for 2 min. 30 cycles of 94°C for 1 min, 61°C for 1 min, 72°C for 1 min. Final elongation step of 10 min at 72°C	This study
	poxtA	*poxtA*-F *poxtA*-R	TATTGTCGGCGTGAACGGAG TCTGCGTTTCTGGGTCAAGG	1355	90-109[f] 1425-1444[f]		

Table 2.2 continued overleaf

Primer purpose	Gene amplified	Primer pair	Nucleotide Sequence (5'-3')	Product size (bp)	Nucleotide coordinates	PCR conditions	Reference
Multiplex PCR used in NMRSARL to screen linezolid-resistant enterococci	*cfr*	*cfr*-F *cfr*-R	TGCTACAGGCGACATTGGAT GACGGTTGGCTAGAGCTTCA	137	357-376[g] 474-493[g]	95ºC for 2 min. 25 cycles of 95ºC for 15 s, 53ºC for 15 s, 68ºC for 90 s. Final elongation step of 5 min at 68ºC	NMRSARL in-house primers
	optrA	*optrA*-F *optrA*-R	ACCGGTGTCCTCTTTGTCAG TCAATGGAGTTACGATCGCCTT	369	1374-1393[e] 1721-1742[e]		
	poxtA	*poxtA*-F *poxtA*-R	TCAGAGCCGTACTGAGCAAC CGTTTCTGGGTCAAGGTGGT	167	1274-1293[f] 1421-1440[f]		

[a] Primer designed in-house, due to error in original manuscript which was followed by publication of a correction (Dutka-Malen *et al.*, 1995b).

[b] Nucleotide coordinates based on *E. faecium* Aus0004, GenBank accession number CP003351.

[c] Nucleotide coordinates based on *E. faecalis* V583, GenBank accession number NC_004668.

[d] Nucleotide coordinates based on *E. faecium* V24, GenBank accession number KX574671.

[e] Nucleotide coordinates based on *optrA* gene, GenBank accession number KY579372.

[f] Nucleotide coordinates based on *poxtA* gene, GenBank accession number MF095097.

[g] Nucleotide coordinates based on *cfr* gene, GenBank accession number NC_023913.1.

2.5 Whole-genome sequencing

2.5.1 Second generation WGS (Illumina MiSeq)

2.5.1.1 Library preparation

Whole-genome sequencing was performed using the Illumina 500-cycle v2 or 600-cycle v3 MiSeq Reagent Kit and a MiSeq bench-top sequencer (Illumina, Eindhoven, Netherlands). Reagents supplied with the Illumina Nextera DNA Flex library preparation kit were used to fragment DNA, attach adaptors and amplify diluted DNA, in a process termed tagmentation.

For each sample, 5 µl of bead-linked transposome (BLT), 5µl of tagmentation buffer (TB1) was mixed with 15 µl of genomic DNA and incubated at 55ºC for 15 min on a thermocycler. Tagmentation was ceased following the addition of 5 µl neutralising tagment stop buffer (TSB) and incubated at 37ºC for 15 min on a thermocycler. The libraries were cleaned in a Thermowell 96-well polycarbonate PCR plate (Corning, Flintshire, UK), using a magnetic stand (ThermoFisher Scientific). The cleared supernatant was carefully removed without disturbing the pellet and 50 µl of tagment wash buffer (TWB) was used to resuspend the bead pellet. This was repeat for three washes with fresh TWB used for each repetition. Following the final wash step the remaining TWB was removed and discarded and the cleaned libraries were resuspended in a freshly made PCR master mix (10 µl enhanced PCR mix [EPM] with 10 µl nuclease free water per sample). Each sample was indexed by the addition of 5 µl of a unique index from the Nextera™ DNA CD Indexes 96-well plates (Illumina). This PCR reaction was then placed on a thermocycler using the following conditions; 68ºC for 3 min, 98ºC for 3 min, 6 cycles of 98ºC for 45 s, 62ºC for 30 s and 68ºC for 2 min, followed by final step of 68ºC for 1 min. A final size-selection clean-up was then performed on the libraries using samples purification beads (SPBs) supplied by Illumina. The PCR reaction for each library was transferred into a well of an Abgene™ 96 well 0.8 mL polypropylene deepwell storage plate (ThermoFisher Scientific) and placed onto the magnetic stand (ThermoFisher Scientific) until the magnetised beads settled at the magnet, causing the solution to clear. The supernatant (22.5 µl) was removed into a fresh well and this was mixed with 42.5 µl of diluted SPBs (22.5 µl SPB with 20 µl nuclease free water per sample). This was mixed by pipetting solution gently and incubated at room temperature for 5 min and placed back onto the magnetic stand until the solution became clear. From this 62.5 µl of the supernatant was transferred into a fresh well and mixed with 7.5 µl of undiluted SPBs,

again this was mixed by pipetting gently and incubated at room temperature for 5 min. The plate was placed back onto the magnetic stand until the solution became clear. The supernatant was removed without disrupting the bead pellet and the bead pellet was washed with 100 µl of freshly prepared 80% (v/v) ethanol; this process was repeated twice. Following the wash steps the beads were resuspended in the supplied resuspension buffer (RSB) and incubated at room temperature for two min. The plate was placed back onto the magnetic stand until the solution became clear. The supernatant (15 µl) was removed into a fresh labelled 0.2 ml PCR tube (Molecular BioProducts) and the concentration assessed using the Qubit 3.0 fluorometer (ThermoFisher Scientific). Each sample library concentration was adjusted to 10 nM and then an equal volume (5 µl) of each library was pooled. The 10 nM pooled library was diluted to 4 nM before denaturing. Freshly diluted 1 nM NaOH was diluted to 0.2 nM NaOH and added to the 4 nM pool at a ratio of 1:1 to denature the library. The resulting solution was diluted to a concentration of 12 pM using the hybridisation buffer (HT1) provided with the Illumina MiSeq reagent kit and spiked with 1% PhiX sequencing control (Illumina) prior to MiSeq loading and WGS.

2.5.1.2 Post-sequencing processing

Upon MiSeq loading, the demultiplexing and adapter trimming functions were enabled using Illumina experiment manager. Following each sequencing run, the quality of the data generated was assured by cluster density and Q30 assessment, according to the manufacturer's (Illumina) instructions. The MiSeq software demultiplexed the reads and trimmed off primer and adapter sequences, leaving a pair of FASTQ files for each isolate sequenced, stored on Illumina's BaseSpace Sequencing Hub (Available at: https://basespace.illumina.com/).

2.5.2 Third generation WGS (MinION)

2.5.2.1 Library preparation

Third generation WGS was performed using a SpotON Flow Cell (R9.4) and MinION device (Oxford Nanopore Technologies, Oxford, UK) (Figure 2.1). Long-read third generation sequencing was performed in multiplex using the one-dimensional (1D) genomic DNA sequencing kit (SQK-LSK108 or SQK-LSK109) and ID native barcoding kit (EXP-NBD103 or EXP-NBD104). As protocols are updated regularly the latest available version of this protocol was always used (Available at: https://nanoporetech.com/community). Briefly, high molecular weight genomic DNA (1-

10µg) ends were repaired and prepared for adapter attachment. Followed by ligation of native barcodes (ONT) and sequencing adapters to the DNA strands. Finally, a clean-up step is used to remove any unprepared DNA and other regents from the library preparation. The prepared library was loaded onto the flowcell via the SpotON sample port in a dropwise fashion. The ports were closed gently along with the MinION device and the sequencing run was commenced using the MinKNOW graphical user interface (GUI). The MinKNOW GUI allows for real-time viewing of reads processed and sequencing run metrics.

2.5.2.2 Post-sequencing processing

Raw-read FAST5 files were base called using Guppy v3.1.5 (Oxford Nanopore). Demultiplexing and adaptor trimming was performed using qCat v1.0.1 (https://github.com/nanoporetech/qcat) or Porechop v0.2.4 (https://github.com/rrwick/Porechop). Following post-sequencing processing a single FASTQ file remained for each isolate sequenced.

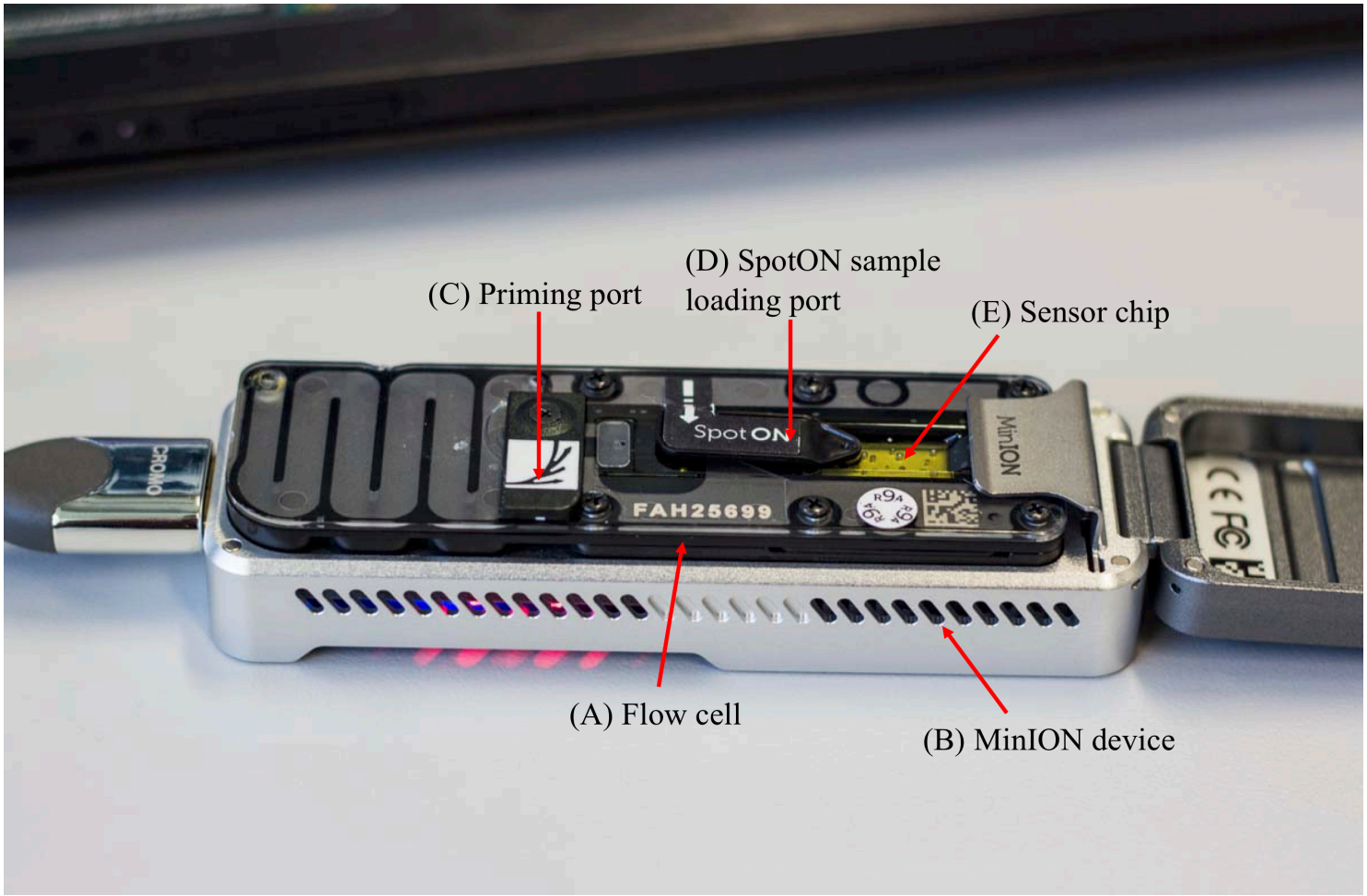


Figure 2.1 Anatomy of a MinION device and flow cell (Oxford Nanopore Technologies, Oxford, UK). The flow cell (A) sits onto the MinION device (B) which contains a heating element required for sequencing runs. The priming port (C) is used to introduce flow cell priming mix before a run and to introduce wash solutions to reuse the flowcell. Sequencing libraries are loaded in a dropwise fashion via the SpotON sample loading port (D). This port allows the library to flow onto the sensor chip (E) which contains thousands of pores which read the sequence via electrical current density flux.

2.7 WGS data processing and analysis

2.7.1 *de novo* assembly

Isolate reads were assembled using the SPAdes genome assembler v3.7.1 (Bankevich *et al.,* 2012). SPAdes automatically identifies the optimal assembly parameters for each assembly. Contigs were stored as FASTA files. Contigs under 1000 bp were considered unreliable and were therefore removed

2.7.2 MLST

Traditional MLSTs were extract in silico from assembled WGS contigs using Ridom SeqSphere+ (version 7.0.4, Ridom GmbH, Münster, Germany) and on BioNumerics (version 7.7, Applied Maths, Ghent, Belgium), both of which connect to the publicly available MLST schemes provided by PubMLST (https://pubmlst.org). Newly identified MLST alleles and profiles were submitted to PubMLST for incorporation into the PubMLST system via SeqSphere+.

2.7.3 cgMLST/wgMLST analysis

For *E. faecium* isolates, the MiSeq generated reads associated with each isolate were directly imported from Illumina's BaseSpace cloud computing analysis and storage hub to BioNumerics (v 7.7, Applied Maths) cloud-based calculation engine. FASTQ files were assembled using the SPAdes *de-novo* assembler (Section 2.7.1). The FASTQ files and associated assembled genomes for each isolate were then submitted to the BioNumerics *E. faecium* whole-genome (wg) MLST scheme (5,489 loci). Two algorithms were used to generate a consensus wgMLST profile for each isolate, an assembly-free algorithm that determined locus presence/absence and an allelic identity algorithm using an assembly-free k-mer approach. The assembly-based algorithm, used a BLAST approach to detect alleles on contigs assembled using SPAdes, all using default parameters. Assembled reads were also imported to Ridom SeqSphere+ v7.0.4 (Ridom GmbH) and underwent cgMLST consisting of 1,423 alleles (de Been *et al.*, 2015). Interpretations of isolate relatedness were based on a combination of allele differences as determined by cgMLST and/or wgMLST, as detailed in each chapter. An overview of this process is outlined in Figure 2.2.

For *E. faecalis* isolates, again the MiSeq generated reads associated with each isolate were directly imported from Illumina's BaseSpace to BioNumerics. FASTQ files were assembled using the SPAdes *de-novo* assembler. The assembled genomes for each

isolate were then submitted to the BioNumerics *E. faecalis* wgMLST scheme consisting of 5,285 loci, for assembly-free and assembled-based allele calling (Figure 2.2).

2.7.4 Quality assurance of WGS data

The quality of the sequence read sets, *de novo* assemblies, and assembly-free and assembly-based allele calls, were assessed using the quality statistics window in BioNumerics v7.7 (Applied Maths) (Figure 2.2). Sequence reads were assessed based on their average quality score. Assemblies were assessed based on their N50 (i.e. the minimum contig length required to cover 50% of the genome), average coverage and the number of contigs produced. WGS was repeated for samples in which quality score <30, average coverage <50x and if less than 95% of core-genome loci were identified.

2.7.5 Phylogenetic tree generation

In Chapter 3, neighbour-joining trees (NJTs) were generated using distances matrices based on cgMLST analysis in Ridom SeqSphere+ v7.0.4. The NJT was visualised and annotated using iTOL version 5 (https://itol.embl.de/). In Chapters 4 and 5, minimum spanning trees (MSTs) were generated using distance matrices based on cgMLST or wgMLST, as specified per chapter. All MSTs were generated in BioNumerics v7.7 (Applied Maths) with permutation resampling (1000 replicates).

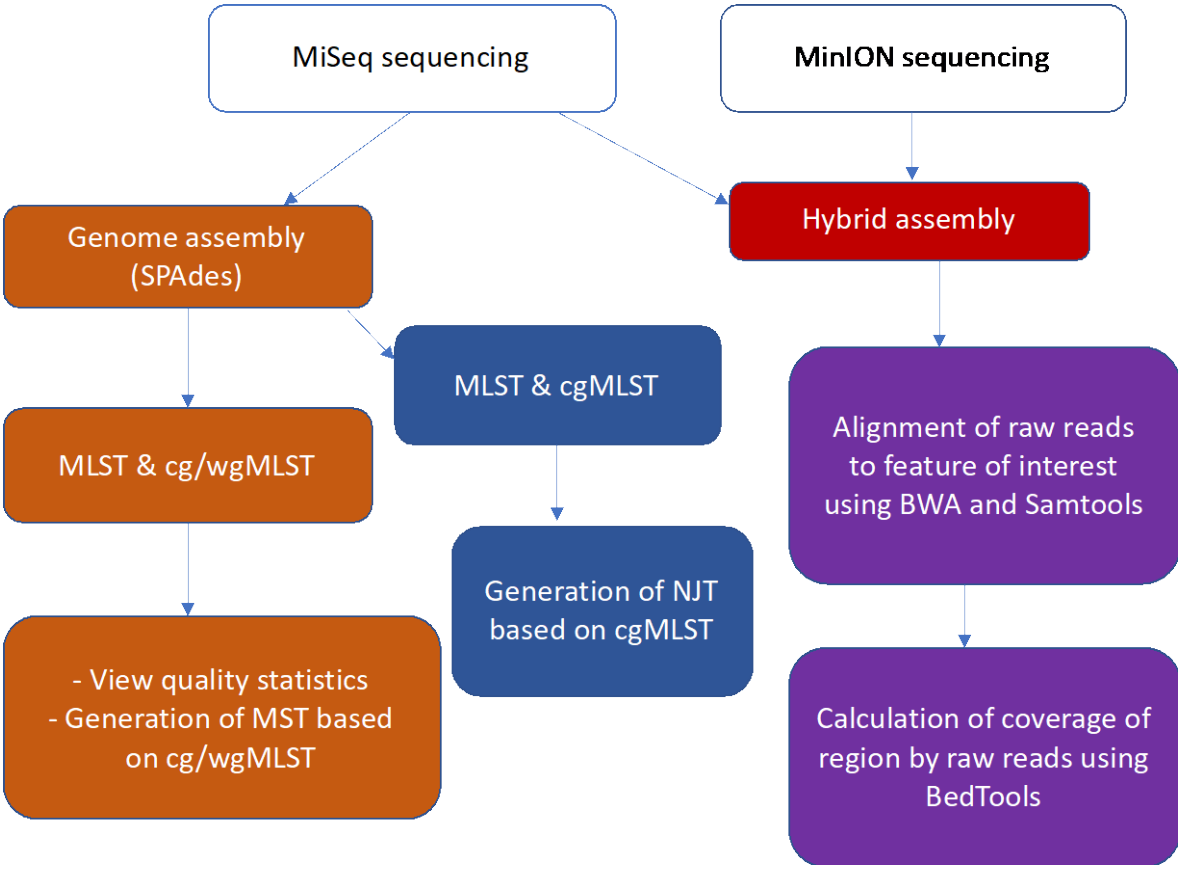


Figure 2.2 Summary of the whole-genome sequence data analysis methods used in the present study for population analysis and plasmid/transposon characterisation and comparisons. The software used during each analysis is represented with a different colour; orange denote BioNumerics version 7.7 (Applied Maths, Belgium), blue denotes Ridom SeqSphere+ version 7.0.4 (Ridom GmbH, Germany) which was only used for *E. faecium* isolates, red denotes UniCycler (Wick *et al.*, 2017) and purple denotes command line analysis.

Abbreviations: MLST, multilocus sequence typing; cgMLST, core-genome multilocus sequence typing; wgMLST, whole-genome multilocus sequence typing; MST, minimum spanning tree; NJT, neighbour-joining tree.

2.7.6 LRE-Finder

Following WGS, all linezolid-resistant isolate sequences were screened for 23S rRNA mutations, o*ptrA*, *cfr* and *poxtA* using the Centre for Genomic Epidemiology LRE-Finder 1.0. This is an online tool which maps raw Illumina reads against a reference sequence to screen for linezolid resistance genes and associated linezolid resistance 23S mutations (specifically G2505A and G2576T) and was used to calculate the copy number of these mutations (https://cge.cbs.dtu.dk/services/LRE-finder/).

2.7.7 Hybrid assembly

Long-read FASTQ files generated from MinION sequencing were used in combination with MiSeq-generated paired-end short-read FASTQ files to perform a hybrid assembly using UniCycler (Wick *et al.*, 2017). Resulting closed plasmids of interest (i.e. those containing *optrA* and/or *poxtA*) and closed *vanA* transposon regions were used for more detailed analysis, outlined in Figure 2.2 and Section 2.7.8.

2.7.8 Analysis of MiSeq generated raw-reads against reference produced by hybrid assembly

Illumina MiSeq paired-end raw reads were mapped against a reference sequence created using hybrid assembly outlined in Section 2.7.7. Burrows-wheel aligner (Li and Durbin, 2009) was used to map Illumina reads against the reference sequence and Samtools (Li *et al.*, 2009) was used to view alignments, sort and index for input into BedTools (Quinlan and Hall, 2010). BedTools bamtobed command was used to convert the sorted Samtools output file (.BAM) to a file (.BED) which was then readable for other BedTools commands. The BedTools coverage command was used to calculate both the depth and breadth percentage coverage of the areas of interest (i.e. reference sequence plasmids) by the Illumina raw reads. The percentage coverage was used to determine the similarity of reference sequences to those harboured by all other isolates in the set (Figure 2.2). An in-house Bash shell script (Figure 2.3) was written to perform this on a loop to process batches of samples and allow the process to be reproducible. Alignments were viewed for coverage and quality using Tablet (Milne *et al.*, 2013).

```
#!/bin/bash

readsFolder="PATH TO DIRECTORY CONTAINING ILLUMINA PAIRED-END READ FILES"
refRead="PATH TO REFERENCE FASTA FILE"
referenceID=PATH TO REFERENCE BED FILE

bwa index $refRead

for i in $(seq 1 291)

do

echo SJ$i

curRead1=$readsFolder/SJ*${i}*/SJ${i}_*R1*.fastq.gz
curRead2=$readsFolder/SJ*${i}*/SJ${i}_*R2*.fastq.gz

bwa mem $refRead $curRead1 $curRead2 > SJ${i}-aln.sam

samtools view -bS SJ${i}-aln.sam > SJ${i}-aln.bam
samtools sort -o SJ${i}_sorted.bam SJ${i}-aln.bam
samtools index SJ${i}_sorted.bam SJ${i}_sorted.bai

bamToBed -i SJ${i}_sorted.bam > SJ${i}_b2_spades.bed
sort -k 1,1 -n SJ${i}_b2_spades.bed > SJ${i}.temp.sorted
mv SJ${i}.temp.sorted  SJ${i}_b2_spades.bed

coverageBed -b SJ${i}_b2_spades.bed -a ${referenceID} > SJ${i}_b2_spades.cov.bed.out

rm SJ${i}_b2_spades.bed

done
```

Figure 2.3 Bash script for mapping Illumina short reads against reference sequences. Text within " " and in capital letters represent instructions for use, rather than executable script text. The "For i in $(seq 1 291)" indicates the start of a loop, i.e. insert 1 for all locations of ${i} in script, then 2 until it reaches 291, in this script example.

2.9 Plasmid analysis

2.9.1 Filter mating

Conjugative transfer of plasmids encoding the oxazolidinone resistance genes *optrA* and *poxtA* or the vancomycin resistance gene *vanA* harboured by clinical enterococcal isolates were undertaken by filter mating experiments using the plasmid-free rifampicin- and fusidic acid-resistant strains *E. faecium* 64/3 and *E. faecalis* OG1RF as plasmid recipients (Werner *et al.*, 2011). Each donor isolate and recipient strain was cultured separately on Brain Heart Infusion agar (BHIA) (Sigma-Aldrich [Merck]) and incubated for 18 h at 37ºC in a static incubator. Following incubation, a single colony was selected and inoculated into 5 ml of BHIB and incubated for 18 h at 37ºC in a shaking incubator at 200 rpm. A 50 µl aliquot of this culture was then used to inoculate 5 ml of fresh BHIB followed by incubation for 2 h at 200 rpm to allow the cells to reach the mid-logarithmic phase of growth. Then a 50 µl volume of each donor and recipient culture was mixed gently in a sterile 1.5. ml Safelock microfuge (Eppendorf) tube and then applied dropwise onto a sterile filter (0.45 µm pore size, Merck Millipore Ltd.), which was then placed on a BHIA plate and incubated overnight at 37ºC in a static incubator. Following incubation, the filter was then transferred into a sterile 50 ml polypropylene conical bottom tube (Greiner Bio-One, Kremsmünster, Austria) containing 1 ml BHIB and was agitated gently to release the culture. The filter was then removed and the tube centrifuged at 2000 × *g* for 5 min. The supernatant was removed and resuspended in 500 µl of BHIB and incubated at 37ºC for 1-2 h at 200 rpm. Subsequently, the culture was plated onto BHIA without antibiotics and onto BHIA containing 5 mg/L chloramphenicol, 30 mg/L rifampicin and 20 mg/L fusidic acid (all from Merck Sigma Aldrich [Merck]), to select for transconjugants harbouring *optrA* and/or *poxtA*. Alternatively, the culture was plated onto BHIA containing 5 mg/L vancomycin, 30 mg/L rifampicin and 20 mg/L fusidic acid, to select for transconjugants harbouring *vanA*. Both selection methods were followed by incubation at 37ºC for 48 h. Presumptive transconjugant colonies growing on BHIA plates supplemented with all three antibiotics were purified by subculturing on CBA and underwent PCR screening for enterococcal species and *vanA/vanB* or *optrA*/*poxtA,* as described in Section 2.4.2.3 and WGS, as described in Section 2.5.1. Confirmed transconjugants were stored on Microbank™ cryogenic beads (Pro-Lab Diagnostics) at -80ºC.

Chapter 3

The problem of healthcare-associated *Enterococcus faecium* in Ireland; spread of vancomycin-resistance via IS*1216E*-mediated transposition and conjugative plasmid transfer of *vanA* through diverse genetic lineages

3.1 Introduction

Enterococci form part of the normal gastrointestinal flora of healthy humans. *Enterococcus faecium* has emerged as an increasingly important nosocomial pathogen responsible for bacteraemia, abdominal, urinary tract and intravenous catheter related infections (Gilmore *et al.*, 2014). Acquired resistance to ampicillin, gentamicin (high level), linezolid and vancomycin has increased worldwide among hospital-associated *E. faecium*, narrowing treatment options for enterococcal infections (Arias and Murray, 2012; Miller *et al.*, 2014; European Centre for Disease Prevention and Control, 2019). Patients that are asymptomatically colonized with vancomycin-resistant *E. faecium* (VREfm) in the gastrointestinal (GI) tract act as both a reservoir and a source for dissemination of VREfm into the hospital environment (Cattoir and Leclercq, 2013), which is a significant challenge for infection control and prevention. Previous studies have identified 2–10 asymptomatic VREfm carriers for each patient with a VREfm infection (Cattoir and Leclercq, 2013). For over a decade, the Republic of Ireland has consistently reported one of the highest rates of invasive VREfm infections in Europe, ranging between 32.5%-45.8% (2006-2019) (European Centre for Disease Prevention and Control, 2019).

Molecular typing of *E. faecium* has proven troublesome due to its highly recombinant genome and methods that have been used effectively with other nosocomial pathogens (e.g. MRSA) including MLST and PFGE were shown to be unreliable for *E. faecium* compared to higher resolution WGS typing methods, such as cgMLST (de Been *et al.*, 2015; Raven *et al.*, 2016; Pinholt *et al.*, 2017). Many studies have reported an additional challenge in typing VREfm in particular, relating to the dissemination of plasmids encoding vancomycin-resistance through diverse genetic lineages (Pinholt *et al.*, 2017, Pinholt *et al.*, 2019). This adds an additional layer of complexity to understanding the population structure of VREfm in particular settings or geographic regions, as genetically unrelated isolates recovered from separate patients may harbour an identical plasmid, the identification of which may be construed, incorrectly, as indicating a possible transmission event.

Previous WGS studies on *E. faecium* isolates have revealed a well-defined hospital endemic population, termed clade A1 (Lebreton *et al.*, 2013; Raven *et al.*, 2017). Clade A1 isolates have been repeatedly shown to be distinct from animal (Clade A2) and community (Clade B) isolates. Clade A1 is also characterised by its enrichment of MGEs, insertion sequences and pathogenicity islands (Lebreton *et al.*, 2013; Gouliouris *et al.*, 2018). Many studies of *E. faecium* have shown that VREfm and VSEfm share a highly related core-

genome and that VREfm can arise *de novo* following the acquisition of an MGE harbouring *vanA* (Raven *et al.*, 2016; Pinholt *et al.*, 2019). Interestingly, the *de novo* emergence of VREfm harbouring *vanB* in the GI tract of humans has previously been described following the acquisition of the *vanB* transposon Tn*1549* from anaerobic bacteria (Howden *et al.*, 2013). This has yet to be shown for *vanA*, but acquisition from other organisms within the GI tract and/or the environment is likely. WGS studies on VREfm have also shown that the population present in hospitals is highly polyclonal, with evidence of both the intra- and inter-hospital spread of particular clones and in some instances the circulation of predominant *vanA* plasmids throughout the population (Raven *et al.*, 2016; Pinholt *et al.*, 2017, Pinholt *et al.*, 2019). A study by Raven *et al.* (2016) identified two clusters geographically restricted to Ireland/Northern Ireland, although the numbers of isolates investigated were low (17 isolates from 2007–2011 and 18 isolates from 2004–2010). Evidence of a unique *vanA* transposon with a truncated transposase gene was noted in 21 isolates included from multiple hospital sites in Ireland, indicating a local source of *vanA* in the Irish VREfm population when compared to that seen in the UK (Raven *et al.*, 2016).

The purpose of this part of the present study was to investigate the population structure of VRE*fm* from a large teaching hospital in Dublin, Ireland, to investigate the genetic regions encoding *vanA* and to investigate their diversity within this population in order to determine if there is a unique feature(s) associated with high levels of invasive VREfm infection in Ireland.

3.2 Materials and Methods

3.2.1 *E. faecium* isolates

Between June 2017 and July 2019, 365 *E. faecium* isolates were collected at the clinical microbiology laboratory in a large Irish teaching hospital in Dublin. These included VREfm screening isolates (n=286) collected over two-week periods each quarter for one year (June 2018, October 2018, March 2019 and June 2019) and all *E. faecium* BSI's (both VREfm and VSEfm) (n=79) recovered during the study period (Table 3.1). Three hundred and sixty-two isolates (99.1%) were recovered from specimens taken from patients housed on 32 wards within the hospital and three isolates (0.8%) were from patient specimens originating from general practitioners (GPs) within the hospital catchment area. A total of 331 isolates were vancomycin-resistant, while the remaining 34 isolates were vancomycin susceptible (Table 3.1).

3.2.2 Species identification and vancomycin resistance gene detection

The identity of enterococcal species and presence of *vanA*/*vanB* resistance genes was confirmed using PCR screening, as described in Chapter 2, Section 2.4.2.2.

3.2.3 Phenotypic susceptibility testing

All isolates were tested for susceptibility to vancomycin and teicoplanin using the VITEK®2 system (bioMérieux) using the EUCAST interpretive criteria, as described in Chapter 2, Section 2.3.3.

3.2.4 Filter mating

Conjugative transfer of plasmids encoding *vanA* for four VREfm isolates from patient screening including SJ11 (non-typeable, CT2), SJ82 (ST203, CT20), SJ245 (ST117, CT2929), SJ267 (ST18, CT1898) and the BSI isolate SJ40 (ST80, CT1598) was undertaken by filter mating using the plasmid-free recipient strains *E. faecium* 64/3 and *E. faecalis* OG1RF, as described in Chapter 2, Section 2.9.1.

3.2.5 Whole-genome sequencing

A total of 365 clinical isolates and 11 transconjugant derivatives of *E. faecium* 64/3 harbouring *vanA* underwent short-read WGS as described in Chapter 2, Section 2.5.1. For isolates selected for hybrid assembly, DNA was extracted as described in Chapter 2, Section 2.4.1.3. Long-read sequencing was performed as described in Chapter 2, Section 2.5.2.

3.2.5.1 Analysis of WGS data

WGS data underwent quality trimming, *de novo* assembly and was analysed using the *E. faecium* cgMLST scheme available in SeqSphere+ (version 7.0.4), as described in Chapter 2, Sections 2.7.1, 2.7.3 and 2.7.4. Conventional MLST was also applied as described in Chapter 2, Section 2.7.2. Neighbour-joining trees (NJTs) were created as described in Chapter 2, Section 2.7.5. In parallel, all isolates had core genome SNPs (cgSNPs) called using Snippy (v4.6.0; available at https://github.com/tseemann/snippy) with the *E. faecium* complete genome Aus0004 (GenBank: CP003351.1) used as the reference for all isolates. The core genome SNP phylogeny was inferred using Gubbins (v2.4.1; available at https://github.com/sanger-pathogens/gubbins) using default parameters and a maximum-

likelihood tree was constructed using IQ-TREE (v1.6.12; available at http://www.iqtree.org/) with 1000 bootstrap replicates and correction made for ascertainment bias.

Isolates suspected to belong to Clade B based on highly distant relationship to all other isolates were compared against the genomes of known reference Clade B *E. faecium* isolates (JE1, GenBank accession: CP033041.1; E1636, GenBank accession: GCA_000172835.1; DT1-1, GenBank accession: CP050255.1) by k-mer distance estimation using MASH (v2.2; available at: https://github.com/marbl/Mash). All Clade B isolates were VSEfm from BSIs, therefore infection was likely caused by commensal *E. faecium* clone in these cases. Clade B isolates were removed from further analysis, as the focus of this study was to examine hospital-associated Clade A1 *E. faecium* in an Irish hospital.

3.2.5.2 Assembly and analysis of mobile genetic elements encoding vancomycin resistance

MinION-generated FASTQ files and MiSeq-generated FASTQ files were used to perform hybrid assembly as described in Chapter 2, Section 2.7.7. The completed sequences were annotated using RAST v2.0 (http://rast.nmpdr.org/) and visualised using SnapGene Viewer v5.2.4 (https://www.snapgene.com/snapgene-viewer/). The sequence of plasmids (pSJ82*vanA* and pSJ245*vanA*), transposon regions (SJ10*vanA*) and a plasmid-like sequence (SJ10*vanA* plasmid-like) encoding *vanA,* were used as a reference sequences to compare against all other VREfm isolates in the collection, as described in Chapter 2, Section 2.7.8. Sequences resolved by hybrid assembly have been deposited in GenBank in BioProject number: PRJNA734127

3.3 Results

3.3.1 Genomic epidemiology of *E. faecium* in an Irish Hospital

A total of 365 *E. faecium* isolates were collected from a large Dublin teaching hospital. Isolates were from routine screening (n=286), collected over two-week periods each quarter for one-year (June 2018, October 2018, March 2019 and June 2019) and all *E. faecium* BSI's (n=79) recovered between September 2017 and October 2019. A total of 331 (90.7%) isolates were vancomycin-resistant (vancomycin MIC > 4 mg/L; 286 screening and 45 BSI isolates), while the remaining 34 (9.3%) isolates (all BSIs) were vancomycin susceptible (vancomycin MIC < 4 mg/L). Of these 365 isolates, 360 (98.6%) belonged to clade A1. The five remaining isolates (1.4%) (all VSEfm BSI) were queried as

belonging to the community-adapted clade B and this was confirmed by comparison with clade B reference sequences, as outlined in Section 3.2.5.1. Only clade A1 isolates were investigated further (Table 3.1).

The majority of the clade A1 *E. faecium* isolates belonged to ST80 (207/360, 57.5%) based on conventional MLST, with the majority of the remaining isolates belonging to ST117 (n=21), ST202 (n=3), ST203 (n=42), ST612 (n=24), ST787 (n=22) and ST789 (n=9). Three isolates were non-typeable due to the previously described deletion of the *pstS* housekeeping gene (Carter *et al.*, 2016). The remaining seven isolates were deemed "novel" as they could not be assigned to a known ST. These have been submitted to PubMLST.org and are awaiting ST assignment (Table 3.1). Using cgMLST, the 360 clade A1 isolates divided into 33 clusters and 63 singletons, with an inter-cluster allelic difference range of 25-1201. Clusters contained mixtures of screening and BSI isolates, as well as mixtures of vancomycin-resistant and vancomycin-susceptible isolates. (Table 3.1, Figure 3.1). The two largest clusters of related isolates in the collection belonged to the conventional MLST ST80 and were defined by their predominant cgMLST complex types (CT). The largest cluster group ST80 CT1598, consisted of 47 isolates (13%, 47/360), 46/47 of which were VREfm and consisted of a mixture of screening and BSI isolates. The single VSEfm isolate in this cluster was from a BSI. One isolate (SJ258) in this cluster belonged to CT3173. The ST80 CT1598 cluster also consisted of isolates recovered from patients housed on 14 different hospital wards between September 2017 and June 2019 (Table 3.1). The second largest ST80 cluster consisted of 46 isolates (12.7%, 46/360), 45 of which belonged to ST80 CT1911 and one isolate (BSI_SJ42) belonged to ST80 CT3153. This cluster also consisted of a mixture of screening and BSI isolates, recovered from patients housed on 15 separate wards and was also predominantly composed of VREfm isolates, with only two VSEfm BSI isolates within this cluster.

While the largest cluster groups belong to ST80, there were other significant cluster groups across other ST's. Three major groups included ST203 CT1599 (n=29), ST612 CT2942/CT2962 (n=22) and ST117 CT2283 (n=18) (Figure 3.1). The ST117 CT2283 cluster consisted of 15 VREfm and three VSEfm, isolated from 10 wards between May 2018 and June 2019. Within this cluster one isolate belonged to ST1478, which is a single-locus variant (SLV) of ST117. Within the cluster group ST203 CT1599, 26 isolates assigned to this CT, one isolate to CT2277, one isolate CT3155 and one isolate did not assign to a CT. The isolates in this group were recovered between September 2017-June 2019, from 14 ward locations and one isolate originated from a GP (Table 3.1). An

interesting large cluster group was seen among ST612 isolates, with the cluster made up of 10 isolates assigned to CT2942 and 12 assigned to CT2962. All isolates were recovered between March 2019-June 2019 from 10 wards, with 45% (10/22) of isolates recovered from Ward 3 (Table 3.1, Figure 3.1)

The majority of clusters contained isolates of the same ST, with only three clusters containing two STs; in all three clusters the second ST within the cluster was found to be a SLV of the predominant ST of that cluster (Table 3.1, Figure 3.1). The entire population in this hospital was polyclonal, with highly related isolates spread across multiple wards and persisting over extended periods of time and evidence of VREfm and VSEfm isolates clustering together.

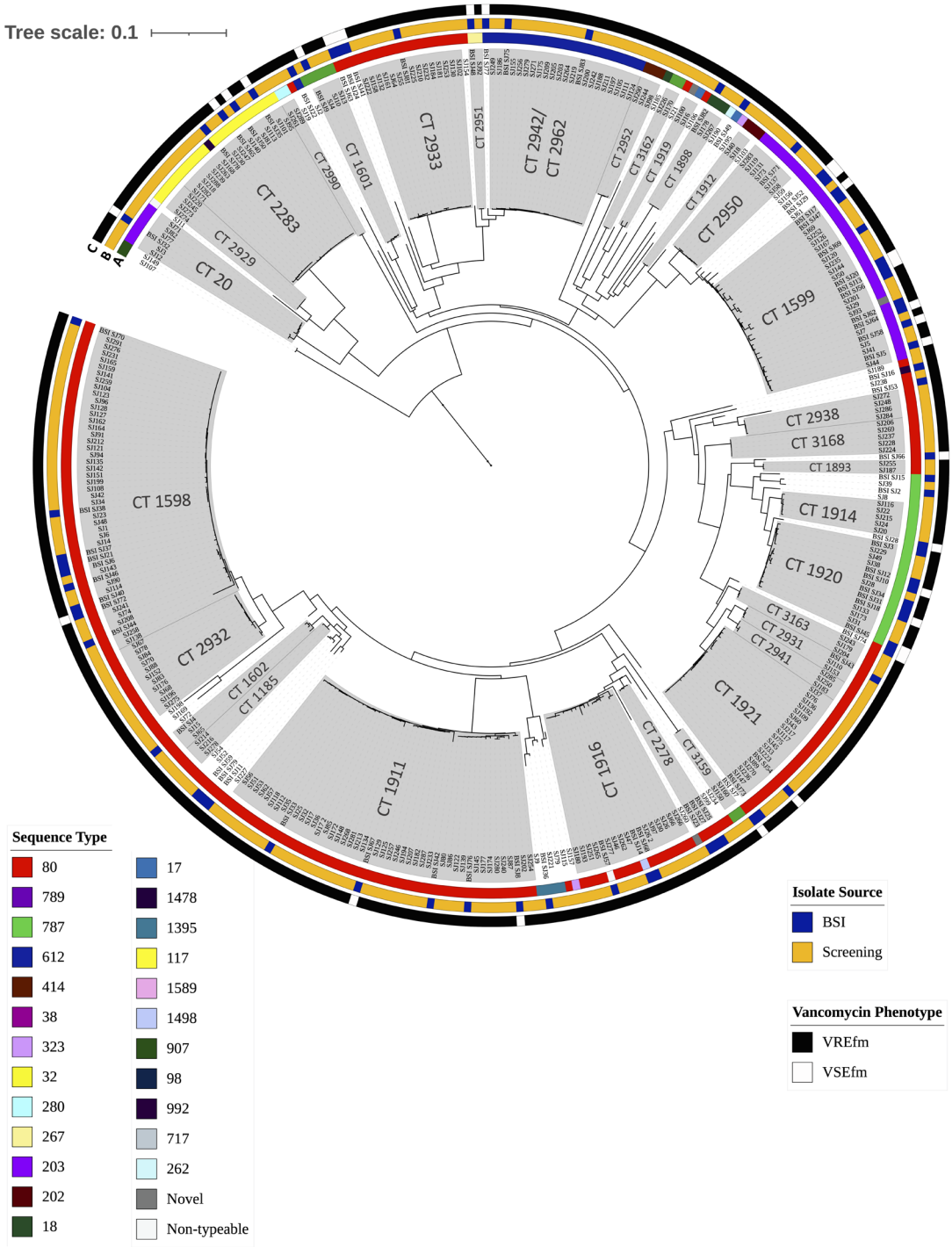


Figure 3.1. Neighbour-joining tree (NJT) based on cgMLST of 360 clade A1 *E. faecium* isolates, recovered between June 2017 and July 2019 from the clinical microbiology laboratory in a large Dublin teaching hospital. The 360 isolates divided into 33 clusters and 63 singletons, with an inter-cluster allelic differences range of 25-1201. Clusters are shaded in grey and predominant complex types (CT) labelled accordingly. Scale bar represents the phylogenetic distance between isolates based on cgMLST. Metadata is represented encircling the NJT as denoted by the colour legends, data represented is A) sequence types, B) source of isolate and C) vancomycin phenotype.

Parallel to the cgMLST analysis, cgSNP analysis was performed on all 360 clade A1 isolates. A maximum-likelihood tree was calculated based on a total of 15,715 cgSNPs (Figure 3.2). Pairwise cgSNP analysis revealed a minimum of zero cgSNP differences between isolates and a maximum of 199 SNPs between the most distantly related isolates. The population was highly polyclonal, with a mean SNP difference of 35 (median=34) and all clusters corresponding with clusters identified by cgMLST (Figure 3.1). Within the predominant ST80 CT1598 cluster, isolates were very closely related with a mean cgSNP difference of 2.2 (median=2) and an intra-cluster cgSNP range of 0-8. Supportive of the cgMLST data, the cgSNP data also showed mixing of screening and BSI isolates within clusters, along with mixing of VREfm and VSEfm isolates (Figure 3.2).

The ST80 group was found to be the predominant ST in this Irish hospital, accounting for 57.5% (207/360) of all clade A1 isolates investigated. Further examination of ST80, confirmed the polyclonal nature of *E. faecium* within STs. The ST80 population divided into 16 clusters of two or more isolates (Figure 3.3). The majority of isolates were found within clusters (88.8%, 184/207), but clusters differed by a wide range, with inter-cluster allelic difference ranging from 22-248 allelic differences. The remaining 23 isolates were deemed singletons as they were unrelated to any other isolates in the population. Within clusters, there was further evidence of relatedness between screening isolates and BSI isolates and between VREfm and VSEfm isolates (Figure 3.3). The significant variation found within the predominant ST80 population further highlights the polyclonal nature of clade A1 *E. faecium* isolates in Ireland.

