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Emergence of an Australian-like *pstS*-null vancomycin resistant *Enterococcus faecium* clone in Scotland

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Abstract

Multi-locus sequencing typing (MLST) is widely used to monitor the phylogeny of microbial outbreaks. However, several strains of vancomycin-resistant *Enterococcus faecium* (VREfm) with a missing MLST locus (*pstS*) have recently emerged in Australia, with a few cases also reported in England. Here, we identified similarly distinct strains circulating in two neighbouring hospitals in Scotland. Whole genome sequencing of five VREfm strains isolated from these hospitals identified four *pstS*-null strains in both hospitals, while the fifth was multi-locus sequence type (ST) 262, which is the first documented in the UK. All five Scottish isolates had an insertion in the *tetM* gene, which is associated with increased susceptibility to tetracyclines, providing no other tetracycline-resistant gene is present. Such an insertion, which encompasses a *dfrG* gene and two currently uncharacterised genes, was additionally identified in all tested *vanA*-type *pstS*-null VREfm strains (5 English and 68 Australian). Phylogenetic comparison with other VREfm genomes indicates that the four *pstS*-null Scottish isolates sequenced in this study are more closely related to *pstS*-null strains from Australia rather than the English *pstS*-null isolates. Given how rapidly such *pstS*-null strains have expanded in Australia, the emergence of this clone in Scotland raises concerns for a potential outbreak.

Introduction

Vancomycin-resistant *Enterococcus* spp. (VRE) was first identified about three decades ago and has now become a major nosocomial pathogen. It typically infects immunocompromised patients and can cause endocarditis, bloodstream, urinary tract, and skin and skin structure infections [1]. VRE infections are generally more serious than those caused by vancomycin-susceptible enterococci, and are associated with higher mortality rates [2]. Among all VRE

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species, vancomycin-resistant *Enterococcus faecium* (VREfm) is responsible for the majority of hospital infections. VREfm has been recently listed as a high priority pathogen for research and development of new antibiotics by the World Health Organisation [3]. Various measures have been implemented to monitor the spread of VREfm infections, including multi-locus sequence typing (MLST); this relies on characterising the allelic profile of seven "house-keeping" genes, located in the *E. faecium* chromosome [4]. Although useful, this MLST scheme is of limited resolution to accurately capture the clonal type of isolated *E. faecium* strains [5–7]. Furthermore, VREfm isolates lacking the *pstS* MLST-gene locus have recently emerged in both England [7] and Australia [8].

Here, we have used whole genome sequencing to identify four VREfm isolates from two Scottish hospitals, which lack the *pstS* locus, and another Scottish VREfm isolate with a MLST profile that, to the best of our knowledge, has not been previously reported in the UK. Additionally, we provide information regarding their resistance profiles and epidemiology.

Materials and methods

VRE strains were isolated from five patients in two Scottish hospitals, between January to October 2017. These patients developed complications following either biliary or colonic surgery, and had been treated with various combinations of penicillin, amoxicillin, flucloxacillin, Tazocin, gentamicin, metronidazole and vancomycin during their hospital stay. The VRE strains were cultured on horse blood agar with single colonies transferred to Mueller Hinton broth (Oxoid) liquid. These were subsequently placed on Mueller Hinton agar. Single colony cultures of these were subsequently used for DNA isolation. Genomic DNA was extracted using an Isolate II genomic DNA kit (Bioline), using the manufacturer's instructions for difficult to lyse Gram-positive bacteria. DNA libraries were then prepared using the NEBNext Fast DNA Fragmentation and Library Prep Set for Ion Torrent (New England Biolabs): briefly, 1 μg of genomic DNA was fragmented, and Ion Xpess barcode adapters (Life Technologies) were ligated to the DNA fragments; after clean-up using Agencourt AMPure XP beads (Beckman Coulter), 400 bp target fragments were isolated following 18 min electrophoresis on E-gel SizeSelect agarose gels (Life Technologies); these were subsequently amplified by PCR and, following another clean-up with Agencourt AMPure XP beads, the quality of the resulting DNA libraries was assessed on a 2100 Bioanalyzer (Agilent Technologies), using high sensitivity DNA chips (Agilent Technologies).Template positive Ion Sphere particles (ISPs) for semiconductor sequencing were prepared using the Ion Touch 2 System (Life Technologies). Enriched ISPs were loaded into ion v2 BC 316 chips (2 genomes per chip) and sequenced on an Ion PGM system (Life Technologies). Low quality reads (quality score threshold: 0.05) were trimmed using CLC genomics Workbench (Qiagen, version 9.5.2), and resulting reads were assembled using SPAdes (St. Petersburg genome assembler, version 3.9). Contigs having less than 1000 bp sequences were discarded. The remaining contigs were reordered on Mauve (version 20150226) using the complete *E. faecium* Aus0004 genome [9] as reference, and resulting genome sequences were submitted to GenBank under the accessions PJZU00000000 (VREF001), PJZT00000000 (VREF002), PJZS00000000 (VREF003), PJZR00000000 (VREF 004) and PJZQ00000000 (VREF005). Antibiotic resistant genes in VREF001-5 genomes were predicted using the Resistance Gene Identifier tool within the comprehensive antibiotic resistance database (CARD) (https://card.mcmaster.ca/analyze/rgi). This tool utilizes algorithms for identification of perfect, strict or loose matches against resistant genes of the comprehensive antibiotic resistance database, based on homology and SNP models [10]. Sequence reads of *pstS*-null genomes from England and Australia were obtained from European Nucleotide Archive, and reads were assembled using CLC genomics Workbench; contigs having less than