Table 3.1 Phenotypic and genotypic characteristics and whole-genome sequencing data analysis of the 360 clade A1 *E. faecium* isolates investigated

Specimen no.	Date of sample	Specimen	Van[a]	Teic[a]	Ward	ST	cgMLST CT[b]	SJ10 *vanA*[c]	SJ10*vanA* p-like[c]	pSJ82 *vanA*[c]	pSJ245 *vanA*[c]
SJ29	10/06/18	Screening	R	R	W8	203	1599	100.0%	96.5%	38.2%	70.0%
BSI_SJ16	19/12/17	BSI	R	R	W19	992	2274	97.5%	97.1%	56.0%	72.7%
SJ216	18/06/19	Screening	R	R	W12	80	1185	100.0%	95.4%	64.0%	71.8%
SJ278	25/06/19	Screening	R	R	W12	80	1185	100.0%	95.1%	63.5%	69.8%
SJ214	18/06/19	Screening	R	R	W1	80	1185	100.0%	95.7%	58.6%	67.8%
SJ72	22/10/18	Screening	R	R	W7	80	1201	100.0%	93.7%	60.3%	66.9%
SJ2	06/06/17	Screening	R	R	W2	789	1596	100.0%	99.4%	38.3%	54.9%
SJ9	25/07/17	Screening	R	R	W1	1395	1597	100.0%	98.4%	54.4%	56.7%
SJ241	22/06/19	Screening	R	S	W3	80	1598	100.0%	99.0%	64.5%	78.3%
SJ276	25/06/19	Screening	R	R	W12	80	1598	100.0%	98.2%	64.2%	80.0%
SJ90	03/03/19	Screening	R	R	W3	80	1598	100.0%	98.6%	64.0%	78.7%
SJ91	03/03/19	Screening	R	R	W3	80	1598	100.0%	97.6%	63.8%	79.3%
SJ114	06/03/19	Screening	R	R	W3	80	1598	100.0%	98.5%	63.7%	77.1%
SJ159	13/03/19	Screening	R	R	W3	80	1598	100.0%	97.7%	63.6%	78.7%
BSI_SJ40	26/06/18	BSI	R	R	W19	80	1598	100.0%	98.9%	63.6%	78.8%
SJ291	28/06/19	Screening	R	R	W12	80	1598	100.0%	92.2%	63.6%	78.6%
BSI_SJ37	29/05/18	BSI	R	R	W19	80	1598	99.4%	98.8%	63.4%	85.1%
SJ143	11/03/19	Screening	R	R	W25	80	1598	100.0%	98.3%	62.1%	73.2%
SJ42	19/06/18	Screening	R	R	W13	80	1598	100.0%	98.9%	61.9%	74.2%
BSI_SJ21	01/01/18	BSI	R	R	W5	80	1598	100.0%	99.0%	61.6%	73.9%

Table 3.1 continued overleaf

Specimen no.	Date of sample	Specimen	Van[a]	Teic[a]	Ward	ST	cgMLST CT[b]	SJ10 *vanA*[c]	SJ10*vanA* p-like[c]	pSJ82 *vanA*[c]	pSJ245 *vanA*[c]
SJ142	10/03/19	Screening	R	R	W3	80	1598	100.0%	97.7%	61.4%	73.8%
SJ14	05/09/17	Screening	R	R	W2	80	1598	100.0%	99.0%	61.2%	73.0%
SJ1	05/09/17	Screening	R	R	W2	80	1598	100.0%	98.8%	61.1%	73.1%
SJ6	24/07/17	Screening	R	R	W2	80	1598	100.0%	98.9%	61.0%	72.9%
SJ162	14/03/19	Screening	R	R	W3	80	1598	100.0%	98.1%	61.0%	73.8%
BSI_SJ38	10/06/18	BSI	R	R	W1	80	1598	100.0%	98.7%	61.0%	72.7%
SJ208	19/06/19	Screening	R	R	W3	80	1598	100.0%	98.7%	61.0%	72.6%
SJ104	05/03/19	Screening	R	R	W2	80	1598	100.0%	98.0%	60.9%	74.4%
SJ231	20/06/19	Screening	R	R	W23	80	1598	100.0%	97.8%	60.8%	73.7%
BSI_SJ46	27/07/18	BSI	R	R	W1	80	1598	100.0%	98.8%	60.8%	74.0%
SJ48	20/06/18	Screening	R	R	W3	80	1598	100.0%	98.8%	60.8%	73.4%
SJ141	10/03/19	Screening	R	R	W18	80	1598	100.0%	97.7%	60.7%	72.4%
SJ96	03/03/19	Screening	R	R	W3	80	1598	100.0%	97.9%	60.5%	74.0%
SJ165	15/03/19	Screening	R	R	W8	80	1598	100.0%	97.9%	60.4%	72.5%
SJ135	08/03/19	Screening	R	R	W23	80	1598	100.0%	97.4%	60.4%	73.1%
BSI_SJ70	27/02/19	BSI	R	R	W16	80	1598	100.0%	97.5%	60.4%	72.8%
BSI_SJ6	09/10/17	BSI	R	R	W12	80	1598	100.0%	99.2%	60.4%	73.1%
SJ128	06/03/19	Screening	R	R	W3	80	1598	100.0%	98.0%	60.4%	72.5%
SJ164	14/03/19	Screening	R	R	W8	80	1598	100.0%	98.1%	60.4%	72.4%
SJ74	23/10/18	Screening	R	R	W3	80	1598	100.0%	98.2%	60.4%	72.1%
SJ127	06/03/19	Screening	R	R	W3	80	1598	100.0%	97.8%	60.4%	72.4%

Table 3.1 continued overleaf

Specimen no.	Date of sample	Specimen	Van[a]	Teic[a]	Ward	ST	cgMLST CT[b]	SJ10 *vanA*[c]	SJ10*vanA* p-like[c]	pSJ82 *vanA*[c]	pSJ245 *vanA*[c]
SJ123	06/03/19	Screening	R	R	W3	80	1598	100.0%	98.1%	60.3%	72.9%
SJ151	12/03/19	Screening	R	R	W3	80	1598	100.0%	97.5%	60.3%	72.4%
SJ212	19/06/19	Screening	R	R	W24	80	1598	100.0%	97.5%	60.3%	72.3%
SJ23	12/06/18	Screening	R	R	W13	80	1598	100.0%	98.7%	60.3%	73.0%
SJ138	09/03/19	Screening	R	R	W3	80	1598	100.0%	98.6%	60.2%	71.6%
SJ34	17/06/18	Screening	R	R	W17	80	1598	100.0%	98.8%	60.2%	72.9%
SJ121	06/03/19	Screening	R	R	W3	80	1598	97.0%	97.2%	60.2%	72.1%
BSI_SJ44	15/07/18	BSI	R	R	W6	80	1598	100.0%	98.9%	60.1%	72.8%
SJ94	03/03/19	Screening	R	R	W3	80	1598	97.0%	97.3%	59.7%	71.8%
SJ199	18/06/19	Screening	R	R	W1	80	1598	100.0%	98.0%	59.4%	73.8%
SJ108	05/03/19	Screening	R	R	W2	80	1598	100.0%	98.6%	57.2%	69.6%
SJ259	22/06/19	Screening	R	R	W2	80	1598	100.0%	94.3%	52.3%	66.9%
BSI_SJ72	14/03/19	BSI	S	S	W6	80	1598	26.7%	82.0%	45.7%	43.8%
SJ93	03/03/19	Screening	R	R	W3	Novel	1599	100.0%	98.9%	43.4%	69.1%
BSI_SJ62	08/01/19	BSI	R	R	W24	203	1599	100.0%	97.0%	45.5%	73.0%
BSI_SJ47	21/08/18	BSI	R	R	W3	203	1599	100.0%	95.0%	44.1%	66.1%
SJ201	18/06/19	Screening	R	R	W6	203	1599	100.0%	97.4%	43.5%	70.5%
SJ144	11/03/19	Screening	R	R	GP	203	1599	100.0%	98.9%	41.9%	70.0%
SJ120	06/03/19	Screening	R	R	W3	203	1599	88.8%	94.8%	41.7%	65.6%
BSI_SJ56	21/12/18	BSI	R	R	W18	203	1599	98.9%	93.0%	41.5%	69.0%
SJ50	20/06/18	Screening	R	R	W3	203	1599	99.5%	99.0%	41.4%	71.8%

Table 3.1 continued overleaf

Specimen no.	Date of sample	Specimen	Van[a]	Teic[a]	Ward	ST	cgMLST CT[b]	SJ10 *vanA*[c]	SJ10*vanA* p-like[c]	pSJ82 *vanA*[c]	pSJ245 *vanA*[c]
SJ41	19/06/18	Screening	R	R	W18	203	1599	100.0%	97.0%	41.4%	71.8%
SJ7	03/09/17	Screening	R	R	W9	203	1599	100.0%	96.1%	41.2%	69.7%
SJ235	20/06/19	Screening	R	R	W14	203	1599	100.0%	99.0%	41.2%	69.5%
SJ44	20/06/18	Screening	R	R	W3	203	1599	100.0%	96.3%	41.0%	71.5%
SJ167	15/03/19	Screening	R	R	W6	203	1599	100.0%	96.1%	41.0%	64.7%
SJ156	12/03/19	Screening	R	R	W23	203	1599	100.0%	97.1%	41.0%	70.1%
SJ5	08/08/17	Screening	R	R	W8	203	1599	100.0%	96.9%	40.9%	69.8%
BSI_SJ69	12/02/19	BSI	R	R	W28	203	1599	85.2%	94.7%	40.8%	63.2%
SJ252	24/06/19	Screening	R	R	W18	203	1599	100.0%	98.4%	40.4%	67.0%
SJ126	06/03/19	Screening	R	R	W6	203	1599	100.0%	98.7%	40.4%	68.0%
SJ61	17/10/18	Screening	R	R	W1	203	1599	98.6%	95.9%	38.9%	69.7%
SJ69	19/10/18	Screening	R	R	W2	203	1599	95.4%	96.0%	38.8%	62.6%
BSI_SJ17	20/12/17	BSI	S	S	W3	203	1599	66.5%	89.2%	36.3%	55.5%
BSI_SJ64	13/01/19	BSI	S	S	W21	203	1599	27.6%	80.9%	28.1%	41.6%
BSI_SJ5	07/10/17	BSI	S	S	W6	203	1599	21.0%	14.2%	27.1%	41.8%
BSI_SJ13	28/11/17	BSI	S	S	W1	203	1599	23.3%	14.8%	26.7%	42.8%
BSI_SJ58	03/01/19	BSI	S	S	W6	203	1599	23.4%	20.5%	26.2%	42.0%
SJ8	20/08/17	Screening	R	R	W1	787	1600	61.9%	87.0%	51.3%	56.1%
SJ4	30/08/17	Screening	R	R	W9	789	1601	100.0%	99.3%	39.7%	54.7%
SJ10	30/08/17	Screening	R	R	W3	789	1601	100.0%	98.8%	39.0%	55.7%
SJ13	29/08/17	Screening	R	R	W3	789	1601	100.0%	99.3%	37.6%	54.1%

Table 3.1 continued overleaf

Specimen no.	Date of sample	Specimen	Van[a]	Teic[a]	Ward	ST	cgMLST CT[b]	SJ10 *vanA*[c]	SJ10*vanA* p-like[c]	pSJ82 *vanA*[c]	pSJ245 *vanA*[c]
BSI_SJ9	30/10/17	BSI	S	S	W3	789	1601	29.6%	17.0%	22.5%	36.5%
SJ15	04/09/17	Screening	R	R	W9	80	1602	97.5%	96.3%	70.5%	72.2%
BSI_SJ4	03/10/17	BSI	R	S	W6	80	1602	97.7%	97.6%	69.6%	72.8%
SJ65	19/10/18	Screening	R	R	W1	80	1602	97.5%	96.8%	60.4%	72.4%
BSI_SJ41	29/06/18	BSI	S	S	W25	80	1697	22.4%	15.1%	21.8%	38.8%
SJ255	24/06/19	Screening	R	R	W6	80	1893	100.0%	96.5%	55.1%	70.1%
SJ187	17/06/19	Screening	R	R	W6	80	1893	100.0%	96.4%	52.4%	67.2%
SJ267	26/06/19	Screening	R	R	W3	18	1898	100.0%	97.6%	52.0%	68.1%
SJ178	12/06/19	Screening	R	R	W3	18	1898	100.0%	96.9%	50.4%	64.8%
BSI_SJ82	09/06/19	BSI	R	R	W28	18	1898	100.0%	97.0%	48.6%	64.3%
SJ86	26/10/18	Screening	R	R	W5	80	1911	100.0%	96.8%	61.9%	70.2%
SJ122	06/03/19	Screening	R	R	W3	80	1911	100.0%	97.0%	61.4%	67.6%
SJ145	12/03/19	Screening	R	R	W1	80	1911	100.0%	96.5%	61.4%	71.6%
SJ254	24/06/19	Screening	R	R	W6	80	1911	92.8%	94.8%	60.6%	65.1%
SJ177	12/06/19	Screening	R	R	W7	80	1911	100.0%	97.0%	60.5%	67.1%
SJ139	10/03/19	Screening	R	R	W1	80	1911	100.0%	96.3%	60.2%	67.7%
SJ174	12/06/19	Screening	R	R	W6	80	1911	100.0%	96.9%	60.2%	66.8%
SJ85	26/10/18	Screening	R	R	W5	80	1911	97.9%	96.5%	60.1%	65.7%
SJ202	18/06/19	Screening	R	R	W6	80	1911	92.8%	95.3%	59.9%	65.0%
BSI_SJ76	17/04/19	BSI	R	R	W3	80	1911	100.0%	96.4%	59.9%	66.4%
SJ80	26/10/18	Screening	R	R	W2	80	1911	99.4%	96.9%	59.1%	62.5%

Table 3.1 continued overleaf

Specimen no.	Date of sample	Specimen	Van[a]	Teic[a]	Ward	ST	cgMLST CT[b]	SJ10 *vanA*[c]	SJ10*vanA* p-like[c]	pSJ82 *vanA*[c]	pSJ245 *vanA*[c]
SJ87	26/10/18	Screening	R	R	W5	80	1911	100.0%	96.8%	58.8%	70.3%
SJ17.2	16/06/18	Screening	R	R	W3	80	1911	97.5%	96.7%	58.8%	62.5%
SJ17	13/06/18	Screening	R	R	W3	80	1911	97.5%	95.8%	58.6%	62.1%
SJ36	18/06/18	Screening	R	R	W3	80	1911	97.5%	96.5%	58.5%	62.1%
SJ280	27/06/19	Screening	R	R	W27	80	1911	100.0%	96.7%	58.4%	69.6%
SJ240	21/06/19	Screening	R	R	W3	80	1911	100.0%	97.0%	57.9%	69.7%
SJ148	12/03/19	Screening	R	R	W2	80	1911	97.6%	96.9%	57.4%	63.0%
SJ35	17/06/18	Screening	R	R	W15	80	1911	97.5%	96.9%	57.3%	62.8%
SJ125	06/03/19	Screening	R	R	W3	80	1911	97.5%	96.8%	57.0%	62.6%
SJ281	26/06/19	Screening	R	R	W3	80	1911	97.6%	96.9%	56.8%	62.3%
SJ194	18/06/19	Screening	R	R	W1	80	1911	97.6%	97.3%	56.8%	63.0%
SJ134	08/03/19	Screening	R	R	W9	80	1911	97.5%	96.7%	56.8%	62.9%
SJ172	11/06/19	Screening	R	R	W7	80	1911	97.7%	96.5%	56.7%	63.8%
SJ25	10/06/18	Screening	R	R	W6	80	1911	97.4%	96.5%	56.7%	62.1%
SJ118	06/03/19	Screening	R	R	W2	80	1911	97.6%	96.5%	56.6%	62.2%
SJ207	18/06/19	Screening	R	R	W4	80	1911	97.6%	96.5%	56.5%	64.7%
SJ268	26/06/19	Screening	R	R	W3	80	1911	97.5%	96.6%	56.5%	62.6%
BSI_SJ33	11/05/18	BSI	R	R	W2	80	1911	97.4%	97.0%	56.5%	62.6%
SJ182	11/06/19	Screening	R	R	W1	80	1911	97.6%	96.8%	56.5%	62.6%
SJ213	20/06/19	Screening	R	R	W3	80	1911	97.6%	97.0%	56.5%	63.7%
SJ112	06/03/19	Screening	R	R	W12	80	1911	97.5%	96.9%	56.4%	62.9%

Table 3.1 continued overleaf

Specimen no.	Date of sample	Specimen	Van[a]	Teic[a]	Ward	ST	cgMLST CT[b]	SJ10 *vanA*[c]	SJ10*vanA* p-like[c]	pSJ82 *vanA*[c]	pSJ245 *vanA*[c]
SJ129	06/03/19	Screening	R	S	W22	80	1911	97.5%	96.7%	56.4%	63.3%
SJ257	23/06/19	Screening	R	R	W1	80	1911	97.6%	96.9%	56.3%	62.4%
SJ246	25/06/19	Screening	R	R	W4	80	1911	97.7%	97.0%	56.2%	62.4%
SJ287	30/06/19	Screening	R	R	W3	80	1911	97.7%	97.2%	56.2%	62.3%
SJ57	16/10/18	Screening	R	R	W7	80	1911	97.5%	96.8%	56.0%	61.9%
SJ32	13/06/18	Screening	R	R	W15	80	1911	97.4%	96.8%	55.9%	62.1%
SJ62	18/10/18	Screening	R	R	W3	80	1911	97.6%	96.7%	55.9%	61.9%
SJ233	20/06/19	Screening	R	R	W25	80	1911	97.5%	96.8%	55.8%	62.4%
BSI_SJ8	16/10/17	BSI	S	S	W26	80	1911	59.2%	87.2%	54.8%	61.1%
SJ51	19/10/18	Screening	R	R	W1	80	1911	97.5%	96.4%	54.5%	62.1%
SJ56	15/10/18	Screening	R	R	W1	80	1911	97.5%	96.2%	54.3%	62.0%
SJ53	15/10/18	Screening	R	R	W1	80	1911	97.5%	95.8%	54.3%	62.0%
BSI_SJ67	04/02/19	BSI	S	S	W30	80	1911	33.3%	82.0%	45.2%	44.1%
SJ18	11/06/18	Screening	R	R	W12	202	1912	100.0%	96.9%	54.0%	59.0%
SJ40	19/06/18	Screening	R	R	W12	202	1912	100.0%	97.0%	53.8%	58.0%
SJ19	12/06/18	Screening	R	R	W5	80	1913	97.5%	97.1%	51.7%	56.0%
SJ215	18/06/19	Screening	R	R	W1	787	1914	100.0%	96.7%	59.0%	77.4%
SJ116	06/03/19	Screening	R	R	W23	787	1914	100.0%	96.4%	58.8%	74.1%
SJ20	12/06/18	Screening	R	R	W1	787	1914	100.0%	98.9%	57.6%	75.3%
SJ22	14/06/18	Screening	R	R	W7	787	1914	97.6%	96.7%	56.4%	68.3%
SJ24	11/06/18	Screening	R	R	W6	787	1914	93.2%	33.0%	56.0%	67.7%

Table 3.1 continued overleaf

Specimen no.	Date of sample	Specimen	Van[a]	Teic[a]	Ward	ST	cgMLST CT[b]	SJ10 *vanA*[c]	SJ10*vanA* p-like[c]	pSJ82 *vanA*[c]	pSJ245 *vanA*[c]
SJ21	15/06/18	Screening	R	R	W6	80	1915	100.0%	96.4%	70.9%	90.5%
BSI_SJ57	27/12/18	BSI	R	R	W6	Novel	1916	100.0%	88.2%	55.7%	64.8%
BSI_SJ14	08/12/17	BSI	R	R	W18	Novel	1916	100.0%	96.2%	54.2%	63.2%
SJ97	04/03/19	Screening	R	R	W3	80	1916	100.0%	94.7%	55.4%	66.7%
SJ262	24/06/19	Screening	R	R	GP	80	1916	100.0%	87.8%	55.3%	64.9%
SJ26	11/06/18	Screening	R	R	W3	80	1916	100.0%	93.9%	55.0%	65.7%
SJ30	12/06/18	Screening	R	R	W14	80	1916	100.0%	94.3%	55.0%	66.1%
SJ193	18/06/19	Screening	R	R	W1	80	1916	100.0%	88.2%	54.9%	65.4%
SJ277	25/06/19	Screening	R	R	W1	80	1916	100.0%	87.1%	54.8%	65.9%
BSI_SJ68	10/02/19	BSI	R	R	W6	80	1916	98.9%	98.5%	54.8%	66.2%
SJ251	20/06/19	Screening	R	R	W1	80	1916	100.0%	87.8%	54.2%	63.9%
SJ26.2	15/06/18	Screening	R	R	W3	80	1916	100.0%	94.7%	54.1%	64.3%
SJ66	19/10/18	Screening	R	R	W1	80	1916	100.0%	96.3%	54.0%	63.8%
SJ180	12/06/19	Screening	R	R	W1	80	1916	100.0%	88.4%	54.0%	63.0%
SJ265	26/06/19	Screening	R	R	W3	80	1916	100.0%	87.7%	53.8%	63.1%
SJ46	20/06/18	Screening	R	R	W3	80	1916	100.0%	98.7%	52.9%	68.1%
SJ100	04/03/19	Screening	R	R	W9	Novel	1919	100.0%	95.2%	54.6%	67.3%
SJ16	12/06/18	Screening	R	R	W1	17	1919	100.0%	99.1%	56.8%	87.0%
SJ31	12/06/18	Screening	R	R	W3	NT	1920	86.1%	95.5%	58.4%	68.4%
BSI_SJ12	19/11/17	BSI	R	R	W1	NT	1920	100.0%	94.1%	58.3%	70.0%
SJ28	11/06/18	Screening	R	R	W3	787	1920	100.0%	96.2%	59.4%	75.0%

Table 3.1 continued overleaf

Specimen no.	Date of sample	Specimen	Van[a]	Teic[a]	Ward	ST	cgMLST CT[b]	SJ10 *vanA*[c]	SJ10*vanA* p-like[c]	pSJ82 *vanA*[c]	pSJ245 *vanA*[c]
SJ173	11/06/19	Screening	R	R	W3	787	1920	100.0%	96.1%	58.8%	74.9%
SJ229	19/06/19	Screening	R	R	W2	787	1920	100.0%	96.4%	58.7%	73.8%
SJ49	20/06/18	Screening	R	R	W3	787	1920	100.0%	96.8%	58.4%	72.1%
BSI_SJ3	28/09/17	BSI	R	R	W6	787	1920	100.0%	95.6%	58.2%	71.1%
BSI_SJ31	04/05/18	BSI	R	R	W4	787	1920	100.0%	98.2%	57.6%	77.5%
SJ38	18/06/18	Screening	R	R	W1	787	1920	100.0%	98.2%	57.6%	73.4%
BSI_SJ34	11/05/18	BSI	R	R	W1	787	1920	100.0%	98.7%	57.3%	75.3%
SJ133	07/03/19	Screening	R	S	W3	787	1920	100.0%	97.6%	57.3%	77.8%
BSI_SJ18	22/12/17	BSI	S	S	W1	787	1920	26.3%	81.3%	45.2%	45.4%
BSI_SJ45	26/07/18	BSI	S	S	W8	787	1920	23.7%	20.3%	35.5%	32.3%
BSI_SJ10	03/11/17	BSI	S	S	W22	787	1920	21.7%	14.3%	29.5%	28.6%
SJ43	19/06/18	Screening	R	R	W16	80	1921	88.1%	95.3%	59.3%	68.8%
SJ147	12/03/19	Screening	R	S	W25	80	1921	100.0%	98.3%	59.1%	72.6%
SJ76	23/10/18	Screening	R	R	W3	80	1921	100.0%	96.4%	58.1%	72.7%
SJ45	20/06/18	Screening	R	R	W3	80	1921	85.6%	95.4%	58.1%	67.3%
SJ89	03/03/19	Screening	R	R	W23	80	1921	100.0%	96.4%	58.0%	73.7%
SJ236	20/06/19	Screening	R	R	W3	80	1921	100.0%	97.6%	57.4%	72.2%
SJ117	06/03/19	Screening	R	R	W3	80	1921	85.2%	94.3%	57.3%	65.6%
SJ223	19/06/19	Screening	R	R	W3	80	1921	100.0%	97.5%	57.3%	72.3%
SJ270	26/06/19	Screening	R	R	W3	80	1921	100.0%	97.4%	57.3%	72.7%
SJ75	23/10/18	Screening	R	R	W3	80	1921	85.2%	95.0%	57.2%	65.7%

Table 3.1 continued overleaf

Specimen no.	Date of sample	Specimen	Van[a]	Teic[a]	Ward	ST	cgMLST CT[b]	SJ10 *vanA*[c]	SJ10*vanA* p-like[c]	pSJ82 *vanA*[c]	pSJ245 *vanA*[c]
BSI_SJ73	20/03/19	BSI	R	R	W1	80	1921	100.0%	98.4%	57.0%	72.4%
SJ33	12/06/18	Screening	R	R	W16	80	1921	87.4%	94.8%	57.0%	67.3%
SJ60	16/10/18	Screening	R	R	GP	80	1921	100.0%	98.4%	56.9%	72.1%
SJ136	08/03/19	Screening	R	R	W7	80	1921	93.9%	95.8%	56.4%	66.2%
SJ217	18/06/19	Screening	R	R	W12	80	1921	97.6%	96.5%	56.3%	64.7%
SJ192	15/06/19	Screening	R	R	W22	80	1921	97.5%	96.5%	56.1%	65.6%
SJ37	19/06/18	Screening	R	R	W6	80	1921	97.6%	96.9%	55.9%	66.6%
SJ109	05/03/19	Screening	R	R	W1	80	1921	77.6%	92.4%	52.0%	61.0%
BSI_SJ54	09/12/18	BSI	S	S	W21	80	1921	66.5%	88.5%	51.8%	66.1%
SJ12	14/08/17	Screening	R	R	W11	203	20	82.7%	30.7%	100.0%	60.7%
SJ3	04/09/17	Screening	R	R	W7	203	20	100.0%	96.9%	100.0%	70.2%
SJ71	19/10/18	Screening	R	R	W4	203	20	81.4%	27.0%	100.0%	59.2%
SJ77	23/10/18	Screening	R	R	W6	203	20	81.5%	27.7%	100.0%	59.7%
SJ82	26/10/18	Screening	R	R	W1	203	20	81.5%	30.3%	100.0%	62.4%
BSI_SJ32	05/05/18	BSI	S	S	W4	203	20	33.3%	18.7%	68.8%	44.6%
SJ47	20/06/18	Screening	R	R	W1	80	2023	100.0%	96.6%	55.0%	64.5%
BSI_SJ11	03/11/17	BSI	R	R	W6	80	2272	100.0%	95.3%	69.1%	80.4%
BSI_SJ15	15/12/17	BSI	R	R	W23	787	2273	97.4%	96.9%	60.2%	75.0%
BSI_SJ19	23/12/17	BSI	S	S	W21	Novel	2275	28.3%	18.9%	25.8%	18.5%
BSI_SJ20	27/12/17	BSI	S	S	W23	203	2276	25.5%	15.8%	28.3%	43.1%
BSI_SJ22	08/02/18	BSI	R	R	W8	612	2277	100.0%	98.1%	69.5%	69.1%

Table 3.1 continued overleaf

Specimen no.	Date of sample	Specimen	Van[a]	Teic[a]	Ward	ST	cgMLST CT[b]	SJ10 *vanA*[c]	SJ10*vanA* p-like[c]	pSJ82 *vanA*[c]	pSJ245 *vanA*[c]
BSI_SJ23	23/02/18	BSI	R	R	W20	80	2278	100.0%	90.4%	63.7%	73.9%
BSI_SJ27	19/03/18	BSI	R	R	W8	80	2278	100.0%	99.2%	63.1%	71.6%
BSI_SJ25	08/03/18	BSI	R	R	W20	80	2278	100.0%	97.1%	55.3%	74.3%
BSI_SJ24	03/03/18	BSI	S	S	W1	80	2279	23.3%	15.6%	33.4%	41.7%
BSI_SJ29	17/04/18	BSI	S	R	W6	203	2280	74.8%	91.2%	35.6%	63.7%
BSI_SJ2	20/09/17	BSI	R	R	W1	787	2281	100.0%	97.8%	62.3%	80.3%
BSI_SJ30	18/04/18	BSI	S	S	W21	32	2282	30.3%	15.6%	22.0%	25.6%
SJ168	09/06/19	Screening	R	R	W3	1478	2283	100.0%	97.0%	69.1%	73.6%
SJ95	03/03/19	Screening	R	R	W3	117	2283	100.0%	96.8%	75.1%	75.2%
SJ140	10/03/19	Screening	R	S	W18	117	2283	100.0%	98.4%	74.5%	75.2%
SJ230	20/06/19	Screening	R	R	W1	117	2283	100.0%	96.8%	74.3%	77.4%
SJ113	06/03/19	Screening	R	R	W3	117	2283	100.0%	96.6%	74.3%	74.8%
SJ81	26/10/18	Screening	R	R	W4	117	2283	100.0%	96.4%	74.2%	77.1%
SJ247	25/06/19	Screening	R	R	W4	117	2283	97.5%	96.4%	73.1%	71.2%
SJ218	18/06/19	Screening	R	R	W12	117	2283	100.0%	97.1%	70.1%	76.1%
SJ171	11/06/19	Screening	R	R	W5	117	2283	100.0%	96.3%	69.4%	74.4%
SJ288	30/06/19	Screening	R	R	W3	117	2283	100.0%	96.9%	69.3%	75.7%
SJ263	25/06/19	Screening	R	R	W27	117	2283	100.0%	96.3%	69.1%	73.9%
SJ239	23/06/19	Screening	R	R	W6	117	2283	100.0%	97.1%	69.0%	73.7%
SJ282	29/06/19	Screening	R	R	W18	117	2283	98.5%	96.6%	69.0%	75.2%
BSI_SJ78	19/04/19	BSI	R	R	W1	117	2283	100.0%	98.1%	68.9%	73.7%

Table 3.1 continued overleaf

Specimen no.	Date of sample	Specimen	Van[a]	Teic[a]	Ward	ST	cgMLST CT[b]	SJ10 *vanA*[c]	SJ10*vanA* p-like[c]	pSJ82 *vanA*[c]	pSJ245 *vanA*[c]
BSI_SJ50	02/11/18	BSI	S	S	W3	117	2283	42.4%	31.4%	64.3%	59.0%
BSI_SJ65	17/01/19	BSI	S	S	W19	117	2283	36.8%	25.3%	63.1%	54.0%
BSI_SJ35	12/05/18	BSI	S	S	W24	117	2283	27.6%	17.9%	60.8%	52.1%
SJ101	04/03/19	Screening	R	R	W12	117	2283	100.0%	97.6%	56.4%	76.4%
BSI_SJ36	15/05/18	BSI	R	R	W6	1395	2284	100.0%	99.3%	68.6%	75.0%
BSI_SJ39	12/06/18	BSI	S	S	W21	38	2285	16.3%	6.6%	10.3%	15.7%
BSI_SJ7	11/10/17	BSI	S	S	W3	80	2286	31.5%	79.9%	49.2%	52.7%
SJ249	25/06/19	Screening	R	R	W1	612	2362	100.0%	96.2%	49.4%	55.9%
SJ219	19/06/19	Screening	R	R	W1	612	2362	100.0%	97.2%	41.4%	58.4%
SJ155	12/03/19	Screening	R	R	W32	612	2362	100.0%	98.1%	38.8%	57.1%
SJ188	15/06/19	Screening	R	R	W21	612	2362	100.0%	97.2%	37.6%	55.6%
SJ200	17/06/19	Screening	R	R	W7	612	2362	100.0%	97.6%	37.5%	56.7%
SJ197	14/06/19	Screening	R	R	W1	612	2362	100.0%	97.1%	37.2%	55.8%
BSI_SJ75	31/03/19	BSI	R	R	W8	612	2362	100.0%	97.0%	37.1%	56.0%
SJ186	16/06/19	Screening	R	R	W6	612	2362	100.0%	96.3%	36.7%	55.3%
SJ211	19/06/19	Screening	R	R	W3	612	2362	100.0%	97.4%	35.9%	56.2%
BSI_SJ83	11/06/19	BSI	R	R	W1	612	2362	100.0%	96.6%	35.9%	54.2%
SJ242	23/06/19	Screening	R	R	W7	612	2362	100.0%	97.2%	35.9%	55.4%
SJ105	05/03/19	Screening	R	R	W1	612	2362	100.0%	97.5%	35.6%	55.2%
SJ11	22/08/17	Screening	R	R	W10	NT	24	100.0%	99.1%	72.3%	87.2%
SJ274	25/06/19	Screening	R	R	W2	117	2929	95.5%	37.6%	76.2%	100.0%

Table 3.1 continued overleaf

Specimen no.	Date of sample	Specimen	Van[a]	Teic[a]	Ward	ST	cgMLST CT[b]	SJ10 *vanA*[c]	SJ10*vanA* p-like[c]	pSJ82 *vanA*[c]	pSJ245 *vanA*[c]
SJ220	20/06/19	Screening	R	R	W2	117	2929	95.5%	39.1%	75.6%	100.0%
SJ245	22/06/19	Screening	R	R	W2	117	2929	95.5%	32.9%	74.9%	100.0%
SJ273	27/06/19	Screening	R	R	W7	117	2929	95.5%	34.4%	74.7%	99.1%
SJ153	12/03/19	Screening	R	R	W9	80	2931	100.0%	96.6%	58.2%	71.8%
BSI_SJ43	08/07/18	BSI	R	R	W8	80	2931	100.0%	96.2%	57.4%	72.0%
SJ110	05/03/19	Screening	R	R	W23	80	2931	100.0%	96.7%	57.4%	71.7%
SJ88	26/10/18	Screening	R	R	W5	80	2932	100.0%	99.0%	70.5%	73.9%
SJ196	18/06/19	Screening	R	R	W1	80	2932	100.0%	98.7%	65.6%	73.0%
SJ84	23/10/18	Screening	R	R	W2	80	2932	100.0%	98.5%	65.4%	72.5%
SJ152	12/03/19	Screening	R	R	W5	80	2932	100.0%	98.3%	65.4%	72.5%
SJ83	26/10/18	Screening	R	R	W1	80	2932	100.0%	98.7%	65.3%	73.5%
SJ275	26/06/19	Screening	R	R	W1	80	2932	100.0%	98.2%	65.2%	72.6%
SJ176	12/06/19	Screening	R	R	W3	80	2932	100.0%	98.4%	65.2%	73.0%
SJ225	19/06/19	Screening	R	R	W3	80	2933	100.0%	95.4%	46.0%	67.9%
SJ102	04/03/19	Screening	R	R	W6	80	2933	100.0%	96.1%	45.3%	66.9%
SJ158	13/03/19	Screening	R	R	W3	80	2933	100.0%	97.3%	44.8%	67.4%
SJ222	20/06/19	Screening	R	R	W1	80	2933	100.0%	97.9%	44.5%	68.0%
SJ161	14/03/19	Screening	R	R	W8	80	2933	100.0%	97.4%	44.1%	66.4%
SJ184	14/06/19	Screening	R	R	W27	80	2933	100.0%	95.7%	44.1%	66.1%
SJ132	07/03/19	Screening	R	R	W18	80	2933	100.0%	97.6%	43.8%	66.2%
SJ181	12/06/19	Screening	R	R	W12	80	2933	100.0%	95.6%	43.5%	66.4%

Table 3.1 continued overleaf

Specimen no.	Date of sample	Specimen	Van[a]	Teic[a]	Ward	ST	cgMLST CT[b]	SJ10 *vanA*[c]	SJ10*vanA* p-like[c]	pSJ82 *vanA*[c]	pSJ245 *vanA*[c]
SJ130	06/03/19	Screening	R	R	W2	80	2933	100.0%	95.6%	43.5%	66.4%
BSI_SJ81	06/05/19	BSI	R	R	W31	80	2933	100.0%	94.8%	43.5%	67.0%
SJ253	24/06/19	Screening	R	R	W6	80	2933	100.0%	95.8%	43.4%	66.1%
SJ232	19/06/19	Screening	R	R	W3	80	2933	98.2%	95.2%	41.6%	69.2%
SJ210	19/06/19	Screening	R	R	W3	80	2933	100.0%	96.1%	40.7%	71.0%
BSI_SJ49	16/09/18	BSI	R	R	W6	17	2934	98.7%	47.2%	51.5%	63.8%
SJ115	06/03/19	Screening	R	R	W3	80	2936	100.0%	98.3%	62.6%	64.9%
SJ284	30/06/19	Screening	R	R	W3	80	2938	100.0%	96.2%	57.7%	71.9%
SJ272	27/06/19	Screening	R	R	W1	80	2938	100.0%	96.5%	57.5%	70.8%
SJ248	25/06/19	Screening	R	R	W1	80	2938	100.0%	96.7%	57.5%	71.2%
SJ286	28/06/19	Screening	R	R	W3	80	2938	100.0%	95.8%	56.9%	71.9%
BSI_SJ59	05/01/19	BSI	R	R	W6	80	2939	100.0%	96.3%	68.4%	74.6%
BSI_SJ53	07/12/18	BSI	R	R	W1	80	2940	100.0%	96.7%	57.4%	71.8%
SJ183	13/06/19	Screening	R	R	W1	80	2941	100.0%	97.2%	57.4%	72.0%
SJ250	25/06/19	Screening	R	R	W1	80	2941	100.0%	97.5%	57.3%	72.2%
SJ285	28/06/19	Screening	R	R	W7	80	2941	100.0%	97.0%	57.1%	72.3%
SJ271	26/06/19	Screening	R	R	W3	612	2942	100.0%	97.1%	39.5%	54.9%
SJ203	19/06/19	Screening	R	R	W3	612	2942	100.0%	97.2%	37.2%	55.5%
SJ124	06/03/19	Screening	R	R	W3	612	2942	100.0%	97.4%	37.2%	54.9%
SJ205	19/06/19	Screening	R	R	W3	612	2942	100.0%	97.4%	36.9%	58.1%
SJ111	05/03/19	Screening	R	R	W7	612	2942	100.0%	97.4%	36.9%	59.6%

Table 3.1 continued overleaf

Specimen no.	Date of sample	Specimen	Van[a]	Teic[a]	Ward	ST	cgMLST CT[b]	SJ10 *vanA*[c]	SJ10*vanA* p-like[c]	pSJ82 *vanA*[c]	pSJ245 *vanA*[c]
SJ175	12/06/19	Screening	R	R	W3	612	2942	100.0%	97.5%	36.8%	56.7%
SJ279	27/06/19	Screening	R	R	W3	612	2942	100.0%	97.3%	36.8%	56.3%
SJ264	26/06/19	Screening	R	R	W3	612	2942	100.0%	97.5%	36.4%	55.2%
SJ256	23/06/19	Screening	R	R	W3	612	2942	100.0%	97.7%	35.5%	56.2%
SJ209	19/06/19	Screening	R	R	W3	612	2942	100.0%	97.0%	35.3%	57.1%
SJ103	05/03/19	Screening	R	R	W2	202	2945	100.0%	99.1%	62.3%	84.4%
SJ106	05/03/19	Screening	R	R	W4	80	2947	96.9%	96.7%	40.7%	58.8%
SJ149	12/03/19	Screening	R	R	W1	907	2948	100.0%	98.2%	40.4%	53.7%
SJ107	05/03/19	Screening	R	R	W1	907	2948	100.0%	97.8%	35.1%	50.7%
SJ137	08/03/19	Screening	R	R	W7	203	2950	100.0%	98.3%	66.0%	81.9%
BSI_SJ71	06/03/19	BSI	R	R	W21	203	2950	100.0%	98.4%	65.0%	81.7%
SJ283	29/06/19	Screening	R	R	W18	203	2950	95.5%	35.4%	52.7%	61.3%
SJ131	06/03/19	Screening	R	R	W3	203	2950	100.0%	96.5%	37.3%	52.3%
SJ119	06/03/19	Screening	R	R	W3	203	2950	100.0%	97.7%	36.7%	56.4%
SJ92	03/03/19	Screening	R	R	W3	267	2951	100.0%	96.8%	37.5%	53.6%
BSI_SJ48	14/09/18	BSI	S	S	W8	267	2951	26.4%	19.7%	20.8%	29.7%
SJ98	04/03/19	Screening	R	R	W6	414	2952	100.0%	97.5%	62.3%	67.9%
SJ290	01/07/19	Screening	R	R	W6	414	2952	100.0%	97.1%	60.2%	64.2%
SJ244	24/06/19	Screening	R	R	W6	414	2952	100.0%	97.5%	60.2%	63.9%
SJ99	04/03/19	Screening	R	R	W9	80	2953	100.0%	97.3%	55.4%	66.0%
SJ289	30/06/19	Screening	R	R	W6	280	2990	100.0%	97.7%	61.6%	76.5%

Table 3.1 continued overleaf

Specimen no.	Date of sample	Specimen	Van[a]	Teic[a]	Ward	ST	cgMLST CT[b]	SJ10 *vanA*[c]	SJ10*vanA* p-like[c]	pSJ82 *vanA*[c]	pSJ245 *vanA*[c]
SJ261	25/06/19	Screening	R	R	W6	280	2990	100.0%	97.0%	61.5%	76.4%
BSI_SJ42	02/07/18	BSI	R	R	W1	80	3153	97.5%	96.7%	55.0%	63.1%
BSI_SJ51	13/11/18	BSI	S	S	W29	98	3154	53.9%	34.5%	40.9%	38.4%
BSI_SJ52	16/11/18	BSI	S	S	W6	203	3155	54.1%	35.7%	37.3%	55.7%
BSI_SJ55	13/12/18	BSI	S	S	W1	717	3156	22.6%	10.3%	26.2%	22.7%
BSI_SJ63	09/01/19	BSI	S	S	W3	80	3157	36.1%	24.4%	56.3%	50.2%
BSI_SJ66	22/01/19	BSI	S	S	W1	80	3158	44.0%	83.2%	48.7%	55.8%
SJ160	13/03/19	Screening	R	R	W12	789	3159	100.0%	96.2%	42.7%	58.0%
SJ150	12/03/19	Screening	R	R	W12	789	3159	100.0%	95.8%	42.2%	58.6%
SJ154	12/03/19	Screening	R	R	W2	80	3160	100.0%	97.0%	40.4%	62.5%
SJ169	11/06/19	Screening	R	R	W2	80	3161	100.0%	97.6%	58.8%	82.5%
SJ170	07/06/19	Screening	R	R	W1	789	3162	100.0%	98.4%	43.4%	69.9%
SJ226	19/06/19	Screening	R	R	W23	789	3162	100.0%	98.3%	43.1%	68.7%
SJ179	12/06/19	Screening	R	R	W3	80	3163	100.0%	97.9%	57.4%	72.2%
SJ204	19/06/19	Screening	R	R	W3	80	3163	100.0%	97.8%	57.0%	71.8%
SJ243	23/06/19	Screening	R	R	W16	80	3163	96.1%	85.2%	56.5%	70.4%
SJ185	09/06/19	Screening	R	R	W6	18	3164	100.0%	93.5%	36.8%	57.8%
SJ189	15/06/19	Screening	R	R	W23	80	3165	100.0%	98.3%	58.1%	79.8%
SJ190	17/06/19	Screening	R	R	W7	262	3166	100.0%	97.8%	67.4%	62.5%
SJ198	18/06/19	Screening	R	R	W2	80	3167	100.0%	98.3%	57.5%	78.3%
SJ206	18/06/19	Screening	R	R	W2	80	3168	100.0%	98.4%	58.9%	73.8%

Table 3.1 continued overleaf

Specimen no.	Date of sample	Specimen	Van[a]	Teic[a]	Ward	ST	cgMLST CT[b]	SJ10 *vanA*[c]	SJ10*vanA* p-like[c]	pSJ82 *vanA*[c]	pSJ245 *vanA*[c]
SJ224	19/06/19	Screening	R	R	W3	80	3168	100.0%	98.8%	57.8%	73.1%
SJ237	21/06/19	Screening	R	R	W3	80	3168	100.0%	98.8%	57.4%	72.3%
SJ228	18/06/19	Screening	R	R	W2	80	3168	100.0%	98.8%	57.1%	72.7%
SJ269	26/06/19	Screening	R	R	W3	80	3168	100.0%	99.0%	56.9%	73.4%
SJ221	18/06/19	Screening	R	R	W6	1395	3169	100.0%	98.0%	68.5%	75.0%
SJ227	20/06/19	Screening	R	R	W27	80	3170	100.0%	89.4%	61.1%	77.1%
SJ234	20/06/19	Screening	R	R	W23	80	3171	100.0%	98.9%	57.7%	60.0%
SJ238	21/06/19	Screening	R	R	W7	80	3172	100.0%	96.2%	57.5%	75.2%
SJ258	24/06/19	Screening	R	R	W23	80	3173	100.0%	98.7%	58.6%	72.3%
SJ260	24/06/19	Screening	R	R	W26	Novel	3174	97.2%	92.8%	52.5%	60.9%
SJ266	26/06/19	Screening	R	R	W3	80	3175	100.0%	95.8%	54.2%	63.3%
BSI_SJ74	24/03/19	BSI	S	S	W1	80	3225	26.6%	80.7%	42.5%	43.9%
BSI_SJ77	17/04/19	BSI	S	S	W1	612	3226	27.0%	80.4%	50.5%	42.3%
BSI_SJ79	28/04/19	BSI	R	R	W6	80	3227	100.0%	96.3%	65.2%	67.9%
SJ195	18/06/19	Screening	R	R	W1	323	561	100.0%	96.2%	70.2%	74.2%
SJ39	18/06/18	Screening	R	R	W6	787	680	98.9%	94.6%	62.2%	78.4%
SJ157	13/03/19	Screening	R	R	W9	Novel	N/A	100.0%	98.7%	64.1%	70.7%
SJ70	19/10/18	Screening	R	R	W2	80	N/A	100.0%	98.8%	66.1%	72.6%
SJ78	23/10/18	Screening	R	R	W2	80	N/A	100.0%	98.9%	65.2%	72.9%
SJ67	22/10/18	Screening	R	R	W2	80	N/A	100.0%	98.4%	65.1%	72.5%
SJ68	19/10/18	Screening	R	R	W2	80	N/A	100.0%	98.5%	65.1%	73.1%

Table 3.1 continued overleaf

Specimen no.	Date of sample	Specimen	Van[a]	Teic[a]	Ward	ST	cgMLST CT[b]	SJ10 *vanA*[c]	SJ10*vanA* p-like[c]	pSJ82 *vanA*[c]	pSJ245 *vanA*[c]
SJ54	15/10/18	Screening	R	R	W4	80	N/A	100.0%	96.1%	60.5%	72.2%
SJ52	16/10/18	Screening	R	R	W1	80	N/A	100.0%	96.2%	60.4%	72.2%
SJ55	15/10/18	Screening	R	R	W2	80	N/A	97.5%	96.3%	42.8%	60.1%
SJ64	19/10/18	Screening	R	R	W10	80	N/A	21.7%	13.8%	28.5%	37.4%
BSI_SJ28	13/04/18	BSI	S	S	W7	787	N/A	28.2%	81.4%	47.0%	53.3%
SJ58	16/10/18	Screening	R	R	W3	203	N/A	100.0%	96.1%	49.9%	82.2%
SJ73	23/10/18	Screening	R	R	W3	203	N/A	100.0%	96.6%	49.7%	81.7%
SJ59	16/10/18	Screening	R	R	W1	203	N/A	100.0%	96.4%	40.5%	69.6%
SJ79	23/10/18	Screening	R	R	W1	1395	N/A	100.0%	98.4%	68.9%	74.7%

[a] Interpreted using clinical breakpoints taken from the European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST, 2019).

[b] The cgMLST complex type (CT) as determined following cgMLST typing. Seven isolates had no CT assigned and thus were marked with N/A. Table organised by CT and therefore related CT's are grouped together.

[c] Coverage (%) of isolates against the reference sequences resolved by hybrid assembly, the transposon region SJ10*vanA*, the SJ10*vanA* plasmid-like (p-like) sequence, and the resolved plasmid sequence pSJ82*vanA* and pSJ245*vanA.* Sequences submitted to GenBank BioProject: PRJNA734127

Abbreviations: Van, vancomycin; teic, teicoplanin; ST, sequence type; CT, complex type; BSI, bloodstream infection; R, resistant; S, susceptible; W, ward; NT, non-typeable; N/A, not assigned.