200 bp were discarded. *In silico* MLST analysis was performed using the PubMLST website (http://pubmlst.org/) [11]. Alignments of genomes were done using the REALPHY (Reference sequence Alignment based Phylogeny builder) online tool (version 1.12) [12], with *E. faecium* Aus0004 [9], Aus0085 [13] as references, using default options (read length: 20, seed length: 22, polymorphism threshold: 0.95) and merging of reference alignments. All input genome sequences were assembled contigs in FASTA format and the two different reference genome alignments were combined to produce the resulting phylogenetic tree. Minimum inhibitory concentrations (MICs; μg/ml) for vancomycin, streptomycin, spectinomycin, tetracycline, oxytetracycline, doxycycline, minocycline and rifampicin were calculated using the microdilution method in cation adjusted Mueller-Hinton broth media (BD Biosciences), whereas MICs (μg/ml) for all other antibiotics were obtained using VITEK 2 (bioMerieux).

Results

The Scottish VRE strains sequenced (VREF001-5) had 2.9–3.0 Mb genomes with 37.6–37.7% GC content (Table 1). Analysis of average nucleotide identity and Tetra Correlation Search (TCS) against database genome sequences, using JSpeciesWS (version 3.0.12) [14], showed that all five VRE isolates were *E. faecium*. VREF001, VREF002, VREF004 and VREF005 were highly related with over 99.9% identity among aligned (>97%) sequences, whereas VREF003 displayed around 16% unique sequences compared with the other four genomes. *In silico* MLST analysis of VREF001-5 genomes identified VREF003 as sequence type (ST) 262, which has not been previously reported in the UK. The other four genomes had exact matches for six MLST alleles (*atpA*-9, *ddl*-1, *gdh*-1, *purK*-1, *gyd*-12, *adk*-1), but no match for *pstS*. This MLST profile has now been assigned as ST1424 in the *E. faecium* MLST database (https://pubmlst.org/efaecium/). However, despite the missing *pstS* in these four genomes, all VREfm strains sequenced in this study did have a *pstS* homologue within a *pst* operon (also referred to as *pstS2*), which is thought to be the actual *pstS* housekeeping gene in *E. faecium* [7,8].

We then searched for antibiotic resistance genes with perfect or strict matches to reference genes of the comprehensive antibiotic resistance database (CARD), but also considering loose matches with exceptional low e-value ($<10^{-100}$) and/or very high identity (>66%) to the reference gene. Analysis of the gene profiles of these five isolates identified the multi-aminoglycoside resistance gene *aac(6')-Ii*, in all five strains, and *aac(6')-aph(2'')*, in all but VREF004. The isolates had at least one macrolide-lincosamide-streptogramin resistance gene (*ermB*), a *pbp5* variant (designated *pbp5-R*) conferring resistance to β-lactams [15], for which no reference gene exists in CARD, and the vancomycin-teicoplanin resistance gene, *vanA*. With the exception of VREF003, all strains had the spectinomycin resistance gene *ant(9)-Ia*. Lastly, VREF003 was the only strain with a tetracycline transporter gene (*tetL*) (Table 2). It is notable that all isolates had two loose matches for the tetracycline resistance gene *tetM*. By further examining the

Table 1. Features of assembled sequences of the five isolates.

Isolate ID	VREF001	VREF002	VREF003	VREF004	VREF005
Accession	**SAMN08196781**	**SAMN08196782**	**SAMN08196783**	**SAMN08196784**	**SAMN08196785**
Size (bp)	2,987,950	2,990,107	2,913,585	2,999,027	2,976,182
GC Content (%)	37.6	37.6	37.7	37.6	37.6
N50	39407	36771	32649	43809	38134
L50	23	22	28	21	22
Contigs	185	185	188	184	190
Depth of coverage	194.91	131.85	170.17	182.45	218.18

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Table 2. MIC values (μg/ml) of selected antibiotics against the five Scottish VREfm isolates. Susceptibility to a given antibiotic is indicated in bold. ND: MIC value not determined. Resistant genes identified which correspond to strict or perfect matches against antibiotic resistance genes of the comprehensive antibiotic resistance database (CARD) are shown. Additionally, some loose matches (underlined) to CARD reference genes, which either had > 99% identity with reference CARD gene (*tetL*), or had zero e-value (*rpoB2*), or were the sole genes that could explain differences in antimicrobial susceptibility among the five isolates (*ant(9)-Ia*, *marA* and *mtrR*), are shown. The variant of a full-length penicillin-binding protein 5 gene conferring resistance to penicillins (*pbp5-R*), for which no reference gene in CARD exists, was found in all genomes. Superscripted numbers at top right of resistant genes (far-right column) correspond to the sequenced strains (last digit of isolate ID) carrying these genes.

		VREF001	VREF002	VREF003	VREF004	VREF005	Resistance genes
Beta-lactams	Amoxicillin	> 32	> 32	> 32	> 32	> 32	*pbp5-R* [1–5]
	Co-Amoxiclav	> 8	> 8	> 8	> 8	> 8	*pbp5-R* [1–5]
Glycopeptides	Vancomycin	64	512	> 512	512	128	*vanA* [1–5], *vanHA* [1–3, 5], *vanRA* [1–5], *vanSA* [1–4], *vanXA* [1–5], *vanYA* [2–4], *vanZA* [3]
	Teicoplanin	> 32	> 32	> 32	> 32	4	*vanA* [1–5], *vanHA* [1–3, 5], *vanRA* [1–5], *vanSA* [1–4], *vanXA* [1–5], *vanYA* [2–4], *vanZA* [3]
Fluoroquinolones	Ciprofloxacin	> 4	> 4	> 4	> 4	ND	*efmA* [1–5]
Aminoglycosides	Gentamicin	> 128	> 128	> 128	> 128	> 128	*aac(6′)-aph(2″)* [1–3, 5]
	Kanamycin	> 128	> 128	> 128	> 128	> 128	*aac(6′)-aph(2″)* [1–3, 5], *aph(3′)-IIIa* [1–2, 4–5], *aac (6′)-Ii* [1–5]
	Streptomycin	32	32	32	32	32	None
	Spectinomycin	512	512	32	> 512	512	*ant(9)-Ia* [1–2, 4–5]
Tetracyclines	Tetracycline	**≤ 0.25**	**≤ 0.25**	> 128	**≤ 0.25**	**≤ 0.25**	*tetL* [3]
	Oxytetracycline	**1**	**≤ 0.25**	> 128	**0.5**	**≤ 0.25**	*tetL* [3]
	Doxycycline	**≤ 0.25**	**≤ 0.25**	16	**≤ 0.25**	**≤ 0.25**	*tetL* [3]
	Minocycline	**≤ 0.13**	**≤ 0.13**	16	**≤ 0.13**	**≤ 0.13**	*tetL* [3]
	Tigecycline	**≤ 0.13**	**≤ 0.13**	**≤ 0.13**	**≤ 0.13**	**≤ 0.13**	None
Macrolides, Lincosamides, Streptogramins	Clarithromycin	> 4	> 4	> 4	> 4	> 4	*ermA* [1–2, 4–5], *ermB* [1–5]
	Quinupristin-Dalfopristin	**0.5**	**0.5**	**0.5**	**1**	**0.5**	None
Others	Chloramphenicol	**8**	**8**	**≤ 4**	**8**	**8**	None
	Linezolid	**2**	**2**	**2**	**2**	**2**	None
	Nitrofurantoin	128	256	128	256	256	Unknown
	Trimethoprim	> 16	> 16	> 16	> 16	> 16	*dfrG* [1–5]
	Rifampicin	16	16	**1**	16	16	*rpoB2* [1–5], ***marA*** [1-2. 4–5], ***mtrR*** [1-2. 4–5]

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region where these *tetM*-like sequences are located, we found that the five genomes had a *Tn5801*-like transposon (full-length or fragment) containing a sequence similar to *Staphylococcus aureus* Mu50 *tetM* gene [16], but with a 3,229 bp insertion. The trimethoprim-resistance gene *dfrG* and two more genes with unknown function were located within this insertion. A similar insertion has been reported previously in some ST17 and ST18 *E. faecium* strains [16] which is thought to result in defective protection against tetracyclines [17]. Lastly, all strains had a homologue of the *E. faecalis IsaA* gene which confers resistance to quinupristin-dalfopristin. However, the product of this gene (known as EatA), unlike the >99% identical $EatA_v$ product found in some *E. faecium* strains, has a threonine at position 450, which is associated with increased susceptibility towards quinupristin-dalfopristin [18,19].

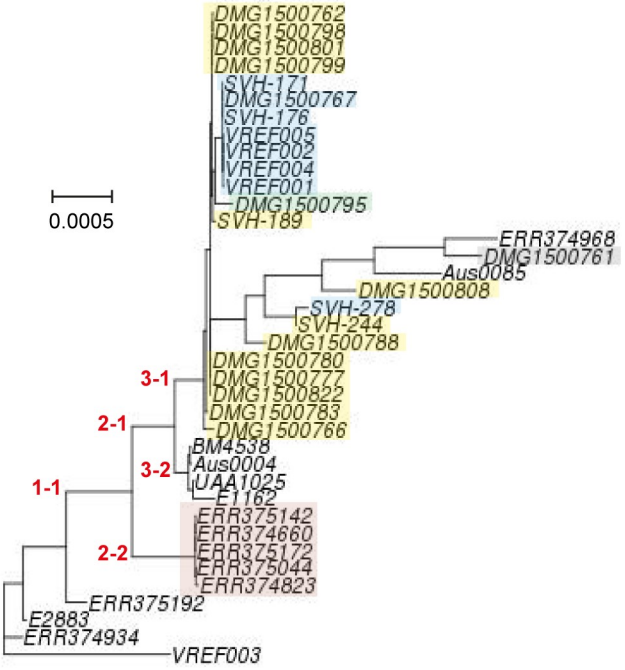
All strains exhibited high level resistance to vancomycin; 4/5 had high level resistance to teicoplanin, associated with the presence of *vanSA* (absent from VREF005, which displayed low level resistance) and 4/5 had high level resistance to spectinomycin (apart from VREF003 which displayed low level resistance). The strains had low level resistance to streptomycin, as well as resistance to a variety of other drugs. The low level resistance to streptomycin and spectinomycin, in the absence of any gene coding for resistance to those antibiotics, is due to decreased uptake of these drugs by *E. faecium* [20]. All isolates were susceptible to linezolid,

chloramphenicol, tigecycline and Synercid (quinupristin-dalfopristin). VREF003 was the only isolate found to be resistant to tetracycline, doxycycline, oxytetracycline and minocycline, but unlike the other four isolates, it was susceptible to rifampicin (Table 2). Since all five isolates had an identical *rpoB2* variant (assumed to exert some level of rifampicin-resistance), the decreased resistance of VREF003 towards rifampicin, compared to the other four strains, may be due to the lack of any *mtrR* and *marA* homologues in the VREF003 genome. Conversely, loose matches of these were present in the other four isolates, and such genes have been shown to confer resistance to multiple drugs in other bacteria, including rifampicin [21,22]. Alternatively, VREF001-2 and VREF004-5 may contain an *mtrR*-independent mechanism of reduced permeability for rifampicin compared with VREF003 [23]. Antibiotic susceptibility tests indicated that all five isolates are classified as multi-drug resistant enterococci, according to standardised international terminology [24].