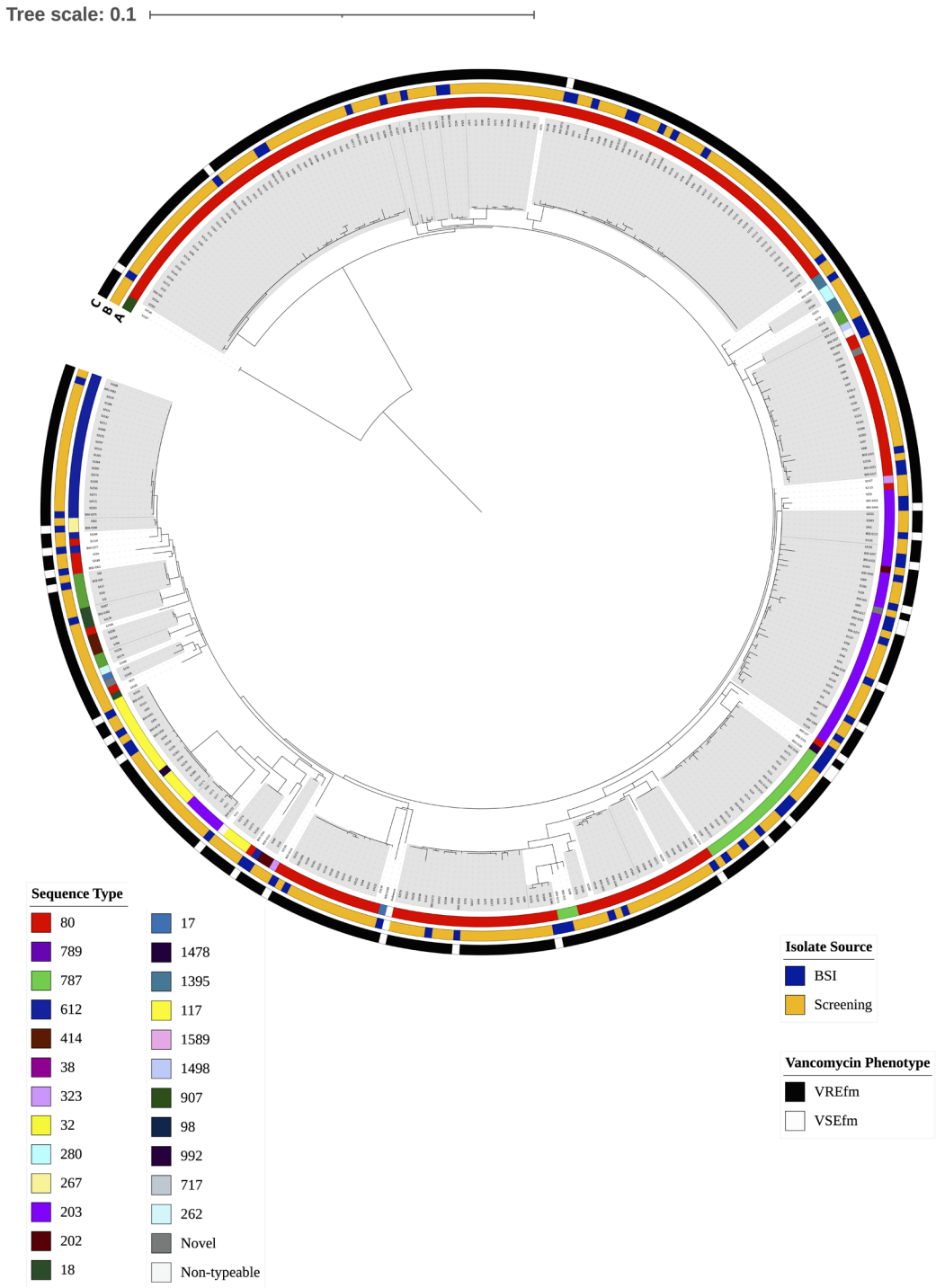


Figure 3.2. Maximum-likelihood tree (MLT) based on 15,715 cgSNPs from 360 clade A1 *E. faecium* isolates, collected between June 2017 and July 2019 from the clinical microbiology laboratory in a large Dublin teaching hospital. The 360 isolates divided into 32 clusters and 63 singletons, with a mean SNP difference of 35 (median=34). Clusters are shaded in grey and correspond to clusters derived using cgMLST (Figure 3.1). Scale bar represents the phylogenetic distance between isolates based on cgSNP data. Metadata is represented encircling the NJT as denoted by the colour legends, data represented is A) sequence types, B) source of isolate and C) vancomycin phenotype.

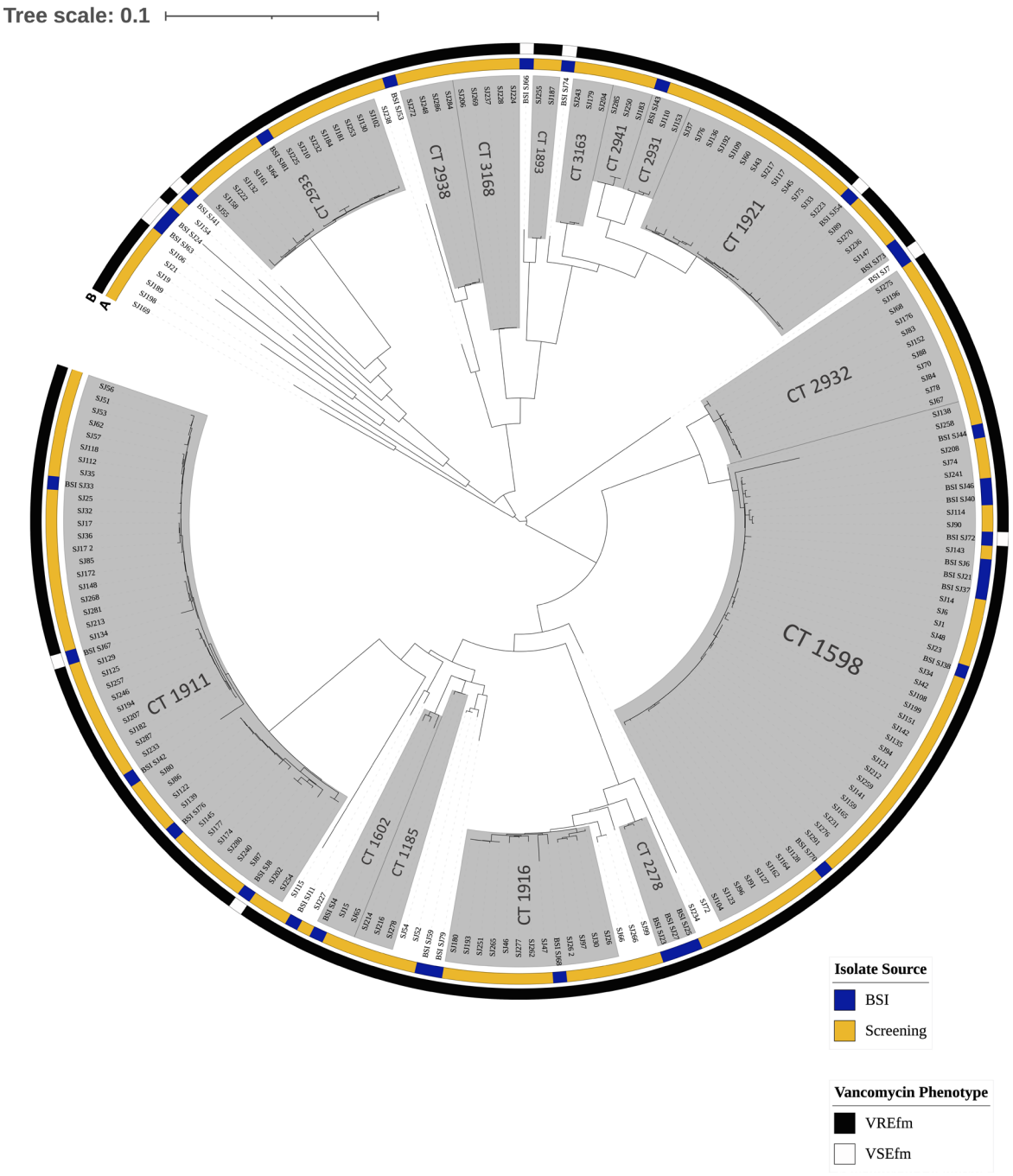


Figure 3.3. Neighbour-joining tree (NJT) based on cgMLST of 207 ST80 *E. faecium* isolates, recovered between June 2017 and July 2019 from the clinical microbiology laboratory in a large Dublin teaching hospital. The 207 isolates divided into 16 clusters and 23 singletons, with an inter-cluster allelic differences range of 22-248. Clusters are shaded in grey and predominant complex types (CT) labelled accordingly. Scale bar represents the phylogenetic distance between isolates based on cgMLST. Metadata is represented encircling the NJT as denoted by the colour legends, data represented is A) source of isolate and B) vancomycin phenotype.

3.3.2 Structural organisation of the *vanA* transposon in Irish VREfm

The *vanA* regions of three selected VREfm isolates were resolved using hybrid assembly, from isolates SJ10 (SJ10*vanA*; 11,210 bp), SJ11 (SJ11*vanA;* 13,252 bp) and BSI_SJ40 (BSI_SJ40*vanA*; 13,269 bp). These isolates were chosen at random at the beginning of the study, to represent different ST's, with SJ10 belonging to ST789 CT1601, SJ11 being non-typeable by conventional MLST belonging to CT24 and BSI_SJ40 belonging to ST80 CT1598. The three *vanA* regions differed from the original *vanA* operon prototype described for the wild-type VREfm strain BM4147 at the Institut Pasteur, in Paris (Arthur *et al.*, 1993; GenBank accession number M97297). The isolates SJ10, SJ11 and BSI_SJ40 all harboured *vanA* differing from the prototype Tn*1546* by multiple insertions of *IS*1216E, differing orientations of the *vanA* operon genes and in SJ11*vanA* and BSI_SJ40*vanA* the insertion of a cadmium efflux accessory protein gene (Figure 3.4). The SJ10*vanA* showed 87.8% sequence coverage to SJ11*vanA* and 86.9% sequence coverage to BSI_SJ40*vanA,* the disparities were due to the presence of the cadmium efflux accessory protein genes and additional non-coding DNA sequences, such as repeat regions between genes (Figure 3.4). Both SJ11 and BSI_SJ40 showed 100% sequence identity to SJ10*vanA.* The SJ10*vanA* was therefore used as a reference sequence to compare the *vanA* region across all VREfm isolates in the population investigated; 95.5% (316/331) of isolates harboured a *vanA* region with >90% sequence identity to the SJ10*vanA* reference.

Attempts to close plasmids harbouring SJ10*vanA,* SJ11*vanA* and BSI_SJ40*vanA* by hybrid assembly were unsuccessful, with all attempts yielding *vanA* on a single large contig that failed to circularise. In the isolate SJ10 this large *vanA* contig was 99,894 bp, in SJ11 *vanA* was located on a 116,749 bp contig and in BSI_SJ40 *vanA* was found on a large 131,341 bp contig. These contigs are likely to be plasmids and using the SJ10 *vanA* contig as a reference plasmid-like sequence, it was clear this element was highly similar throughout all VREfm lineages, with 97.2% (322/331) of isolates having >85% sequence identity to this 99,894 bp plasmid-like contig. Comparison to the SJ10*vanA* plasmid-like contig demonstrated SJ11had 99.1% and BSI_SJ40 had 98.9% sequence similarity, indicating all isolates harboured *vanA* on highly similar plasmid-like regions.

Two plasmids encoding *vanA* were successfully resolved using hybrid assembly from isolates SJ82 (ST203, CT20) and SJ245 (ST117, CT2929) and the plasmids were named pSJ82*vanA* (48,934 bp) and pSJ245*vanA* (40,559 bp), respectively, (Figures 3.5 and 3.6). In addition to vancomycin resistance, both plasmids encoded resistance to different classes of antibiotics. Plasmid pSJ82*vanA* encoded resistance to chloramphenicol (*catA7*),

erythromycin (*erm(B)*), aminoglycosides (*ANT(6)-I*) and streptothricin (*sat-4*) (Figure 3.5). Plasmid pSJ82*vanA* was identified in five screening isolates (SJ3, SJ12, SJ71, SJ77 and SJ82), all of which exhibited 100% sequence coverage identity to pSJ82*vanA*. All five of these isolates and SJ82 were indistinguishable by cgMLST, belonging to ST203, CT20. Interestingly, these six isolates were recovered from five different wards over a period of more than a year (first isolate 14th August 2017, last isolate 26th October 2018) (Table 3.1), indicating both spread between wards and persistence over time.

In addition to *vanA*, plasmid pSJ245*vanA* also encoded genes encoding resistance to other antibiotic classes, including erythromycin (*erm(B)*) and aminoglycosides (*ANT(6)-I and APH(3')-III*) (Figure 3.6). The *vanA* region encoded by pSJ245*vanA* exhibited 95.5% similarity to SJ10*vanA*, but there was much rearrangement in the *vanA* region in this isolate, including the additional insertion of IS*Efa5*. These findings reveal that the method of mapping used may only identify that all aspects of the reference are present, but ultimately hybrid assembly is required to determine the fine detail of the structural organisation. Interestingly, plasmid pSJ245*vanA* (from isolate SJ245, ST117) was identified in three other ST117 isolates with >99% sequence identity observed (isolates SJ220, SJ273 and SJ274), all four of which were recovered within seven days of each other, from two separate wards (Table 3.1). Furthermore, all four isolates were indistinguishable by cgMLST and formed a phylogenetic cluster, with all isolates belonging to ST117 CT2929 (Figure 3.1). This indicates the spread of a single clone in this setting and highlights the usefulness of pairing cgMLST and plasmid analysis data.

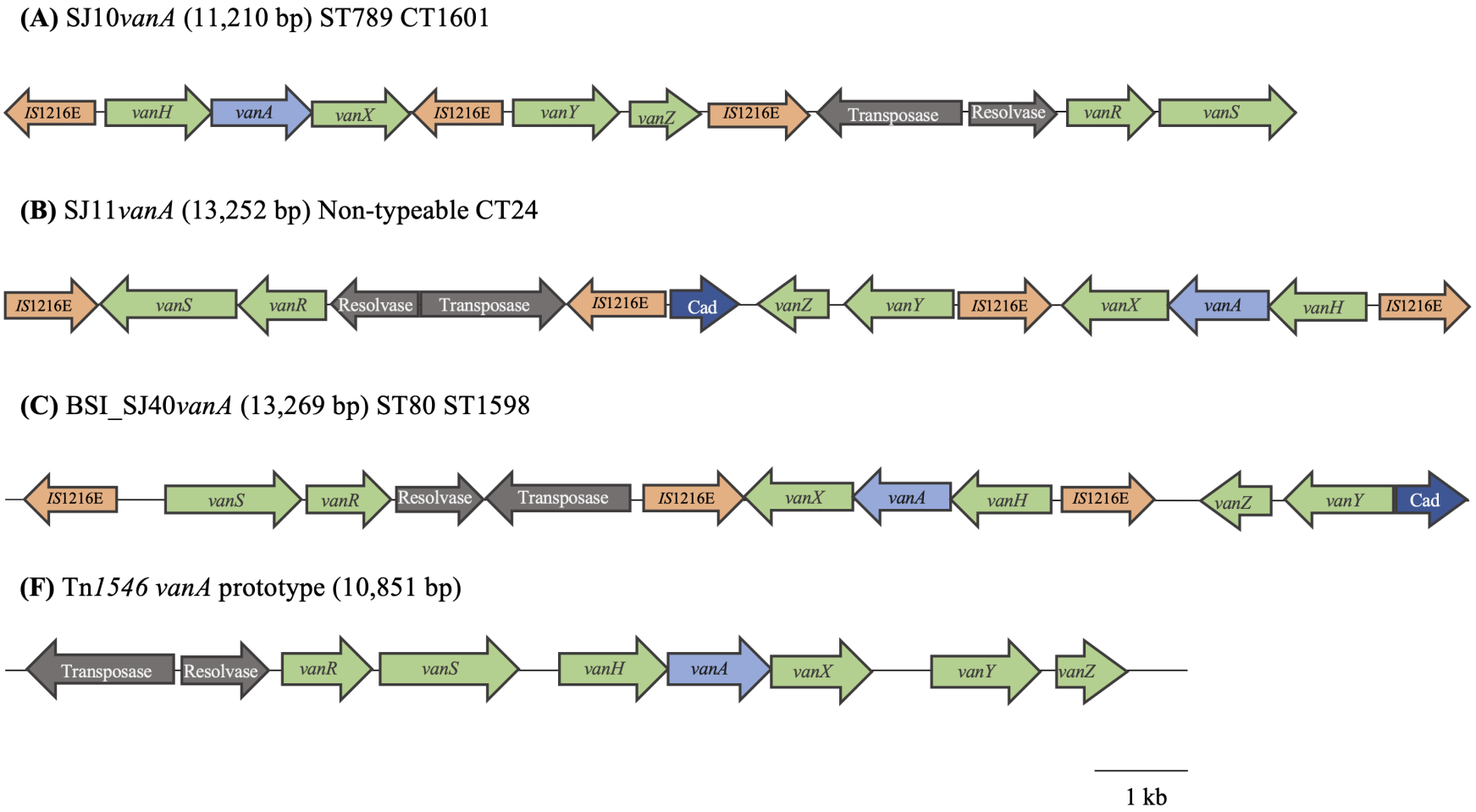


Figure 3.4 Schematic diagram showing the structural organisation of *vanA* transposon regions. (Continued overleaf)

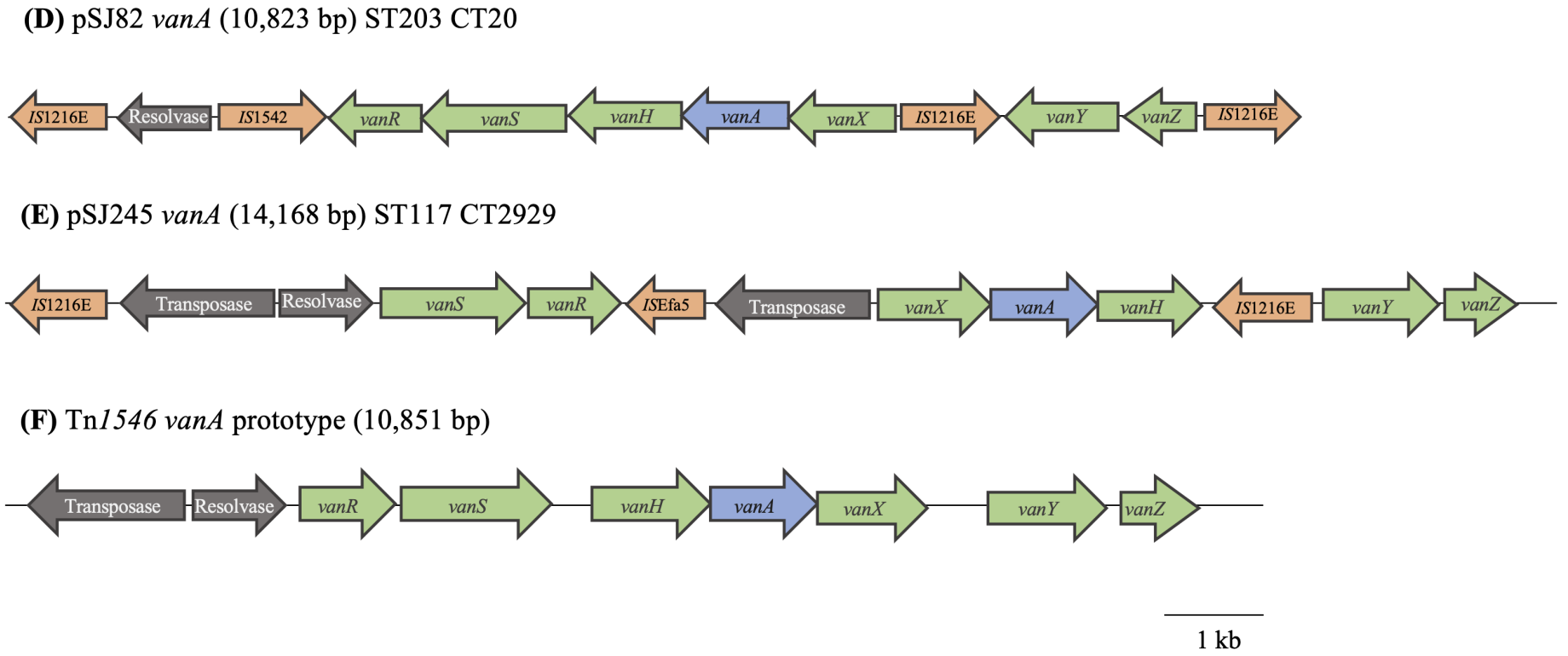


Figure 3.4 Schematic diagram showing the structural organisation of *vanA* transposon regions. Closed by hybrid assembly from Irish vancomycin-resistant *E. faecium* isolates SJ10 (A) (SJ10*vanA* 11,210 bp region), SJ11 (B) (SJ11*vanA* 13,252 bp region), BSI_SJ40 (C) (BSI_SJ40*vanA*; 13,269 bp), SJ82 (10,823 bp *vanA* region from the plasmid pSJ82*vanA*) and SJ245 (14,168 bp *vanA* region from the plasmid pSJ245*vanA*) determined in the present study, and (F) the structural organisation of the prototype *vanA* transposon Tn*1546* first described by Arthur *et al.*, 1993. Genes and their orientation are denoted by directional arrows and labelled with corresponding genes name. A reference size scale bar is shown at the bottom of the diagram. Abbreviation: Cad, cadmium efflux accessory protein.

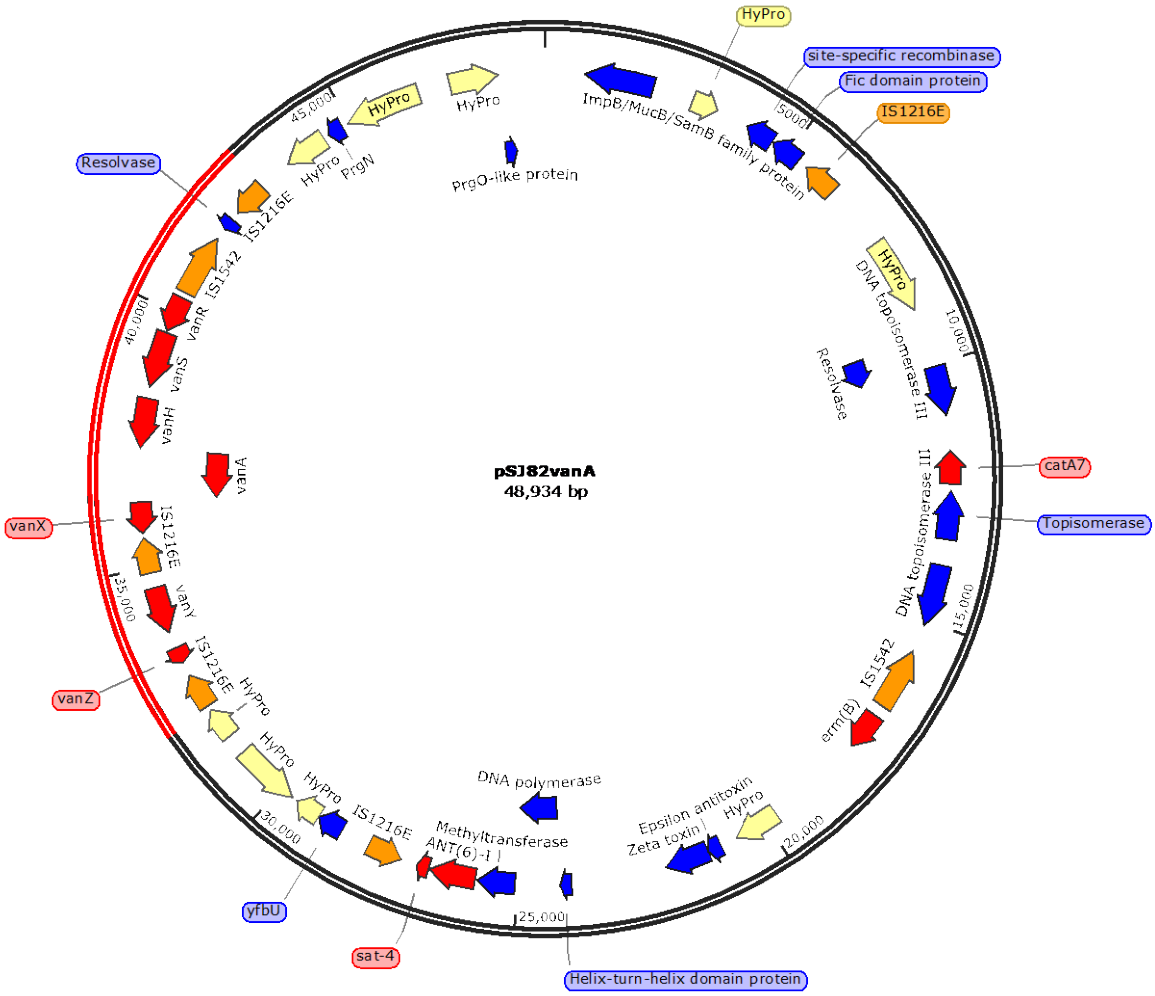


Figure 3.5 Schematic diagram of the structural organisation of plasmid pSJ82*vanA* from the vancomycin-resistant *E. faecium* screening isolate SJ82 encoding *vanA* resolved by hybrid assembly of paired-end Illumina MiSeq short reads with Oxford Nanopore Technologies long reads. The *vanA* region is highlighted in red. Genes of interest and their orientation are represented by arrows as follows: red indicates antibiotic resistance genes, orange indicates insertion sequences/transposases, blue indicates protein described in label and yellow indicates sequences encoding hypothetical proteins. Abbreviation: HyPro; hypothetical protein.

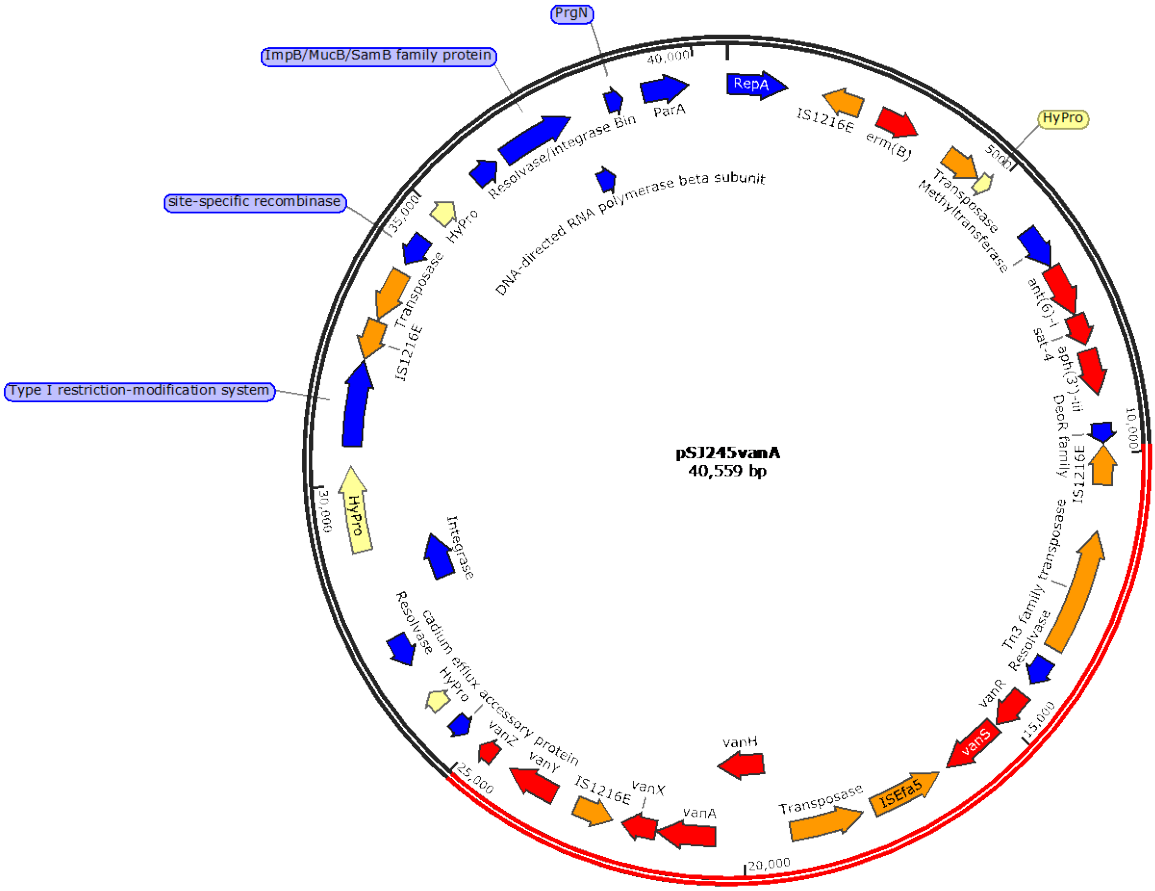


Figure 3.6 Schematic diagram of the structural organisation of plasmid pSJ245*vanA* from the vancomycin-resistant *E. faecium* screening isolate SJ245 encoding *vanA* resolved by hybrid assembly of paired-end Illumina MiSeq short reads with Oxford Nanopore Technologies long reads. The *vanA* harbouring region is highlighted in red. Genes of interest and their orientation are represented by arrows as follows: red indicates antibiotic resistance genes, orange indicates insertion sequences/transposases, blue indicates protein described in label and yellow indicates sequences encoding hypothetical proteins. Abbreviation: HyPro; hypothetical protein.

3.3.4 Transmission of *vanA* via IS*1216E*-mediated transposition and plasmid conjugation

Conjugation of *vanA* was successful in all four isolates investigated from patient screening including SJ11 (non-typeable, CT2), SJ82 (ST203, CT20), SJ245 (ST117, CT2929), SJ267 (ST18, CT1898) and the BSI isolate BSI_SJ40 (ST80, CT1598) to the recipient *E. faecium* 64/3 strain. Isolates were chosen to represent a range of ST's and to include both screening and BSI isolates. All attempts to conjugate *vanA* to the recipient *E. faecalis* OGR1F strain were unsuccessful. All transconjugants (TCs) were phenotypically resistant to vancomycin and teicoplanin (vancomycin MIC > 4 mg/L, teicoplanin MIC >2 mg/L) (Table 3.2). All transconjugants also showed the identical sequence coverage to SJ10*vanA* as their corresponding donor strain, 81.5-100%, indicating an identical element was transferred from the donor strain to the recipient in each case (Table 3.2). The donor isolates SJ11, SJ267 and BSI_SJ40 and their corresponding *E. faecium* 64/3 transconjugant derivatives showed near identical coverage to the SJ10*vanA* plasmid-like contig, indicating this region is conjugative (Table 3.2). Plasmid pSJ245 present in donor isolate SJ245 was also shown to be conjugative, with the corresponding *E. faecium* 64/3 transconjugant derivatives SJ245:Efm 64/3 TC1 and SJ245:Efm 64/3 TC2 exhibiting 100% sequence coverage to pSJ245. Both transconjugants also showed 95.5% sequence coverage to the reference SJ10*vanA,* identical to the coverage in the donor isolate SJ245 (Table 3.2)

The transconjugant SJ82:Efm 64/3 TC1 also underwent hybrid assembly and interestingly the fully resolved plasmid harbouring the *vanA* operon identified in the transconjugant was found to be much larger (175,921 bp) than pSJ82*vanA* present in the parental donor isolate SJ82 (48,934 bp). The 175,921 bp plasmid from the transconjugant SJ82:Efm 64/3 TC1 was named pSJ82_TC (Figure 3.7). Examination of the hybrid assembly of the donor isolate SJ82 revealed the presence of another large plasmid termed pSJ82_B (232,026 bp) encoding two copies of the aminoglycoside resistance gene *AAC(6')-Ib* in addition to pSJ82*vanA* (48,934 bp) encoding *vanA*. Analysis of the 175,921 bp *vanA*-encoding plasmid pSJ82_TC from the transconjugant SJ82:Efm 64/3 TC1 revealed that it was composed of the *vanA* operon flanked on either side by IS*1216E* (9,826 bp) from pSJ82*vanA* inserted within a large section of pSJ82_B (166,095 bp) from the parental donor isolate SJ82 (Figure 3.7). The movement of the *vanA* operon from pSJ82*vanA* into pSJ82_B is evident by the identical sequence coverage to the reference SJ10*vanA*, with both the donor and transconjugant having 81.5% sequence coverage (Table 3.2). It appears that the *vanA* operon from pSJ82*vanA* likely moved via

transposition to pSJ82_B present in the donor isolate SJ82 and there was loss of a 65,931 bp portion of pSJ82_B during/after conjugation into the *E. faecium* 64/3 recipient strain giving rise to the hybrid plasmid pSJ82_TC (Figure 3.7). Two additional SJ82:Efm 64/3 transconjugants from the same conjugation experiment (SJ82:Efm 64/3 TC2 and SJ82:Efm 64/3 TC3) were found to have 100% sequence coverage to the pSJ82_TC (175,921 bp) hybrid plasmid identified in SJ82:Efm 64/3 TC1. These findings highlight the highly recombinant nature of clade A1 *E. faecium* isolates, along with their success at transferring *vanA* operon efficiently.

3.3.5 IS*1216E* associated with instability of the *vanA* operon in Irish VREfm

Multiple insertions of IS*1216E* within and around the *vanA* operon were observed in SJ10*vanA* (Figure 3.4), with 95.5% (316/331) of all VREfm isolates investigated harbouring a highly similar *vanA* region as demonstrated by >90% sequence identity. This enrichment for IS*1216E* was also observed in the hybrid assemblies of the screening isolates SJ11 (non-typeable, CT2), SJ82 (ST203, CT20), SJ245 (ST117, CT2929) and the BSI isolate BSI_SJ40 (ST 80, CT 1598). This repetitive insertion of IS*1216E* in the *vanA* operon observed in Irish VREfm is a likely source of instability. As has been reported previously, insertion sequences (particularly IS*1216E*) can form transposable elements and excise from the genome in different ways causing instability in areas of their enrichment (Vandecraen *et al.*, 2017; Shan *et al.*, 2020). Two examples of this instability were evident in isolates BSI_SJ8 and BSI_SJ29, both VSEfm from BSIs. Isolate BSI_SJ8 was PCR-positive for *vanA*, but phenotypically susceptible to vancomycin and teicoplanin (vancomycin MIC < 4 mg/L, teicoplanin MIC < 2 mg/L) (Table 3.1). Further interrogation of the WGS data revealed that BSI_SJ8 harboured the *vanH, vanA* and *vanX* genes of the *vanA* operon flanked by *IS*1216E on both sides. This isolate had lost the sensor-regulator genes (*vanR, vanS*), along with the *vanY* and *vanZ* (associated with teicoplanin resistance) genes. In contrast, isolate BSI_SJ29 was PCR-negative for *vanA*, phenotypically susceptible to vancomycin, but resistant to teicoplanin (vancomycin MIC < 4 mg/L, teicoplanin MIC > 2 mg/L) (Table 3.1). On examination of the WGS data, the inverse occurred in BSI_SJ29, with the loss of the *vanH, vanA, vanX* genes, but the retention of *vanR* and *vanS* sensor-regulator genes. The pheontypic resistance to teicoplanin could be explained by the expression of *vanZ* in this isolate.

Table 3.2 MIC profiles of *vanA*-positive transconjugant derivatives of *E. faecium* 64/3

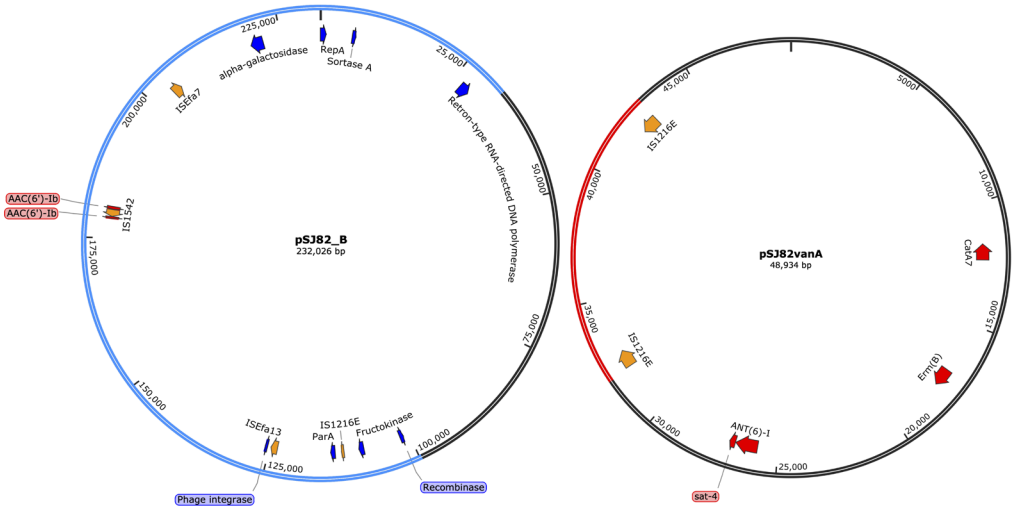
Strain/isolate/transconjugant	Gene conjugated	Vancomycin MIC (mg/L) (R > 4 mg/L)[a]	Teicoplanin MIC (mg/L) (R >2 mg/L)[a]	SJ10*vanA*[b]	SJ10*vanA* plasmid-like[b]
Donor: VREfm SJ11 (non-typeable)	N/A	>256	64	100%	99.1%
Recipient: *E. faecium* 64/3	N/A	≤1	≤1	N/A	N/A
Transconjugant: SJ11:*Efm* 64/3 TC1	*vanA*	64	16	100%	99.9%
Transconjugant: SJ11:*Efm* 64/3 TC2	*vanA*	128	16	100%	99.8%
Donor: VREfm SJ82 (ST203)	N/A	>256	>256	81.5%	30.3%
Recipient: *E. faecium* 64/3	N/A	≤1	≤1	N/A	N/A
Transconjugant: SJ82:*Efm* 64/3 TC1	*vanA*	>256	8	81.5%	31.6%
Transconjugant: SJ82:*Efm* 64/3 TC2	*vanA*	>256	16	81.5%	31.6%
Transconjugant: SJ82:*Efm* 64/3 TC3	*vanA*	>256	128	81.5%	31.6%
Donor: VREfm SJ245 (ST117)	N/A	>256	128	95.5%	32.9%
Recipient: *E. faecium* 64/3	N/A	≤1	≤1	N/A	N/A
Transconjugant: SJ245:*Efm* 64/3 TC1	*vanA*	>256	16	95.5%	34%
Transconjugant: SJ245:*Efm* 64/3 TC2	*vanA*	>256	16	95.5%	30%
Donor: VREfm SJ267 (ST18)	N/A	>256	32	100%	97.6%
Recipient: *E. faecium* 64/3	N/A	≤1	≤1	N/A	N/A
Transconjugant: SJ267:*Efm* 64/3 TC1	*vanA*	256	16	100%	98.4%
Transconjugant: SJ267:*Efm* 64/3 TC2	*vanA*	256	16	100%	98.3%
Donor: VREfm BSI_SJ40 (ST80)	N/A	>256	32	100%	98.9%
Recipient: *E. faecium* 64/3	N/A	≤1	≤1	N/A	N/A
Transconjugant: BSI_SJ40:*Efm* 64/3 TC1	*vanA*	64	16	100%	98.4%
Transconjugant: BSI_SJ40:*Efm* 64/3 TC2	*vanA*	64	16	100%	98.5%

[a] Clinical breakpoint taken from the European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST, 2019).

[b] Coverage (%) of isolates against the reference sequences resolved by hybrid assembly, the transposon region SJ10*vanA* and the SJ10*vanA* plasmid-like.

Abbreviations: MIC, minimum inhibitory concentration; R, resistant; N/A, not applicable.

A) Donor (SJ82)



B) Transconjugant (SJ82:Efm 64/3 TC1)

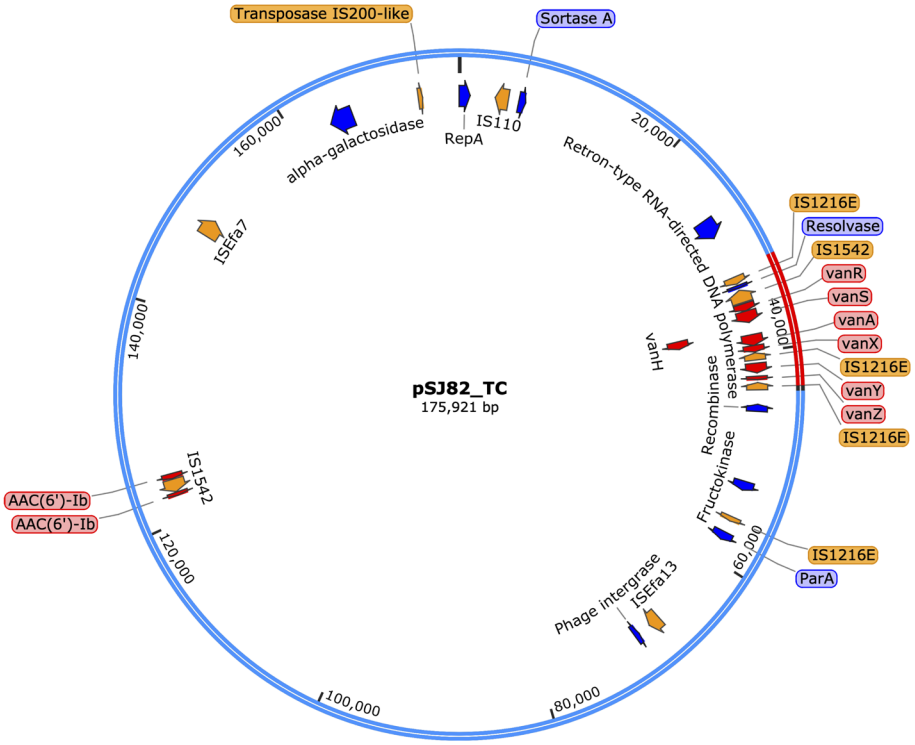


Figure 3.7 Schematic diagrams of the structural organisation of A) plasmids pSJ82_B and pSJ82*vanA* from VREfm donor strain SJ82 and B) the vancomycin resistance plasmid pSJ82_TC from the transconjugant SJ82:Efm 64/3 TC1. Diagrams are partially annotated for clarity. Genes of interest and their orientation are represented by arrows: red indicates antibiotic resistance genes, orange indicates insertion sequences/transposases and blue indicates genes encoding known proteins. The regions from plasmids present in the donor isolate SJ82 (i.e. pSJ82_B and pSJ82*vanA*) that are present in the hybrid transconjugant plasmid pSJ82_TC are marked as follows: pSJ82_B region highlighted in blue and pSJ82*vanA* region highlighted in red. Plasmid regions in pSJ82*vanA* and pSJ82_B shaded in black were not present in pSJ82_TC.

3.4 Discussion

This study represents the first to investigate the population structure of *E. faecium* isolates from primarily hospitalised patients in Ireland using WGS technology. During this study, VREfm screening isolates (n=286) recovered over two-week periods each quarter for one year (June 2018, October 2018, March 2019 and June 2019) from the clinical microbiology laboratory in a large Irish teaching hospital were investigated. All *E. faecium* BSI's (n=79) recovered between June 2017 and July 2019 from the same hospital were also investigated. A total of 365 isolates were investigated, of which 360 (98.6%) belonged to the hospital-endemic clade A1 (both VREfm (331/360) and VSEfm (29/360) and five belonged to the community-adapted clade B (all VSEfm).

Analysis of WGS data revealed the overall population structure of clade A1 *E. faecium* in the hospital concerned was highly polyclonal. Isolates belonging to the same ST as determined by conventional MLST were shown to be distantly related to each another using highly discriminatory cgMLST (Figure 3.1). This polyclonal nature of clade A1 *E. faecium* agrees with what has been shown previously by WGS studies, mainly from Europe (Lebreton *et al.*, 2013; Raven *et al.*, 2016; Pinholt *et al.*, 2017; Gorrie *et al.*, 2019). The use of cgMLST provides a uniform, yet highly discriminatory molecular typing method that is standardised and yields results that are comparable across countries. A threshold of ≤ 20 allelic differences has been recommended in previous studies to describe *E. faecium* isolates as indistinguishable or highly related (de Been *et al.*, 2015; Pinholt *et al.*, 2017). In the present study, it was evident that clusters of related isolates could be defined by the CT to which the majority of isolates in that cluster belonged to. In some clusters there was evidence of additional CT's or the presence of an isolate which did not assign to any CT; a good example of this was observed with isolates assigned to ST203 CT1599 (n=29), in which 26 isolates assigned to cluster CT1599, one isolate to CT2277, one isolate to CT3155 and one isolate did not assign to any CT (Table 3.1, Figure 3.1). Using the predominant CT to define a cluster of related isolates provides a common naming system that permits isolates from different geographic locations to be compared. In contrast, results of SNP based typing is often not comparable across different laboratories and countries, as the methods, computational tools and reference sequences used for performing SNP based typing can be highly variable (de Been *et al.*, 2015; Schürch *et al.*, 2018).

A large proportion of the VREfm isolates investigated were found to harbour identical or highly related *vanA* MGEs, with 95.5% (316/331) of VREfm isolates

exhibiting >90% sequence identity to the SJ10*vanA* reference (Table 3.1, Figure 3.4). This finding of identical *vanA* MGEs in both highly related and otherwise genetically unrelated isolates adds an additional challenge in terms of infection control and prevention. Along with clonal expansion of existing VREfm clones in the hospital environment, the transfer of a promiscuous *vanA* MGE could lead to the generation of new VREfm clones from existing VSEfm. Analysis of the spread of *vanA* encoding MGEs and their similarity across lineages as undertaken in the present study has added to the current knowledge of how VREfm spread/arise. The circulation of a common *vanA* element among diverse lineages of VREfm was also observed previously in Denmark, where 81% of isolates investigated harboured an identical *vanA* plasmid (Pinholt *et al.*, 2019). It is important to note that the reference mapping method used to examine the similarity of *vanA* among isolates has an important limitation; it gives a good indication of how common a genetic region is in the population, but it cannot account for additional genetic material inserted within the region in question. The additional resolution provided by hybrid assembly of short read Illumina and long read Oxford nanopore sequencing data was used to provide additional clarity in the variability of *vanA* in some isolates.