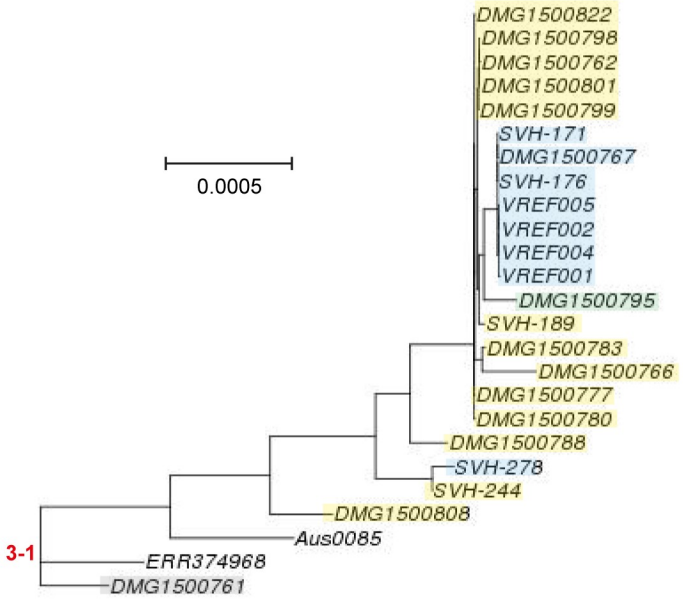
We then performed a phylogenetic comparison of VREF001-5 genomes with other known VREfm genomes reported to have a missing *pstS* locus; these included: 14 (out of 66 reported) Australian strains isolated from 9 different hospitals between 2014 and 2015 [8], 5 Australian strains (out of 202 reported within 6 local health districts) isolated from 2 different local health districts (LHD-1 and LHD-2) in New South Wales during 2016 [25], and all five English *pstS*-null *E. faecium* strains isolated from a single hospital (Kathy Raven, personal communication) in 2005 [7]. In our phylogenetic analysis we also included: five hospital-associated isolates of different MLST sequence type each, three isolates (BM4538, UAA1025 and E2883) previously reported to have an insertion in the *tetM*-locus [16] and the first complete *E. faecium* genome, Aus0004 [9]. The 14 Australian *pstS*-null genomes were carefully selected to represent *pstS*-null isolates from the 66 totally reported across Australia by Carter and co-workers [8], covering all hospitals, health jurisdictions and importantly, phylogenetically distinct clades.

This analysis indicated that Scottish *pstS*-null isolates are most closely related to Australian *pstS*-null isolates (Fig 1A; subclade 3–1), and particularly to certain ST1424 clones isolated from New South Wales (hospital 3 and Local Health District 1). Due to the existence of long branches within subclade 3–1 (Fig 1A), the phylogeny of that subclade was further refined by analysis of genomes belonging only to this subclade [12] (Fig 1B). Within this subclade, all ST1421, ST1422 and ST1424 clones were found to be closely related, whereas the sole ST1423 clone (*vanB* type DMG1500761) was found to diverge the most among all Australian and Scottish *pstS*-null strains (Fig 1A–1C). English *pstS*-null isolates, which are all ST1477, all cluster together (Fig 1A; subclade 2–2) and are distinct form Australian and Scottish *pstS*-null strains. Nevertheless, all *pstS*-null clones appear to be related to ST17 clones (Fig 1A; subclade 3–2 and Fig 1C), which suggests that the former may have derived from ST17 strains. Most of the Australian *pstS*-null genomes we analysed were ST1421 (Fig 1C), in agreement with recent data showing that ST1421 is the most common and widespread *pstS*-null VRE strain in Australia, accounting for more than 70% of cases [25,26]. Since both Australian [8] and Scottish (this study) VREfm *pstS*-null strains were found in different hospitals (Fig 1C), it is likely that such clones have spread in the community. Our phylogenetic analysis further supports an intercontinental ST1424 clone spread between hospitals of New South Wales and Scotland (Fig 1). On the other hand, all English VREfm strains with missing *pstS* locus [7] cluster together and are likely to have spread within the hospital from which they were isolated. VREF003 is more phylogenetically related to ST18 *E. faecium* isolates (Fig 1A), in agreement with a previous study showing a phylogenetic relationship between ST262 and ST18 *E. faecium* strains [27]. Unlike the sole pstS-null *vanB* type VREfm which had an intact *tetM* gene sequence in its genome, all the *pstS*-null *vanA* type VREfm strains included in our phylogenetic analysis (4/4 Scottish, 18/18 Australian and 5/5 English) (Fig 1C) had a *Tn5801*-like transposon with an insertion in their *tetM* locus. To identify how common this insertion is, we additionally tested the

A.



B.



C.