In the present study, filter mating experiments demonstrated the transmissibility of *vanA* directly via conjugative plasmids, such as pSJ245 (Table 3.2). The ability of *vanA* to transpose from one plasmid to another in a clinical isolate and then to translocate to a new recipient by conjugative plasmid transfer was also demonstrated. This was seen in the hybrid plasmid (pSJ82_TC) in transconjugant SJ82:Efm TC1, where the *vanA* operon flanked on either side by *IS*1216E (9,826 bp) from pSJ82*vanA* from the parental isolate SJ82 inserted into pSJ82_B (166,095 bp) also present in the donor isolate. Following conjugation, the hybrid plasmid pSJ82_TC was found to have retained the 9,826 bp *vanA* element but to have lost a 65,931 bp segment of pSJ82_B (Figure 3.7). As vancomycin was used in the selection of transconjugants, selective pressure was present for the mobilisation of *vanA*, which was likely achieved by transposition of *vanA* to a conjugative plasmid.

A major concern in the infection prevention and control of VREfm is the potential for new VREfm either arising *de novo* within the GI tract of humans by the acquisition of *vanA* from other gut microbes or by the aquistion of *vanA* by VSEfm, either directly from other VREfm or an environmental source of *vanA*. The emergence of VREfm in the GI tract *de novo* has been described previously for *vanB*, with enterococci acquiring *vanB* from gut anaerobes via Tn*1549* (Howden *et al.*, 2013). The same has yet to be described

for *vanA*, but the finding that clade A1 VSEfm in the present study were indistinguishable from VREfm suggests trafficking of *vanA*. For example, in the ST80 CT1598 cluster there were zero allelic differences between the VSEfm isolate BSI_SJ72 and the VREfm screening isolates SJ1, SJ6, SJ14 and the VREfm BSI isolate BSI_SJ37 (Figure 3.3). This indicates that the VSEfm isolates in this study could readily acquire *vanA* as they are genetically indistinguishable from the VREfm. Another risk factor for the potential spread/transmission of VREfm in the hospital environment is the possibility of environmental sources of *vanA*. This has been shown by its presence in hospital wastewater and nearby water treatment plants in the UK (Gouliouris *et al.*, 2018). Similar evidence of this environmental contamination was previously shown in Ireland, with evidence of VREfm in rural drinking water supplies, along with its presence in wastewater (Morris *et al.*, 2012). Environmental sources pose a double threat for VREfm spread; they may act as a reservoir to transmit *vanA* or as a route for direct acquisition of VREfm in the community, i.e presence in drinking water in rural Ireland (Morris *et al.*, 2012). Currently in Ireland, patients are not routinely screened for VREfm upon hospital admission, whereas routine screening is undertaken in high-risk areas, such as ICUs and oncology wards. Therefore, some patients may act as unidentified reservoirs for the spread of VREfm in hospitals in Ireland.

Another notable characteristic of the clade A1 *E. faecium* isolates is the persistence of clones in hospitals over extended periods of time; for example, in the present study the ST80 CT1598 cluster contained highly related isolates recovered from 14 different wards between September 2017 to June 2019 (21 months) (Table 3.1). There were only 3 allelic differences between the earliest isolate SJ6 (24th July 2017) from Ward 2 and the latest isolate SJ291 (28th June 2019) from Ward 12. The distribution of these highly related isolates across multiple wards in one hospital indicates widespread trafficking of isolates throughout the hospital, possibly by healthcare staff, contaminated equipment and/or hospital fixtures and fittings including contaminated toilet/bathroom facilities. The persistence of these strains over 21 months is also concerning as it is likely that they have survived in the hospital environment despite current cleaning/decontamination protocols. A study undertaken in two haematology wards in a large UK hospital over a six-month period in 2015, reported that 48% (447/992) of environmental swab samples were positive for VREfm, with a positive proportion ranging from 36% for medical devices to 76% for non-touch areas (air vents and high-efficiency particulate air (HEPA) filters). Of particular concern, was the ability of VREfm to persist in the environment following routine cleaning

after patient discharge, with 20% (9/41) of bedroom/bathroom areas remaining contaminated. (Gouliouris *et al.*, 2021). Persistence of VREfm in the hospital environment provides an additional level of complexity in the challenge to control VREfm.

The isolates investigated in the present study are the first in Ireland to be investigated using WGS, and interestingly share the same current predominating prevalence of *vanA* encoding ST80 VREfm with Denmark. Studies of isolates mainly from the Copenhagen area of Denmark (11 hospitals) revealed a substantial increase in VREfm between 2011 and 2015, with only nine patients infected or colonized with VREfm in 2011 rising to more than 1500 VREfm patients identified between 2012 and 2015. Detailed WGS studies revealed that this was caused by the introduction of a single clone of a *vanA* encoding ST80 VREfm and its subsequent expansion and spread (Pinholt *et al.*, 2015, Pinholt *et al.*, 2017, Pinholt *et al.*, 2019). Findings from Denmark and from the present study contrast starkly with findings from other European countries where other STs prove to be more prevalent and no clonal patterns have been described. The 2018 EARS-Net report by the ECDC stated "*Corresponding increasing trends highlight the need for close monitoring to better understand the epidemiology, clonal diversity and risk factors associated with infection. Contrary to many other species under surveillance, no distinct geographical pattern could be seen for vancomycin-resistant E. faecium, as high percentages were reported from both southern, eastern and northern Europe*." (ECDC, 2019b), further highlighting the variety in *E. faecium* clones when compared with how other microorganisms spread.

In Germany, a 30-year review of VRE by the National Reference Centre for Staphylococci and Enterococci gave an extended insight into VRE epidemiology over time and revealed a shift in predominant strains over each decade. ST117 *vanA* encoding VREfm predominated in the 1990s, which changed to ST203 *vanA* VREfm in the early 2000s. Interestingly at the beginning of the 2010s, there was a shift to ST192 and ST117 *vanB* encoding VREfm. This shift to predominant *vanB* VREfm persists to the present day. There was a persistent large outbreak of ST80 *vanB* VREfm affecting 2,900 patients in two hospitals in southwest Germany noted between 2015-2017, with ST80 CT1013 identified as the dominant clone (Werner *et al.*, 2020). This latter finding may be an indicator of the success of ST80 and its ability to spread and cause outbreaks. Recent reports from Sweden detailed outbreaks of VREfm primarily due to ST80 *vanB*-encoding VREfm, which was described as the predominant VREfm clone in 2018 and 2019, accounting for 75% and 46% of new cases, respectively. (Fang *et al.*, 2021). This was followed by a rapid and

sharp decline in January to May 2020 (a total of seven cases) and no ST80 cases were identified in June 2020 (Fang *et al.*, 2021). The findings of the present study reveal the predominance of ST80 in the hospital concerned between June 2017 and July 2019. Whether ST80 has predominated prior to this period requires to be investigated. Furthermore, whether ST80 predominates in other Irish hospitals is also an issue. Initial studies including five other regional hospitals in Ireland from Q4 2019 (October-December), indicate that the majority of VREfm isolates investigated (58/103, 56.3%) belong to ST80, indicating ST80 is also predominant across the country (Kavanagh N. 2021, personal communication, 28 January 2021).

This study also reports the first VREfm *vanA* with multiple insertions of an identical IS element, IS*1216E*. Previous reports of different iterations of *vanA* have shown a single insertion of an IS element into the middle of the *vanA* operon (Pinholt *et al.*, 2017). To date no previous studies have described this multiple division of the *vanA* operon by IS*1216E*. The *vanA* operon described in SJ10 is also the first to date to show a rearrangement in the order of the *van* genes, with the transposase, resolvase, *vanR* and *vanS* being encoded following an insertion of IS*1216E* and downstream of *vanY* and *vanZ* at the end of the operon (Figure 3.4). It has previously been reported that clade A1 *E. faecium* harbouring the linezolid-resistance gene *poxtA* flanked by IS*1216E* can loop out and form small transposable elements (TEs) (Shan *et al.*, 2020). There are indications in the present study that multiple insertions of IS*1216E* throughout the *vanA* operon could lead to instability. The rearrangement observed of the *van* genes (the transposase, resolvase, *vanR* and *vanS* encoded downstream of *vanY* and *vanZ*) in SJ10*vanA* is likely due to instability caused by IS*1216E*. Other examples of this instability can be seen in the VSEfm isolate BSI_SJ8, which harboured *vanH, vanA* and *vanX* genes flanked by *IS*1216E on both sides, but which lacked the sensor-regulator system and *vanY, vanZ* genes. This may be due to instability and the possibility that these genes were lost by the formation of small TEs. It is conceivable that VSEfm isolates like BSI_SJ8 could revert to VREfm, either by reacquiring the lost genes within the patients GI tract, or by an alternative activation of transcription of *vanH, vanA* and *vanX* under the pressure of vancomycin. The opposite was observed in VSEfm isolate BSI_SJ29, which had lost the *vanH, vanA* and *vanX* genes possibly by *IS*1216E forming a TE. A source of real interest in this isolate was that the operon appears to still function based on the retention of phenotypic resistance to teicoplanin but loss of vancomycin resistance, likely due to the activation of *vanR, vanS*

and subsequent transcription of the remaining *vanY* and *vanZ* genes. Further work is required to fully confirm this hypothesis.

Overall, VREfm in Irish hospitals is highly prevalent, evident by the fact the Republic of Ireland has consistently reported one of the highest rates of invasive VREfm infections in Europe for over ten years, ranging between 32.5%-45.8% (2006-2019) (European Centre for Disease Prevention and Control, 2019). This high prevalence of VREfm in Ireland could be due to several factors. Current Irish national guidelines do not recommend screening of all patients admitted to hospitals for VREfm, so we currently do not know the true prevalence of VREfm carriage. One study from the South of Ireland in Cork, estimated the ~19% of people carry VREfm in their GI tract (Whelton *et al.*, 2016). It is also clear that the ongoing rates of invasive VREfm infections need to be addressed as Ireland has consistently reported the highest rates in Europe for over a decade with no significant decreases recorded. (European Centre for Disease Prevention and Control, 2018). Further studies, including a larger number of Irish hospitals around the country are required to get a better understanding of the population structure of VREfm across Ireland. Investigating stored historical VREfm isolates would also be beneficial to determine if the predominance of ST80 is recent or has persisted for many years. Finally possible environmental sources/selective pressure should be examined, as likely VREfm is constantly being shed into our wastewater and may possibly get into drinking water, as shown in Ireland in 2012 (Morris *et al.*, 2012). It is clear Ireland needs to act urgently on the problem of VREfm in healthcare settings, as these infections lead to increased morbidity, mortality and an increase in bed days used.

Chapter 4

Linezolid resistance in *Enterococcus faecium* and *Enterococcus faecalis* from hospitalized patients in Ireland: high prevalence of the multidrug resistance genes *optrA* and *poxtA* in isolates with diverse genetic backgrounds

4.1 Introduction

Linezolid is an antibiotic used to treat infections caused by MDR Gram-positive bacteria, including VRE. For the past decade, Ireland had the highest rate of VREfm bloodstream infections in Europe between 2007-2018 (European Centre for Disease Prevention and Control, 2019). Although no published data is available exclusively on linezolid usage in Ireland, an almost 10% increase in the overall use of antimicrobials was noted between 2007-2017 (HSE/HPSC, 2018). A linezolid usage increase of 40% was reported in one large Irish acute hospital between 2012-2013 (Lazaris *et al.*, 2017). Also, in Chapter 5, a 40.3% increase of linezolid consumption in a North Dublin hospital was seen, from 0.46 DDD/100 BDU in Q4 2016 to 1.14 DDD/100 BDU by Q4 2019. Furthermore, the emergence of linezolid-resistant enterococci (LRE) during or after linezolid exposure has been well described (Gonzales *et al.*, 2001; Cai *et al.*, 2015; Wang *et al.*, 2015; Bi *et al.*, 2018; Bai *et al.*, 2019).

Linezolid binds in the V domain of the 23S rRNA component of the 50S ribosomal subunit and inhibits protein synthesis. This binding site overlaps with that of other antimicrobials, such as chloramphenicol, clindamycin, tiamulin, and streptogramin A (Lin *et al.*, 1997; Swaney *et al.*, 1998; Diekema and Jones, 2000; Long and Vester, 2012). Enterococcal linezolid resistance can be due to G2576T or G2505A mutations in the 23S rRNA binding site or mutations in the genes encoding ribosomal proteins L3 and/or L4 (Bi *et al.*, 2018). However, linezolid-resistance can develop following acquisition of the resistance genes *optrA*, *poxtA* and variants of the *cfr* gene, which have been described in detail in Chapter 1.

The *optrA* gene was first described in plasmid pE349 from a clinical *E. faecalis* in China and subsequently identified in *E. faecium* and *E. faecalis* from humans and food-producing animals throughout European, American and Asian countries (Cai *et al.*, 2015; Wang *et al.*, 2015; Cavaco *et al.*, 2016; Mendes *et al.*, 2016, 2018; Bi *et al.*, 2018). Although the number of *E. faecium* and *E. faecalis* isolates harbouring these genes reported to date is low, *optrA* has been reported recently with increased frequency. In 2014, it was reported that 3/9 linezolid non-susceptible isolates (MIC > 4 mg/L) were *optrA*-positive *E. faecalis* (two from Ireland) (Mendes *et al.*, 2016). This increased to 8/17 in 2016 (Mendes *et al.*, 2018) German researchers reported that 6% of 698 LRE recovered between 2007-2017 harboured *optrA* (Bender *et al.*, 2018b)

The *poxtA* gene was originally identified in an Italian clinical MRSA in 2018 and subsequently in a porcine *E. faecium* (Antonelli *et al.*, 2018; Brenciani *et al.*, 2019). More

recently, *optrA* and *poxtA* were co-located on a conjugative plasmid in a porcine *E. faecalis* (Hao *et al.*, 2019). Prior to this study, *poxtA* had only been reported in a Greek clinical *E. faecium* in 2018 (Papagiannitsis *et al.*, 2019). The *cfr* gene, and its variants *cfr(B)* and *cfr(D)* (GenBank: MG707078.1) have been reported in clinical *E. faecium*, whereas only *cfr* has been reported in *E. faecalis* (Diaz *et al.*, 2012; Bender *et al.*, 2016; Lazaris *et al.*, 2017; Guerin *et al.,* 2020).

The first reported linezolid-resistant VREfm outbreak in Ireland occurred in 2014 involving 15 patients and was identified as a clonal outbreak using PFGE (O'Driscoll *et al.*, 2015). All isolates harboured the G2576T 23S mutation and were *cfr*-negative. Other linezolid resistance genes were not investigated (O'Driscoll *et al.*, 2015). In 2014, the first two *optrA*-positive VREfm were recovered in separate Irish hospitals (Mendes *et al.*, 2016). In 2016, the Irish Health Protection Surveillance Centre requested that all LRE identified in Irish hospitals should be sent to the NMRSARL for linezolid resistance gene screening. In 2017, a VREfm clinical isolate harbouring a *cfr*- and *optrA*-encoding plasmid, was reported from an Irish hospital (Lazaris *et al.*, 2017).

The purpose of this part of the study was to investigate the molecular mechanisms and spread of linezolid resistance in LRE from Irish hospitals sent to the NMRSARL for linezolid resistance gene screening between June 2016 and August 2019. All isolates harbouring *optrA*, *poxtA* or *cfr* and a selection of isolates without these genes were investigated using WGS.

4.2 Materials and Methods

4.2.1 Linezolid-resistant enterococcal isolates

Between June 2016 and August 2019, 154 LRE from patients in Irish hospitals were sent to the NMRSARL for linezolid resistance gene PCR screening, as described in Chapter 2, Section 2.4.2.2. Thirty-five of these harboured at least one of the *optrA* and *poxtA* genes and were investigated in detail. Of the remaining 119 isolates without linezolid resistance genes, 20 representatives from a range of isolation dates and hospital locations were also investigated (Table 4.1). These 55 isolates investigated in detail were recovered in 14 Irish hospitals (H1-H14) (Figure 4.1). The remaining 99 LRE isolates lacking resistance genes were not investigated further.

4.2.2 Phenotypic and genotypic testing

All isolates were tested for susceptibility to linezolid, vancomycin, chloramphenicol and tetracycline using the VITEK®2 system (bioMérieux) and E-tests (bioMérieux) were used to assess linezolid MICs between 8-256 mg/L, as described in Chapter 2, Section 2.3.3. PCRs for enterococcal species and linezolid resistance genes were performed as described in Chapter 2, Section 2.4.2.

4.3.3 Conjugation

Conjugative transfer of plasmids encoding *optrA* and/or *poxtA* harboured by all 35 LRE was undertaken by filter mating as described in Chapter 2, Section 2.9.1.

4.2.4 Whole-genome sequencing

The 55 LRE and transconjugants underwent short-read WGS as described in Chapter 2, Section 2.5.1. For isolates selected for hybrid assembly DNA was extracted as described in Chapter 2, Section 2.4.1.3. Long-read sequencing was performed as described in Chapter 2, Section 2.5.2.

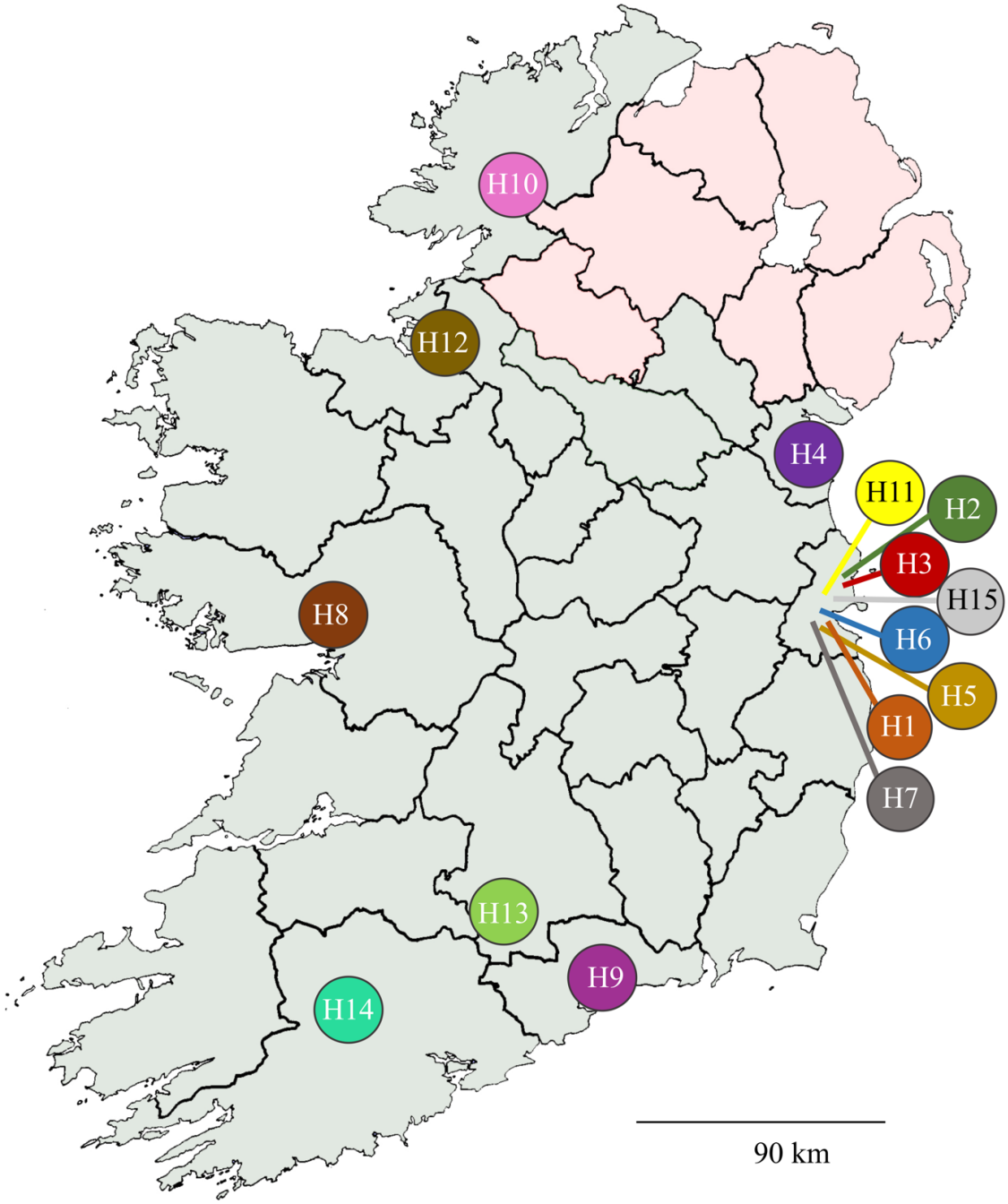


Figure 4.1 Map of Ireland with geographic locations of the hospitals (H1-H14) from which the linezolid-resistant enterococcal isolates investigated in the present study were recovered between June 2016 and August 2019.

4.2.5 Analysis of WGS data

WGS data were analysed using the *E. faecium* wgMLST scheme available in BioNumerics v7.7 (Applied Maths), as described in Chapter 2, Section 2.7.3. A filter was applied to include only the 1,423 core-genome cgMLST loci (de Been *et al.*, 2015). Conventional MLST was also applied as described in Chapter 2, Section 2.7.2. Minimum-spanning trees (MSTs) were created as described in Chapter 2, Section 2.7.5. All isolates were processed through LRE-Finder as described in Chapter 2, Section 2.7.6.

4.2.6 Assembly and analysis of plasmids encoding resistance genes

MinION-generated FASTQ files and MiSeq-generated FASTQ files were used to perform a hybrid assembly as described in Chapter 2, Section 2.7.7. The completed sequences were annotated using RAST v2.0 (http://rast.nmpdr.org/). The sequence of plasmid pM16/06594 encoding *poxtA,* was used as a reference sequence to compare against all other LRE isolates, as described in Chapter 2, Section 2.7.8.

Sequences of plasmids resolved by hybrid assembly (pM16/0594, pM18/0011 and pM17/0314) and DNA regions encoding *optrA* variants have been deposited in GenBank under accession numbers MN831410, MN831411, MN831412, MN831413, MN831414, MN831415, MN831416, MN831417, MN831418, MN831419.

4.3 Results

4.3.1 Linezolid-resistant isolates

A total of 35/154 (22.7%) LRE (23 *E faecalis* and 12 *E. faecium*) submitted to the NMRSARL between June 2016 and August 2019 harboured *optrA* (two *E. faecium* and 13 *E. faecalis)*, *poxtA* (nine *E. faecium* and 10 *E. faecalis*), or both *optrA* and *poxtA* (one *E. faecium).* All 35 isolates were from hospitalised patients in 11 Irish hospitals (H1-H11) (Figure 4.1) and were phenotypically resistant to linezolid, chloramphenicol and tetracycline (Table 4.1). Six *E. faecium* isolates were vancomycin-resistant, harbouring *vanA.* The remaining 29 LRE isolates were vancomycin-susceptible and lacked *vanA* (Table 4.1). The majority (19/35) of LRE harboured *poxtA* only. The remaining LRE harboured *optrA* (14/35), *optrA* and *poxtA* (1/35) or *optrA* and *cfr(D)* (1/35). One VREfm (M19/0595) harboured *poxtA* and the G2576T 23S mutation (4/6 copies mutated). The remaining 34 LRE lacked 23S mutations. All 20/119 LRE investigated that lacked *cfr, optrA* and/or *poxtA* (two *E. faecalis*, 18 *E. faecium*) were phenotypically resistant to linezolid and chloramphenicol (Table 4.1). All 20 LRE exhibited a varying copy number [1-5] of the G2576T 23S mutation. The majority of the LRE (15/18 *E. faecium*) harboured *vanA* and exhibited vancomycin MICs ≥ 32 mg/L. The remaining five *vanA*-negative isolates (two *E. faecalis* and three *E. faecium*) were vancomycin-susceptible (Table 4.1).

Table 4.1 Phenotypic and genotypic characteristics of the 55 linezolid-resistant enterococcal clinical isolates recovered in Irish hospitals between June 2016 and August 2019

Isolates	Hospital (H)	Sites	Patient age (years)	Date recovered[a]	Sequence type (ST)	Linezolid resistance gene(s) detected	Location/ environment of resistance genes	23S mutation copy number (G2576T)	Lnz MIC mg/L (R>4 mg/L)[b]	Vanc MIC mg/L (R>4 mg/L)[b,c]	Chl MIC mg/L (R> 32 mg/L)[b]	Tet MIC mg/L (R> 2 mg/L)[b]
E. faecalis												
M16/0419	H8	N/A	73	08/06/2016	480	*poxtA*	pM160594-like plasmid	0	8	1	32	≥16
M16/0420	H8	N/A	74	14/06/2016	21	*optrA*	Plasmid pE349 (*optrA*_WT)	0	≥256	4	≥64	≥16
M16/0513	H11	N/A	81	21/07/2016	480	*poxtA*	pM160594-like plasmid	0	8	1	≥64	≥16
M16/0516	H10	N/A	60	18/07/2016	480	*poxtA*	pM160594-like plasmid	0	8	1	≥64	≥16
M16/0633	H8	N/A	73	16/09/2016	480	*poxtA*	pM160594-like plasmid	0	8	2	32	≥16
M17/0144	H6	N/A	76	13/02/2017	768	*optrA*	Plasmid pE349 (*optrA*_WT)	0	32	2	≥64	≥16
M17/0145	H8	N/A	90	15/02/2017	768	*optrA*	Plasmid pE349 (*optrA*_WT)	0	32	2	≥64	≥16
M17/0149	H2	N/A	49	10/02/2017	768	*optrA*	Plasmid pE349 (*optrA*_WT)	0	128	2	≥64	≥16
M17/0240	H4	Urine	49	21/03/2017	890	*optrA*	Plasmid (*optrA*_I)	0	32	2	≥64	≥16
M17/0261	H8	Urine	55	13/04/2017	480	*poxtA*	pM160594-like plasmid	0	32	1	≥64	≥16

Table 4.1 continued overleaf

Isolates	Hospital (H)	Sites	Patient age (years)	Date recovered[a]	Sequence type (ST)	Linezolid resistance gene(s) detected	Location/ environment of resistance genes	23S mutation copy number (G2576T)	Lnz MIC mg/L (R> 4mg/L)[b]	Vanc MIC mg/L (R> 4 mg/L)[b,c]	Chl MIC mg/L (R> 32 mg/L)[b]	Tet MIC mg/L (R> 2 mg/L)[b]
M17/0574	H3	N/A	64	29/07/2017	480	*poxtA*	pM160594-like plasmid	0	8	2	32	≥16
M17/0590	H8	Urine	75	14/08/2017	480	*poxtA*	pM160594-like plasmid	0	8	1	16	≥16
M17/0603	H5	CV line tip	<1	12/08/2017	19	*optrA*	Plasmid pE349 (*optrA*_WT)	0	48	2	≥64	≥16
M17/0913	H6	Drain fluid	76	31/10/2017	480	*poxtA*	pM160594-like plasmid	0	4	1	16	≥16
M17/0986	H3	N/A	51	16/11/2017	21	*optrA*	Plasmid pE349 (*optrA*_WT)	0	128	2	≥64	≥16
M18/0011	H2	Groin abscess	57	22/12/2017	480	*poxtA*	pM160594-like plasmid	0	8	1	16	≥16
M18/0108	H7	N/A	53	23/01/2018	480	*poxtA*	pM160594-like plasmid	0	4	FTG	FTG	FTG
M18/0173	H6	N/A	49	06/02/2018	480	*optrA*	Plasmid (*optrA*_II)	0	16	1	≥64	≥16
M18/0497	H6	Screening[d]	60	01/05/2018	16	*optrA*	Chromosome (*optrA*_VI)	0	32	1	32	≥16
M18/0581	H9	N/A	49	29/05/2018	166	*optrA*	Plasmid pE349 (*optrA*_WT)	0	≥256	1	≥64	≥16
M18/0582	H9	N/A	43	30/05/2018	480	*optrA*	Plasmid (*optrA*_I)	0	16	1	≥64	≥16
M18/0906	H6	Blood	70	12/09/2018	32	*optrA*	Chromosome (*optrA*_IV)	0	8	4	≥64	2
M19/0596	H6	Screening[d]	44	02/06/2019	480	*optrA*	Plasmid (*optrA*_I)	0	8	1	32	≥16

Table 4.1 continued overleaf

Isolates	Hospital (H)	Sites	Patient age (years)	Date recovered[a]	Sequence type (ST)	Linezolid resistance gene(s) detected	Location/ environment of resistance genes	23S mutation copy number (G2576T)	Lnz MIC mg/L (R> 4 mg/L)[b]	Vanc MIC mg/L (R> 4 mg/L)[b,c]	Chl MIC mg/L (R> 32 mg/L)[b]	Tet MIC mg/L (R> 2 mg/L)[b]
M17/0558	H2	Urine	70	22/07/2017	6	ND	N/A	3	64	1	≥64	≥16
M18/0601	H2	N/A	71	06/06/2018	6	ND	N/A	5	≥256	1	32	≥16
E. faecium												
M16/0427	H4	N/A	90	26/06/2016	202	*optrA*	Plasmid pE349 (*optrA*_WT)	0	≥256	1	≥64	≥16
M16/0594	H9	N/A	73	05/09/2016	18	*optrA poxtA*	Chromosome pM160594	0	16	1	16	≥16
M17/0311	H3	N/A	21	08/05/2017	787	*poxtA*	pM160594-like plasmid	0	64	≤0.5	8	≥16
M17/0314	H3	N/A	52	12/05/2017	80	*optrA, cfr(D)*	Plasmid (*optrA*_III)	0	64	1	16	≥16
M17/0351	H3	N/A	21	16/05/2017	203	*poxtA*	pM160594-like plasmid	0	16	≥32	8	≥16
M17/0798	H8	Urine	36	07/10/2017	202	*poxtA*	pM160594-like plasmid	0	16	1	16	≥16
M17/0987	H10	Screening[d]	58	21/11/2017	202	*poxtA*	pM160594-like plasmid	0	16	≥32	16	≥16
M18/0012	H2	Screening[d]	57	22/12/2017	323	*poxtA*	pM160594-like plasmid	0	12	≥32	≥64	≥16
M18/0732	H9	Umbilical swab	86	09/07/2018	18	*poxtA*	pM160594-like plasmid	0	8	≤0.5	8	≥16
M18/1163	H3	Screening[d]	72	11/12/2018	1588	*poxtA*	pM160594-like plasmid	0	32	≥32	16	≥16
M19/0357	H1	Screening[d]	86	22/03/2019	80	*poxtA*	pM160594-like plasmid	0	128	≥32	16	2

Table 4.1 continued overleaf

Isolates	Hospital (H)	Sites	Patient age (years)	Date recovered[a]	Sequence type (ST)	Linezolid resistance gene(s) detected	Location/ environment of resistance genes	23S mutation copy number (G2576T)	Lnz MIC mg/L (R> 4 mg/L)[b]	Vanc MIC mg/L (R> 4 mg/L)[b,c]	Chl MIC mg/L (R> 32 mg/L)[b]	Tet MIC mg/L (R> 2 mg/L)[b]
M19/0595	H3	Screening[d]	56	05/06/2019	80	*poxtA*	pM160594-like plasmid	4	≥256	≥32	16	≥16
M16/0360	H12	N/A	50	10/05/2016	80	ND	N/A	2	≥256	≥32	8	≥16
M16/0367	H4	N/A	68	20/05/2016	203	ND	N/A	3	≥256	≥32	≥64	≥16
M16/0411	H9	N/A	68	18/06/2016	787	ND	N/A	3	≥256	≤0.5	16	≥16
M16/0479	H10	N/A	54	01/07/2016	203	ND	N/A	2	≥256	≥32	≥64	≥16
M17/0344	H4	Screening[d]	54	17/05/2017	203	ND	N/A	2	16	≥32	8	≥16
M17/0345	H3	N/A	67	16/05/2017	203	ND	N/A	2	48	≥32	8	≥16
M17/0347	H6	Drain fluid	68	15/05/2017	17	ND	N/A	4	48	1	8	≥16
M17/0348	H6	Screening[d]	68	15/05/2017	17	ND	N/A	5	64	≥32	16	≥16
M17/0427	H6	Screening[d]	50	17/07/2017	787	ND	N/A	1	32	≥32	≥16	≥16
M17/0563	H13	Screening[d]	54	31/07/2017	787	ND	N/A	3	32	≥32	8	≥16
M18/0369	H2	N/A	80	19/03/2018	789	ND	N/A	3	≥256	≥32	8	≥16
M18/0597	H1	N/A	38	08/06/2018	787	ND	N/A	2	≥256	≥32	8	≥16
M18/0874	H2	N/A	66	08/09/2018	789	ND	N/A	4	≥256	≥32	8	≥16
M18/1149	H1	Drain fluid	40	06/12/2018	117	ND	N/A	2	32	≤0.5	8	≤1
M18/1152	H9	Screening[d]	50	05/12/2018	80	ND	N/A	3	≥256	≥32	8	≤1
M19/0180	H4	Screening[d]	57	31/01/2019	80	ND	N/A	2	64	≥32	8	≥16
M19/0193	H1	N/A	71	06/02/2019	80	ND	N/A	3	16	≥32	8	≥16
M19/0359	H14	Urine	53	21/03/2019	80	ND	N/A	3	64	≥32	8	≥16

Table 4.1 continued overleaf

[a] Isolates positive for linezolid resistance genes/total LRE submitted to NMRSARL per year are as follows; June 2016-May 2017: 15/62 (24.2%), June 2017-May 2018: 14/38 (36.8%), June 2018-May 2019: 6/48 (12.5%).

[b] Clinical breakpoints taken from the European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST, 2019).

[c] Vancomycin susceptibility was only determined for the 55 linezolid-resistant enterococcal isolates investigated in detail in the present study, including the 35 isolates harbouring linezolid resistance genes (12 *E. faecium* [six vancomycin resistant] and 23 *E. facealis*) and the 20 isolates without linezolid resistance genes (two *E. faecalis* and 18 *E. faecium* [15 vancomycin resistant]). The vancomycin susceptibility of the remaining 99 linezolid-resistant enterococci sent to the National MRSA Reference Laboratory between June 2016-August 2019 and which lacked linezolid resistance genes was not determined.

[d] Screening: from rectal swab or stool sample.

Abbreviations: MIC, minimum inhibitory concentration; Lnz, Linezolid; R, resistance; Vanc, Vancomycin; Chl, Chloramphenicol; Tet, Tetracyline; N/A, not available; ND, not detected; CV, central venous; FTG; failed to grow.

4.3.2 Relatedness of LRE based on WGS

Of the 55 LRE investigated, the 30 *E. faecium* included from 11 hospitals were assigned to 10 sequence types (STs) using traditional MLST, with ST80 predominating (8/30, 26.6%). Seventeen *E. faecium* isolates were differentiated into seven clusters (CI–CVII) using cgMLST (Figure 4.2a). Clusters CI-CVI contained isolates of the same STs (ST17, ST787, ST789, ST202, ST203), while cluster CVII consisted of ST203 and ST1588 isolates (a single locus variant of ST203) (Figure 4.2a). Clusters were differentiated by intra-cluster and inter-cluster allelic differences of 0-22 and 38-394, respectively. Isolates exhibiting ≤22 allelic differences were deemed closely related, based on previous work (de Been *et al.*, 2015). Clusters CI and CIII contained isolates from the same hospitals (H2 and H6), all with the G2576T 23S mutation. The remaining five clusters consisted of isolates from two or more hospitals, and a mixture of isolates exhibiting linezolid resistance associated with G2576T mutations or a resistance gene (Figure 4.2a).

Of the 55 LRE investigated, the 25 *E. faecalis* included originated from 10 hospitals and belonged to nine STs using traditional MLST with ST480 predominating (13/25). Twenty of the *E. faecalis* isolates differentiated into four clusters (CI, CII, CIII, CIV) using wgMLST (Figure 4.2b). The remaining five isolates were distantly related to any other isolate, exhibiting between 339-1897 allelic differences. Clusters were defined by the contrasting tight value of intra-cluster differences (0-43). Each cluster contained isolates from the same STs (ST6, ST21, ST480 & ST768). Only cluster, CIV, contained two isolates from the same hospital (H2); both with the G2576T 23S mutation. The remaining three clusters contained isolates from 2-8 hospitals. Clusters CII (*n*=3) and CIII (*n*=2) contained only *optrA*-positive isolates, whereas cluster CI (*n*=13) contained only ST480 isolates, 10 and three of which harboured *poxtA* or *optrA*, respectively. Isolates within cluster CI exhibited an average allelic difference of 29 (range: 7 – 90) (Figure 4.2b).

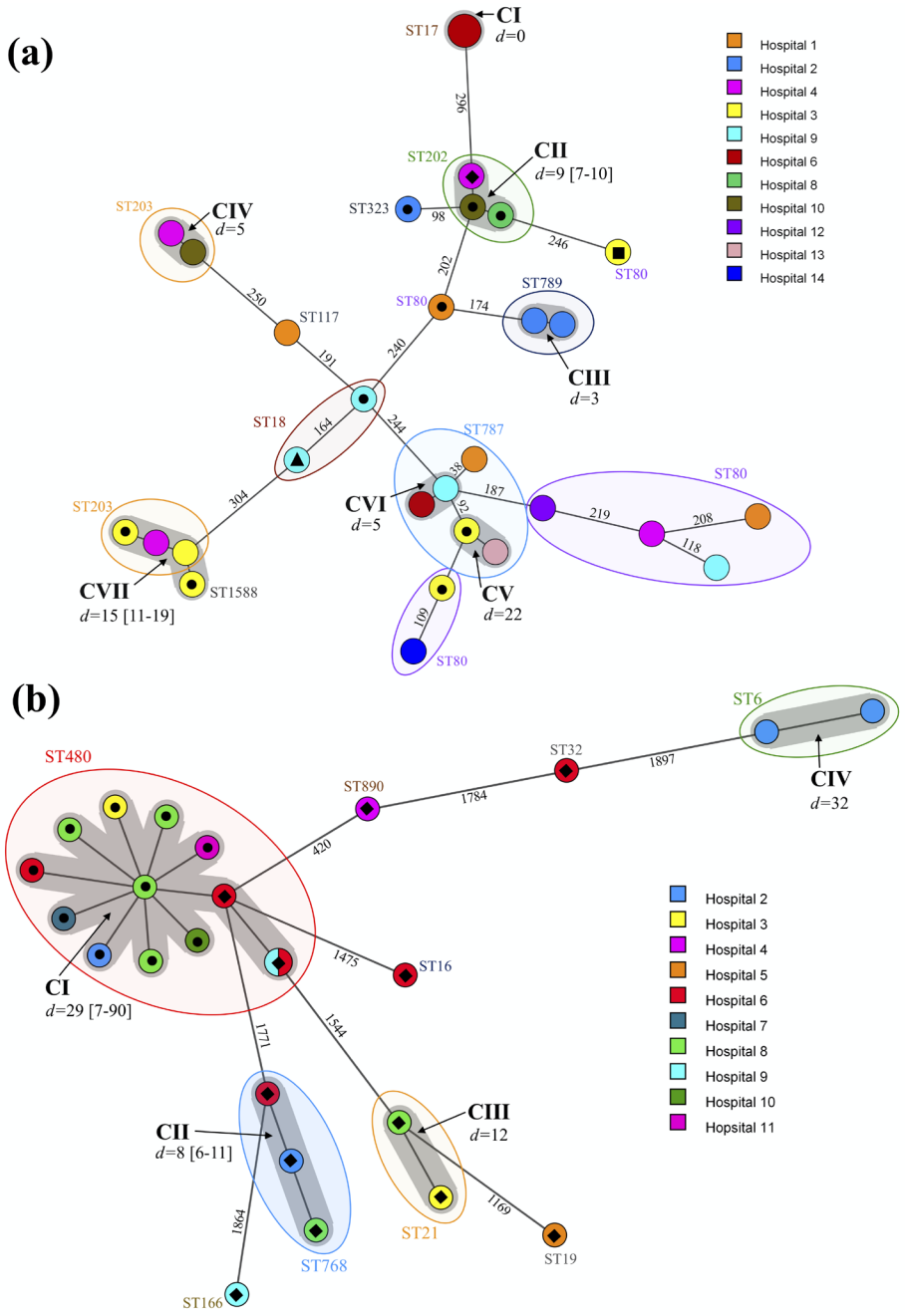


Figure 4.2 Minimum spanning trees based on (a) core-genome multilocus sequence typing (cgMLST) data from 30 linezolid-resistant clinical *E. faecium* isolates, (b) whole-genome multilocus sequence typing (wgMLST) data from 25 linezolid-resistant *E. faecalis* clinical isolates. All isolates were recovered between June 2016 and August 2019 from 16 Irish hospitals, as denoted by the colour legends. The numbers on the branches represent the number of cgMLST/wgMLST allelic differences. Sequence types (ST) are shown encircled by coloured ovals. Grey shadowing around nodes indicates clusters of related isolates and are labelled in bold and denoted CI – CVII; *d*= indicates average allelic differences and the range in square brackets. Isolate designations are as follows: filled black circle, *poxtA*-positive; filled black diamond, *optrA*-positive; filled black square, *optrA*- and *cfr(D)*-positive and filled black triangle, *optrA*- and *poxtA*-positive. Isolates not marked with a symbol were negative for linezolid resistant genes and harboured a varying copy numbers (1-5) of the G2576T 23S mutation associated with linezolid resistance.

4.3.3 The genetic environment surrounding *optrA*

The WGS data of a selection of isolates encoding *optrA* underwent hybrid assembly, namely two *E. faecalis* (M17/0149 and M17/0240) and six *E. faecium* (M16/0594, M17/0314, M18/0173, M18/0497, M18/0582, M18/0906 and M19/0596). The first plasmid resolved was a 36,331 bp *optrA*-encoding plasmid (pM17/0149) from *E. faecalis* M17/0149, which was the same size and exhibited 100% DNA sequence identity to plasmid pE349.

Of the 16 *optrA*-positive isolates, half (one *E. faecium* and seven *E. faecalis*) harboured plasmids exhibiting 100% sequence coverage to pM17/0149 (pE349-like). Multiple *E. faecalis* STs (ST19, ST21, ST166, ST768) harboured this pE349-like plasmid, suggesting independent acquisition by different genetic backgrounds (Table 4.1). For these eight isolates, the genetic environment surrounding *optrA* was designated *optrA*_WT (Figure 4.3). The remaining eight *optrA-positive* isolates exhibited <35% sequence identity to pM17/0149 and were selected for WGS hybrid assembly. The genetic environment surrounding *optrA* was examined and *optrA* was identified on plasmids in five isolates (M17/0240, M18/0582, M19/0596, M18/0173 and M17/0314), and within the chromosome in three isolates (M18/0497, M16/0594 and M18/0906) (Figure 4.3). Three variations surrounding *optrA* were evident on the plasmid encoded regions: *optrA*_I (M17/0240, M18/0582, M19/0596), *optrA*_II (M18/0173) and *optrA*_III (M17/0314). In *optrA*_I and *optrA*_II, *fexA* was encoded around 750 bp upstream of *optrA,* similarly to *optrA*_WT, whereas *optrA*_III lacked *fexA*. Variations were distinguished by various flanking IS elements and other genes encompassed between these elements (Figure 4.3a). Interestingly, isolate M17/0314, harbouring *optrA*_III, also encoded the linezolid resistance gene *cfr(D)* and the macrolide, lincosamide and streptogramin B resistance genes *erm(A)* and *erm(B)*, on a 103,600 bp plasmid (pM17/0314) (Figure 4.4). In the case of 1/8 *optrA*_WT isolates (M16/0427), 2/3 isolates harbouring *optrA*_I (M17/0240 and M19/0596), and one *optrA*_III isolate (M17/0314), *optrA* was successfully conjugated into a recipient strain of the same species (Table 4.2).

Three variations of the genetic environment surrounding *optrA* in the chromosome, designated *optrA*_IV, *optrA*_V and *optrA*_VI, were identified in isolates M18/0906, M16/0594 and M18/0497, respectively (Table 4.1). Both the *optrA*_IV and o*ptrA*_V variants harboured *optrA* flanked by *tnpA* and *tnpB* from Tn*554* on one end, and by *IS*L3 on the other end. The *optrA*_IV variant encoded *fexA* and *optrA* in the same orientation as *optrA*_WT, but in *optrA*_V, *fexA* was encoded around 2800 bp upstream from *optrA* in the

opposite orientation to the arrangement in *optrA*_WT (Figure 4.3b). In *optrA*_VI *optrA* was flanked by IS*Enfa5* and IS*Efa5* and exhibited the same *optrA* and f*exA* arrangement present in *optrA*_WT (Figure 4.3b).

Attempts to transfer *optrA* in the three isolates encoding chromosomal *optrA* by conjugation were unsuccessful.

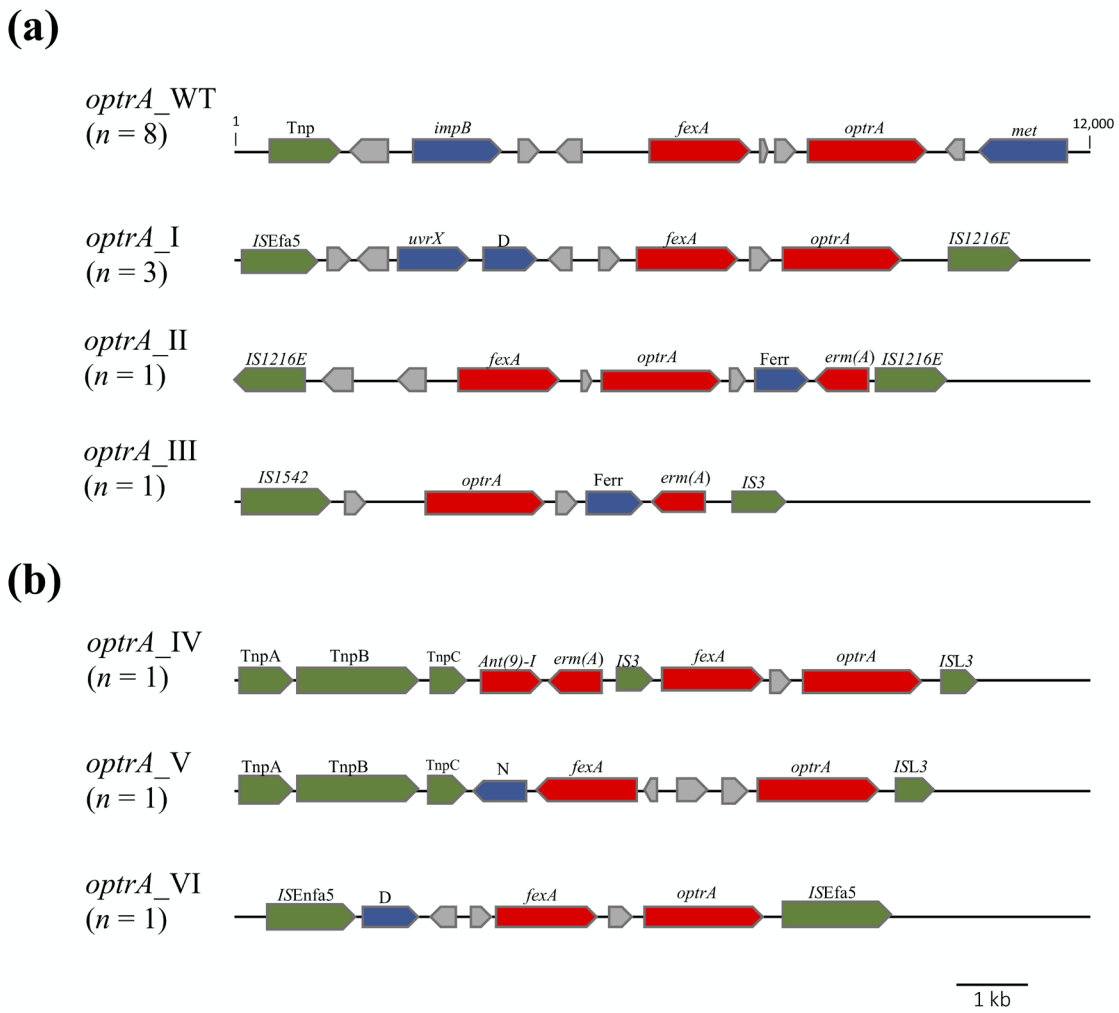


Figure 4.3 Schematic representation of the *optrA* gene loci in *E. faecalis* and *E. faecium* clinical isolates from Ireland. Different variants of the genetic environment surrounding *optrA* were detected in linezolid-resistant enterococci encoded on plasmids (a) and within the chromosome (b) and were aligned against the prototype *optrA*_WT, first described by Wang *et al.,* in 2015. Genes of interest and their orientation are represented by arrows and labelled; red indicates antibiotic resistance genes, green indicates insertion sequences/transposases, blue indicates known proteins and grey indicates hypothetical proteins. Abbreviations: Tnp, transposase; *met*, truncated DNA adenine methylase; *uvrX*, putative UV-damage repair protein, D, DNA-directed RNA polymerase beta subunit; Ferr, ferredoxin; N, NAD(P)H oxidoreductase.

Table 4.2 MIC profiles for transconjugants of recipient *E. faecium* 64/3 and *E. faecalis* OG1RF

Strain	Gene conjugated	Linezolid MIC (mg/L) (R > 4 mg/L)[a]	Vancomycin MIC (mg/L) (R > 4 mg/L)[a]	Chloramphenicol MIC (mg/L) (R > 32 mg/L)[a]	Tetracycline MIC (mg/L) (R > 4 mg/L)[a]
Recipient *Efm* 64/3	None	4	≤1	8	≤0.5
Recipient *Efs* OG1RF	None	≤2	2	≤4	≤0.5
Donor M16/0427		≥256	1	≥64	≥16
M16/0427:*Efm* 64/3 TC1	*optrA*	32	≤0.5	≥64	≥16
M16/0427:*Efm* 64/3 TC1	*optrA*	64	≤0.5	32	≥16
Donor M16/0594		16	1	16	≥16
M16/0594:*Efm* 64/3 TC1	*poxtA*	4	≤0.5	16	≥16
M16/0594:*Efm* 64/3 TC2	*poxtA*	4	≤0.5	16	≥16
Donor M17/0240		32	2	>64	>16
M17/0240:*Efs* OG1RF TC1	*optrA*	32	1	32	≤1
Donor M17/0314		64	1	16	>16
M17/0314:*Efm* 64/3 TC1	*optrA*	32	≤0.5	8	≤1
Donor M19/0596		8	1	32	>16
M19/0596:*Efs* OG1RF TC1	*optrA*	32	1	32	≤1
Donor M16/0419		8	1	32	>16
M16/0419:*Efs* OG1RF TC1	*poxtA*	32	1	16	>16
M16/0419:*Efs* OG1RF TC2	*poxtA*	24	1	16	>16

Table 4.2 continued overleaf

Strain	Gene conjugated	Linezolid MIC (mg/L) (R > 4 mg/L)[a]	Vancomycin MIC (mg/L) (R > 4 mg/L)[a]	Chloramphenicol MIC (mg/L) (R > 32 mg/L)[a]	Tetracycline MIC (mg/L) (R > 4 mg/L)[a]
Donor M16/0633		8	2	32	>16
M16/0633:*Efs* OG1RF TC1	*poxtA*	16	1	16	>16
M16/0633:*Efs* OG1RF TC2	*poxtA*	8	1	16	>16
Donor M18/0011		8	1	16	>16
M18/0011:*Efm* 64/3 TC1	*poxtA*	8	≤0.5	16	≤1
M18/0011:*Efs* OG1RF TC1	*poxtA*	16	1	16	≤1
Donor M19/0357		128	>32	16	<1
M19/0357:*Efm* 64/3 TC1	*poxtA*	16	≤0.5	16	≤1

[a] Clinical breakpoint taken from the European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST, 2019).

Abbreviations: MIC, minimum inhibitory concentration; R, resistant; *Efm*, Enterococcus faecium; *Efs, Enterococcus faecalis;* TC, transconjugant.

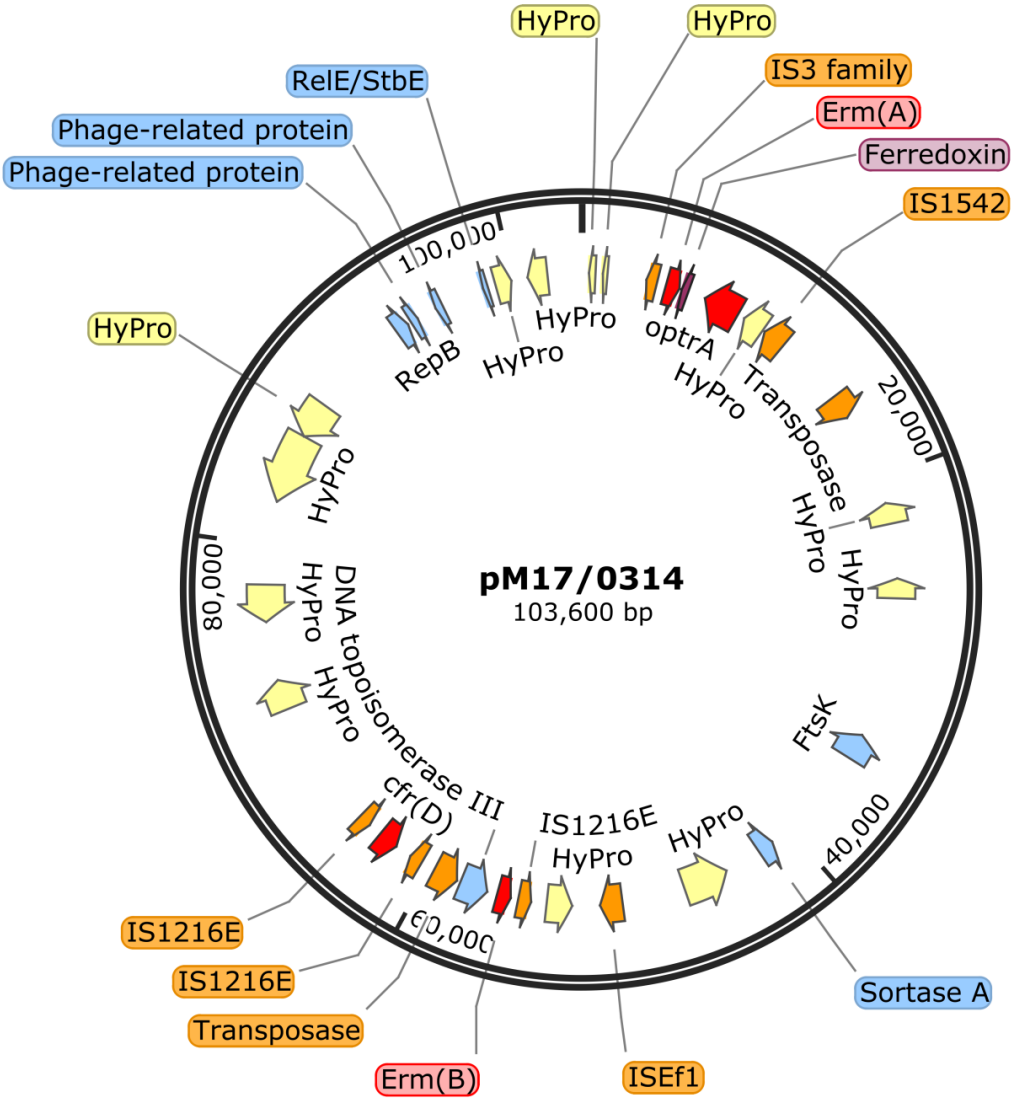


Figure 4.4 Schematic diagram of the structural organisation of plasmid pM17/0314 from *E. faecium* isolate M17/0314 encoding the *poxtA* linezolid resistance gene resolved by hybrid assembly of paired-end Illumina MiSeq short reads with Oxford Nanopore Technologies long reads. pM17/0314 harbours the *optrA*_III variation of the environment around *optrA*. Genes of interest and their orientation are represented by arrows as follows: red indicates antibiotic resistance genes, orange indicates insertion sequences/transposases, blue indicates known proteins and yellow indicates hypothetical proteins. The plasmid size is labelled indicating number of base pairs (bp). Abbreviations: HyPro; hypothetical protein.

4.3.4 Characterisation of isolates encoding *poxtA*

The *E. faecium* isolate M16/0594 harboured *optrA*_V in its chromosome and *poxtA, tet*(M) and *tet*(L) on a 21,849 bp plasmid (pM16/0594) (Figure 4.5). Plasmid pM16/0594 was conjugative and transconjugant derivatives were obtained with the *E. faecium* 64/3 recipient. Of the remaining 19 *poxtA*-positive LRE (nine *E. faecium* and 10 *E. faecalis*) 5/19 (all *E. faecium*) exhibited 100% sequence coverage to pM16/0594, while 14/19 (four *E. faecium* and ten *E. faecalis)* exhibited between 72.9%-99.3% coverage. All isolates exhibited 100% sequence coverage to a 4001 bp *poxtA*-encoding region, flanked by two *IS*1216E elements in parallel orientation.

The *poxtA*-positive *E. faecalis* isolate M18/0011 exhibited 73.7% sequence coverage to pM16/0594 was also resolved using hybrid assembly. The plasmid encoding *poxtA* in M18/0011 (pM18/0011) was 3,570 bp smaller than pM16/0594 (18,279 bp) and lacked *tet*(M) and *tet*(L). An identical 4001 bp region encoding *poxtA* flanked by two IS1216E elements in parallel orientation was observed in pM18/0011 (Figure 4.6), albeit in the reverse orientation to the identical region in pM16/0594. Plasmid pM18/0011 was also conjugative and transconjugant derivates were obtained with the *E. faecalis* OG1RF and *E. faecium* 64/3 recipients (Table 4.2).

In total, 5/20 *poxtA*-positive LRE yielded transconjugant derivatives including *E. faecium* M16/0594 (harbouring pM16/0594) and *E. faecalis* M18/0011 (harbouring pM18/0011), two *E. faecalis* donors (M16/0419, 75.2% like pM16/0594; M16/0633, 74.5% like pM16/0594) conjugated to *E. faecalis* OG1RF and one *E. faecium* donor (M19/0357, 77.9% like pM16/0594) conjugated to *E. faecium* 64/3 (Table 4.2). For the remaining 15 *poxtA*-positive LRE conjugation was unsuccessful.

All 10 *poxtA*-positive *E. faecalis* LRE identified belonged to ST480 and were closely related with an average of 22 (range 7-54) wgMLST allelic differences (Figure 4.2b). The ST480 clone was recovered in seven hospitals (Table 4.1). In contrast, *poxtA* was found in *E. faecium* in various STs (ST18, ST80, ST202, ST203, ST323, ST787 & ST1588), spread across six hospital sites (Table 4.1).

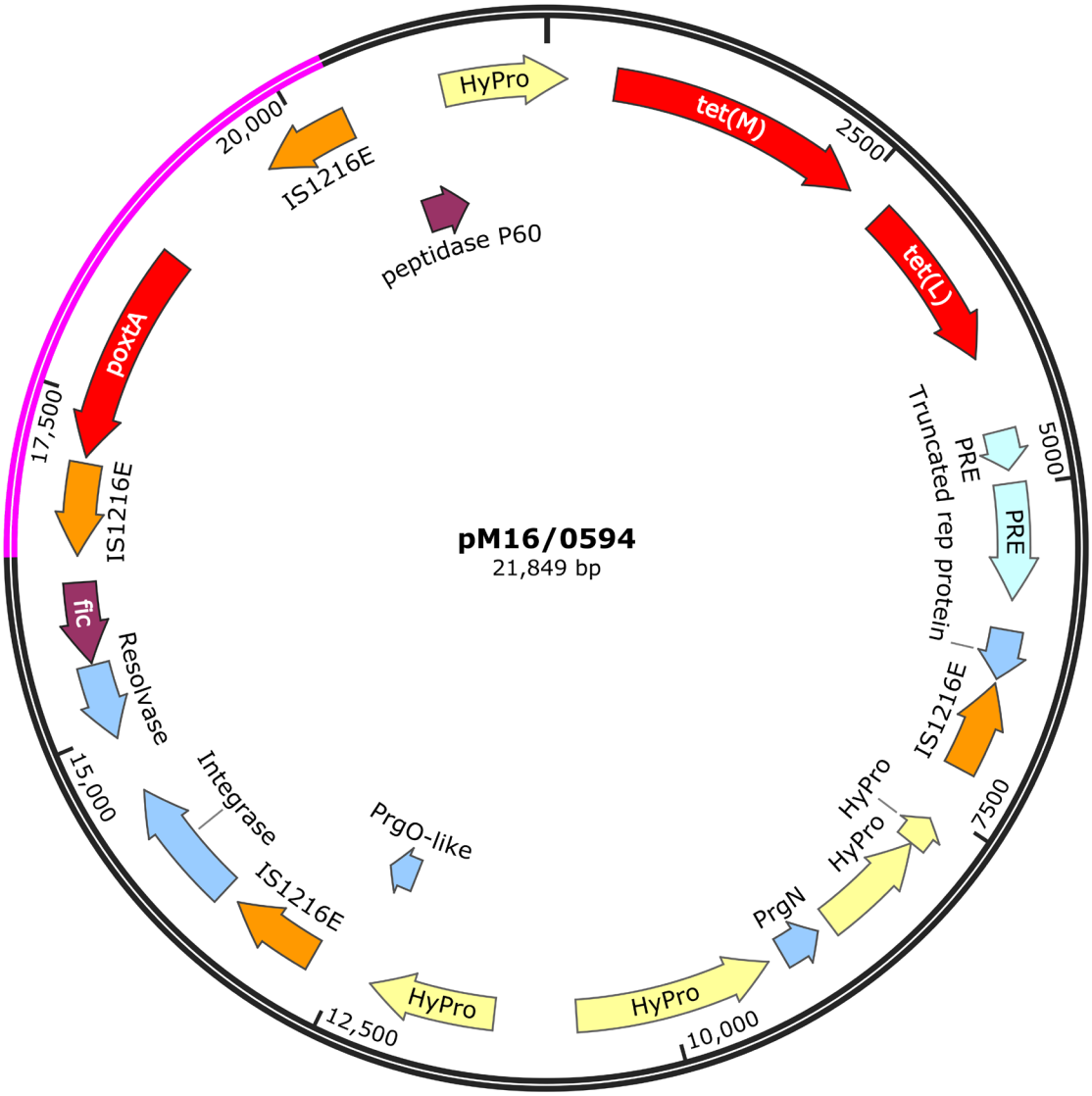


Figure 4.5 Schematic diagram of the structural organisation of plasmid pM16/0594 from *E. faecium* isolate M16/0594 encoding the *poxtA* linezolid resistance gene resolved by hybrid assembly of paired-end Illumina MiSeq short reads with Oxford Nanopore Technologies long reads. The 4001 bp highly conserved region around *poxtA* flanked by two IS*1216E* in parallel orientation is highlighted in pink. Genes of interest and their orientation are represented by arrows as follows: red indicates antibiotic resistance genes, orange indicates insertion sequences, blue indicates rep proteins, purple indicates other known proteins and yellow indicates hypothetical proteins. The plasmid size is labelled indicating number of base pairs (bp). Abbreviations: PRE; plasmid recombination enzyme, fic; cell filamentation protein, HyPro; hypothetical protein.

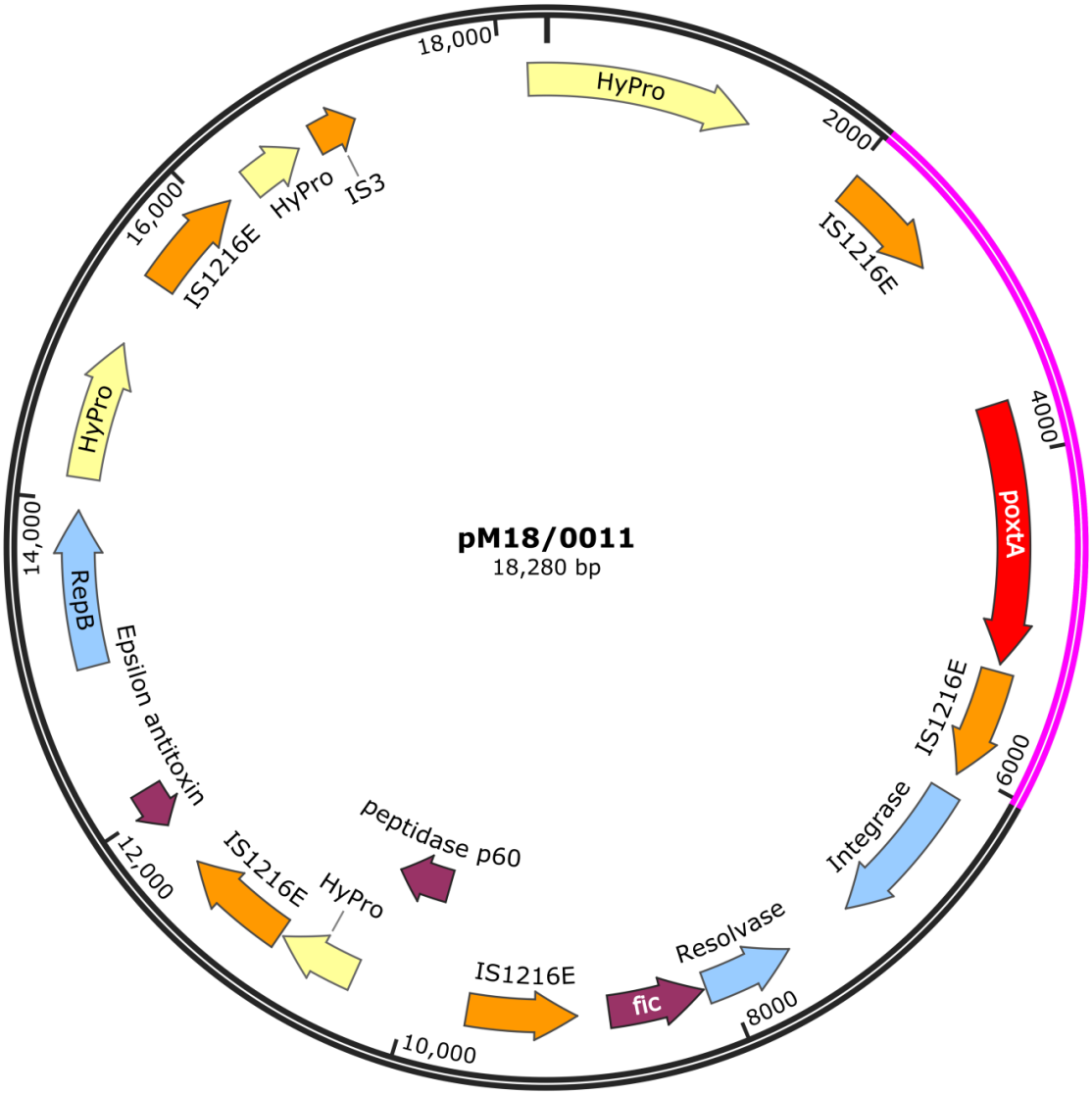


Figure 4.6 Schematic diagram of the structural organisation of plasmid pM18/0011 from *E. faecalis* isolate M18/0011, encoding *poxtA* linezolid resistance, resolved from WGS hybrid assembly of paired-end Illumina MiSeq reads with Oxford Nanopore Technologies long reads. The 4001 bp highly conserved region around *poxtA* flanked by two IS1216E in parallel orientation, is highlighted by pink of plasmid structure. Genes of interest and their orientation are represented by arrows and labelled, red indicates antibiotic resistance genes, orange indicates insertion sequences, blue indicates rep proteins, other known proteins are in purple and yellow indicates hypothetical proteins. The plasmid size is labelled indicating number of base pairs (bp). Abbreviations: fic; cell filamentation protein, HyPro; hypothetical protein.

4.4 Discussion

A total of 35/154 (22.7%) LRE (23 *E faecalis* and 12 *E. faecium*) submitted to the NMRSARL between June 2016 and August 2019 harboured *optrA* or both *optrA* and *poxtA,* this is the highest prevalence of *optrA* and/or *poxtA* in human enterococci reported to date. This collection of *poxtA* positive isolates was also the largest of clinical origin reported to date, with only a single other *poxtA* positive clinical isolate being reported in Greece, prior to this study (Papagiannitsis *et al.*, 2019). This study also is the first report of mutational and gene-encoded linezolid resistance in a single enterococcal isolate, (M19/0595) harboured *poxtA* and the G2576T 23S mutation (4/6 copies mutated). The combination of mutational and acquired linezolid resistance in a single isolate indicates an environment in which is linezolid resistance is allowed to flourish under selective pressures. These findings emphasise the problem with LRE in Ireland.

Overall, the population structure of LRE was polyclonal, but the presence of highly related strains in different hospitals was evident. The polyclonal nature of *E. faecium* has been well described in Chapter 3, and what is evident from this study is that the *E. faecium* population of LRE are much more diverse than that of the *E. faecalis*.

The variation surrounding *optrA* in the LRE investigated combined with the identification of multiple linezolid resistance genes (*optrA*/*poxtA* and *optrA/cfr(D)*) in individual isolates is indicative again of an environment with a high selective pressure for linezolid resistance. It is interesting to note that even though *optrA* was likely introduced or possibly present previously on the pE349-like plasmid, only half of the isolates contained this conjugative plasmid. The remaining half had variable *optrA* environments, which is likely as a result of selective pressure and also the surrounding of *optrA* by varying *IS*-elements has been shown to aid in its dissemination and also recombination of the surrounding environment (He *et al.*, 2016).

The 4001 bp *poxtA*-encoding region, flanked by two *IS*1216E elements in parallel orientation; this conserved *poxtA* element has previously been shown to be responsible for horizontal gene transfer of *poxtA* (Antonelli *et al.*, 2018; Huang *et al.*, 2019; Lei *et al.*, 2019). These findings indicate the spread of *poxtA* in human *E. faecium* and *E. faecalis* in Ireland via this 4001 bp element or by a very similar *poxtA* plasmid. One limitation of the method of read mapping against pM16/0594 as comparison against all other isolates, is the risk of missing larger plasmids. This is due to the fact that 100% coverage will indicate the query sequence is identical to the reference used but cannot indicate if the plasmid is larger. Therefore, isolates with 100% sequence coverage to pM16/0594 have at least a

21,849 bp plasmid region identical to that of pM16/0594, which may be contained within larger plasmids.

The spread of a single clone was indicated in all 10 *poxtA*-positive *E. faecalis* LRE, all of which belong to ST480 is one of the predominant *optrA*-positive clinical *E. faecalis* clones in France and Germany (Bender *et al.*, 2018b; Sassi *et al.*, 2019). In contrast, *poxtA* was found to have spread in *E. faecium* via the 4001 bp mobile element or a promiscuous plasmid to multiple STs (ST18, ST80, ST202, ST203, ST323, ST787 & ST1588). This highlights the importance at looking at the movement of MGEs as well as the population biology, as like in Chapter 3, MGEs in Enterococci have shown to be transmissible across varying genetic lineages.

The MDR genes *optrA* and *poxtA* have been widely reported in livestock and food producing animals, mainly in Asia, but also in Europe (Cai *et al.*, 2015; Tamang *et al.*, 2017; Brenciani *et al.*, 2019; Huang *et al.*, 2019; Lei *et al.*, 2019). Oxazolidinones are not used in food-producing animals. Nevertheless, the detection of LRE in these animals harbouring MDR plasmids can be linked to the colocation of resistance genes for antibiotics commonly used in animals (phenicols, tetracyclines, lincosamides, and aminoglycosides) (Lobritz *et al.*, 2003; Schwarz *et al.*, 2004; Torres *et al.*, 2018). Furthermore, the crossover of resistance to various groups of antimicrobials encoded by *optrA* (resistance to oxazolidinones and phenicols) and *poxtA* (resistance to oxazolidinones, phenicols and tetracycline) may contribute to enrichment for linezolid resistance in livestock isolates (Cai *et al.*, 2015; Antonelli *et al.*, 2018). Thus, the livestock industry and animal husbandry may play an important role in the indirect selection of multidrug resistant enterococci, including LRE. This poses a risk for public health by providing a reservoir of linezolid-resistance genes that could be transmitted into human enterococcal lineages by HGT or by the direct acquisition of animal LRE strains by humans.

According to the European Medicines Agency, the most commonly purchased antimicrobial used in animals in Ireland is tetracycline, accounting for 39.7% (39.2 tonnes) of total veterinary antimicrobials (European Medicines Agency, 2020). Interestingly, coinciding with this, the current study reports the highest prevalence of *poxtA* among clinical enterococci reported to date, which may be indirectly selected for by high tetracycline use in agriculture. In contrast, veterinary amphenicols make up 3.3% (3.3 tonnes) of antimicrobials used in animals, again as both *optrA* and *poxtA* encode phenicol resistance, along with the fact *optrA* is often co-located with *fexA* (in the present study

15/16 *optrA*-positive enterococci harboured *optrA* co-located with *fexA)*, this could further play a role in the indirect selection of these genes in Ireland (European Medicines Agency, 2020). Indirect selection for linezolid resistance through animal husbandry and agriculture is likely to play a role in its exponential increase in hospitals, along with a rise in the overall consumption of antimicrobials in healthcare. The spread of *optrA* and/or *poxtA* from animal strains is a very likely source of linezolid-resistance in human enterococcal strains and potentially we have seen the spread of these AMR encoding genes via MGEs into the clinical environment. This highlights the crucial need for a "One Health" approach to AMR.

The results of this study revealed the high prevalence and spread of *optrA* and *poxtA* among enterococci in Irish hospitals. A major cause for concern is that 26.3% (5/19) of isolates harbouring a *poxtA*-encoding plasmid also harboured *vanA*, which poses a significant risk for hospitalised patients, as treatment options for such strains are very limited. Linezolid consumption in Irish hospitals is currently not specifically recorded as part of annual national hospital antimicrobial consumption surveillance (HSE/HPSC, 2018). This needs to change, and linezolid consumption in hospitals needs to be more rigidly controlled. Also, this study further highlights the need for a "One Health" approach, to examine all aspects of the increase in these AMR genes, to include monitoring of antimicrobials in healthcare, agriculture and also the release of antimicrobials into the environment (wastewater and agricultural runoff from slurry).

Chapter 5

A hospital outbreak of linezolid-resistant and vancomycin-resistant ST80 *Enterococcus faecium* harbouring an *optrA*-encoding conjugative plasmid investigated by whole-genome sequencing

5.1 Introduction

High resolution molecular typing of *E. faecium* has been shown to be crucial in understanding its population dynamics, due to the nature of its highly recombinant genome. As detailed in Chapters 3 and 4, *E. faecium* in Ireland have demonstrated a polyclonal population structure. Additionally, the transfer of identical MGEs encoding antimicrobial resistance determinants into unrelated genetic backgrounds has also been shown. Therefore, in an outbreak setting it is preferable to use both a WGS-based typing approach, along with typing and comparison of the MGE harbouring the resistance gene of interest. Both approaches used in conjunction can give a clearer and more precise overview of an outbreak.

As described in Chapter 1, *E. faecium* has emerged as an important nosocomial pathogen causing bacteraemia, abdominal, urinary tract and intravenous catheter-related infections (Arias and Murray, 2012). Acquired resistance to ampicillin, gentamicin (high level) and vancomycin has increased worldwide among hospital-associated *E. faecium*, narrowing treatment options. Ireland had one of the highest rates of vancomycin-resistant *E. faecium* (VREfm) bloodstream infections in Europe between 2006-2018 (European Centre for Disease Prevention and Control, 2019). Furthermore, the population-weighted mean percentage of VREfm across Europe increased significantly from 10.4% in 2014 to 17.3% in 2018 (European Centre for Disease Prevention and Control, 2018).

Conventional MLST for *E. faecium* was first described in 2002, consisting of seven housekeeping genes with derived nomenclature managed and assigned via PubMLST.org (Homan *et al.*, 2002). However, conventional MLST has been deemed to lack the resolution necessary for typing VREfm following the advent of high-throughput WGS and has largely been replaced by cgMLST (de Been *et al.*, 2015; Pinholt *et al.*, 2017; Werner *et al.*, 2020). Clinical VREfm worldwide assign to conventional STs belonging to the epidemic hospital-adapted lineage, clade A1. These strains are generally enriched in MGEs, putative virulence determinants, and antibiotic resistance determinants (Willems *et al.*, 2005; Wurster *et al.*, 2016). Whole-genome sequencing studies have further revealed a VREfm polyclonal population structure, with evidence of hospital transmission and inter- and intra-regional spread of VREfm clones (Howden *et al.*, 2013; Raven *et al.*, 2016; Pinholt *et al.*, 2017) and no distinct geographical patterns have emerged. Enhanced surveillance is required to better understand the epidemiology, clonal diversity and risk factors associated with VREfm (European Centre for Disease Prevention and Control, 2018).

Linezolid is an antibiotic used to treat infections caused by multi-drug resistant Gram-positive bacteria, including VREfm. The emergence of linezolid-resistant enterococci (LRE) during or after linezolid exposure has been well described, with the first description of resistance noted during initial clinical trials (Gonzales *et al.*, 2001; Cai *et al.*, 2015; Wang *et al.*, 2015; Bi *et al.*, 2018; Bai *et al.*, 2019). However, linezolid resistance in VREfm was first reported in Greece in 2004 (Bersos *et al.*, 2004; Zahedi Bialvaei *et al.*, 2017). Linezolid binds in the V domain of the 23S rRNA component of the 50S ribosomal subunit and inhibits protein synthesis (Swaney *et al.*, 1998). Enterococcal linezolid resistance results mainly from G2576T or G2505A mutations in the 23S rRNA binding site or mutations in genes encoding ribosomal proteins L3 and/or L4 (Bi *et al.*, 2018). Linezolid-resistance can also develop following acquisition of the *optrA, poxtA* genes and variants of the *cfr* gene, which are frequently encoded on conjugative plasmids (Bender *et al.*, 2018a).

The *optrA* gene was first described from a clinical *E. faecalis* isolate in China and was subsequently identified in *E. faecium* and *E. faecalis* isolates from humans and food-producing animals throughout European, American and Asian countries (Cai *et al.*, 2015; Wang *et al.*, 2015; Cavaco *et al.*, 2016; Mendes *et al.*, 2016, 2018; Bi *et al.*, 2018). The OptrA protein belongs to the ATP-binding cassette (ABC)-F protein subfamily and mediates resistance to oxazolidinones and phenicols. A recent study indicated the mechanism of *optrA*-mediated antibiotic resistance does not involve active efflux, like other ABC transporters (Wang *et al.*, 2018). Current evidence indicates that (ABC)-F proteins like OptrA bind to the ribosome and effect the release of ribosome-targeted antibiotics, thereby rescuing the translation machinery from antibiotic-mediated inhibition (Sharkey and O'Neill, 2018). Although the number of *optrA*-positive enterococci reported to date is low, these have increased recently. In 2014, 3/9 linezolid-resistant isolates (linezolid MIC > 4 mg/L) were *optrA*-positive *E. faecalis* (two from Ireland); this increased to 8/17 in 2016 (Mendes *et al.*, 2016, Mendes *et al.* 2018).

In Ireland in 2014, the first reported linezolid-resistant VREfm (LVREfm) clonal outbreak was reported involving 15 patients and was investigated using PFGE. All isolates harboured the G2576T 23S rRNA mutation and were *cfr*-negative. However, other linezolid resistance genes were not investigated at that time (O'Driscoll *et al.*, 2015). That same year, two *optrA*-positive VREfm were recovered in separate Irish hospitals (Mendes *et al.*, 2016). Chapter 4, describes that the population structure of LRE in Ireland since centralised screening commenced in 2016. This study revealed the highest reported

prevalence of gene-encoded linezolid resistance, with *optrA* and/or *poxtA* identified in 22.7% (35/154) isolates. Linezolid resistance genes in Ireland were found to be predominantly encoded on conjugative plasmids and identified in diverse enterococcal lineages.

In October 2019, a LVREfm isolate was recovered from a patient in a north Dublin hospital. Enhanced patient screening and environmental sampling yielded additional VREfm isolates including LVREfm suggesting an outbreak. This part of the present study aimed to confirm the occurrence of an LVREfm outbreak and investigate its dynamics using WGS, and to review the control measures implemented to terminate the outbreak.

5.2 Material and Methods

5.2.1 Hospital setting

The outbreak occurred in a level 2, 107-bed hospital in North Dublin and was primarily associated with two wards and the X-Ray department. Ward 1 (W1) was a 26-bed unit with single patient en-suite rooms, linked with ward 2 (W2), an oncology day-unit (Figure 5.1). The hospital specialities include general medical and oncology with a large proportion of patients requiring extensive care.

5.2.2 VREfm surveillance

In October 2019, patient A was admitted to W1 with chronic leg ulcers, cellulitis and an extensive medical history including colon cancer. Patient A had also been admitted the previous month to the high dependency unit and to W1. While an inpatient, patient A visited other departments including X-Ray and required a high level of care. On re-admission 13 days later, patient A was screened and placed on contact isolation precautions due to a history of carriage of multi-drug resistant organisms (MDRO) including VREfm. An LVREfm isolate was recovered from rectal screening on this admission, after which additional emphasis was placed on contact precautions, hand hygiene and equipment decontamination. Nine days later, patient B (W1) also yielded LVREfm following rectal screening. This prompted the infection prevention and control team (IPCT) to request that all patients on W1 be screened for VREfm carriage, after

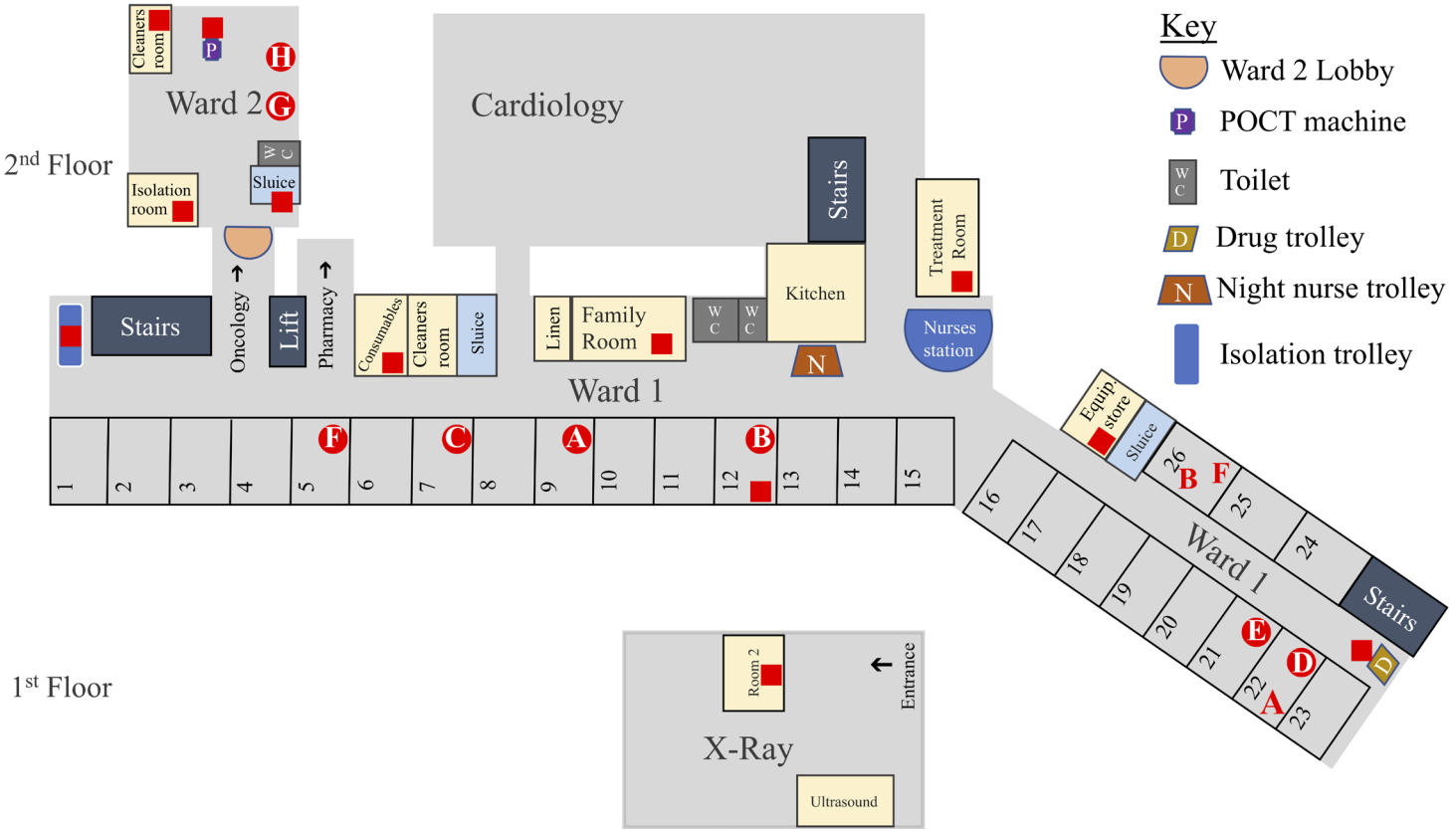


Figure 5.1 Schematic diagram showing the layout of hospital ward 1, ward 2 and X-ray involved in *optrA*-positive vancomycin-resistant *E. faecium* outbreak. The single en-suite rooms in Ward 1 are labelled 1-26. Other areas of interest are labelled accordingly, or details are provided using the key. Locations where patients (denoted by capital letters A-H) that yielded LVREfm isolates were housed are denoted by a filled red circle. Environmental sites that yielded LVREfm isolates are denoted by a filled red square. Patients A, B and F were transferred during the course of the outbreak and their movements are denoted by corresponding red letter in alternate rooms. Patient G, an oncology in-patient housed on ward 3 (not shown), is shown on ward 2, as this is the likely location for acquisition of LVREfm. Abbreviations: POCT machine, point-of-care-testing machine; Equip. store, equipment storage; consumables, consumable storeroom.

which three more LVREfm-positive patients were identified (patients C-E, Figure 5.1, Table 5.1). Additional weekly and discharge screening was introduced. As two LVREfm-positive patients (A & B) were oncology patients, the IPCT introduced screening of all patients attending the oncology out-patient W2, which identified two further LR-VREfm-positive patients (patients G & H, Figure 1, Table SI). Patient G was an in-patient on a different hospital ward (W3) who attended W2 every few weeks from early 2019 until the start of the outbreak. Extended rectal screening across the hospital identified VREfm in a further nine patients (Table 5.1). All VREfm patient screening isolates from the hospital recovered during the suspected outbreak timeline were investigated.

5.2.3 Patient and environmental screening

Extensive environmental screening was undertaken in all inpatient wards and other areas where patients had attended during hospitalisation. Environmental sites were sampled using regular FLOQSwabs® (Copan Diagnostics Inc., California, USA), pre-moistened with sterile water. Individual swab tips were placed into 5 ml of BHI broth (Fannin Ltd), incubated for 16-18 h at 37ºC, after which the cultures were inoculated onto CHROMID® VRE (bioMérieux) agar with a 10 µg linezolid disc (Oxoid). Patient rectal screening swabs were also inoculated onto CHROMID® VRE (bioMérieux) agar with a 10 µg linezolid disc (added following identification of the index case), and onto CHROMID® CPS® Elite (bioMérieux) agar to ensure bowel flora were present on the swab.

5.2.4 Decontamination and control measures

For the duration of the outbreak W1 was closed to patient admissions and transfers, strict visitor restrictions were implemented, specific staff members were dedicated to W1, cleaning of all equipment in patient areas was increased to twice daily and cleaning of bathroom facilities was increased to four-times daily, using Actichlor Plus (Ecolab Limited, Cheshire, UK) with 1,000 ppm available chlorine. Patient rooms that yielded LVREfm were decontaminated with hydrogen peroxide vapour (HPV) following patient transfer or discharge using a Bioquell Rapid Bio-Decontamination Unit (Bioquell Ireland Ltd., Limerick, Ireland).

Table 5.1 Phenotypic and genotypic characteristics of the 38 vancomycin-resistant *E. faecium* isolates recovered in an outbreak setting in an Irish hospital over four weeks in October 2019, with the addition of one isolate from the index patient from 2018

***E. faecium* isolate No.**	**Ward/ Room[a]**	**Day since first isolate recovered**	**Source[c]**	**Clinical history[d]**	**Lnz MIC mg/L (R≥ 4 mg/L)[e]**	**Vanc MIC mg/L (R≥ 4 mg/L)[e]**	**Chl MIC mg/L (R≥ 8 mg/L)[e]**	***optrA***	**ST**	**cgMLST cluster[f]**	**Plasmid sequence similarity (%) to pEfmO_03**
O_01	W1	N/A[b]	Patient A		4.0	≥32	32	-	80	C1	N/A
O_02	W1 9 > 22	0	Patient A	Colon cancer, diabetes, COPD, chronic leg ulcers, multiple MDRO including VRE carriage	8.0	≥32	≥256	+	80	C1	100
O_03	W1 12 > 26	8	Patient B	Metastatic cancer, palliative care	16.0	≥32	≥256	+	80	C1	100
O_04	W1	13	Room 12		16.0	≥32	≥256	+	80	C1	100
O_05	W2	13	Sluice room		16.0	≥32	≥256	+	80	C1	100
O_06	W1	13	Isolation trolleys		16.0	≥32	≥256	+	80	C1	100
O_07	W1 7	13	Patient C	COPD, arthritis, malignancy	8.0	≥32	16	+	80	C1	100
O_08	W1	13	Treatment room		16.0	≥32	≥256	+	80	C1	100
O_09	W1 22	14	Patient D	Infected leg ulcers, recurrent UTI's, rheumatoid arthritis	16.0	≥32	≥256	+	80	C1	99.98
O_10	W1	14	Patient		2.0	≥32	16	-	SLV of ST80	N/A	N/A
O_11	W1	14	Patient		2.0	≥32	32	-	80	C4	N/A
O_12	W1	14	Patient		2.0	≥32	32	-	80	C1	N/A

Table 5.1 continued overleaf

E. faecium isolate No.	Ward/ Room[a]	Day since first isolate recovered	Source[c]	Clinical history[d]	Lnz MIC mg/L (R≥ 4 mg/L)[e]	Vanc MIC mg/L (R≥ 4 mg/L)[e]	Chl MIC mg/L (R≥ 8 mg/L)[e]	*optrA*	ST	cgMLST cluster[f]	Plasmid sequence similarity (%) to pEfmO_03
O_13	W1 21	15	Patient E	Metastatic malignancy, palliative care	16.0	≥32	≥256	+	SLV of ST80	C1	100
O_14	W1	16	Equipment store		16.0	≥32	≥256	+	80	C1	99.98
O_15	W1	16	Consumable store		8.0	≥32	32	+	80	C1	100
O_16	W1	16	Family room		32.0	≥32	≥256	+	80	C1	100
O_17a	W1	16	Drug trolley		1.0	≥32	16	-	80	C1	N/A
O_17b	W1	16	Drug trolley		16.0	≥32	32	+	80	C1	100
O_18	W1	16	Linen room		2.0	≥32	16	-	80	C1	N/A
O_19	W1	16	Night nurse trolley		1.0	≥32	16	-	80	C4	N/A
O_20	W1	16	Cleaners store		2.0	≥32	16	-	80	C1	N/A
O_21	W4	16	Patient		2.0	≥32	32	-	80	C1	N/A
O_22	W2	20	POCT machine		8.0	≥32	≥256	+	80	C1	100
O_23	W2	20	Isolation room		8.0	≥32	≥256	+	80	C1	100
O_24	W1 5 > 26	20	Patient F	Congestive cardiac failure and COPD	16.0	≥32	≥256	+	80	C1	44.1
O_25	W2	20	Cleaner room		8.0	≥32	≥256	+	80	C1	100
O_26	X-ray	20	Room 2		8.0	≥32	≥256	+	80	C1	100
O_27	W2	20	Lobby		2.0	≥32	16	-	80	C4	N/A
O_28	W1	20	Patient		2.0	≥32	64	-	80	C3	N/A
O_29	W1	20	Patient		2.0	≥32	32	-	80	C4	N/A
O_30	W4	20	Patient		1.0	≥32	32	-	80	C1	N/A

Table 5.1 continued overleaf

***E. faecium* isolate No.**	**Ward/ Room[a]**	**Day since first isolate recovered**	**Source[c]**	**Clinical history[d]**	**Lnz MIC mg/L (R≥ 4 mg/L)[e]**	**Vanc MIC mg/L (R≥ 4 mg/L)[e]**	**Chl MIC mg/L (R≥ 8 mg/L)[e]**	***optrA***	**ST**	**cgMLST cluster[f]**	**Plasmid sequence similarity (%) to pEfmO_03**
O_31	W1	20	New treatment room (room 10)		4.0	≥32	64	-	80	C3	N/A
O_32	W6	20	Bathroom		2.0	≥32	32	-	80	C2	N/A
O_33	X-ray	20	Ultrasound		2.0	≥32	32	-	80	N/A	N/A
O_34	W3	21	Patient		2.0	≥32	32	-	80	C2	N/A
O_35	W5	22	Patient		1.0	≥32	16	-	80	C4	N/A
O_36	W3	23	Patient G	Metastatic malignancies, gastrointestinal upset	8.0	≥32	≥256	+	80	C1	99.98
O_37a	W2	23	Patient H	Breast cancer	2.0	≥32	16	-	80	C1	N/A
O_37b	W2	23	Patient H	Breast cancer	32.0	≥32	≥256	+	80	C1	100

[a] Room numbers have been changed to maintain patient anonymity, x > y indicates room transfers during course of outbreak.
[b] This isolate was recovered from the index case (patient A) in October 2018. one year before the outbreak.
[c] All isolates recovered from patients were recovered from rectal swabs. Environmental isolates were recovered from pre-moistened FLOQSwabs® (Copan Diagnostics Inc., California, USA) used to swab environmental sites.
[d] Clinical history only provided for patients found to harbour LVREfm, all other patient clinical history was not available.
[e] Clinical breakpoints taken from the European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST, 2019).
[f] Thirty-seven of the 39 VREfm outbreak isolates were differentiated into four clusters (C1–C4) using cgMLST (Figure 5.2).
Abbreviations: Lnz, Linezolid; Vanc, Vancomycin; Chl, Chloramphenicol; W, Ward; N/A, Not applicable; ST, sequence type; MDRO, multiple drug-resistant organisms; COPD, chronic obstructive pulmonary disease; SLV, single-locus variant.