		MLST alleles											
Isolate ID	**ST**	**atpA**	**ddl**	**gdh**	**purK**	**gyd**	**pstS**	**adk**	**Origin**	**Hospital**	***tetM*- insertion**	***van*- genotype**	**Year**
Aus0004	17	1	1	1	1	1	1	1	Australia	Unknown	YES	*van* B	1998
Aus0085	203	15	1	1	1	1	20	1	Australia	Unknown	NO**	*van* B	2009
BM4538	17	1	1	1	1	1	1	1	Australia	Unknown	YES	*van* B	2004
DMG1500761	1423	4	1	1	1	1	0	1	Australia	Unknown	NO	*van* B	2014
DMG1500762	1421	1	1	1	1	1	0	1	Australia	3	YES	*van* A	2014
DMG1500766	1421	1	1	1	1	1	0	1	Australia	1	YES	*van* A	2015
DMG1500767	1424	9	1	1	1	12	0	1	Australia	3	YES	*van* A	2015
DMG1500777	1421	1	1	1	1	1	0	1	Australia	1	YES	*van* A	2015
DMG1500780	1421	1	1	1	1	1	0	1	Australia	5	YES	*van* A	2015
DMG1500783	1421	1	1	1	1	1	0	1	Australia	4	YES	*van* A	2015
DMG1500788	1421	1	1	1	1	1	0	1	Australia	6	YES	*van* A	2015
DMG1500795	1422	1	1	1	1	12	0	1	Australia	3	YES	*van* A	2015
DMG1500798	1421	1	1	1	1	1	0	1	Australia	3	YES	*van* A	2015
DMG1500799	1421	1	1	1	1	1	0	1	Australia	9	YES	*van* A	2014
DMG1500801	1421	1	1	1	1	1	0	1	Australia	8	YES	*van* A	2014
DMG1500808	1421	1	1	1	1	1	0	1	Australia	2	YES	*van* A	2015
DMG1500822	1421	1	1	1	1	1	0	1	Australia	7	YES	*van* A	2015
E1162	17	1	1	1	1	1	1	1	France	Unknown	NO	negative	1997
E2883	18	7	1	1	1	5	1	1	Netherlands	Unknown	YES	negative	2002
ERR374660	1477	7	2	1	1	1	0	1	England	X	YES	*van* A	2005
ERR374823	1477	7	2	1	1	1	0	1	England	X	YES	*van* A	2005
ERR374934	18	7	1	1	1	5	1	1	UK	Unknown	YES	*van* A	2009
ERR374968	78	15	1	1	1	1	1	1	UK	Unknown	N/A	*van* A	2009
ERR375044	1477	7	2	1	1	1	0	1	England	X	YES	*van* A	2005
ERR375142	1477	7	2	1	1	1	0	1	England	X	YES	*van* A	2004
ERR375172	1477	7	2	1	1	1	0	1	England	X	YES	*van* A	2004
ERR375192	80	9	1	1	1	12	1	1	UK	Unknown	YES	*van* A	2011
SVH-171	1424	9	1	1	1	12	0	1	Australia	LHD-1*	YES	*van* A	2016
SVH-176	1424	9	1	1	1	12	0	1	Australia	LHD-1*	YES	*van* A	2016
SVH-189	1421	1	1	1	1	1	0	1	Australia	LHD-1*	YES	*van* A	2016
SVH-244	1421	1	1	1	1	1	0	1	Australia	LHD-5*	YES	*van* A	2016
SVH-278	1424	9	1	1	1	12	0	1	Australia	LHD-5*	YES	*van* A	2016
UAA1025	17	1	1	1	1	1	1	1	France	Unknown	YES	*van* A	1996
VREF001	1424	9	1	1	1	12	0	1	Scotland	W	YES	*van* A	2017
VREF002	1424	9	1	1	1	12	0	1	Scotland	H	YES	*van* A	2017
VREF003	262	7	1	1	1	5	7	1	Scotland	H	YES	*van* A	2017
VREF004	1424	9	1	1	1	12	0	1	Scotland	H	YES	*van* A	2017
VREF005	1424	9	1	1	1	12	0	1	Scotland	H	YES	*van* A	2017

Fig 1. Scottish *pstS*-null VREfm isolates are related to *vanA*-type Australian *pstS*-null VREfm isolates. (A) Phylogenetic tree of selected *E. faecium* genomes, constructed using the REALPHY (Reference sequence Alignment based Phylogeny builder) online tool, with Aus0004 and Aus0085 used as reference genomes. *pstS*-null genomes are present in subclades 2–2 (English isolates) and 3–1 (Australian and Scottish isolates), (B) The phylogeny of strains clustering in subclade 3–1 (A) was further refined using REALPHY with Aus0085 and ERR374968 as references. (C) The MLST sequence type (ST) and alleles, country of origin, *van* genotype, hospital and year of isolation are given for each sequenced genome. * Only the Local Health District (LHD) is known for Australian strains isolated in New South Wales hospitals during 2016. Hospitals 3 and 9 are also in New South Wales jurisdiction, but their LHD is not known. Information is given on whether an insertion encompassing *dfrG* and two uncharacterised genes was found within *tetM* in these genomes. N/A: not applicable (no *tetM* sequences found). ** Although no such insertion was identified in Aus0085, there was a frameshift mutation within its *tetM* gene. *pstS*-null genomes of same ST are highlighted with the same colour.

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remaining *pstS*-null genomes (50 *vanA*-type and one *van*-negative) from the ones reported by Carter et al. All these, without exception, were identified to be ST1421 and to have the same *tetM* insertion within a *Tn5801*-like transposon. Such insertion, which has been previously documented in the MLST-typeable Aus0004, BM4538, E2883 and UAA1025 [16] (also included in our phylogenetic analysis), was further observed in VREF003, ERR374934 and ERR374968. Collectively, it appears that the *tetM* insertion in VREfm is strongly associated with *vanA* type strains missing *pstS* (STs: 1421, 1422, 1424 and 1477), but may also be found in some VREfms with STs: 17, 18, 80 and 262 (Fig 1C).