5.2.5 Phenotypic and genotypic analysis

All isolates were tested for susceptibility to linezolid and vancomycin using the VITEK®2 system (bioMérieux), as described in Chapter 2, Section 2.3.3. All VREfm and LVREfm isolates were referred to the NMRSARL, where gradient strips (E-test, bioMérieux) were used to assess linezolid and chloramphenicol MICs, as described in Chapter 2, Section 2.3.3. PCRs for identification of enterococcal species and detection of resistance genes were performed as described in Chapter 2, Section 2.4.2.3. One additional stored linezolid-susceptible VREfm isolate recovered in October 2018 from patient A, who was deemed the index case in the current outbreak, was also investigated.

5.2.6 Whole-Genome Sequencing

Thirty-nine enterococcal isolates (Table 5.1) and several transconjugant derivatives underwent WGS, as described in Chapter 2, Section 2.5.1.

LVREfm isolate O_03 (patient B) was selected for hybrid assembly to determine the genetic organisation of an *optrA*-encoding conjugative plasmid it harboured. For this isolate, DNA was extracted as described in Chapter 2, Section 2.4.1.2. Long-read sequencing was performed as described in Chapter 2, Section 2.5.2.

5.2.6.1 Analysis of WGS data

WGS data were analysed using the *E. faecium* wgMLST scheme available in BioNumerics v7.7 (Applied Maths, Belgium), as described in Chapter 2, Section 2.7.3. A filter was applied to include only the 1,423 core-genome cgMLST loci (de Been *et al.*, 2015). Conventional MLST was also applied as described in Chapter 2, Section 2.7.2. Minimum-spanning trees (MSTs) were created as described in Chapter 2, Section 2.7.5. All isolate sequences were processed through LRE-Finder as described in Chapter 2, Section 2.7.6.

All isolate sequences were also compared with an in-house database of 245 VREfm whole-genome sequences from isolates recovered in two other Dublin hospitals between September 2017-October 2019. Data was stored in Ridom SeqSphere+ and analysed using cgMLST as described in Chapter 2, Section 2.7.3.

5.2.6.2 Hybrid assembly of an optrA-encoding plasmid

The genetic organisation of the *optrA*-encoding plasmid pEfmO_03 from LR-VREfm outbreak isolate O_03 (patient B) was determined following hybrid assembly as described in Chapter 2, Section 2.7.7. The completed plasmid was annotated using RAST v2.0

(http://rast.nmpdr.org/). This was used as a reference sequence to compare against all other outbreak isolates, as described in Chapter 2, Section 2.7.8. The sequence of pEfmO_03 has been deposited in GenBank, accession number: MT261365.

5.2.7 Conjugation

Conjugative transfer of plasmids encoding *optrA* harboured by LVREfm was undertaken by filter mating as described in Chapter 2, Section 2.9.1.

5.3 Results

5.3.1 Patient VREfm

Eight patients were found to be colonised with LVREfm over a 4-week period. The patients were located in or visited hospital wards W1 and W2 (oncology), with one oncology patient located in W3 at the time of screening (Figure 5.1). A further nine patients yielded VREfm during a period of enhanced screening (Table 5.1). Patient A, from whom the first LVREfm was recovered, had previously been admitted to the hospital high dependency unit the previous month and to W1. The patient was discharged and 13 days later readmitted to W1. Readmission screens yielded LVREfm. A review of patients who had previously occupied the same bed as patient A revealed no further VREfm. In addition all VREfm isolates recovered over the previous year were reviewed on the VITEK®2 system and no linezolid resistance was found. Patient H yielded both a VREfm (O_37a) and a LVREfm (O_37b) from their screening sample (Table 5.1). A review of antimicrobial prescribing for each patient (A-H) involved in the LVREfm outbreak revealed that only patient A had received linezolid.

5.3.2 Environmental screening

Following swab based sampling of 129 environmental sites throughout the hospital, with particular focus on W1, W2 and X-ray, 20 VREfm were recovered, including 14 LVREfm (Figure 5.1, Table 5.1). In W1, room 12 yielded a LVREfm five days after admission of patient B who also yielded LVREfm. The isolation and drug trolleys, which are moved throughout W1, also yielded LVREfm. The drug trolley in W1 yielded both an VREfm (O_17a) and an LVREfm (O_17b) (Table SI). The treatment room, equipment storage room and consumables store room, all of which have a high volume of staff traffic, all yielded LVREfm. The family room on W1 also yielded LR-VREfm (Figure 5.1, Table 5.1). In W2, the sluice room, point-of-care-testing (POCT) machine, cleaners store room and the isolation room all yielded LVREfm, between 13-20 days after recovery of the first

LR-VREfm from patient A (index case). In X-ray, only room 2 yielded LVREfm (Figure 5.1, Table 5.1).

5.3.3 Antimicrobial consumption

Analysis of hospital antimicrobial prescribing audit data revealed that linezolid consumption increased from 0.46 defined daily doses per 100 bed days used (DDD/100 BDU) beginning Q4 2016 to 1.14 DDD/100 BDU by Q4 2019 (range; 0.15-1.39 DDD/100 BDU). A steady rise in linezolid consumption was noted from Q2 of 2019 (1.04 DDD/100 BDU) to Q3 of 2019 (1.23 DDD/100 BDU). Consumption of other antimicrobials (vancomycin and daptomycin) also rose in Q3 2019, reflecting increased complexity of patients and increased numbers of patients colonised with MDROs. All prescriptions were deemed appropriate and compliant with the hospital's restricted antimicrobials policy.

5.3.4 Whole genome sequencing

5.3.4.1 Population structure of outbreak isolates

A total of 37/39 VREfm investigated belonged to ST80; the remaining two isolates were a single-locus variant (SLV) of ST80. Twenty isolates (51.3%) were resistant to linezolid (MICs > 4 mg/L) (Table 5.1), all of which harboured *optrA*, but lacked *poxtA*, *cfr* and the 23S rRNA G2576T or G2505A mutations. The remaining 19 VREfm lacked linezolid-resistance genes and were susceptible to linezolid (Table 5.1). Thirty-seven of the VREfm differentiated into four clusters (C1–C4) using cgMLST (Figure 5.2). The majority of isolates (*N*=28) belonged to C1 and were closely related (average allelic difference of two, range=0-10). C1 consisted of ST80 (*N*=27) isolates, and one isolate deemed a SLV of ST80 and consisted of a mixture of patient (*N*=12) and environmental (*N*=16) isolates. C1 also contained LVREfm isolate O_02, the first *optrA*-positive LVREfm outbreak isolate, recovered from the suspected index case (patient A). A stored VREfm *optrA*-negative isolate (O_01) from patient A recovered a year previously also clustered in C1 (Table 5.1, Figure 5.2). Isolates O_01 and O_02 exhibited only three allelic differences. Two samples (patient H and the W1 drug trolley) each yielded pairs of *optrA*-postive LVREfm and *optrA*-negative VREfm isolates all of which clustered in C1; O_17a (VREfm) and isolate O_17b (LVREfm) from the drug trolley exhibited one allelic difference, whereas isolate O_37a (VREfm) and isolate O_37b (LVREfm) from patient H were indistinguishable.

Clusters C2-C4 consisted of *optrA*-negative ST80 VREfm and were deemed unrelated to C1 isolates with intra-cluster allelic differences of 57-388 (Figure 5.2).

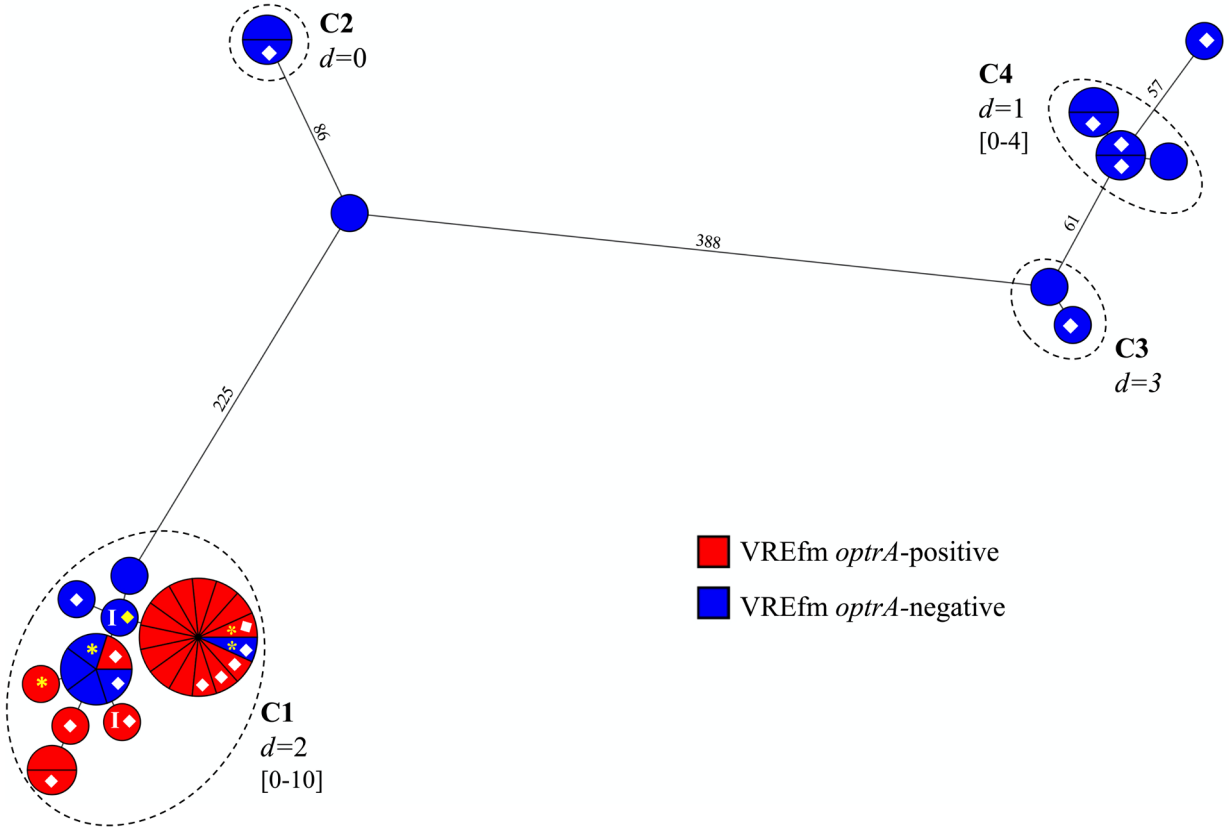


Figure 5.2 Minimum spanning tree based on core-genome multilocus sequence typing (cgMLST) data from the 39 ST80 vancomycin-resistant *E. faecium* (VREfm) isolates recovered from patient rectal screening swabs (19 isolates, 17 patients, denoted by a filled white diamond) and hospital environmental sites (20 isolates) during the hospital outbreak between the 8th of October and the 1st of November 2019. Twenty of the isolates were linezolid-resistant (LR) and harboured *optrA* as denoted by the colour legend. The first LVREfm outbreak isolate recovered from the suspected index patient in October 2019 is denoted by I. A stored linezolid-susceptible VREfm isolate lacking *optrA* recovered from the same patient a year earlier is denoted by a filled yellow diamond and an I. A yellow asterisk denotes pairs of isolates; one isolate of each pair was LVREfm (harbouring plasmid pEfmO_03) and the other VREfm (lacking plasmid pEfmO_03). Pairs of isolates included O_17a and O_17b recovered from a drug trolley, also O_37a and O_37b recovered from patient H. The numbers on the branches represent the number of cgMLST allelic differences. Clusters of related isolates are encircled and labelled C1 – C4; d= indicates average allelic differences and the range is shown in square brackets.

5.3.4.2 Outbreak isolates compared to other isolates from other Irish hospitals

A further comparison with a database of sequencing reads from 245 VREfm recovered in two other Dublin hospitals between September 2017-October 2019, revealed that all C1 outbreak isolates clustered within a larger cluster of ST80 isolates. The majority of database isolates (146/245, 59.9%) belonged to ST80, which divided into 11 clusters and 26 singletons, with an inter-cluster allelic differences range of 25-257 (Figure 5.3). The large cluster, termed ST80 complex type 2933, consisted of the 28 C1 outbreak isolates and five additional VREfm from another Dublin hospital (Hospital 2) recovered between March-October 2019. This cluster had an average allelic difference of three (range=0-15).

5.3.4 A plasmid encoding *optrA*

The WGS data of the LVREfm outbreak isolate O_03 (patient B) underwent hybrid assembly and a 56,684 bp plasmid (pEfmO_03) encoding *optrA* and the chloramphenicol resistance gene *fexA* was resolved. The *optrA* gene was flanked by *Tnp*A and *Tnp*B from *Tn*554, and by *IS*Efa15 (Figure 5.4). This differed from all the *optrA* types described in Chapter 4. A total of 19/20 *optrA*-positive outbreak LVREfm harboured plasmids exhibiting ≥99.98% sequence coverage to pEfmO_03. The remaining isolate exhibited 44.1% sequence coverage to pEfmO_03, with 100% coverage across the entire *optrA*-encoding region. Plasmid pEfmO_03 was conjugative; transconjugant derivatives of the *E. faecium* 64/3 recipient were obtained with four LVREfm isolates (O_03, O_04, O_13, O_23) and with the *E. faecalis* OG1RF recipient using the LVREfm isolate O_23 as donor (Table 5.2). All transconjugants harboured pEfmO_03 and were resistant to linezolid and chloramphenicol (MIC >4 and >32 mg/L, respectively), (Table 5.2).

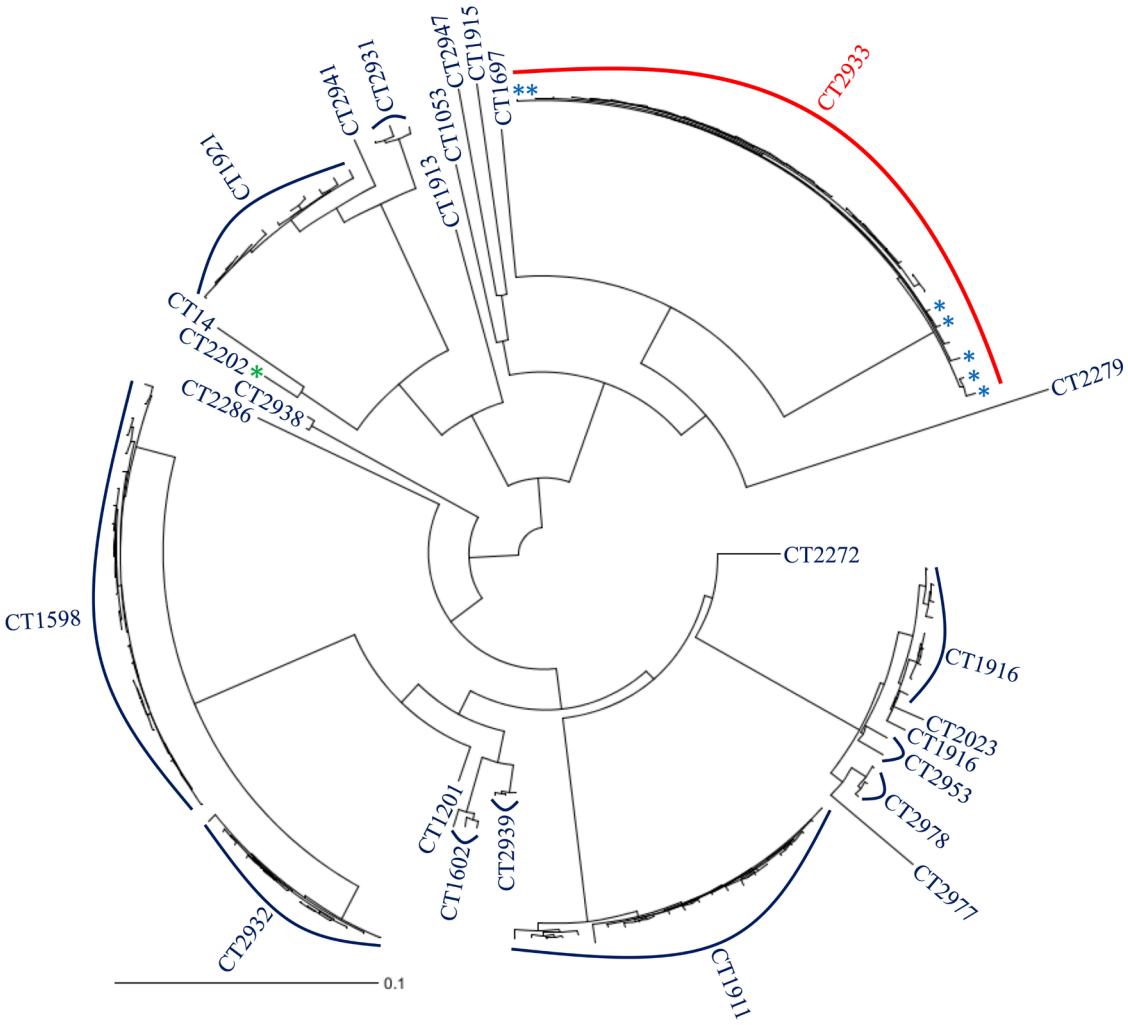


Figure 5.3 Neighbour-joining tree based on core genome multi-locus sequence typing (cgMLST) of 174 ST80 vancomycin-resistant *E. faecium* (VREfm) isolates, including the 28 outbreak cluster (C1) isolates from the present study (Hospital 1) and 146 isolates from two other Irish hospitals (Hospitals 2 and 3), recovered between September 2017 and October 2019. The 174 isolates divided into 11 clusters and 26 singletons, with an intercluster allelic differences range of 25–257. All of the isolates in the outbreak cluster (C1) from the present study grouped into complex type (CT) 2933 along with seven linezolid-susceptible VREfm from another Dublin hospital (Hospital 2). CT2933 is highlighted in red, with isolates from Hospital 2 indicated by a blue asterisk. Isolates within CT2933 had an average allelic difference of three (range 0–15). All other complex types are labelled and highlighted in blue. Outside of CT2933, the majority of isolates were from Hospital 2 (*N*=145), except for one isolate from Hospital 3 indicated by a green asterisk. Scale bar represents the phylogenetic distance between isolates based on cgMLST.

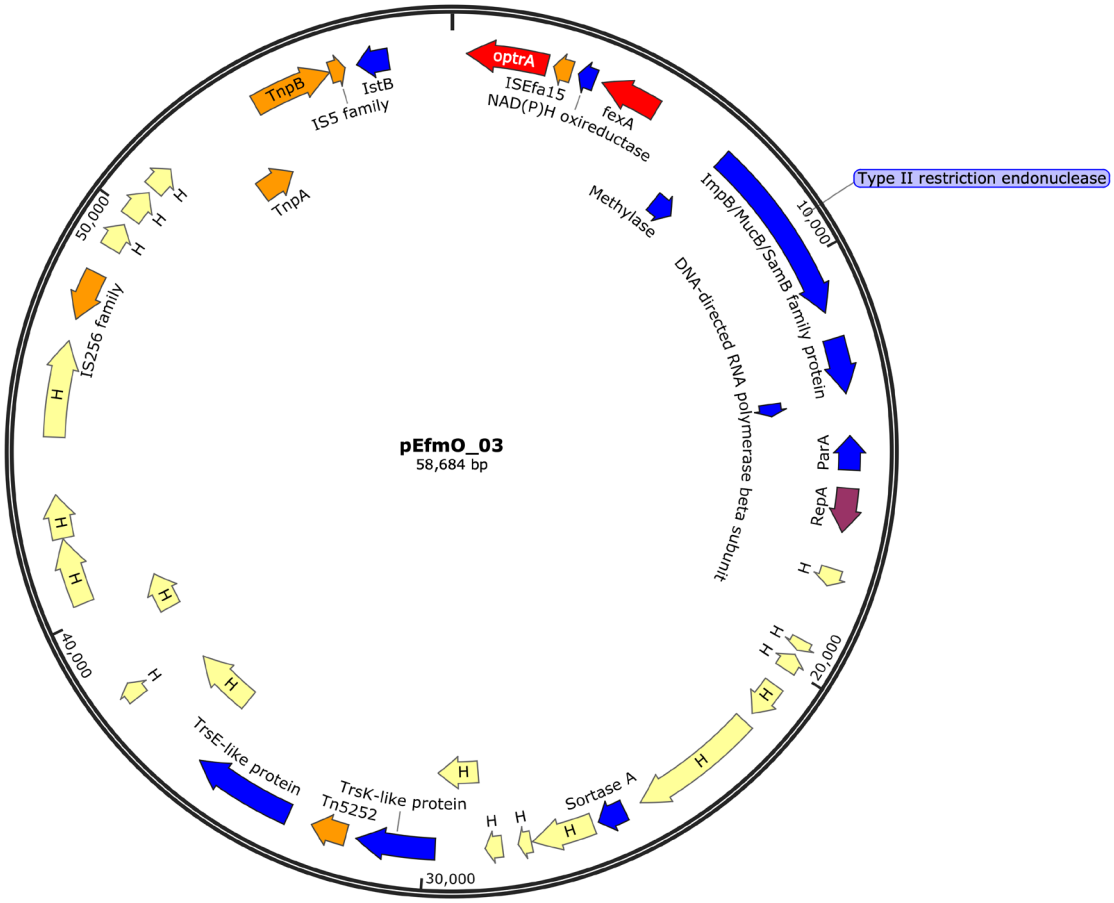


Figure 5.4 Schematic diagram of the structural organisation of plasmid pEfmO_03 from vancomycin-resistant *E. faecium* isolate O_03 encoding the *optrA* linezolid resistance gene resolved by hybrid assembly of paired-end Illumina MiSeq short reads with Oxford Nanopore Technologies long reads. Genes of interest and their orientation are represented by arrows as follows: red indicates antibiotic resistance genes, orange indicates insertion sequences/transposases, blue indicates known proteins and yellow indicates hypothetical proteins. The plasmid size is labelled indicating number of base pairs (bp). Abbreviations: H; hypothetical protein.

Table 5.2 MIC profiles of transconjugant derivatives of *E. faecium* 64/3 and *E. faecalis* OG1RF

Strain/isolate/transconjugant	Gene conjugated	Linezolid MIC (mg/L) (R ≥ 4 mg/L)[a]	Vancomycin MIC (mg/L) (R ≥ 4 mg/L)[a]	Chloramphenicol MIC (mg/L) (R ≥ 8 mg/L)[a]
Donor: VREfm isolate O_03	N/A	16	≥32	≥256
Recipient: *E. faecium* 64/3	N/A	4	≤1	8
Transconjugant: O_03:*Efm* 64/3 TC2	*optrA*	32	≤1	≥256
Donor: VREfm isolate O_04	N/A	16	≥32	≥256
Recipient: *E. faecium* 64/3	N/A	4	≤1	8
Transconjugant: O_04:*Efm* 64/3 TC2	*optrA*	16	≤1	≥256
Donor: VREfm isolate O_13	N/A	16	≥32	≥256
Recipient: *E. faecium* 64/3	N/A	4	≤1	8
Transconjugant: O_13:*Efm* 64/3 TC1	*optrA*	16	≤1	≥256
Donor: VREfm isolate O_23	N/A	8	≥32	≥256
Recipient: *E. faecium* 64/3	N/A	4	≤1	8
Transconjugant: O_23:*Efm* 64/3 TC2	*optrA*	32	1	≥256
Donor: VREfm isolate O_23	N/A	8	≥32	≥256
Recipient: *E. faecalis* OG1RF	N/A	≤2	2	≤4
Transconjugant: O_23:*Efs* OG1RF TC2	*optrA*	48	4	≥256

[a] Clinical breakpoints taken from the European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST, 2019).
Abbreviations: MIC, minimum inhibitory concentration; R, resistant; N/A, not applicable.

5.4 Discussion

Linezolid-resistant enterococci harbouring acquired resistance genes have been reported with increasing frequency year on year, since 2014 (Mendes *et al.*, 2016, 2018; Bender *et al.*, 2018b; Sassi *et al.*, 2019). The study detailed in Chapter 4, described the highest prevalence of *optrA* and *poxtA* among LRE reported to date, with *optrA* identified in vancomycin susceptible *E. faecalis* and *E. faecium* isolates with diverse genetic backgrounds. The *poxtA* gene was also identified in nine *E. faecium* isolates, including five LVREfm deemed unrelated by cgMLST, with isolates belonging to several STs (ST80, ST202, ST203 and ST1588). Previously, *optrA* was reported in four French VREfm recovered between 2013 and 2015, three of which were ST80 and one ST17 (Sassi *et al.*, 2019). The present study represents the first reported hospital outbreak involving *optrA*-positive VREfm, with all isolates belonging to ST80 (or a SLV of ST80) of the hospital-adapted clade A1. All 28 outbreak isolates formed a single cgMLST cluster (C1) and all were highly related (average allelic difference of 2; range = 0-10) (Figure 5.2). The majority of C1 isolates (20/28) were LVREfm, 19/20 of which harboured a 56,684 bp conjugative plasmid (pEfmO_03) encoding *optrA* and *fexA* (Figure 5.4). The remaining eight C1 isolates were VREfm and lacked pEfmO_03 but were otherwise indistinguishable or very closely related to the LVREfm.

An allelic difference of ≤20 has been previously proposed as the threshold for determining *E. faecium* isolates as closely related based on cgMLST (de Been *et al.*, 2015). Interrogation of a WGS database of 245 VREfm isolates from two other Irish hospitals revealed that the majority of isolates belonged to ST80 (*N*=146), which further divided into 11 clusters, with 27 different complex types. All of the isolates in the outbreak cluster (C1) grouped into complex type 2933, along with seven VREfm from another Dublin hospital (Figure 5.3). These findings demonstrate that VREfm clones can persist over long periods and in different hospital locations, which has been reported previously (Pinholt *et al.*, 2017, 2019). The average allelic difference between isolates within complex type 2933 was three (range=0-15), showing the closely related nature of the outbreak isolates to isolates from another Dublin hospital. The frequent transfer of patients between hospitals in Ireland (especially in Dublin) could contribute to trafficking of individual strains between hospitals.

The first *optrA*-positive LVREfm outbreak isolate (O_02) recovered from the suspected index patient in October 2019 exhibited only three allelic differences to an *optrA*-negative VREfm (O_01) from the same patient a year earlier. These findings

indicate that the index patient harboured the same VREfm strain for a year. When this strain acquired the *optrA*-encoding plasmid pEfmO_03 was not determined; the patient had no animal/farm exposure and no source of *optrA* was identified in the hospital. The highly related nature of all isolates in cluster C1, together with the finding of an identical *optrA*-encoding conjugative plasmid in all but one LVREfm outbreak isolates indicates the spread of a single strain over the four-week outbreak period. The remaining LVREfm outbreak isolate (O_24) exhibited 44.1% sequence identity to pEfmO_03, with 100% coverage around the entire region surrounding *optrA* and *fexA*, suggesting the loss of some plasmid sequence. The findings of the present study contrast with the previous study described in Chapter 4 of LRE from Irish hospitals, which revealed the presence of the mobile linezolid resistance genes *optrA* and *poxtA* in enterococci with diverse genetic backgrounds. During the present study, two samples (patients H and the drug trolley on W1) yielded isolate pairs, each consisting of an *optrA*-postive LVREfm and *optrA*-negative VREfm isolate. One pair of ST80 isolates (O_37a and O_37b), from patient H, exhibited zero allelic differences and the other pair of ST80 isolates (O_17a and O_17b) exhibited one allelic difference. The *optrA*-positive isolate of each pair harboured pEfmO_03. These findings indicated the loss/gain of the pEfmO_03 plasmid in individual samples.

The suspected index patient, patient A, had previously been treated with linezolid four weeks prior to the recovery of the first LVREfm outbreak isolate from this patient in October 2019. No other patient involved in the outbreak had a history of linezolid treatment. Based on this, the close similarity of all the LVREfm outbreak isolates and the presence of an identical *optrA*-encoding plasmid (pEfmO_03) in 95% (19/20) of LVREfm, strongly suggests that the outbreak was due to the recent transmission of the LVREfm from patient A, either by indirect contact with other patients via the hands of healthcare workers (HCWs) and/or by shedding of the LVREfm into the hospital environment. Interestingly, pEfmO_03 was unique to this outbreak and showed minimal sequence identity (7.8%-18.2%) to the *optrA* genetic environments, both chromosomal and plasmid, described previously in LRE isolates detailed in Chapter 4, Section 4.3.3.

LVREfm environmental isolates in C1 were identified up to 20 days following the initial isolate recovery from patient A, even following enhanced environmental decontamination and increased awareness of the importance of hand hygiene among HCWs. A review of hand hygiene audit records revealed the hospital was compliant with national standards on hand hygiene and achieved >95% compliance. Nonetheless,

extensive environmental screening also revealed that sites such as treatment and supply rooms harboured LVREfm. These findings highlight the critical importance of hand hygiene in hospitals and highlight a significant need for ongoing improvements. The appointment of local hand hygiene champions may be beneficial in this regard. The implementation of enhanced IPC measures (improved cleaning of the environment, the use of HPV decontamination, the scheduling and recording of equipment cleaning, ceasing ward admissions and staff dedicated to W1) was successful in the rapid termination of the outbreak, which was deemed over four weeks after the last LVREfm patient isolate was recovered.

It is likely that linezolid usage was a contributory factor in the emergence of the LVREfm in the outbreak hospital as from Q4 2016, linezolid consumption increased from 0.46 DDD/100 BDU to 1.14 DDD/100 BDU by Q4 2019. This increased linezolid usage reflected increased complexity of patients and colonisation with MDRO's. All prescriptions were deemed appropriate and compliant with the restricted antimicrobials policy of the hospital. This highlights the challenging requirement for more prudent antimicrobial treatment of medically complex patients harbouring MDRO's. Plasmid encoded *optrA* has been reported previously in animal staphylococcal isolates (Fan *et al.*, 2016; Guo *et al.*, 2018). It is worrying to consider the possibility that the *optrA*-encoding plasmid identified in the LVREfm isolates in the present study may eventually transfer into staphylococci (e.g. MRSA), or indeed other enterococci, in the hospital environment, further limiting options for treating infections caused by these organisms.

In conclusion, WGS and epidemiological data analysis was central in the rapid identification and characterisation of a clonal ST80 outbreak of LVREfm harbouring a 56,684 bp conjugative plasmid (pEfmO_03) encoding *optrA.* The team approach adopted in the management of this outbreak directed the rapid implementation of enhanced IPC measures including the early detection and aggressive environmental decontamination, which resulted in the timely containment and termination of the outbreak.

Chapter 6

General Discussion

6.1 Population structure of hospital-adapted enterococci with a focus on *Enterococcus faecium* in a large acute Irish hospital

This study provides the first detailed insight into the population structure of hospital-adapted *E. faecium* in a large acute Irish hospital using the high-resolution typing methods provided by WGS. From the results presented in Chapter 3, it is evident that *E. faecium* isolates in this Irish hospital are highly polyclonal following cgMLST analysis, both by the variety of conventional STs identified and by the diversity of isolates belonging to the same ST. The resulting polyclonal nature of hospital-adapted *E. faecium* was unsurprising, as this has previously been demonstrated in other studies across Europe where WGS has been used (Raven *et al.*, 2016; Pinholt *et al.*, 2017; Gouliouris *et al.*, 2018; Werner *et al.*, 2020). Nonetheless, there are still only limited WGS-based studies on hospital-adapted *E. faecium*, but these are increasing as the costs associated with high-throughput WGS reduce. The polyclonal nature of *E. faecium* provides a challenge for typing and tracking clones across Europe and in 2018 the ECDC EARS-Net report stated "*Corresponding increasing trends highlight the need for close monitoring to better understand the epidemiology, clonal diversity and risk factors associated with infection. Contrary to many other species under surveillance, no distinct geographical pattern could be seen for vancomycin-resistant E. faecium, as high percentages were reported from both southern, eastern and northern Europe*" (ECDC, 2019b). This further highlights the challenges in understanding the dissemination of *E. faecium* compared to how other significant hospital pathogens such as MRSA spread.

The findings presented in Chapter 3 clearly demonstrated that the population structure of VREfm was polyclonal in a single large Dublin acute hospital. In Chapter 4, the population structure of linezolid-resistant *E. faecium* from multiple Irish hospitals was also shown to be polyclonal, but here the presence of highly related strains in different hospitals in different geographical locations was evident. This suggests trafficking of strains between hospitals occurs, either by staff/patient transfer or local sources of enterococci in the community. It was also evident from the study of LRE that in Ireland *E. faecium* are much more diverse relative to the *E. faecalis* isolates studied. In the linezolid-resistant *E. faecalis* isolates investigated, the majority 52% (13/25; 10 *poxtA*-positive and 3 *optrA*-positive) of isolates were found to be highly related ST480 clones. The ST480 clone has been previously reported as the predominant ST of *optrA*-positive clinical *E. faecalis* clones in France and Germany (Bender *et al.*, 2018b; Sassi *et al.*, 2019). A very recent study

suggests that hospital-adapted *E. faecalis* strains existed in the pre-antibiotic era (isolates recovered between dates ranging from 1936 up to 2018). The population is cohesively connected through homologous recombination, metabolic flexibility and a stable large core genome, suggesting adaptations seen in these "hospital-adapted" strains likely occurred in an alternative niche(s) (Pöntinen *et al.*, 2021).

Findings presented in Chapter 3 revealed that there was an absence of a clear divide between vancomycin-resistant and vancomycin-susceptible *E. faecium* clones. Many VSEfm isolates were indistinguishable from or closely related to VREfm isolates, which suggests that the former can readily acquire *vanA*. For example, in the ST80 CT1598 cluster identified in Chapter 3, there were zero allelic differences between the VSEfm isolate BSI_SJ72 and the VREfm screening isolates SJ1, SJ6, SJ14 and the VREfm BSI isolate BSI_SJ37 (Figure 3.3). This indicates that the VSEfm isolates in this study could readily acquire *vanA,* as they are genetically indistinguishable from the VREfm. This ability to acquire *vanA* is advantageous for the survival of enterococci in the hospital environment, as it is well known that enterococci can harbour pheromone-responsive plasmids, which can be swiftly disseminated among enterococcal species via HGT (Palmer *et al.*, 2010). In contrast in Chapter 4, a highly similar *poxtA*-encoding conjugative plasmid (pM16/0594, 21,849 bp) was identified in multiple genetic backgrounds (both *E. faecium* and *E. faecalis*), showing how efficiently enterococci can spread AMR genes across species. Additionally, it has been well described that enterococci may harbour AMR genes encoded on broad host range conjugative plasmids that can be disseminated to and from other bacterial species via HGT. This ability could facilitate enterococci to act as a vehicle for disseminating AMR, most worryingly to other Gram-positive bacteria, such as staphylococci (Palmer *et al.*, 2010).

6.1.1 Predominance of ST80 VREfm in Ireland, a cause for concern?

Interestingly, the isolates investigated in Chapter 3 demonstrated a clear predominance of ST80 *vanA* encoding VREfm in a large Dublin acute hospital. Furthermore, the outbreak of LVREfm described in Chapter 5, was also due to an ST80 VREfm clone that acquired an *optrA*-encoding linezolid resistance plasmid. In Chapter 5, there was convincing evidence that the ST80 VREfm strain from the index patient was persistent in the GI tract for at least one year prior to the acquisition of the *optrA*-encoding plasmid. This suggests ST80 are efficient at both colonisation and persisting for extended periods of time in the human GI

tract. Denmark has also reported the predominant prevalence of *vanA* encoding ST80 VREfm. Studies of isolates mainly from the Copenhagen area of Denmark (11 hospitals) revealed a substantial increase in VREfm between 2011 and 2015. Only nine patients were infected or colonized with VREfm in 2011, rising to more than 1500 VREfm patients identified between 2012 and 2015. The increase in VREfm was traced back to the introduction of a single clone of a *vanA*-encoding ST80 VREfm and its subsequent expansion and spread (Pinholt *et al.*, 2015, Pinholt *et al.*, 2017, Pinholt *et al.*, 2019). Denmark had the advantage of being able to apply WGS-based typing to all of their VREfm isolates, from the beginning of the increase. The findings from Denmark, along with the predominance and evidence of prolonged colonisation by ST80 in the present study suggest that *E. faecium* isolates within the ST80 lineage are more efficient at surviving and spreading. Possible explanations for the success of ST80 clones may involve the evolution of strain-specific traits, such as those enabling more efficient and persistent colonization of the GI tract of humans and animals. Additionally, ST80 clones could have additional adaptations which strengthen their survival and persistence in the environment, which is evident from Chapter 3, where highly related ST80 isolates were identified over a 21-month period, suggesting a persistent environmental reservoir. Gut-commensal *E. faecium* strains have also been shown to be an important reservoir for traits that can be transferred successfully to clinical *E. faecium* strains (de Been *et al.*, 2013). Therefore, another scenario worth considering is that hospital-adapted ST80 clones are more efficient at acquiring necessary traits for survival from other gut-commensals. Finally, ST80 clones may also be more efficient at acquiring MGE via HGT, as evident in Chapter 5 by the acquisition of *optrA* by an ST80 VREfm.

In Germany, a persistent large outbreak of ST80 *vanB* VREfm affecting 2,900 patients in two hospitals in southwest Germany between 2015-2017 was reported (Werner *et al.*, 2020). These findings may be an additional indicator of the success of ST80 and its ability to spread and cause outbreaks. Sweden has also reported outbreaks of VREfm primarily due to ST80 *vanB*-encoding VREfm (Fang *et al.*, 2021). A predominant ST80 population in Ireland was indicated from the present study by its predominance in a large Dublin hospital (Chapter 3). This was supported by findings from the outbreak of LVREfm mediated by rapid clonal expansion of an ST80 clone in a separate hospital (Chapter 5). Along with initial further studies in other regional hospitals in Ireland from Q4 2019 (October-December), which also indicates the majority of VREfm isolates investigated

(58/103, 56.3%) also belong to ST80 (Kavanagh N. 2021, personal communication, 28 January 2021). Based on this evidence, the predominance of ST80 in Irish hospitals is a significant concern, as studies indicate that ST80 is very successful at spreading and also causing outbreaks. Whether ST80 has predominated in Irish hospitals prior to this study period also requires investigation, along with further work to examine the fitness and success of ST80 over other STs present in Ireland.

6.2 Rethinking VREfm and LRE infection control and prevention

The ECDC reported that each year, HCAI/AMR is responsible for about 33,000 deaths and adds about €1.1 billion in associated costs to the health care systems of EU/European Economic Area (EEA) countries (ECDC, 2019a). In Ireland, a 2013 National Clinical Effectiveness Committee report estimated the burden on HCAI to the Health Service Executive (HSE), with over 25,000 patients affected annually at a cost of around €118 million (National Clinical Effectiveness Committee, 2013). It is clear that effective IPC and decreasing levels of HCAIs is both good for patients and their families, but also good for the overall economy of the country.

Whether current Irish national guidelines are still appropriate was a major question at the outset of this project. The most current national IPC guidelines on VRE infections from the Royal College of Physicians of Ireland were published in 2014. These guidelines recommend active surveillance screening for VRE in the following patient groups: patients admitted to high-risk areas (ICU, haematology/oncology, transplantation) with weekly screening thereafter and patients known to be previously VRE-positive upon re-admission to hospital. Additionally, patients transferred from other Irish hospitals or patients transferred from any hospital abroad, and where appropriate, ‘at risk’ patients who have been contacts of known VRE positive patients during an outbreak (Royal College of Physicians of Ireland, 2014). It is also recommended that ideally every patient who is colonised or infected with VRE should be isolated in a single room with en-suite facilities and that Contact Precautions should be applied. Isolation is not recommended for known VRE-positive patients in long-term care facilities, nor is active surveillance advised (Royal College of Physicians of Ireland, 2014). The ability to implement best practice IPC in relation to VRE colonisation or infection in hospitals is a major challenge in Ireland, because of the sub-optimal infrastructure present in many hospitals. In 2009, the advisory group Strategy for the control of Antimicrobial Resistance in Ireland (SARI),

recommended that newly built hospital in-patient accommodation should comprise 100% single-patient rooms and that existing multiple bedded rooms contain no more than three beds (SARI, 2009). However, little progress has been made in implementing these recommendations since then. In a 2011-2012 European hospital study, the country median proportion of single-bed rooms was 24.2% across Europe, with Irish hospitals falling significantly below this at between 10-20% (ECDC, 2013). Of 60 Irish acute hospitals surveyed in May 2017, the average proportion of single patient rooms in public hospitals ranged from 15% to 29%, with 52% in private hospitals. (Health Protection Surveillance Centre, 2017). It is clear that hospital infrastructure has played a significant role in Ireland's inability to effectively manage VRE patients due to a widespread shortage of availability of single-bed rooms.

In addition to sub-optimal infrastructure, there is evidence that current hospital cleaning regimes are inadequate for the effective eradication of VRE and/or LRE. In Chapter 3, findings showed that highly related ST80 VREfm clones were present over 21-months in the large hospital concerned, indicating a possible environmental source for persistence of these clones, which is being missed during current cleaning/decontamination regimes. In the UK, a study undertaken in two haematology wards over a six-month period provided evidence of the role of the environment in VREfm persistence. It reported that 48% (447/992) of environmental swab samples were positive for VREfm, with a positive proportion ranging from 36% for medical devices to 76% for non-touch areas (air vents and high-efficiency particulate air (HEPA) filters) (Gouliouris *et al.*, 2021). The ability of VREfm to persist in the environment following routine cleaning after patient discharge was a particular concern, with 20% (9/41) of bedroom/bathroom areas remaining contaminated. Following deep cleaning of bedroom/bathroom areas, 9% (4/43) of sites sampled remained contaminated with *E. faecium*. The four isolates recovered after cleaning were genetically related to isolates collected just before deep cleaning, demonstrating that bacteria in these four sites persisted through the decontamination process. Additionally, any benefit of deep cleaning was shown to be short-lived, as around half of sampled sites were *E. faecium*-positive within three days of patients return to ward areas (Gouliouris *et al.*, 2021). In Chapter 5, staff hands were suggested as the main source of contamination of environmental sites with LVREfm, as isolates were identified in "clean areas", such as the treatment room. Highlighting the crucial importance of compliance with hand hygiene among all staff, to both prevent environmental

contamination and transfer of organisms from patient to patient. The role of the environment is clearly a factor in VRE/LRE spread and actions to limit spread via the environment may be to increase cleaning frequency. For example, in Chapter 5 cleaning of toilet areas was changed from twice a day to four times a day and this along with other interventions contributed to halting the outbreak. Cleaning regimes should also be applied to "non-touch" areas such as air vents, along with "high-touch non-patient areas" such as staff canteens, nurses' station, store rooms and treatment preparation rooms and cleaning logbooks should be maintained and audited to monitor compliance. Monitoring the effectiveness of enhanced cleaning regimes should also be implemented by use of increased environmental screening. As our infrastructure issues cannot be overcome easily, additional resources need to be provided to improve the effectiveness of cleaning, decontamination and increasing staffing levels, so compliance with enhanced measures can be followed.

The prevalence of VRE carriage among patients in a hospital in Cork was estimated to be 19.1%, following examination of all faecal samples submitted to the clinical microbiology laboratory and was even higher (31.4%) when just inpatient specimens were investigated (Whelton *et al.*, 2016). During the course of the present study, it was estimated that the overall prevalence of VRE colonisation among patients was between 10.6-16.4% in the primary large acute Dublin hospital studied in Chapter 3 (Brennan G. 2021, personal communication, 1 March 2021). Currently routine screening for LRE is not undertaken in Ireland, so there's no data on carriage rates. However, in Chapter 5 it was evident that LVREfm carriage was prevalent among patients in the outbreak hospital and spread during the course of the outbreak, despite the fact all patients were housed in single en-suite rooms. In 2018, the Commission for Hospital Hygiene in Germany recommended screening for LRE in cases of possible transmission events. For example, when more than one case is notified within 3 months in a healthcare facility and an epidemiological link between isolates is suspected (Werner *et al.*, 2019). Subsequently, the German National Reference Centre for Staphylococci and Enterococci developed and validated the use of an appropriate selective and differential screening agar for rapid isolation of LRE from rectal screening swabs (Werner *et al.*, 2019).

Based on the previous, albeit limited, prevalence estimates of VRE carriage in Irish hospitals referred to above, current data indicate that between 1 in 5 and 1 in 10 patients

are colonised with VRE. Under current national screening guidelines, most patients would not be identified as VRE carriers until they enter a "high-risk" area. Thus, unidentified VRE carriers can act as "silent" reservoirs that can lead to the further colonisation of other patients. The lack of screening for LRE in Irish hospitals is also a cause for concern, as the numbers of LRE in Ireland are increasing. It is evident that LRE can disseminate mobile linezolid resistance genes to VRE (i.e. the acquisition of *optrA* by a VREfm in Chapter 5) giving rising to LVREfm, for which very limited treatment options are currently available. It is crucial now to begin routine surveillance for LRE carriage before the problem becomes endemic like VRE already has. Regarding VRE screening in Ireland, it is time to rethink the guidelines and carry out pre-admission screening on all patients to identify these hidden reservoirs at an early stage and ensure best practice contact precautions are applied in all cases to prevent spread to other patients. Improvement of environmental decontamination along with increasing hand hygiene compliance will aid in limiting spread through environmental sources. It was also evident in Chapter 5 that knowledge of reservoirs of MDR organisms along with enhanced deep cleaning can control further spread of these organisms.

6.2.1 Potential impacts of the coronavirus (COVID-19) pandemic on VRE rates

There are indications that the coronavirus (COVID-19) pandemic, mediated by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), will have negative effects on the rates of VRE, both in increased BSIs and increased intestinal colonisation, as hospitals have been forced to divert substantial resources to cope with COVID-19. A study in an ICU in Rome between 1st March and 15th April 2020 indicated VRE colonisation rates increased to 30% (17/57), from a previous prevalence rate of 15% (11/75) recorded during the first six months of 2019 (Cataldo *et al.*, 2020). The study also reported that overall BSIs rates were 3.8-times higher in 2020 than in 2019. Key drivers of an increased incidence of BSIs in COVID-19 patients in this ICU were suggested to be due to several factors including immune dysregulation in severe COVID-19, the extensive use of antimicrobials and reduced adherence to effective IPC measures (Cataldo *et al.*, 2020). In Germany, an outbreak of ST117 *vanB*-encoding VRE was also reported on a COVID-19 ICU, where contaminated surfaces were found to have played a key role in the outbreak, highlighting the importance of hand hygiene and effective surface decontamination. At the time of the outbreak, minimum staffing requirements in German hospitals had been suspended in order to cope with the ongoing COVID-19 pandemic. It is well known that

reduced staffing levels in hospitals is commonly associated with poor adherence to IPC measures (Kampmeier *et al.*, 2020). Diversion of resources in hospitals due to COVID-19 may have a detrimental effect on the rates of VREfm BSIs and increasing colonisation rates in Ireland, which is very concerning due to the fact that VREfm invasive disease in Irish hospitals was one of the worst in Europe prior to the onset of the pandemic.

6.3 Antimicrobial resistance, a OneHealth perspective

A review initiated by the UK government on AMR illustrated the considerable human and economic burden it will have to bear if action is not taken. Initial research shows that with a continued rise in antimicrobial resistance, by 2050 AMR would result in the deaths of 10 million people worldwide every year, which would lead to AMR overtaking cancer as the main cause of death worldwide (O'Neil, 2014). Economically it may lead to a reduction of 2-3.5% in Gross Domestic Product (GDP), costing the nations of the world up to $100 trillion (O'Neil, 2014). If no effective action is undertaken, AMR to second-line antibiotics (e.g. third generation cephalosporins, macrolides and fluoroquinolones) will be 72% higher in 2030 compared to 2005 in the EU/EEA (ECDC, 2019a). In the same period, AMR to last-line antimicrobial treatment agents, such as vancomycin and linezolid will more than double (ECDC, 2019a).

Selective pressure plays a major role in increasing rates of both VRE and LRE in Ireland. Examination of European antimicrobial consumption data reveal that Ireland is the 5th highest consumer of glycopeptides. Ireland's usage is ~76% higher than Denmark, 0.080 versus 0.025 DDD per 1000 inhabitants per day, respectively (ECDC, 2021). Overall Ireland has the 7th highest consumption rate of antibacterials for systemic use in Europe (ECDC, 2021). To date, the figures on consumption of oxazolidinones across Europe are not recorded separately by the ECDC. In Chapter 5, there was anecdotal evidence of increasing trend in consumption of these last-line drugs, with linezolid consumption increasing from 0.46 DDD/100 BDU to 1.14 DDD/100 BDU by Q4 2019. It is clear urgent action is needed to reduce our overall consumption of antimicrobials, especially our consumption of glycopeptides and oxazolidinones to aid in the reduction of our numbers of VRE and LRE in Ireland.

An OneHealth approach is also crucial in the defence against AMR, as there is a complex and connected network for the spread of MDR bacteria but also AMR genes (Figure 6.1). For example, the MDR genes *optrA* and *poxtA* have been widely reported in livestock and food producing animals, mainly in Asia, but also in Europe (Cai *et al.*, 2015; Tamang *et al.*, 2017; Brenciani *et al.*, 2019; Huang *et al.*, 2019; Lei *et al.*, 2019; Elghaieb *et al.*, 2020; Freitas *et al.*, 2020). Limited studies have examined the prevalence of *optrA* and *poxtA* in human isolates, so the true extent of humans as a reservoir of these genes is not fully established. Oxazolidinones are not used in food-producing animals. Nevertheless, the increased detection in animals, primarily boiler chickens and pigs, of LRE carrying the *optrA* gene encoded on transferable plasmids is concerning. As the *optrA* and *poxtA* genes are often co-located with resistance genes for antibiotics commonly used in animals (e.g. phenicols and tetracyclines), this highlights the role of agriculture in the indirect selection of MDR enterococci, which poses a risk for public health (Torres *et al.*, 2018). Exposing animals to low doses of antimicrobials through feed and drinking water supplies may in certain conditions increase their productivity, for example by decreasing morbidity and mortality caused by clinical infections and additionally having a growth promotion effect (Nilsson, 2012). Consequently, this exposure to antimicrobials in animals generates a selective pressure encouraging an increased prevalence of the MDR genes *optrA* and *poxtA*. According to the European Medicines Agency, the most commonly purchased antimicrobial used in animals in Ireland is tetracycline, accounting for 39.7% (39.2 tonnes), with amphenicols accounting for up to 3.3% (3.3 tonnes), of total veterinary antimicrobials (European Medicines Agency, 2020). Coinciding with this, Chapter 4 reported the highest prevalence of *poxtA* (encoding oxazolidinones, phenicol and tetracycline resistance) among clinical enterococci reported to date. Indirect selection for linezolid resistance through animal husbandry and agriculture is likely to play a role in its exponential increase in hospitals, along with a rise in the overall consumption of antimicrobials in healthcare. The spread of *optrA* and/or *poxtA* from animal strains is a very likely source of linezolid-resistance in human enterococcal strains and potentially we have seen the spread of these AMR encoding genes via MGEs into the clinical environment. This further highlights the crucial need for a OneHealth approach to AMR.

During the early 1990s, VREfm encoding *vanA* colonisation in livestock was common. A strong link was made with the use of the glycopeptide avoparcin (first introduced in 1975) as a growth promoter in European agriculture. The chemical structure of avoparcin was

closely related to that of vancomycin and subsequently was found to be selective for the vancomycin-resistant gene *vanA* (Nilsson, 2012). Once the use of avoparcin was banned in the EU in 1997, VRE detected in livestock and in food of animal origin decreased and the prevalence of human colonization by VRE declined massively, confirming avoparcin use conferred significant selective pressure for VRE (Nilsson, 2012). Avoparcin's role in increasing the VRE rate in Europe was further confirmed by comparison to the USA, where avoparcin was never approved for use in animals and thus VRE was not detected in livestock until 2008 (Nilsson, 2012). VRE are often found in wastewater, are persistent in the healthcare environment and have also been found in drinking water in rural Ireland (Morris *et al.*, 2012; Gouliouris *et al.*, 2018, Gouliouris *et al.*, 2021). It is evident that the level of MDR enterococci is increasing, in particular in Ireland and this interplay between human healthcare, agriculture and environmental contamination must be considered holistically in tackling the rising problem of AMR in Ireland (Figure 6.1). To combat the rise of MDR enterococci, a complete ban on the use of antimicrobials in feed and water of food-producing animals worldwide should be considered, prudent antimicrobial stewardship in veterinary medicine and improved farming conditions (e.g. phasing out of factory farming, particularly for chickens). Further education and promotion of antimicrobial stewardship in healthcare may also be beneficial and where possible avoiding the prophylactic use of broad-spectrum antimicrobials prior to diagnosis of bacterial infections.

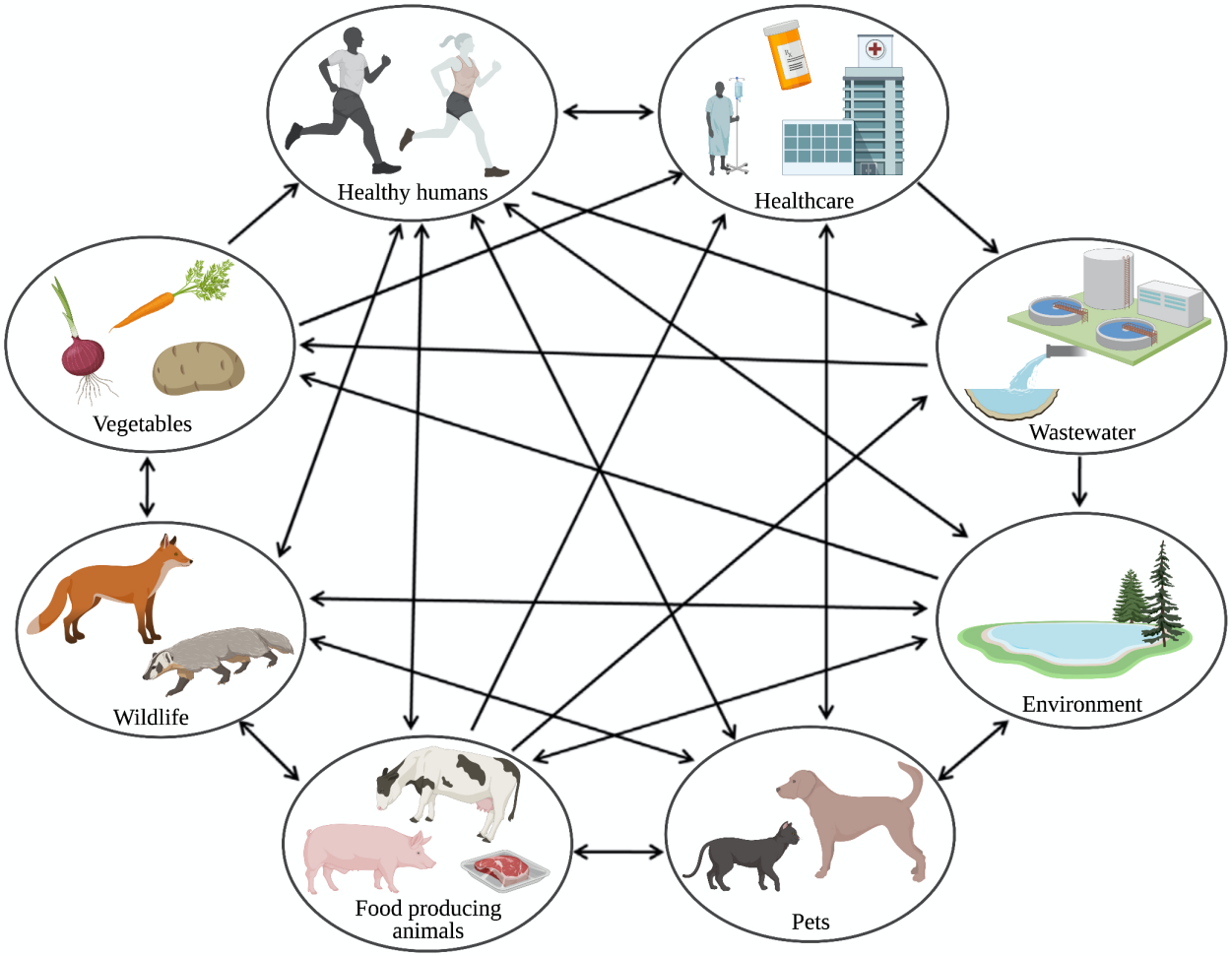


Figure 6.1 Schematic highlighting the various routes by which bacteria and/or antimicrobial resistance genes can spread between animals and humans. Adapted from Nilsson, 2012.

6.4 The potential for pan-resistant *E. faecium* in the near-future

There are increasing reports of linezolid resistance (one of the last-line drugs used to treat VREfm) in both *E. faecium* and VREfm across Europe (Brenciani *et al.*, 2016; Lazaris *et al.*, 2017; Argudín *et al.*, 2019; Sassi *et al.*, 2019). Ireland has also reported the highest levels (22.7%) in Europe to date of the acquired linezolid resistance genes *optrA* and *poxtA* in clinical enterococci (Chapter 4). Ireland was also the first country to report an outbreak of LVREfm that was caused by the acquisition of an *optrA*-encoding plasmid by a VREfm followed by its rapid clonal expansion and dissemination (Chapter 5). One of the main characteristics of enterococci as successful nosocomial pathogens relates to their ability to acquire and spread AMR genes. It has already been reported that VRE have developed resistance to the three remaining "last-line" antibiotics, linezolid, tigecycline and daptomycin (Kamboj *et al.*, 2011; Lazaris *et al.*, 2017; Bender *et al.*, 2018a, 2020). Without effective interventions enterococci will continue to spread uncontrolled and an increasing percentage of these organisms will become resistant to all available drugs. Enterococci are often overlooked as they lack the overt virulence traits present in other pathogens like *S. aureus*, but their ability to disseminate AMR is the biggest concern, most worryingly is the potential transmission of vancomycin or linezolid resistance to *S. aureus* and/or MRSA. It is clear that treatment options for MDR isolates are also becoming very limited and the risk of pan-resistant *E. faecium* is becoming more likely. Immediate action is needed in Ireland to prevent this, as we are already ahead of other countries with our high levels of invasive VREfm and are also reporting the highest levels of LRE.

6.5 The future of WGS in clinical microbiology

Whole-genome sequencing has the potential to revolutionise the clinical microbiology laboratory, by improvement of turn-around-times (TATs), allowing a greater understanding of microbial populations in the hospital environment, along with real-time tracking of outbreaks, providing the informative data required to facilitate effective IPC decisions. The ECDC is supporting and promoting the implementation of WGS in both public reference laboratories, but also in routine clinical laboratories. The ECDC published an expert opinion on WGS for public health surveillance, which outlines a number of pre-requisites necessary before we can have harmonisation of WGS across European hospitals, mainly by making data comparable and of a standardised quality (ECDC, 2016). The first major pre-requisite for inter-laboratory comparability is the establishment of quality assurance parameters. All sequencing data produced by public health laboratories must

reach a minimum standard (ECDC, 2016). Secondly, a WGS analysis strategy must be agreed upon, which will likely involve two steps. The first step will involve standardized genotypic nomenclature (e.g. cgMLST) that is easily comparable across countries. The second step will permit detailed phylogenetic reconstruction within genotypes (e.g. SNV analysis) (ECDC, 2016). Empirical data interpretation criteria must be established, along with the development of international data storage, exchange platforms and a high-security system whereby epidemiological and clinical data can be linked and shared (ECDC, 2016). Finally, significant investments must be made to facilitate access to WGS instruments, validated bioinformatic tools, laboratory infrastructure and ongoing operational funding (ECDC, 2016). Work is ongoing in creating an environment for routine WGS in clinical microbiology, but the harmonisation of typing across Europe will be particularly advantageous in understanding the population of hospital-adapted enterococci. The COVID-19 pandemic has shown the necessity of WGS in the typing and tracing of SARS-Co-V2 variants worldwide and has accelerated the use of WGS in the clinical setting along with global data sharing.

Efforts to date have largely focused on the establishment of routine WGS in surveillance or reference laboratories. The next step will be to introduce this technology into routine hospital laboratories for real-time patient care. The workflow and priorities relating to the implementation of WGS in clinical laboratories differ slightly from those in reference facilities. Mainly the TATs will be of greater importance, as the data generated will be used to inform both treatment and IPC decisions. It is clear the role and need for WGS in medical microbiology laboratories will increase during the next few years, not only for research, but also, and more importantly, for molecular diagnostics, infection prevention and control, the investigation of outbreaks, the characterization and surveillance of pathogens, the detection of novel resistance genes and for the application of a metagenomics approach on clinical samples (Deurenberg *et al.*, 2017). For example, currently in clinical laboratories a major issue is the TAT for blood culture-based diagnostics can take up to 5 days. With the time from blood-culture specimen receipt to identification of the causative organism requiring on average 1–2 days, with an additional 2–3 days after organism identification required to determine phenotypic antibiotic susceptibility pattern (Taxt *et al.*, 2020). During this time patients are treated with broad-spectrum antimicrobials, further adding unnecessary selective pressure for AMR in hospitals. The ideal solution is an approach to rapidly identify the organism causing the

infection along with its predicted AMR profile, in order to ensure patients, receive appropriate, targeted therapy tailored for their individual infection. Research using the Oxford Nanopore Technologies sequencing platform to perform direct WGS on a blood sample from a query septic patient has shown results can be obtained within 5-6 hours. After sample preparation and initialisation of the sequencing run the organism identification can be available within 10 minutes and an AMR profile within one hour (Grumaz *et al.*, 2020; Taxt *et al.*, 2020). This massive reduction in TAT is very promising, as it can lead to better patient care, reduced selective pressure for AMR due to reduced use of broad-spectrum antimicrobials and has the potential to be cost saving. Furthermore, along with faster TATs, hospitals would obtain sequencing data for organisms causing BSIs and can use this downstream to monitor the population of BSI causing organisms in their healthcare environment. Additional costs would initially be required for equipment and staff training, but once implemented may be cost saving in the long term due to the potential to reduce patient morbidity, mortality and bed days used.

6.6 Future work

Understanding the population dynamics and reservoirs of enterococci is of vital importance in their effective control. It is clear that enterococci are unlike other organisms under surveillance by the ECDC, such as *S. aureus*/MRSA which spreads clonally and its movement across the continent can be more readily monitored. For enterococci, much more data is required to fully understand their polyclonal population structure and the dynamics of their spread across Europe i.e. do individual countries have individual populations or are there patterns of transfers of strains between countries. In Ireland, it is crucial to examine the risk of the environment as a source of VRE, LRE and most importantly the resistance elements *vanA*, *optrA* and *poxtA*. Targeting areas for environmental screening such as wastewater, rivers and lakes, which may be contaminated by agricultural run-off. Additionally, examining the carriage rates in livestock and the people who care for them would by significant for understanding enterococci transmission, as a recent study from the USA provides evidence of sharing of antimicrobial resistant *S. aureus* between pigs and farm workers (Randad *et al.*, 2021). Along with environmental and livestock sources, now is the time to continue the ongoing surveillance of the hospital-adapted enterococci and the population structure in Ireland using WGS. This will allow the long-term monitoring of changes in the general population dynamics of enterococci in Ireland and provide insights into the effectiveness of current and/or new IPC measures.

Additionally, the routine use of WGS may act as a tool to flag outbreaks in real-time within healthcare settings.

6.7 Concluding remarks

Vancomycin-resistant and linezolid-resistant enterococci have become a persistent and increasing problem in hospitals in Ireland, which can no longer be ignored in the way our invasive VREfm infection problem has for the past decade. Urgent action is required to understand the true burden of the problem of multidrug resistant enterococci. Implementing admission screening of all patients for VRE/LRE, increasing the frequency of and improving current cleaning regimes, improving hand hygiene compliance in healthcare and improved antimicrobial stewardship both in healthcare and agriculture would contribute significantly to managing this problem A OneHealth approach is necessary to stop the spread of these organisms, in particular within our healthcare environment. It is clear that enterococci act as effective vehicles for movement of antimicrobial resistance genes and may even become pan-resistant themselves. To date the majority of transfer of antimicrobial resistance genes occurs between enterococcal species (i.e. *E. faecium* and *E. faecalis*), but the major concern is that without action increasing levels of these VRE and LRE will lead to an increase in the transfer of antimicrobial resistance genes to other Gram-positive organisms, such as *S. aureus* and/or MRSA.

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Appendix

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Linezolid resistance in *Enterococcus faecium* and *Enterococcus faecalis* from hospitalized patients in Ireland: high prevalence of the MDR genes *optrA* and *poxtA* in isolates with diverse genetic backgrounds

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Objectives: To investigate the prevalence of the *optrA*, *poxtA* and *cfr* linezolid resistance genes in linezolid-resistant enterococci from Irish hospitals and to characterize associated plasmids.

Methods: One hundred and fifty-four linezolid-resistant isolates recovered in 14 hospitals between June 2016 and August 2019 were screened for resistance genes by PCR. All isolates harbouring resistance genes, and 20 without, underwent Illumina MiSeq WGS. Isolate relatedness was assessed using enterococcal whole-genome MLST. MinION sequencing (Oxford Nanopore) and hybrid assembly were used to resolve genetic environments/plasmids surrounding resistance genes.

Results: *optrA* and/or *poxtA* were identified in 35/154 (22.7%) isolates, the highest prevalence reported to date. Fifteen isolates with diverse STs harboured *optrA* only; one *Enterococcus faecium* isolate harboured *optrA* (chromosome) and *poxtA* (plasmid). Seven *Enterococcus faecalis* and one *E. faecium* harboured *optrA* on a 36 331 bp plasmid with 100% identity to the previously described *optrA*-encoding conjugative plasmid pE349. Variations around *optrA* were also observed, with *optrA* located on plasmids in five isolates and within the chromosome in three isolates. Nine *E. faecium* and 10 *E. faecalis* harboured *poxtA*, flanked by IS*1216E*, within an identical 4001 bp region on plasmids exhibiting 72.9%–100% sequence coverage to a 21 849 bp conjugative plasmid. *E. faecalis* isolates belonged to ST480, whereas *E. faecium* isolates belonged to diverse STs. Of the remaining 119 linezolid-resistant isolates without linezolid resistance genes, 20 investigated representatives all harboured the G2576T 23S RNA gene mutation associated with linezolid resistance.

Conclusions: This high prevalence of *optrA* and *poxtA* in diverse enterococcal lineages in Irish hospitals indicates significant selective pressure(s) for maintenance.

Introduction

Linezolid is an antibiotic used for infections caused by MDR Gram-positive bacteria, including VRE. Linezolid resistance was first reported in vancomycin-resistant *Enterococcus faecium* (VREfm) in Greece in 2004.[1,2] Ireland had the highest rate of VREfm bloodstream infections in Europe between 2007 and 2017.[3] Although no data are available on linezolid usage in Ireland, an almost 10% increase in the overall use of antimicrobials was noted between 2007 and 2017.[4] A linezolid usage increase of 40% between 2012 and 2013 was reported in one Irish hospital.[5] The emergence of linezolid-resistant enterococci (LRE) during or after linezolid exposure has been described.[6–10]

Linezolid binds in the V domain of the 23S rRNA component of the 50S ribosomal subunit and inhibits protein synthesis.[11] Enterococcal linezolid resistance can be due to G2576T or G2505A mutations in the 23S rRNA binding site or mutations in the genes encoding ribosomal proteins L3 and/or L4.[7] However, linezolid resistance can develop following acquisition of the resistance genes *optrA*, *poxtA* and variants of the *cfr* gene, which have been described in detail previously.[12] Although the reported number of

E. faecium and *Enterococcus faecalis* isolates harbouring these genes is low, *optrA* has been reported with increased frequency recently. In 2014, it was reported that 3/9 linezolid non-susceptible isolates (MIC ≥4 mg/L) were *optrA*-positive *E. faecalis* (two from Ireland).[13] This increased to 8/17 in 2016.[14] German researchers reported that 6% of 698 LRE recovered between 2007 and 2017 harboured *optrA*.[15]

The *poxtA* gene was originally identified in an Italian clinical MRSA in 2018 and subsequently in a porcine *E. faecium*.[16,17] More recently, *optrA* and *poxtA* were co-located on a conjugative plasmid in a porcine *E. faecalis*.[18] To date, *poxtA* has only been reported in a Greek clinical *E. faecium* in 2018.[19] The *cfr* gene, and its variants *cfr*(B) and *cfr*(D) (GenBank: MG707078.1) have been reported in clinical *E. faecium*,[5] whereas only *cfr* has been reported in *E. faecalis*.[5,20,21] The first reported linezolid-resistant VREfm outbreak in Ireland occurred in 2014; it involved 15 patients and was identified as a clonal outbreak using PFGE.[22] All isolates harboured the G2576T 23S mutation and were *cfr* negative. Other linezolid resistance genes were not investigated.[22] In 2014, the first two *optrA*-positive VREfm were recovered in separate Irish hospitals.[13] In 2016, the Irish Health Protection Surveillance Centre requested that all LRE identified in Irish hospitals should be sent to the National MRSA Reference Laboratory (NMRSARL) for linezolid resistance gene screening. In 2017, a VREfm clinical isolate harbouring a *cfr*- and *optrA*-encoding plasmid was reported from an Irish hospital.[5]

The purpose of this study was to investigate the molecular mechanisms and spread of linezolid resistance in LRE from Irish hospitals sent to the NMRSARL for linezolid resistance gene screening between June 2016 and August 2019. All isolates harbouring *optrA*, *poxtA* or *cfr* and a selection of isolates without these genes were investigated using WGS.

Materials and methods

Isolates

Between June 2016 and August 2019, 154 LRE from patients in Irish hospitals were sent to the NMRSARL for linezolid resistance gene PCR screening (Table S1, available as Supplementary data at *JAC* Online). Thirty-five of these harboured at least one of the genes *optrA* and *poxtA* and were investigated in detail. Of the remaining 119 isolates without linezolid resistance genes, 20 representatives from a range of isolation dates and hospital locations were also investigated (Table S2). These 55 isolates investigated in detail were recovered in 14 Irish hospitals (H1–H14) (Figure S1). The remaining 99 LRE isolates lacking resistance genes were not investigated further.

Phenotypic and genotypic testing

All isolates were tested for susceptibility to linezolid, vancomycin, chloramphenicol and tetracycline using the VITEK®2 system (bioMérieux, France). MICs were interpreted using the EUCAST interpretative criteria.[23] Etests (bioMérieux) were used to assess linezolid MICs between 8 and 256 mg/L. PCRs for enterococcal species and resistance genes (Table S1) were performed using GoTaq DNA polymerase and buffers (Promega Corporation, USA).

Conjugation

Conjugative transfer of plasmids encoding *optrA* and/or *poxtA* harboured by all 35 LRE was undertaken by filter mating using the plasmid-free rifampicin- and fusidic acid-resistant recipient strains *E. faecium* 64/3 and *E. faecalis* OG1RF.[15] Putative transconjugants were screened for enterococcal species, *optrA* and *poxtA* by PCR. Transconjugants harbouring *optrA* or *poxtA* underwent WGS and genomes were assembled using SPAdes v3.7.1 and compared with the corresponding recipient strain genomes.

WGS

The 55 LRE and transconjugants underwent WGS using genomic DNA extracted using the S. aureus Genotyping Kit 2.0 [Abbott (Alere Technologies), Germany] and the QIAGEN DNeasy Blood and Tissue Kit (QIAGEN, UK). Libraries prepared with the Nextera DNA Flex Library Preparation Kit (Illumina, The Netherlands) underwent paired-end sequencing using the 500-cycle MiSeq Reagent Kit v2 (Illumina). Libraries were scaled to yield ≥50× coverage.

For isolates selected for hybrid assembly, DNA was extracted using the GenFind v3 kit (Beckman Coulter, USA). Long-read sequencing was performed in multiplex using MinION sequencing (Oxford Nanopore Technologies, UK), the 1D Genomic DNA sequencing kit (SQK-LSK109) and 1D Native Barcoding Kit (EXP-NBD103). Libraries were sequenced on an Mk1B (MIN101B) MinION platform with a FLO-MIN106D (SpotON R9.4) flow cell and using MinKNOW v1.7.10 (Oxford Nanopore). Basecalls were performed on MinION FAST5 files using Guppy v3.1.5 (Oxford Nanopore) and demultiplexing was performed using qCat v1.0.1 (https://github.com/nanoporetech/qcat).

Analysis of WGS data

WGS data were analysed using the enterococcal whole-genome (wg) MLST schemes available in BioNumerics v7.7 (Applied Maths, Belgium). The *E. faecium* scheme consisted of 5489 wgMLST loci [including 1423 core-genome (cg) MLST loci], while the *E. faecalis* scheme consisted of 5285 wgMLST loci.[24] Two BioNumerics algorithms were used to generate a consensus wgMLST profile for each isolate, one of which determined locus presence/absence and allelic identity using an assembly-free *k*-mer approach. The other, assembly-based, method used a BLAST approach to detect alleles on contigs assembled using SPAdes v3.7.1, all using default parameters. Minimum-spanning trees (MSTs) were created using BioNumerics based on allelic differences. Illumina WGS data for all isolates were examined for 23S rRNA mutations (G2576T and G2505A) using LRE-Finder (https://cge.cbs.dtu.dk/services/LRE-finder/).[25]

Assembly and analysis of plasmids encoding resistance genes

MinION-generated FASTQ files and MiSeq-generated FASTQ files were used to perform a hybrid assembly using Unicycler.[26] The genetic organization of plasmids harbouring *optrA* or *poxtA* was determined following hybrid assembly and annotation using RAST v2.0 (http://rast.nmpdr.org/).[27] These were used as reference sequences for further analysis. MiSeq reads were mapped against reference plasmid sequences and percentage depth and breadth of coverage was calculated using Burrows–Wheeler aligner, SAMtools and BEDTools coverage.[28–30] Alignments were viewed for quality using Tablet.[31]

Sequences of plasmids resolved by hybrid assembly (pM16/0594, pM18/0011 and pM17/0314) and DNA regions encoding *optrA* variants have been deposited in GenBank under accession numbers MN831410, MN831411, MN831412, MN831413, MN831414, MN831415, MN831416, MN831417, MN831418 and MN831419.

Results and discussion

Linezolid-resistant isolates

A total of 35/154 (22.7%) LRE (23 *E. faecalis* and 12 *E. faecium*) submitted to the NMRSARL between June 2016 and August 2019

harboured *optrA* (2 *E. faecium* and 13 *E. faecalis*), *poxtA* (9 *E. faecium* and 10 *E. faecalis*) or both *optrA* and *poxtA* (1 *E. faecium*). All 35 isolates were from hospitalized patients in 11 Irish hospitals (H1–H11) (Figure S1) and were phenotypically resistant to linezolid, chloramphenicol and tetracycline (Table S2). This is the highest prevalence of *optrA* and/or *poxtA* in human enterococci reported to date. Six *E. faecium* isolates were vancomycin resistant and harboured *vanA*. The remaining 29 LRE isolates were vancomycin susceptible and lacked *vanA* (Table S2). The majority (19/35) of LRE harboured *poxtA* only; the largest collection of *poxtA*-positive human isolates reported to date. The remaining LRE harboured *optrA* only (14/35), *optrA* and *poxtA* (1/35) or *optrA* and *cfr*(D) (1/35). One VREfm (M19/0595) harboured *poxtA* and the G2576T 23S mutation (4/6 copies mutated) and is the first report of mutational and gene-encoded linezolid resistance in a single enterococcal isolate. The remaining 34 LRE lacked 23S mutations.

All 20/119 LRE investigated that lacked *cfr, optrA* and/or *poxtA* (2 *E. faecalis*, 18 *E. faecium*) were phenotypically resistant to linezolid and chloramphenicol (Table S2). All 20 LRE exhibited a varying copy number (1–5) of the G2576T 23S mutation. The majority of the LRE (15/18 *E. faecium*) harboured *vanA* and exhibited vancomycin MICs $\geq$32 mg/L. The remaining five *vanA*-negative isolates (two *E. faecalis* and three *E. faecium*) were vancomycin susceptible (Table S2).

Relatedness of LRE based on WGS

Of the 55 LRE investigated, the 30 *E. faecium* included from 11 hospitals were assigned to 10 STs using traditional MLST, with ST80 predominating (8/30, 26.6%). Seventeen *E. faecium* isolates were differentiated into seven clusters (CI–CVII) using cgMLST (Figure 1a). Clusters CI–CVI contained isolates of the same STs (ST17, ST787, ST789, ST202 and ST203), while cluster CVII consisted of ST203 and ST1588 (a single locus variant of ST203) isolates (Figure 1a). Clusters were differentiated by intracluster and intercluster allelic differences of 0–22 and 38–394, respectively. Isolates exhibiting $\leq$22 allelic differences were deemed closely related, based on previous work.[24] Clusters CI and CIII contained isolates from the same hospitals (H2 and H6), all with the G2576T 23S mutation. The remaining five clusters consisted of isolates from two or more hospitals and a mixture of isolates exhibiting linezolid resistance associated with G2576T mutations or a resistance gene (Figure 1a).

Of the 55 LRE investigated, the 25 *E. faecalis* included originated from 10 hospitals and belonged to nine STs using traditional MLST, with ST480 predominating (13/25). Twenty of the *E. faecalis* isolates differentiated into four clusters (CI–CIV) using wgMLST (Figure 1b). The remaining five isolates were distantly related to any other isolate, exhibiting between 339 and 1897 allelic differences. Clusters were defined by the contrasting tight value of intracluster differences (0–43). Each cluster contained isolates from the same STs (ST6, ST21, ST480 and ST768). Only one cluster, CIV, contained two isolates from the same hospital (H2), both with the G2576T 23S mutation. The remaining three clusters contained isolates from 2–8 hospitals. Clusters CII (n = 3) and CIII (n = 2) contained only *optrA*-positive isolates, whereas cluster CI (n = 13) contained only ST480 isolates, 10 and 3 of which harboured *poxtA* or *optrA*, respectively. Isolates within cluster CI exhibited an average allelic difference of 29 (range 7–90) (Figure 1b).

Overall the population structure of LRE was polyclonal, but the presence of highly related strains in different hospitals was evident.

Genetic environment surrounding optrA

The WGS data of a selection of isolates encoding *optrA* underwent hybrid assembly, namely two *E. faecium* (M16/0594 and M17/0314) and seven *E. faecalis* (M17/0149, M17/0240, M18/0173, M18/0497, M18/0582, M18/0906 and M19/0596). The first plasmid resolved was a 36 331 bp *optrA*-encoding plasmid (pM17/0149) from *E. faecalis* M17/0149, which was the same size and exhibited 100% DNA sequence identity to plasmid pE349, first described from a clinical *E. faecalis* in China and subsequently identified in *E. faecium* and *E. faecalis* from humans and food-producing animals throughout European, American and Asian countries.[7,9,13,14,32] Of the 16 *optrA*-positive isolates, half (one *E. faecium* and seven *E. faecalis*) harboured plasmids exhibiting 100% sequence coverage to pM17/0149 (pE349-like). Multiple *E. faecalis* STs (ST19, ST21, ST166 and ST768) harboured this pE349-like plasmid, suggesting independent acquisition by different genetic backgrounds (Table S2). For these eight isolates, the genetic environment surrounding *optrA* was designated *optrA*_WT (Figure 2). The remaining eight *optrA*-positive isolates exhibited <35% sequence identity to pM17/0149 and were selected for WGS hybrid assembly. The genetic environment surrounding *optrA* was examined and *optrA* was identified on plasmids in five isolates (M17/0240, M18/0582, M19/0596, M18/0173 and M17/0314) and within the chromosome in three isolates (M18/0497, M16/0594 and M18/0906) (Figure 2). Three variations surrounding *optrA* were evident on the plasmid-encoded regions: *optrA*_I (M17/0240, M18/0582 and M19/0596); *optrA*_II (M18/0173); and *optrA*_III (M17/0314). In *optrA*_I and *optrA*_II, *fexA* was encoded around 750 bp upstream of *optrA*, similarly to *optrA*_WT, whereas *optrA*_III lacked *fexA*. Variations were distinguished by various flanking IS elements and other genes encompassed between these elements (Figure 2a). Interestingly, isolate M17/0314, harbouring *optrA*_III, also encoded the linezolid resistance gene *cfr*(D) and the macrolide, lincosamide and streptogramin B resistance genes *erm*(A) and *erm*(B), on a 103 600 bp plasmid (pM17/0314) (Figure S2). In the case of 1/8 *optrA*_WT isolates (M16/0427), 2/3 isolates harbouring *optrA*_I (M17/0240 and M19/0596) and 1 *optrA*_III isolate (M17/0314), *optrA* was successfully conjugated into a recipient strain of the same species (Table S3).

Three variations of the genetic environment surrounding *optrA* in the chromosome, designated *optrA*_IV, *optrA*_V and *optrA*_VI, were identified in isolates M18/0906, M16/0594 and M18/0497, respectively (Table S2). Both the *optrA*_IV and *optrA*_V variants harboured *optrA*, flanked by *tnpA* and *tnpB* from Tn*554* on one end and by IS*L3* on the other end. The *optrA*_IV variant encoded *fexA* and *optrA* in the same orientation as *optrA*_WT, but in *optrA*_V, *fexA* was encoded around 2800 bp upstream from *optrA* in the opposite orientation to the arrangement in *optrA*_WT (Figure 2b). In *optrA*_VI, *optrA* was flanked by IS*Enfa5* and IS*Efa5* and exhibited the same *optrA* and *fexA* arrangement present in *optrA*_WT (Figure 2b). Attempts to transfer *optrA* in the three isolates encoding chromosomal *optrA* by conjugation were unsuccessful.

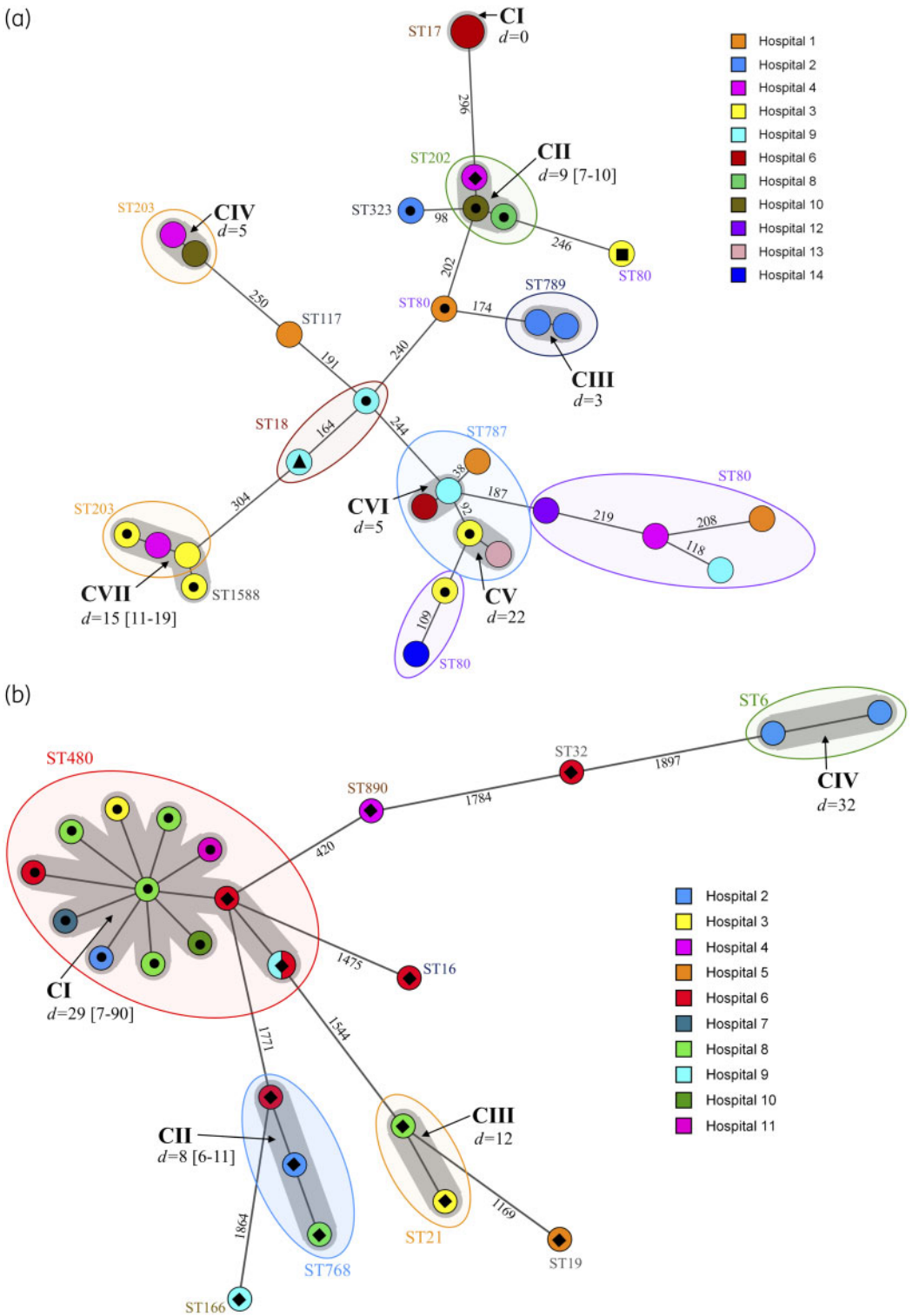

Figure 1. Minimum spanning trees based on (a) cgMLST data from 30 linezolid-resistant clinical *E. faecium* isolates and (b) wgMLST data from 25 linezolid-resistant *E. faecalis* clinical isolates. All isolates were recovered between June 2016 and August 2019 from 14 Irish hospitals, as denoted in the legends. The numbers on the branches represent the number of cgMLST/wgMLST allelic differences. STs are shown in coloured ovals. Grey shadowing around nodes indicates clusters of related isolates, which are labelled in bold and denoted CI–VII; '*d*=' values indicate average allelic differences and the range in square brackets. Isolate designations are as follows: filled black circle, *poxtA* positive; filled black diamond, *optrA* positive; filled black square, *optrA* positive and *cfr*(D) positive; and filled black triangle, *optrA* positive and *poxtA* positive. Isolates not marked with a symbol were negative for linezolid resistance genes and harboured various copy numbers (1–5) of the G2576T 23S mutation associated with linezolid resistance.

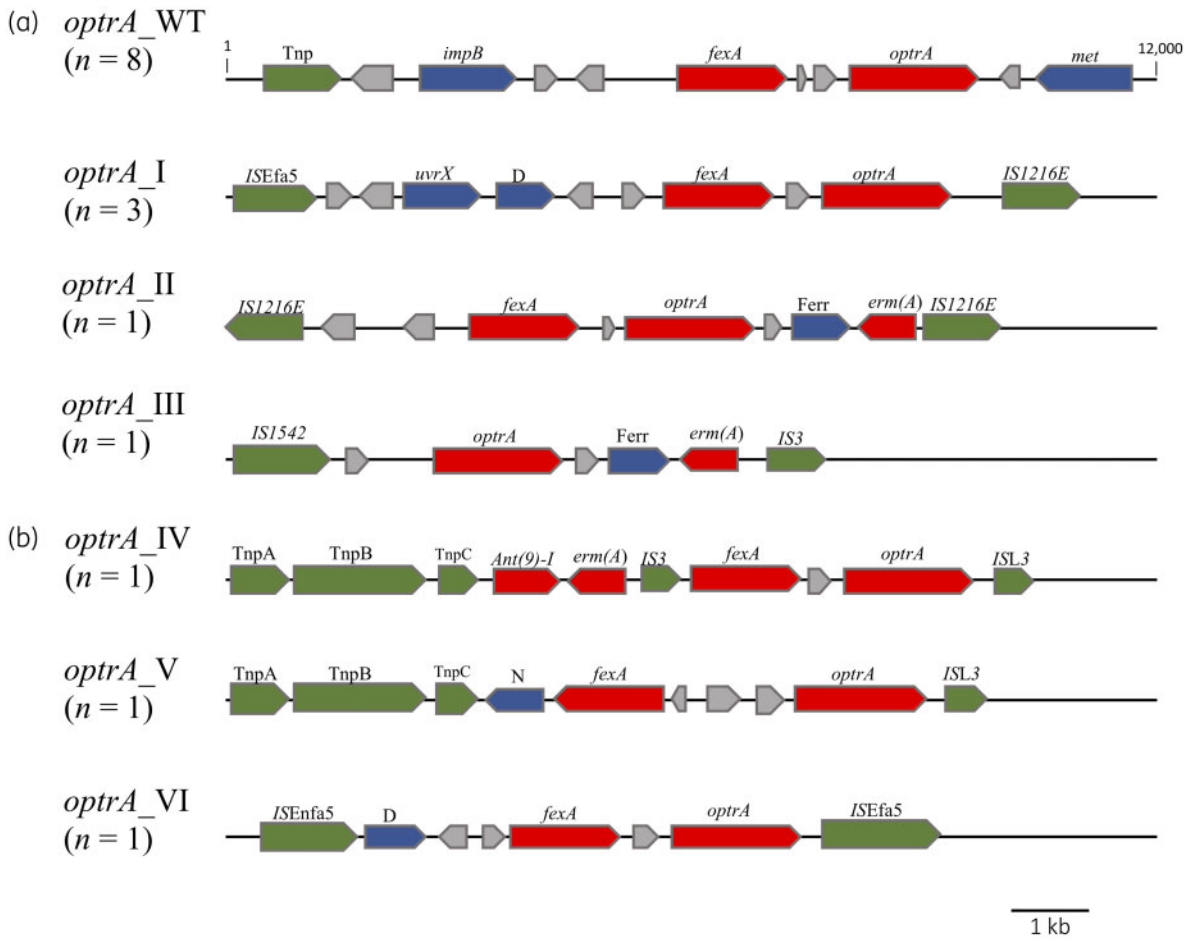

Figure 2. Schematic representation of the *optrA* gene loci in *E. faecalis* and *E. faecium* clinical isolates from Ireland. Different variants of the genetic environment surrounding *optrA* were detected in LRE encoded on plasmids (a) and within the chromosome (b) and were aligned against the prototype *optrA*_WT, first described by Wang *et al.* in 2015.[9] Genes of interest and their orientation are shown with arrows and labelled; red indicates antibiotic resistance genes, green indicates ISs/transposases, blue indicates known proteins and grey indicates hypothetical proteins. Tnp, transposase; *met*, truncated DNA adenine methylase; *uvrX*, putative UV-damage repair protein; D, DNA-directed RNA polymerase β subunit; Ferr, ferredoxin; N, NAD(P)H oxidoreductase.

The variation surrounding *optrA* in the investigated LRE, combined with the identification of multiple linezolid resistance genes [*optrA*/*poxtA* and *optrA*/*cfr*(D)] in individual isolates, is indicative of an environment with a high selective pressure for linezolid resistance.

Characterization of isolates encoding poxtA

The *E. faecium* isolate M16/0594 harboured *optrA*_V in its chromosome and *poxtA*, *tet*(M) and *tet*(L) on a 21 849 bp plasmid (pM16/0594) (Figure 3). Plasmid pM16/0594 was conjugative and transconjugant derivatives were obtained with the *E. faecium* 64/3 recipient. Of the remaining 19 *poxtA*-positive LRE (9 *E. faecium* and 10 *E. faecalis*), 5/19 (all *E. faecium*) exhibited 100% sequence coverage to pM16/0594, while 14/19 (4 *E. faecium* and 10 *E. faecalis*) exhibited between 72.9% and 99.3% coverage. All isolates exhibited 100% sequence coverage to a 4001 bp *poxtA*-encoding region, flanked by two IS*1216E* elements in parallel orientation; this conserved *poxtA* element has previously been shown to be responsible for horizontal gene transfer of *poxtA*.[16,33,34] These findings indicate the spread of *poxtA* in human *E. faecium* and *E. faecalis* in Ireland via this 4001 bp element or by a very similar *poxtA* plasmid. One limitation of this method of read mapping against pM16/0594 is the risk of missing larger plasmids, as 100% coverage will indicate the query sequence is identical to the reference used but cannot indicate if the plasmid is larger. Therefore, isolates with 100% sequence coverage to pM16/0594 have at least a 21 849 bp plasmid region identical to that of pM16/0594, which may be contained within larger plasmids.