Discussion

MLST analysis is inferior to whole genome sequence analysis for outbreak investigations of VREfm [7], due to the high rate of recombination events occurring within the *E. faecium* chromosome that cannot be captured adequately by MLST analysis [27,28]. Furthermore, the emergence of non-typeable VREfm strains poses an extra obstacle in implementing the MLST scheme for VREfm phylogenetic analysis. Non-typeable VREfm strains have recently emerged and have been shown to be very rare in the UK. In a study encompassing whole genome sequencing of nearly 500 *E. faecium* healthcare-associated isolates from 2001–2011 in the UK and Ireland, there were only five cases of *pstS*-null VRE strains, all of which were isolated from a single English hospital, between 2004 and 2005 [7].The emergence of such strains was even more recent in Australia: the first two strains were isolated in 2013, but numbers increased rapidly to a total of 89 cases, by the end of 2015 [8,29,30] and to about 300 cases, by the end of 2016 [25,30,31].

Here, we report four additional non-typeable VREfm strains isolated in 2017, which are the first identified in Scotland. Their striking genomic similarity, combined with the fact that one was isolated from a different hospital, indicates that, although deriving from a single strain type, they are not necessarily hospital-acquired, but may be health-care associated, or spread within the community. Although transmission may have occurred through the frequent transfer of equipment, specimens, staff and patients between these two hospitals (located in the same health board just 14 miles apart), evidence suggests that VRE strains are also transmitted outside hospitals, such as in long term care facilities [6]. Additionally, VRE clones can disperse outside health-care facilities, since strains can be carried in patients for long periods, ranging from a few months to a few years following discharge from hospital [32,33]. For the above reasons VRE infections may spread over long periods and across long distances. Indeed, our phylogenetic analysis is indicative of an inter-continental spread between Australia and Scotland: the closest phylogenetic relationships were identified among Australian and Scottish ST1424 clones. Such six-locus sequence type has been identified in 28.7% (58/202) of the recently sequenced *pstS*-null VREfm strains from New South Wales, Australia [25]. In addition, the spread of such strains in New South Wales is also suggestive of inter-hospital transmission for this VREfm clone [25]. Even within the same hospital, VRE transmission routes can be prolonged and complex with closely related VRE strains re-appearing in the same ward after

several months, or transmitted to patients located in different wards [34]. We also observed re-appearance of a *pstS*-null strain (isolate VREF005) in the same hospital, seven months after discharge of the last carrier patient.

With the exception of one vancomycin-susceptible and one *vanB* type strain, all other (69) previously sequenced *pstS*-null *E. faecium* strains have been found to be *vanA* type [7,8]. Consistent with these studies, the newly sequenced Scottish VREfm strains with the missing *pstS* locus were also *vanA* type. In fact, the vast majority (84.2%) of all sequenced *vanA E. faecium* 2016 isolates from New South Wales, have been identified as *pstS*-null strains [25]. Hence, *pstS*-null VREfm appears to be a very successful *vanA* clone. We further identified that loss of *pstS* in *vanA* type VREfm is strongly associated with an insertion in a *tetM* gene (74/74 cases tested). Such insertion was present in all ST4121, ST1422 and ST1424 strains tested, isolated from around a dozen hospitals across Australia (New South Wales, Victoria and Australian Capital Territory) and the UK (England and Scotland), and was only absent in the sole *vanB* type *pstS*-null *E. faecium* strain identified. Although we cannot exclude the possibility that *vanA*-type *pstS*-null members with an intact or no *tetM* sequence exist (the vast majority of the recently isolated ST1421 and ST1424 stains from New South Wales were excluded in our analysis), our data strongly suggest that the *Tn5801*-like transposon with the *tetM* insertion is an extremely common feature of *vanA*-type *pstS*-null strains. How strongly associated these two features are (missing *pstS* and *tetM* insertion), why they were co-selected in numerous *vanA* type VREs and what is the significance of this association, are issues that need to be investigated further. The insertion within the *tetM* gene apparently leads to a mutant TetM protein which is unable to confer resistance towards tetracyclines, as it can be inferred from relevant susceptibility tests against tetracycline, doxycycline and minocycline, using Aus0004 [17], VREF001-2 and VREF004-5 (this study). All sequenced isolates were resistant to trimethoprim, and the cause of such resistance is most likely the *dfrG* gene inserted into the *tetM* locus. None of the isolates sequenced contained a gene for tigecycline, Synercid, or linezolid resistance, and as a consequence they were susceptible to these three drugs. Since these antibiotics are frequently used for management of multi-drug-resistant enterococcal infections [1,35], they may still be the favourable option for combating VREfm strains with a missing *pstS* locus.

The emergence of *pstS*-null VREfm strains in Scotland raises concerns for a potential outbreak. These strains were found to be highly similar to Australian *pstS*-null strains, which have very rapidly expanded to become the leading strain in Australia (2016). They are causing infections in numerous hospitals and in different health jurisdictions [8,25,26]. Furthermore, the missing *pstS* locus of such strains renders the—limited in resolution—MLST analysis even less suitable for VREfm outbreak investigations. Hence, our study, like previous ones [7,8,25], highlights the need for whole genome sequencing approaches in monitoring VREfm epidemiology.