The *poxtA*-positive *E. faecalis* isolate M18/0011 exhibiting 73.7% sequence coverage to pM16/0594 was also resolved using hybrid assembly. The plasmid encoding *poxtA* in M18/0011 (pM18/0011) was 3570 bp smaller than pM16/0594 (18 279 bp) and lacked *tet*(M) and *tet*(L). An identical 4001 bp region encoding *poxtA*, flanked by two IS*1216E* elements in parallel orientation, was observed in pM18/0011 (Figure S3), albeit in the reverse orientation to the identical region in pM16/0594. Plasmid pM18/0011 was also conjugative and transconjugant derivatives were obtained with the *E. faecalis* OG1RF and *E. faecium* 64/3 recipients (Table S3).

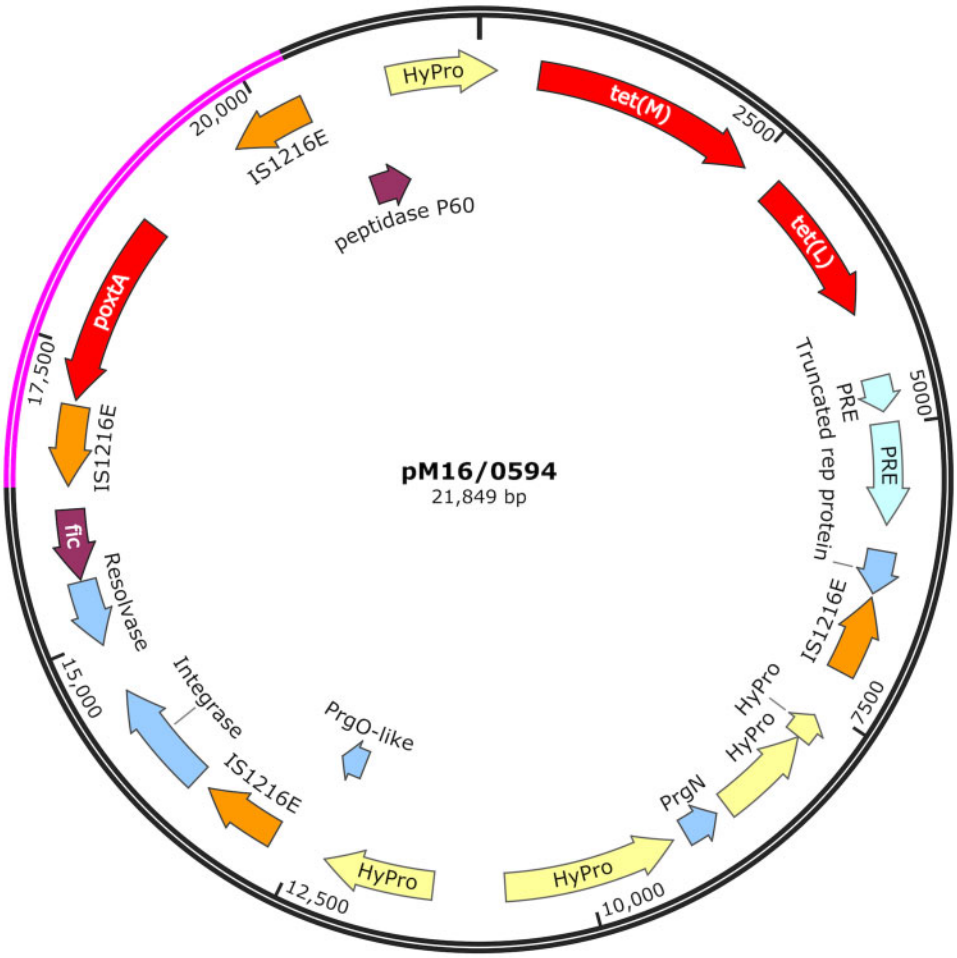

Figure 3. Schematic diagram of the structural organization of plasmid pM16/0594 from *E. faecium* isolate M16/0594, encoding the *poxtA* linezolid resistance gene resolved by hybrid assembly of paired-end Illumina MiSeq short reads with Oxford Nanopore Technologies long reads. The 4001 bp highly conserved region around *poxtA*, flanked by two IS*1216E* in parallel orientation, is highlighted in pink. Genes of interest and their orientation are represented by arrows as follows: red indicates antibiotic resistance genes, orange indicates ISs, blue indicates rep proteins, purple indicates other known proteins and yellow indicates hypothetical proteins. The plasmid size is labelled (bp). PRE, plasmid recombination enzyme; fic, cell filamentation protein; HyPro, hypothetical protein.

In total, 5/20 *poxtA*-positive LRE yielded transconjugant derivatives including *E. faecium* M16/0594 (harbouring pM16/0594) and *E. faecalis* M18/0011 (harbouring pM18/0011), two *E. faecalis* donors (M16/0419, 75.2% like pM16/0594; and M16/0633, 74.5% like pM16/0594) conjugated to *E. faecalis* OG1RF and one *E. faecium* donor (M19/0357, 77.9% like pM16/0594) conjugated to *E. faecium* 64/3 (Table S3). For the remaining 15 *poxtA*-positive LRE, conjugation was unsuccessful. The spread of a single clone was indicated in all 10 identified *poxtA*-positive *E. faecalis* LRE, as all belonged to ST480 and were closely related, with an average of 22 (range 7–54) wgMLST allelic differences (Figure 1b). The ST480 clone was recovered in seven hospitals (Table S2). ST480 is one of the predominant *optrA*-positive clinical *E. faecalis* clones in France and Germany.[15,35] In contrast, *poxtA* was found to have spread in *E. faecium* via the 4001 bp mobile element or a promiscuous plasmid to multiple STs (ST18, ST80, ST202, ST203, ST323, ST787 and ST1588).

Conclusions

The results of this study revealed the high prevalence and spread of *optrA* and *poxtA* among enterococci in Irish hospitals. A major cause for concern is that 26.3% (5/19) of isolates harbouring a *poxtA*-encoding plasmid also harboured *vanA*, which poses a significant risk for hospitalized patients, as treatment options for such strains are limited. Linezolid consumption in Irish hospitals is currently not specifically recorded as part of annual national hospital antimicrobial consumption surveillance.[4] This needs to change and linezolid consumption in hospitals needs to be more rigidly controlled.

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clinical isolates from Irish hospitals. We thank the clinical microbiology staff from all the Irish hospitals that sent clinical enterococcal isolates for investigation of linezolid resistance genes. We wish to acknowledge Dr Mette Pinholt, Professor Henrik Westh and the bioinformatic staff of the Hvidovre University Hospital, Copenhagen, Denmark, for their assistance and guidance in the bioinformatic analysis of clinical enterococci. We thank Dr Peter Flanagan for support with Oxford Nanopore MinION sequencing and analysis. We would also like to thank Dr Jennifer Bender of the Robert Koch Institute for providing us with the recipient strains for filter mating experiments.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 to S3 and Figures S1 to S3 are available as Supplementary data at *JAC* Online

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Hospital outbreak of linezolid-resistant and vancomycin-resistant ST80 *Enterococcus faecium* harbouring an *optrA*-encoding conjugative plasmid investigated by whole-genome sequencing

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SUMMARY

Background: Linezolid is an antibiotic used to treat infections caused by multi-drug-resistant Gram-positive bacteria. Linezolid resistance in enterococci has been reported with increasing frequency, with a recent rise in resistance encoded by *optrA*, *poxtA* or *cfr*.
Aim: To investigate a hospital outbreak of linezolid- and vancomycin-resistant *Enterococcus faecium* (LVREfm) using whole-genome sequencing (WGS).
Methods: Thirty-nine VREfm from patient screening (19 isolates, 17 patients) and environmental sites (20 isolates) recovered in October 2019 were investigated. Isolates were screened using polymerase chain reaction for *optrA*, *poxtA* and *cfr*, and underwent Illumina MiSeq WGS. Isolate relatedness was assessed using *E. faecium* core genome multilocus sequence typing (cgMLST). One LVREfm underwent MinION long-read WGS (Oxford Nanopore Technologies) and hybrid assembly with MiSeq short-read sequences to resolve an *optrA*-encoding plasmid.
Findings: Twenty isolates (51.3%) were LVREfm and *optrA*-positive, including the LVREfm from the index patient. A closely related cluster of 28 sequence type (ST) 80 isolates was identified by cgMLST, including all 20 LVREfm and eight linezolid-susceptible VREfm, with an average allelic difference of two (range 0–10), indicating an outbreak. Nineteen (95%) LVREfm harboured a 56,684-bp conjugative plasmid (pEfmO_03). The remaining LVREfm exhibited 44.1% sequence coverage to pEfmO_03. The presence of pEfmO_03 in LVREfm

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and the close relatedness of the outbreak cluster isolates indicated the spread of a single strain. The outbreak was terminated by enhanced infection prevention and control (IPC) and environmental cleaning measures, ceasing ward admissions and ward-dedicated staff.
Conclusion: WGS was central in investigating an outbreak of ST80 LVREfm. The rapid implementation of enhanced IPC measures terminated the outbreak.

Introduction

Enterococcus faecium is an important nosocomial pathogen causing bacteraemia, and abdominal, urinary tract and intravenous catheter-related infections [1]. Acquired resistance to ampicillin, gentamicin (high level) and vancomycin has increased worldwide among hospital-associated *E. faecium*, narrowing treatment options [1]. Ireland had one of the highest rates of vancomycin-resistant *E. faecium* (VREfm) bloodstream infections in Europe between 2006 and 2018 [2]. Furthermore, the population-weighted mean percentage of VREfm across Europe increased from 10.4% in 2014 to 17.3% in 2018 [3].

Conventional multi-locus sequence typing (MLST) for *E. faecium* was first described in 2002, consisting of seven housekeeping genes with derived nomenclature managed and assigned via PubMLST.org [4]. Clinical VREfm worldwide are assigned to sequence types (STs) belonging to the epidemic hospital-adapted lineage clade A1. These strains are generally enriched in mobile genetic elements, putative virulence determinants and antibiotic resistance determinants [5,6]. Whole-genome sequencing (WGS) studies revealed a polyclonal VREfm population structure with evidence of hospital transmission and inter- and intraregional spread of VREfm clones [7–9] and no distinct geographical patterns [3]. Enhanced surveillance is required to better understand the epidemiology, clonal diversity and risk factors associated with VREfm [3].

Linezolid is an antibiotic used to treat infections caused by multi-drug-resistant Gram-positive bacteria, including VREfm [10]. The emergence of linezolid-resistant enterococci (LRE) during or after linezolid exposure has been well described, with the first description of resistance noted during initial clinical trials [11–15]. Linezolid binds in the V domain of the 23S rRNA component of the 50S ribosomal subunit and inhibits protein synthesis [16]. Enterococcal linezolid resistance results mainly from G2576T or G2505A mutations in the 23S rRNA binding site or mutations in genes encoding ribosomal proteins L3 and/or L4 [13]. Linezolid resistance can also develop following acquisition of the *optrA* and *poxtA* genes and variants of the *cfr* gene, which are frequently encoded on conjugative plasmids [17].

The *optrA* gene was first described from a clinical *Enterococcus faecalis* in China, and was subsequently identified in *E. faecium* and *E. faecalis* from humans and food-producing animals throughout European, American and Asian countries [12,14,15,18–20]. The OptrA protein belongs to the ATP-binding cassette (ABC)-F protein subfamily and mediates resistance to oxazolidinones and phenicols, which share an overlapping binding site at the ribosomal A site. A recent study indicated that the mechanism of *optrA*-mediated antibiotic resistance does not involve active efflux, like other ABC transporters [21]. Current evidence indicates that (ABC)-F proteins such as OptrA bind to the ribosome and effect the release of ribosome-targeted antibiotics, thereby rescuing the translation machinery from antibiotic-mediated inhibition [22]. Although the number of *optrA*-positive enterococci reported to date is low, they have increased recently. In 2014, three of nine linezolid-resistant isolates [linezolid minimum inhibitory concentration (MIC) >4 mg/L] were *optrA*-positive *E. faecalis* (two from Ireland); this increased to eight of 17 in 2016 [18,19].

In Ireland, the first linezolid-resistant VREfm (LVREfm) clonal outbreak was reported in 2014 involving 15 patients. This was investigated using pulsed-field gel electrophoresis. All isolates harboured the G2576T 23S rRNA mutation and were *cfr* negative. However, other linezolid resistance genes were not investigated [23]. That same year, two *optrA*-positive VREfm were recovered in separate Irish hospitals [18]. Since centralized screening commenced in 2016, Ireland has had the highest reported prevalence of gene-encoded linezolid resistance, with *optrA* and/or *poxtA* identified in 22.7% (35/154) of isolates, predominantly encoded on conjugative plasmids in diverse enterococcal lineages [24].

In October 2019, a LVREfm isolate was recovered from a patient in a hospital in Dublin. Enhanced patient screening and environmental sampling yielded additional VREfm isolates including LVREfm, suggesting an outbreak. This study describes the WGS analysis of these LVREfm, identification of an outbreak by an ST80 strain harbouring an *optrA*-encoding conjugative plasmid, and control measures implemented to terminate the outbreak.

Methods

Hospital setting

The outbreak occurred in a level 2, 107-bed hospital in Dublin, and was primarily associated with two wards and the x-ray department. Ward 1 (W1) was a 26-bed unit with single patient en-suite rooms, linked with Ward 2 (W2), an oncology day-unit (Figure 1). The hospital specialities include general medical and oncology, with a large proportion of patients requiring extensive care.

VREfm surveillance

In October 2019, Patient A was admitted to W1 with chronic leg ulcers, cellulitis and an extensive medical history including colon cancer. Patient A had been admitted to the high-dependency unit and W1 during the previous month. While an inpatient, Patient A visited other departments including the x-ray department and required a high level of care. On re-admission 13 days later, Patient A was screened and placed on contact isolation precautions due to a history of carriage of

multi-drug-resistant organisms (MDROs) including VREfm. An LVREfm isolate was recovered from rectal screening on this admission, after which additional emphasis was placed on contact precautions, hand hygiene and equipment decontamination. Nine days later, Patient B (W1) also yielded LVREfm following rectal screening. This prompted the infection prevention and control team (IPCT) to request that all patients on W1 be screened for VREfm carriage, after which three more LVREfm-positive patients were identified (Patients C–E, Figure 1 and Table I). Additional weekly and discharge screening was introduced. As two LVREfm-positive patients (Patients A and B) were oncology patients, the IPCT introduced screening of all patients attending W2 (oncology day-unit), which identified two further LVREfm-positive patients (Patients G and H, Figure 1 and Table I). Patient G was an inpatient on a different hospital ward (W3) who attended W2 every few weeks from early 2019 until the start of the outbreak. Extended rectal screening across the hospital identified VREfm in a further nine patients (Table I). All VREfm patient screening isolates from the hospital recovered during the suspected outbreak timeline were investigated.

Patient and environmental screening

Extensive environmental screening was undertaken in all inpatient wards and other areas that patients had attended during hospitalization. Environmental sites were sampled using regular FLOQSwabs (Copan Diagnostics Inc., Murrieta, CA, USA), pre-moistened with sterile water. Individual swab tips were placed in 5 mL of brain heart infusion broth (Fannin Ltd., Dublin, Ireland), incubated for 16–18 h at 37°C, after which the cultures were inoculated on to CHROMID VRE (bioMérieux, Marcy l'Etoile, France) agar with a 10-μg linezolid disc [Oxoid (Thermo Fisher Scientific),Dublin, Ireland]. Patient rectal screening swabs were also inoculated on to CHROMID VRE (bioMérieux) agar with a 10-μg linezolid disc (added following identification of index case), and on to CHROMID CPS Elite (bioMérieux) agar to ensure that bowel flora were present on the swab.

Decontamination and control measures

For the duration of the outbreak, W1 was closed to patient admissions and transfers, strict visitor restrictions were implemented, specific staff members were dedicated to W1, cleaning of all equipment in patient areas was increased to twice daily, and cleaning of bathroom facilities was increased to four times daily using Actichlor Plus (Ecolab Ltd, Northwich, UK) with 1000 ppm available chlorine. Patient rooms that yielded LVREfm were decontaminated with hydrogen peroxide vapour (HPV) following patient transfer or discharge using the

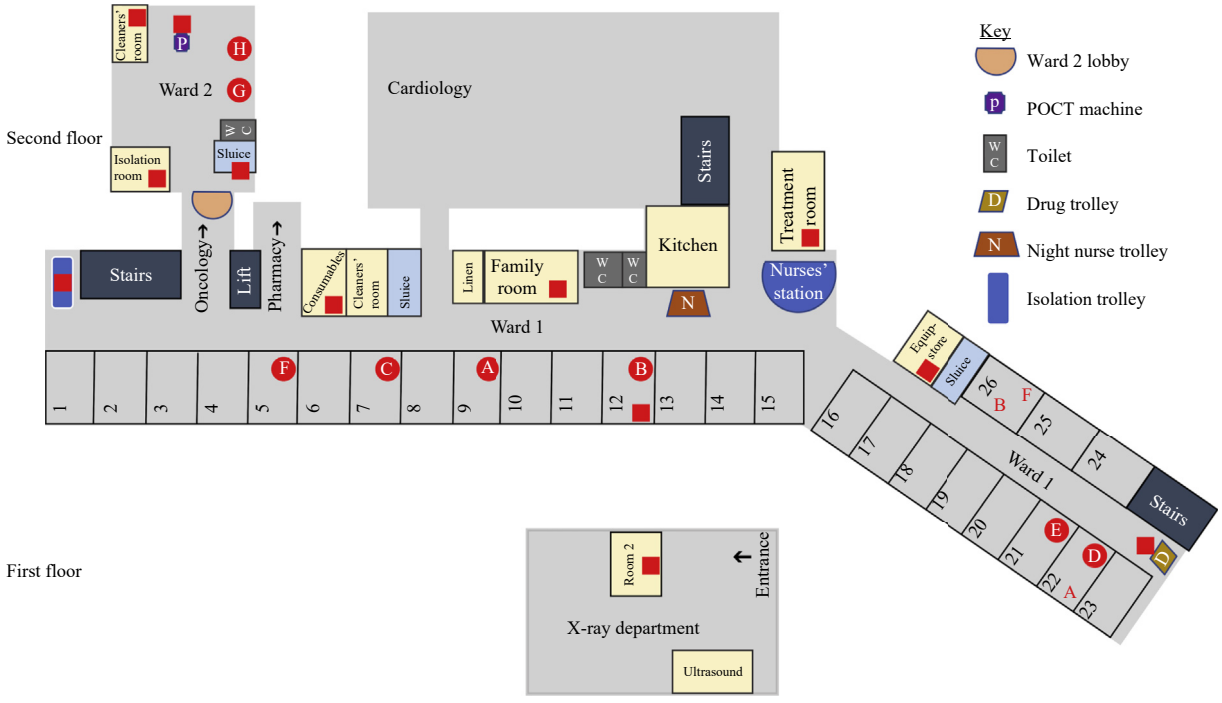

Figure 1. Schematic diagram showing the layout of Ward 1, Ward 2 and the x-ray department involved in the *optrA*-positive vancomycin-resistant *Enterococcus faecium* (VREfm) outbreak. The single en-suite rooms in Ward 1 are labelled 1–26. Room numbers have been changed to maintain patient anonymity. Other areas of interest are labelled accordingly, or details are provided using the key. Locations where patients (denoted by capital letters A–H) that yielded linezolid-resistant VREfm (LVREfm) isolates were housed are denoted by a filled red circle. Environmental sites that yielded LVREfm environmental isolates are denoted by a filled red square. Patients A, B and F were transferred during the course of the outbreak; their movements are denoted by corresponding red letters in alternate rooms. Patient G, an oncology inpatient housed on Ward 3 (not shown), is shown on Ward 2 as this is the likely location for acquisition of LVREfm. Room locations of patients and environmental sites that yielded VREfm are not shown to retain clarity. This information is provided in Table I. POCT machine, point-of-care-testing machine; Equip. store, equipment storage; consumables, consumable storeroom.

Table I
Phenotypic and genotypic characteristics of the 38 vancomycin-resistant *Enterococcus faecium* (VREfm) isolates recovered in an outbreak setting in an Irish hospital over 4 weeks in October 2019, with the addition of one isolate from the index patient from 2018

E. faecium isolate no.	Ward/room[a]	Days since first isolate recovered	Source[c]	Clinical history	LIN MIC mg/L (R>4 mg/L)[d]	VAN MIC mg/L (R>4 mg/L)[d]	CHL MIC mg/L (R>32 mg/L)[d]	*optrA*	ST	cgMLST cluster[e]	Plasmid sequence similarity (%) to pEfmO_03
O_01	W1	N/A[b]	Patient A		4.0	≥32	32	-	80	C1	N/A
O_02	W1 9>22	0	Patient A	Colon cancer, diabetes, COPD, chronic leg ulcers, multiple MDROs including VRE carriage	8.0	≥32	≥256	+	80	C1	100
O_03	W1 12>26	8	Patient B	Metastatic cancer, palliative care	16.0	≥32	≥256	+	80	C1	100
O_04	W1	13	Room 12		16.0	≥32	≥256	+	80	C1	100
O_05	W2	13	Sluice room		16.0	≥32	≥256	+	80	C1	100
O_06	W1	13	Isolation trolleys		16.0	≥32	≥256	+	80	C1	100
O_07	W1 7	13	Patient C	COPD, arthritis, malignancy	8.0	≥32	≥256	+	80	C1	100
O_08	W1	13	Treatment room		16.0	≥32	≥256	+	80	C1	100
O_09	W1 22	14	Patient D	Infected leg ulcers, recurrent UTIs, rheumatoid arthritis	16.0	≥32	≥256	+	80	C1	99.98
O_10	W1	14	Patient		2.0	≥32	16	-	SLV of ST80	N/A	N/A
O_11	W1	14	Patient		2.0	≥32	32	-	80	C4	N/A
O_12	W1	14	Patient		2.0	≥32	32	-	80	C1	N/A
O_13	W1 21	15	Patient E	Metastatic malignancy, palliative care	16.0	≥32	≥256	+	SLV of ST80	C1	100
O_14	W1	16	Equipment store		16.0	≥32	≥256	+	80	C1	99.98
O_15	W1	16	Consumable store		8.0	≥32	≥256	+	80	C1	100
O_16	W1	16	Family room		32.0	≥32	≥256	+	80	C1	100
O_17a	W1	16	Drug trolley		1.0	≥32	16	-	80	C1	N/A
O_17b	W1	16	Drug trolley		16.0	≥32	≥256	+	80	C1	100
O_18	W1	16	Linen room		2.0	≥32	16	-	80	C1	N/A
O_19	W1	16	Night nurses' trolley		1.0	≥32	16	-	80	C4	N/A
O_20	W1	16	Cleaners' store		2.0	≥32	16	-	80	C1	N/A
O_21	W4	16	Patient		2.0	≥32	32	-	80	C1	N/A
O_22	W2	20	POCT machine		8.0	≥32	≥256	+	80	C1	100
O_23	W2	20	Isolation room		8.0	≥32	≥256	+	80	C1	100
O_24	W1 5>26	20	Patient F	Congestive cardiac failure, COPD	16.0	≥32	≥256	+	80	C1	44.1
O_25	W2	20	Cleaners' room		8.0	≥32	≥256	+	80	C1	100
O_26	X-ray	20	Room 2		8.0	≥32	≥256	+	80	C1	100
O_27	W2	20	Lobby		2.0	≥32	16	-	80	C4	N/A
O_28	W1	20	Patient		2.0	≥32	64	-	80	C3	N/A
O_29	W1	20	Patient		2.0	≥32	32	-	80	C4	N/A

(*continued on next page*)

Table I (*continued*)

E. faecium isolate no.	Ward/room[a]	Days since first isolate recovered	Source[c]	Clinical history	LIN MIC mg/L (R>4 mg/L)[d]	VAN MIC mg/L (R>4 mg/L)[d]	CHL MIC mg/L (R>32 mg/L)[d]	*optrA*	ST	cgMLST cluster[e]	Plasmid sequence similarity (%) to pEfmO_03
O_30	W4	20	Patient		1.0	≥32	32	-	80	C1	N/A
O_31	W1	20	New treatment room (room 10)		4.0	≥32	64	-	80	C3	N/A
O_32	W6	20	Bathroom		2.0	≥32	32	-	80	C2	N/A
O_33	X-ray	20	Ultrasound		2.0	≥32	32	-	80	N/A	N/A
O_34	W3	21	Patient		2.0	≥32	32	-	80	C2	N/A
O_35	W5	22	Patient		1.0	≥32	16	-	80	C4	N/A
O_36	W3	23	Patient G	Metastatic malignancies, gastrointestinal upset	8.0	≥32	≥256	+	80	C1	99.98
O_37a	W2	23	Patient H	Breast cancer	2.0	≥32	16	-	80	C1	N/A
O_37b	W2	23	Patient H	Breast cancer	32.0	≥32	≥256	+	80	C1	100

LIN, linezolid; VAN, vancomycin; CHL, chloramphenicol; W, ward; N/A, not applicable; ST, sequence type; MDRO, multi-drug-resistant organism; COPD, chronic obstructive pulmonary disease; UTI, urinary tract infection; cgMLST, core genome multi-locus sequence typing; MIC, minimum inhibitory concentration; R, resistant; SLV, single-locus variant; POCT, point-of-care testing.

[a] Room numbers have been changed to maintain patient anonymity, x>y indicates room transfers during the course of the outbreak.

[b] This isolate was recovered from the index case (Patient A) 1 year prior to the outbreak.

[c] All isolates recovered from patients were recovered from rectal swabs. Environmental isolates were recovered from pre-moistened FLOQSwabs (Copan Diagnostics Inc.) used to swab the area.

[d] Clinical breakpoints (with epidemiological cut-off value used for chloramphenicol) taken from the European Committee on Antimicrobial Susceptibility Testing guidelines [25].

[e] Thirty-seven of the 39 VREfm outbreak isolates were differentiated into four clusters (C1–C4) using cgMLST (Figure 2).

Bioquell Rapid Bio-Decontamination Unit (Bioquell Ireland Ltd, Limerick, Ireland).

Phenotypic and genotypic analysis

All isolates were tested for susceptibility to linezolid and vancomycin using the VITEK2 system (bioMérieux), and results were interpreted using the European Committee on Antimicrobial Susceptibility Testing interpretative criteria [25]. All VREfm and LVREfm isolates were referred to the National MRSA Reference Laboratory, where gradient strips (Etest, bioMérieux) were used to assess linezolid and chloramphenicol MICs. Polymerase chain reactions (PCRs) for identification of enterococcal species and detection of resistance genes (Table S1, see online supplementary material) were performed using GoTaq DNA polymerase and buffers (Promega Corp., Madison, WI, USA). One additional stored linezolid-susceptible VREfm isolate recovered in October 2018 from Patient A, who was deemed to be the index case in the current outbreak, was also investigated.

Whole-genome sequencing

Thirty-nine enterococcal isolates and selected transconjugant derivatives (Table I and Table S2, see online supplementary material) underwent WGS using genomic DNA extracted with the *S. aureus* Genotyping Kit 2.0 [Abbott (Alere Technologies GmbH), Jena, Germany] and the Qiagen DNeasy blood and tissue kit (Qiagen, Hilden, Germany) [24]. Libraries prepared with the Nextera DNA Flex Library Preparation Kit (Illumina, Inc., San Diego, CA, USA) underwent paired-end sequencing using the 500-cycle MiSeq Reagent Kit v2 (Illumina) [24]. Libraries were scaled to yield ≥50x coverage.

LVREfm isolate O_03 (Patient B) was selected for hybrid assembly to determine the genetic organization of an *optrA*-encoding conjugative plasmid it harboured. For this isolate, DNA was extracted using the Qiagen HMW MagAttract kit (Qiagen). Long-read sequencing was performed using MinION sequencing (Oxford Nanopore Technologies, Oxford, UK) using the one-dimensional genomic DNA sequencing kit (SQK-LSK109) and an MK1B (MIN101B) MinION platform with a FLO-MIN106D (SpotON R9.4) flow cell and MinKNOW v1.7.10 (Oxford Nanopore Technologies). Basecalls were performed on MinION FAST5 files using Guppy v3.1.5 (Oxford Nanopore Technologies), and demultiplexing was performed using qCat v1.0.1 (https://github.com/nanoporetech/qcat).

Analysis of WGS data

WGS data were analysed using the *E. faecium* whole genome MLST scheme available in BioNumerics v7.7 (Applied Maths, Sint-Martens-Latem, Belgium), with a filter applied to include only the 1423 core genome (cg) MLST loci [26]. Conventional MLST was also applied using Bionumerics to denote STs. Two BioNumerics algorithms were used to generate a consensus cgMLST profile for each isolate, one of which determined locus presence/absence and allelic identity using an assembly-free k-mer approach. The other assembly-based method used a BLAST approach to detect alleles on contigs assembled using SPAdes v3.7.1, all using default parameters. Minimum-spanning trees were created using BioNumerics based on cgMLST allelic differences. Illumina WGS data for all LVREfm were also

examined for 23S rRNA mutations (G2576T and G2505A) using LRE-finder (https://cge.cbs.dtu.dk/services/LRE-finder/). All isolates were also compared with an in-house database of 245 VREfm whole-genome sequences from isolates recovered in two other Dublin hospitals between September 2017 and October 2019. Data were stored in Ridom SeqSphere+ v6.0.0 and analysed using the *E. faecium* cgMLST scheme [26].

Hybrid assembly of an optrA-encoding plasmid

MinION- and MiSeq-generated FASTQ files were used to perform a hybrid assembly using UniCycler [27]. The genetic organization of the *optrA*-encoding plasmid pEfmO_03 from LVREfm outbreak isolate O_03 (Patient B) was determined and was annotated using RAST v2.0 (http://rast.nmpdr.org/). This was used as a reference sequence for further analysis against which MiSeq reads from other LVREfm were mapped, and percentage depth and breadth of coverage were calculated using Burrows–Wheeler aligner, Samtools and BedTools coverage [28–30]. The sequence of pEfmO_03 has been deposited in GenBank (Accession Number MT261365).

Conjugation

Conjugative transfer of plasmids encoding *optrA* harboured by LVREfm was undertaken by filter mating using the plasmid-free rifampicin- and fusidic acid-resistant recipient strains *E. faecium* 64/3 and *E. faecalis* OG1RF as described previously [31]. Putative transconjugants were screened for enterococcal species and *optrA* by PCR (Table S1, see online supplementary material). Transconjugants harbouring *optrA* underwent WGS, and genomes were assembled using SPAdes v3.7.1 and compared with the corresponding recipient strain genomes.

Results

Patient VREfm

Eight patients were found to be colonized with LVREfm over a 4-week period. The patients were located in or visited W1 and W2 (oncology), with one oncology patient located in W3 at the time of screening (Figure 1). A further nine patients yielded VREfm during a period of enhanced screening (Table I). Patient A, from whom the first LVREfm was recovered, had been admitted to the hospital's high-dependency unit and W1 during the previous month. The patient was discharged, and was re-admitted to W1 13 days later. Re-admission screens yielded LVREfm. A review of patients who had previously occupied the same bed as Patient A revealed no further VREfm. In addition, all VREfm isolates recovered over the previous year were reviewed on the VITEK2 system (bioMérieux) and no linezolid resistance was found. Patient H yielded both VREfm (O_37a) and LVREfm (O_37b) from their screening sample (Table I). A review of antimicrobial prescribing for each patient (Patients A–H) involved in the LVREfm outbreak revealed that only Patient A had received linezolid.

Environmental screening

Following sampling of 129 environmental sites throughout the hospital, with particular focus on W1, W2 and the x-ray department, 20 VREfm were recovered, including 14 LVREfm (Figure 1 and Table I). In W1, Room 12 yielded LVREfm 5 days after admission of Patient B, who also yielded LVREfm. The isolation and drug trolleys, which are moved throughout W1, also yielded LVREfm. The drug trolley in W1 yielded both VREfm (O_17a) and LVREfm (O_17b) (Table I). The treatment room, equipment storage room and consumables store room, all of which have a high volume of staff traffic, all yielded LVREfm. The family room on W1 also yielded LVREfm (Figure 1 and Table I). In W2, the sluice room, point-of-care-testing (POCT) machine, cleaners' store room and the isolation room all yielded LR-VREfm between 13 and 20 days after recovery of the first LVREfm from Patient A (index case). In the x-ray department, only Room 2 yielded LVREfm (Figure 1 and Table I).

Antimicrobial consumption

Analysis of hospital antimicrobial prescribing audit data revealed that linezolid consumption increased from 0.46 defined daily doses per 100 bed-days used (DDD/100 BDU) for Q4 2016 to 1.14 DDD/100 BDU by Q4 2019 (range 0.15–1.39 DDD/100 BDU). A steady increase in linezolid consumption was noted from Q2 2019 (1.04 DDD/100 BDU) to Q3 2019 (1.23 DDD/100 BDU). Consumption of other antimicrobials (vancomycin and daptomycin) also increased in Q3 2019, reflecting increased complexity of patients and increased numbers of patients colonized with MDROs. All prescriptions were deemed appropriate and compliant with the hospital's restricted antimicrobials policy.

WGS of isolates

In total, 37 of 39 VREfm investigated belonged to ST80; the remaining two isolates were a single-locus variant (SLV) of ST80. Twenty isolates (51.3%) were resistant to linezolid (Table I), all of which harboured *optrA*, but lacked *poxtA*, *cfr*, and the 23S rRNA G2576T or G2505A mutations. The remaining 19 VREfm lacked linezolid-resistance genes and were susceptible to linezolid (Table I). Thirty-seven VREfm differentiated into four clusters (C1–C4) using cgMLST (Figure 2). The majority of isolates (N=28) belonged to C1 and were closely related (average allelic difference of two, range 0–10). C1 consisted of ST80 (N=27) isolates and one isolate deemed to be an SLV of ST80, and consisted of a mixture of patient (N=12) and environmental (N=16) isolates. C1 also contained LVREfm isolate O_02, the first *optrA*-positive LVREfm outbreak isolate, recovered from the suspected index case (Patient A). A stored VREfm *optrA*-negative isolate (O_01) from Patient A recovered 1 year previously also clustered in C1 (Figure 1 and Table I). Isolates O_01 and O_02 exhibited three allelic differences. Two samples (Patient H and the W1 drug trolley) yielded pairs of *optrA*-positive LVREfm and *optrA*-negative VREfm isolates, all of which clustered in C1. O_17a (VREfm) and O_17b (LVREfm) from the drug trolley exhibited one allelic difference, whereas O_37a (VREfm) and O_37b (LVREfm) from Patient H were indistinguishable. C2–C4 consisted of *optrA*-negative ST80 VREfm and were deemed to be unrelated to C1 isolates with intracluster allelic differences of 57–388 (Figure 2). Further comparison with a database of sequencing reads from 245 VREfm recovered in two other Irish hospitals revealed that all C1 outbreak isolates grouped in a larger cluster of ST80 isolates. The majority of database isolates (146/245, 59.9%)

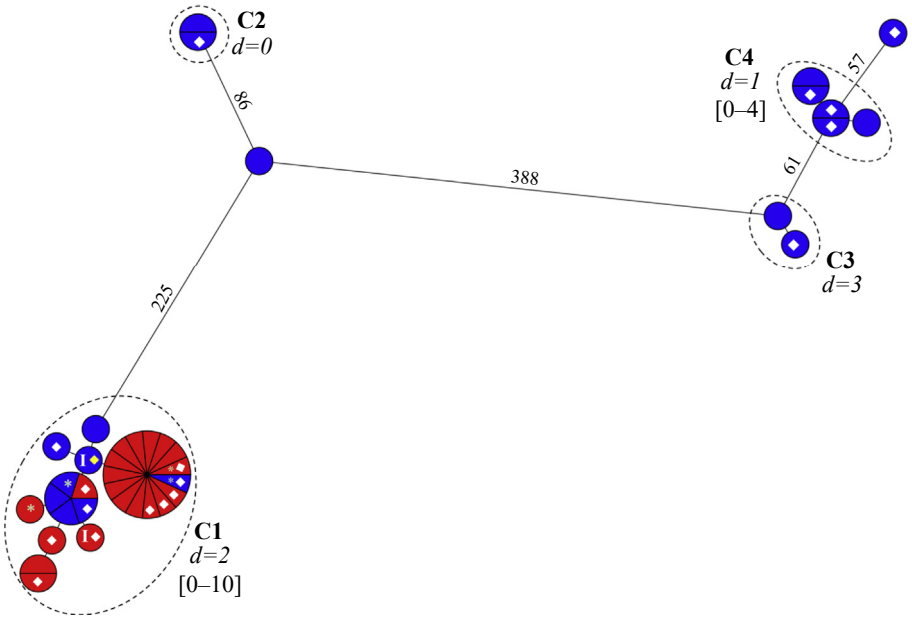

Figure 2. Minimum spanning tree based on core genome multi-locus sequence typing (cgMLST) data from the 39 ST80 vancomycin-resistant *Enterococcus faecium* (VREfm) isolates recovered from patient rectal screening swabs (19 isolates, 17 patients, denoted by a filled white diamond) and hospital environmental sites (20 isolates) during the hospital outbreak between 8th October and 1st November 2019. Twenty of the isolates were linezolid-resistant VREfm (LVREfm) and harboured *optrA*. Red circles indicate VREfm *optrA*-positive isolates, and blue circles indicate VREfm *optrA*-negative isolates. The first LVREfm outbreak isolate recovered from the suspected index patient in October 2019 is denoted by I. A stored linezolid-susceptible VREfm isolate lacking *optrA* recovered from the same patient 1 year earlier is denoted by a filled yellow diamond and an I. A green asterisk denotes pairs of isolates; one isolate of each pair was LVREfm (harbouring plasmid pEfmO_03) and the other isolate of each pair was VREfm (lacking plasmid pEfmO_03). Pairs of isolates included O_17a and O_17b recovered from a drug trolley, and O_37a and O_37b recovered from Patient H. The numbers on the branches represent the number of cgMLST allelic differences. Clusters of related isolates are encircled and labelled C1–C4. *d* indicates average allelic differences and the range is shown in square brackets.

belonged to ST80, which divided into 11 clusters and 26 singletons, with an intercluster allelic difference range of 25–257 (Figure S1, see online supplementary material). The large cluster, termed 'ST80 complex type 2933', consisted of the 28 C1 outbreak isolates and seven additional VREfm from another Dublin hospital (Hospital 2) recovered between March and October 2019. This cluster had an average allelic difference of three (range 0–15).

A plasmid encoding optrA

The WGS data of the LVREfm outbreak isolate O_03 (Patient B) underwent hybrid assembly, and a 56,684-bp plasmid (pEfmO_03) encoding *optrA* and the chloramphenicol resistance gene *fexA* was resolved. The *optrA* gene was flanked by *Tnp*A and *Tnp*B from *Tn*554, and by *IS*Efa15 (Figure S2, see online supplementary material). In total, 19/20 *optrA*-positive outbreak LR-VREfm harboured plasmids exhibiting ≥99.98% sequence coverage to pEfmO_03. The remaining isolate exhibited 44.1% sequence coverage to pEfmO_03, with 100% coverage across the entire *optrA* encoding region. Plasmid pEfmO_03 was conjugative; transconjugant derivatives of the *E. faecium* 64/3 recipient were obtained with four LVREfm isolates (O_03, O_04, O_13, O_23) and with the *E. faecalis* OG1RF recipient using the LVREfm isolate O_23 as donor (Table S2, see online supplementary material). All transconjugants harboured pEfmO_03 and were resistant to linezolid and chloramphenicol (MIC >4 and >32 mg/L, respectively) (Table S2, see online supplementary material).

Discussion

Linezolid-resistant enterococci harbouring acquired resistance genes have been reported with increasing frequency year on year since 2014 [18,19,24,31,32]. A recent Irish study described the highest prevalence of *optrA* and *poxtA* among LRE reported to date, with *optrA* identified in vancomycin-susceptible *E. faecalis* and *E. faecium* isolates with diverse genetic backgrounds. The *poxtA* gene was also identified in nine *E. faecium* isolates, including five LVREfm deemed to be unrelated by cgMLST, with isolates belonging to several STs (ST80, ST202, ST203 and ST1588) [24]. Previously, *optrA* was reported in four French VREfm recovered between 2013 and 2015, three of which were ST80 and one ST17 [32]. The present study represents the first reported hospital outbreak involving *optrA*-positive VREfm, with all isolates belonging to ST80 (or an SLV of ST80) of the hospital-adapted clade A1. All 28 outbreak isolates formed a single cgMLST cluster (C1), and all were highly related (average allelic difference of two, range 0–10) (Figure 2). The majority of C1 isolates (20/28) were LVREfm, and 19 of 20 harboured an 56,684-bp conjugative plasmid (pEfmO_03) encoding *optrA* and *fexA* (Figure S2, see online supplementary material). The remaining eight C1 isolates were

VREfm and lacked pEfmO_03, but were otherwise indistinguishable or very closely related to LVREfm.

An allelic difference ≤20 has been proposed previously as the threshold for determining *E. faecium* isolates as closely related based on cgMLST [26]. Interrogation of a WGS database of 245 VREfm isolates from two other Irish hospitals revealed that the majority of isolates belonged to ST80 (N=146), which further divided into 11 clusters, and 16 singletons, with 27 different complex types. All of the isolates in the outbreak cluster (C1) grouped into complex type 2933, along with seven VREfm from another Dublin hospital (Figure S1, see online supplementary material). These findings demonstrate that VREfm clones can persist over long periods and in different hospital locations, which has been reported previously [8,33]. The average allelic difference between isolates within complex type 2933 was three (range 0–15), showing the closely related nature of the outbreak isolates to isolates from another Dublin hospital. The frequent transfer of patients between hospitals in Ireland (especially in Dublin) could contribute to trafficking of individual strains between hospitals.

The first *optrA*-positive LVREfm outbreak isolate (O_02) recovered from the suspected index patient exhibited only three allelic differences to an *optrA*-negative VREfm (O_01) from the same patient 1 year earlier, indicating that the index patient harboured the same VREfm strain for 1 year. When this strain acquired the *optrA*-encoding plasmid pEfmO_03 was not determined; the patient had no animal/farm exposure and no source of *optrA* was identified in the hospital. The highly related nature of all isolates in C1, together with the finding of an identical *optrA*-encoding conjugative plasmid in all but one LVREfm outbreak isolate indicates the spread of a single strain over the 4-week outbreak period. The remaining LVREfm outbreak isolate (O_24) exhibited 44.1% sequence identity to pEfmO_03, with 100% coverage around the entire region surrounding *optrA* and *fexA*, suggesting the loss of some plasmid sequence. The findings of this study contrast with previous studies of LRE from Irish hospitals, which revealed the presence of the mobile linezolid resistance genes *optrA* and *poxtA* in enterococci with diverse genetic backgrounds [24]. During the present study, two samples (Patients H and the drug trolley on W1) yielded isolate pairs, each consisting of an *optrA*-postive LVREfm and an *optrA*-negative VREfm isolate. One pair of ST80 isolates (O_37a and O_37b), from Patient H, exhibited zero allelic differences and the other pair of ST80 isolates (O_17a and O_17b) exhibited one allelic difference. The *optrA*-positive isolate of each pair harboured pEfmO_03. These findings indicated the loss/gain of the pEfmO_03 plasmid in individual samples.

The suspected index patient (Patient A) had been treated with linezolid 4 weeks prior to recovery of the first LVREfm outbreak isolate in October 2019. No other patient involved in the outbreak had a history of linezolid treatment. Based on this, the close similarity of all the LVREfm outbreak isolates and the presence of an identical *optrA*-encoding plasmid (pEfmO_03) in 95% (19/20) of LVREfm strongly suggests that the outbreak was due to the recent transmission of LVREfm from Patient A, either by indirect contact with other patients via the hands of healthcare workers (HCWs) and/or by shedding of LVREfm into the hospital environment. Interestingly, pEfmO_03 was unique to this outbreak and showed minimal sequence identity (7.8–18.2%) to the *optrA* genetic environments, both chromosomal and plasmid, described previously in LRE from Ireland [24].

LVREfm environmental isolates in C1 were identified up to 20 days following initial recovery of the isolate from Patient A, even following enhanced environmental decontamination and increased awareness of the importance of hand hygiene among HCWs. Review of hand hygiene audit records revealed the hospital was compliant with national standards on hand hygiene and achieved >95% compliance. Nonetheless, extensive environmental screening also revealed that sites such as treatment and supply rooms harboured LVREfm. These findings highlight the critical importance of hand hygiene in hospitals, and highlight a significant need for ongoing improvements. The appointment of local hand hygiene champions may be beneficial in this regard. The implementation of enhanced IPC measures (improved cleaning of the environment, use of HPV decontamination, scheduling and recording of equipment cleaning, ceasing ward admissions and staff dedicated to W1) resulted in the rapid termination of the outbreak, which was deemed over 4 weeks after the last LVREfm patient isolate was recovered.

It is likely that linezolid usage was a contributory factor in the emergence of LVREfm in the outbreak hospital as linezolid consumption increased from 0.46 DDD/100 BDU in Q4 2016 to 1.14 DDD/100 BDU by Q4 2019. This increased linezolid usage reflected increased complexity of patients and colonization with MDROs. All prescriptions were deemed appropriate and compliant with restricted antimicrobials policy. This highlights the challenging requirement for more prudent antimicrobial treatment of medically complex patients harbouring MDROs. Finally, plasmid-encoded *optrA* has been reported previously in animal staphylococci [34,35]. It is worrying to consider the possibility that the *optrA*-encoding plasmid identified in the LVREfm isolates in this study may eventually transfer into staphylococci (e.g. MRSA), or indeed other enterococci, in the hospital environment, further limiting options for treating infections caused by these organisms.

In conclusion, WGS and epidemiological data analysis was central in the rapid identification and characterization of a clonal ST80 outbreak of LVREfm harbouring a 56,684-bp conjugative plasmid (pEfmO_03) encoding *optrA*. The team approach adopted in the management of this outbreak directed the rapid implementation of enhanced IPC measures, including early detection and aggressive environmental decontamination, which resulted in timely containment and termination of the outbreak.

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Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhin.2020.05.013.

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