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References

1. O'Driscoll T, Crank CW, O'Driscoll T, Crank CW. Vancomycin-resistant enterococcal infections: Epidemiology, clinical manifestations, and optimal management. Infect Drug Resist. 2015; 8: 217–230. https://doi.org/10.2147/IDR.S54125 PMID: 26244026
2. Orsi GB, Ciorba V. Vancomycin resistant enterococci healthcare associated infections. Ann Ig. 2013; 25: 485–492. https://doi.org/10.7416/ai.2013.1948 PMID: 24284534
3. WHO. Prioritization of pathogens to guide discovery, research and development of new antibiotics for drug resistant bacterial infections, including tuberculosis [Internet]. Essential medicines and health products. 2017. WHO reference number: WHO/EMP/IAU/2017.12
4. Homan WL, Tribe D, Poznanski S, Li M, Hogg G, Spalburg E, et al. Multilocus sequence typing scheme for Enterococcus faecium. J Clin Microbiol. 2002; 40: 1963–71. https://doi.org/10.1128/JCM.40.6.1963-1971.2002 PMID: 12037049
5. Bonten M, Corander J, Pinholt M, van Schaik W, Westh H, Bletz S, et al. Core Genome Multilocus Sequence Typing Scheme for High-Resolution Typing of Enterococcus faecium. J Clin Microbiol. 2015; 53: 3788–3797. https://doi.org/10.1128/JCM.01946-15 PMID: 26400782
6. Brodrick HJ, Raven KE, Harrison EM, Blane B, Reuter S, Török ME, et al. Whole-genome sequencing reveals transmission of vancomycin-resistant Enterococcus faecium in a healthcare network. Genome Med. 2016; 8: 4. https://doi.org/10.1186/s13073-015-0259-7 PMID: 26759031
7. Raven KE, Reuter S, Reynolds R, Brodrick HJ, Russell JE, Török ME, et al. A decade of genomic history for healthcare-associated Enterococcus faecium in the United Kingdom and Ireland. Genome Res. 2016; 26: 1388–1396. https://doi.org/10.1101/gr.204024.116 PMID: 27527616
8. Carter GP, Buultjens AH, Ballard SA, Baines SL, Tomita T, Strachan J, et al. Emergence of endemic MLST non-typeable vancomycin-resistant Enterococcus faecium. J Antimicrob Chemother. 2016; 71: 3367–3371. https://doi.org/10.1093/jac/dkw314 PMID: 27530751
9. Lam MMC, Seemann T, Bulach DM, Gladman SL, Chen H, Haring V, et al. Comparative Analysis of the First Complete Enterococcus faecium Genome. J Bacteriol. 2012; 194: 2334–2341. https://doi.org/10.1128/JB.00259-12 PMID: 22366422
10. McArthur AG, Waglechner N, Nizam F, Yan A, Azad MA, Baylay AJ, et al. The Comprehensive Antibiotic Resistance Database. Antimicrob Agents Chemother. 2013; 57: 3348–3357. https://doi.org/10.1128/AAC.00419-13 PMID: 23650175
11. Jolley KA, Maiden MCJ. BIGSdb: Scalable analysis of bacterial genome variation at the population level. BMC Bioinformatics. 2010; 11: 595. https://doi.org/10.1186/1471-2105-11-595 PMID: 21143983
12. Bertels F, Silander OK, Pachkov M, Rainey PB, Van Nimwegen E. Automated reconstruction of whole-genome phylogenies from short-sequence reads. Mol Biol Evol. 2014; 31: 1077–1088. https://doi.org/10.1093/molbev/msu088 PMID: 24600054
13. Lam MMC, Seemann T, Tobias NJ, Chen H, Haring V, Moore RJ, et al. Comparative analysis of the complete genome of an epidemic hospital sequence type 203 clone of vancomycin-resistant Enterococcus faecium. BMC Genomics. 2013; 14: 595. https://doi.org/10.1186/1471-2164-14-595 PMID: 24004955
14. Richter M, Rosselló-Móra R, Oliver Glöckner F, Peplies J. JSpeciesWS: A web server for prokaryotic species circumscription based on pairwise genome comparison. Bioinformatics. 2016; 32: 929–931. https://doi.org/10.1093/bioinformatics/btv681 PMID: 26576653
15. Montealegre MC, Roh JH, Rae M, Davlieva MG, Singh K V, Shamoo Y, et al. Differential Penicillin-Binding Protein 5 (PBP5) levels in the enterococcus faecium clades with different levels of ampicillin resistance. Antimicrob Agents Chemother. 2017; 61: e02034–16. https://doi.org/10.1128/AAC.02034-16 PMID: 27821450

16. León-Sampedro R, Novais C, Peixe L, Baquero F, Coque TM. Diversity and evolution of the Tn5801-tet(M)-like integrative and conjugative elements among Enterococcus, streptococcus, and Staphylococcus. Antimicrob Agents Chemother. 2016; 60: 1736–1746. https://doi.org/10.1128/AAC.01864-15 PMID: 26729505

17. Cattoir V, Isnard C, Cosquer T, Odhiambo A, Bucquet F, Guérin F, et al. Genomic analysis of reduced susceptibility to Tigecycline in Enterococcus faecium. Antimicrob Agents Chemother. 2015; 59: 239–244. https://doi.org/10.1128/AAC.04174-14 PMID: 25348531

18. Singh K V., Weinstock GM, Murray BE. An Enterococcus faecalis ABC homologue (Lsa) is required for the resistance of this species to clindamycin and quinupristin-dalfopristin. Antimicrob Agents Chemother. 2002; 46: 1845–1850. https://doi.org/10.1128/AAC.46.6.1845-1850.2002 PMID: 12019099

19. Isnard C, Malbruny B, Leclercq R, Cattoira V. Genetic basis for in vitro and in vivo resistance to lincosamides, streptogramins a, and pleuromutilins (LSAP Phenotype) in Enterococcus faecium. Antimicrob Agents Chemother. 2013; 57: 4463–4469. https://doi.org/10.1128/AAC.01030-13 PMID: 23836170

20. Arias CA, Murray BE. The rise of the Enterococcus: Beyond vancomycin resistance. Nature Reviews Microbiology. 2012. https://doi.org/10.1038/nrmicro2761 PMID: 22421879

21. Cohen SP, McMurry LM, Levy SB. marA locus causes decreased expression of OmpF porin in multiple-antibiotic-resistant (Mar) mutants of Escherichia coli. J Bacteriol. 1988; 170: 5416–5422. https://doi.org/10.1128/jb.170.12.5416-5422.1988 PMID: 2848006

22. Hagman KE, Pan W, Spratt BG, Balthazar JT, Judd RC, Shafer WM. Resistance of Neisseria gonorrhoeae to antimicrobial hydrophobic agents is modulated by the rntrRCDE efflux system. Microbiology. 1995; 141: 611–622. https://doi.org/10.1099/13500872-141-3-611 PMID: 7711899

23. Abadi FJ, Carter PE, Cash P, Pennington TH. Rifampin resistance in Neisseria meningitidis due to alterations in membrane permeability. Antimicrob Agents Chemother. 1996; 40: 646–51. PMID: 8851587

24. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect. 2012; 18: 268–281. https://doi.org/10.1111/j.1469-0691.2011.03570.x PMID: 21793988

25. van Hal SJ, Beukers AG, Timms VJ, Ellem JA, Taylor P, Maley MW, et al. Relentless spread and adaptation of non-typeable vanA vancomycin-resistant Enterococcus faecium: a genome-wide investigation. J Antimicrob Chemother. 2018; 1487–1491. https://doi.org/10.1093/jac/dky074 PMID: 29566173

26. Coombs G, Daley D, on behalf of The Australian Group on Antimicrobial Resistance. Australian Enterococcal Sepsis Outcome Program (AESOP). 2016 Final Rep. 2017; 1–41. Available: http://www.agargroup.org/files/AESOP2016FinalReport2017.pdf

27. van Hal SJ, Ip CLC, Ansari MA, Wilson DJ, Espedido BA, Jensen SO, et al. Evolutionary dynamics of Enterococcus faecium reveals complex genomic relationships between isolates with independent emergence of vancomycin resistance. Microb Genomics. 2015; 2: 48. https://doi.org/10.1099/mgen.0.000048 PMID: 27713836

28. Howden BP, Holt KE, Lam MMC, Seemann T, Ballard S, Coombs GW, et al. Genomic insights to control the emergence of vancomycin-resistant enterococci. MBio. 2013; 4: e00412–13. https://doi.org/10.1128/mBio.00412-13 PMID: 23943759

29. Lee RS, Gonçalves Da Silva A, Baines SL, Strachan J, Ballard S, Carter GP, et al. The changing landscape of vancomycin-resistant Enterococcus faecium in Australia: A population-level genomic study. J Antimicrob Chemother. 2018; 73: 3268–3278. https://doi.org/10.1093/jac/dky331 PMID: 30189014

30. Leong KWC, Cooley LA, Anderson TL, Gautam SS, McEwan B, Wells A, et al. Emergence of Vancomycin-Resistant Enterococcus faecium at an Australian Hospital: A Whole Genome Sequencing Analysis. Sci Rep. 2018; 8: 6274. https://doi.org/10.1038/s41598-018-24614-6 PMID: 29674657

31. Leong KWC, Cooley LA, O'Toole RF. Draft Genome Sequence of New Vancomycin-Resistant Enterococcus faecium Sequence Type 1421. Genome Announc. 2018; 6: e00438–18. https://doi.org/10.1128/genomeA.00438-18 PMID: 29773638

32. Sohn KM, Peck KR, Joo E-J, Ha YE, Kang C-I, Chung DR, et al. Duration of colonization and risk factors for prolonged carriage of vancomycin-resistant enterococci after discharge from the hospital. Int J Infect Dis. 2013; 17: e240–e246. https://doi.org/10.1016/j.ijid.2012.09.019 PMID: 23195640

33. Karki S, Land G, Aitchison S, Kennon J, Johnson PDR, Ballard SA, et al. Long-term carriage of vancomycin-resistant enterococci in patients discharged from hospitals: a 12-year retrospective cohort study. J Clin Microbiol. 2013; 51: 3374–9. https://doi.org/10.1128/JCM.01501-13 PMID: 23926167

34. Raven KE, Gouliouris T, Brodrick H, Coll F, Brown NM, Reynolds R, et al. Complex Routes of Nosocomial Vancomycin-Resistant Enterococcus faecium Transmission Revealed by Genome Sequencing. Clin Infect Dis. 2017; 64: 886–893. https://doi.org/10.1093/cid/ciw872 PMID: 28362945

35. Arias CA, Contreras GA, Murray BE. Management of Multi-Drug Resistant Enterococcal Infections. Clin Microbiol Infect. 2010; 16: 555–62. https://doi.org/10.1111/j.1469-0691.2010.03214.x PMID: 20569266