Genomic Epidemiology of VREfm in Victoria

The changing landscape of VREfm in Victoria, Australia: a State-wide genomic snapshot

Robyn S. Lee^{a#}, Anders Gonçalves da Silva^a, Sarah L. Baines^b, Janet Strachan^a, Susan Ballard^a, Glen P. Carter^a, Jason C. Kwong^b, Mark Schultz^a, Dieter Bulach^a, Torsten Seemann^c, Timothy P. Stinear^b and Benjamin P. Howden^{a#}

The Microbiological Diagnostic Unit Public Health Laboratory, Department of Microbiology and Immunology, The University of Melbourne, at the Peter Doherty Institute for Infection and Immunity, Victoria, Australia ^a; Department of Microbiology and Immunology, The University of Melbourne, at the Peter Doherty Institute for Infection and Immunity, Victoria, Australia ^b; Melbourne Bioinformatics, The University of Melbourne, Victoria, Australia ^c

[#]Address correspondence to:

Dr. Robyn Lee PhD robyn.s.c.lee@gmail.com¹

Prof. Benjamin Howden MD PhD bhowden@unimelb.edu.au

Type of manuscript: Research Article

Word count: Abstract 247, Intro 510, Results and Discussion 2,501

Main tables: 1

Main figures: 4

Key words: Enterococcus; Genomic epidemiology; antimicrobial resistance; transmission; infection control

¹ Robyn Lee is currently at the Center for Communicable Disease Dynamics, at the Harvard T.H. Chan School of Public Health, 677 Huntington Avenue, Boston, MA, USA 02115

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31 **Abstract** (247/250 words)

32 Vancomycin-resistant *Enterococcus faecium* (VREfm) represent a major source of
33 nosocomial infection worldwide. In Australia, the *vanB* genotype is dominant; however there
34 has been a recent increase in the predominantly plasmid-encoded *vanA* genotype, prompting
35 investigation into the genomic epidemiology of VREfm in this context.

36 **Materials and Methods.**

37 A cross-sectional study of VREfm in Victoria, Australia (Nov.10th - Dec.9th, 2015). A total of
38 321 VREfm isolates (from 286 patients) were collected and whole-genome sequenced with
39 Illumina NextSeq. Single nucleotide polymorphisms (SNPs) were used to assess relatedness.
40 Multi-locus sequence types (STs), and genes associated with resistance and virulence were
41 identified. The *vanA*-harbouring plasmid from an isolate from each ST was assembled using
42 long-read data.

43 **Results.**

44 *vanA*-VREfm comprised 17.8% of isolates. ST203, ST80 and a *pstS*(-) clade, ST1421,
45 predominated (30.5%, 30.5% and 37.2% of *vanA*-VREfm, respectively). Most *vanB*-VREfm
46 were ST796 (77.7%). *vanA*-VREfm isolates were closely-related within hospitals vs. between
47 them (core SNPs 10 [interquartile range 1-357] vs. 356 [179-416] respectively), suggesting
48 discrete introductions of *vanA*-VREfm, with subsequent intra-hospital transmission. In
49 contrast, *vanB*-VREfm had similar core SNP distributions within vs. between hospitals, due
50 to widespread dissemination of ST796. Overall, *vanA*-harbouring plasmids differed across
51 STs, and with exception of ST78 and ST796, *Tn1546* transposons also varied.

52 **Conclusions.**

53 *vanA*-VREfm in Victoria is associated with multiple STs, and is not solely mediated by a
54 single shared plasmid/*Tn1546* transposon; clonal transmission appears to play an important

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55 role, predominantly within, rather than between, hospitals. In contrast, *vanB*-VREfm appears
56 to be well-established and widespread across Victorian healthcare institutions.

57

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58 Introduction

59 *Enterococcus faecium* is a leading cause of nosocomial infections world-wide (1, 2).
60 Resistance to glycopeptides, especially vancomycin, is the most clinically-relevant for *E.*
61 *faecium* (1); mortality associated with any *E. faecium* blood-stream infection (BSI) exceeds
62 30% (3), while patients with vancomycin-resistant *E. faecium* (VREfm) bacteremia have
63 ~2.5-fold higher odds of death compared to those with vancomycin-susceptible infection (4).
64 Resistance to vancomycin in *E. faecium* can be conferred by different *van* gene clusters (*A*, *B*,
65 *D*, *E*, *G*, *L*, *M*, and *N* (5-8)), with most infection attributed to *vanA* and *vanB* genotypes (9).
66 VREfm is thought to be transmitted between hospitalized patients, healthcare staff, and/or via
67 the hospital environment (10), with risk of colonization highest among those receiving long
68 courses of antibiotics, the critically-ill and/or immunosuppressed, and those with prolonged
69 hospitalization or history of nursing home residence (11). VREfm has also been shown to
70 arise *de novo* in a patient, following transfer of *vanB* gene cluster from commensal gut
71 anaerobes to *E. faecium* (12, 13).

72 The prevalence of the *vanA* and *vanB* genotypes varies geographically; in North
73 America, *vanA* has been shown to drive the VREfm epidemic (e.g., (14)), while in Europe,
74 both *vanA* and *vanB* genotypes play a role (15). In Australia, the first case of VREfm
75 detected in 1994 carried the *vanA* operon (16); however, it is *vanB*-VREfm that has
76 subsequently predominated.

77 Multiple clones of *vanB*-VREfm have been detected in Australia, mostly from
78 sequence types (STs) within the hospital-associated clonal complex (CC) 17(9, 17-20). A
79 survey of sentinel laboratories from across Australia in 2011 found that 97.2% (104/107
80 isolates) of vancomycin-nonsusceptible isolates had the *vanB* genotype (17), over half of
81 which were ST203 (19, 21). Subsequent country-wide surveys showed this strain dominated

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82 until 2014, when it was surpassed by the ST796 clone (22) - largely due to its widespread
83 dissemination throughout Victoria and southeastern Australia (23).

84 Along with the spread of the *vanB*-associated ST796 clone, there has been a recent,
85 dramatic increase in *vanA*-VREfm; since 2011, the prevalence of *vanA*-VREfm bacteremia,
86 as measured by these sentinel surveys, increased from <1% (2/341) to 21% (90/408 isolates
87 genotyped) in 2016 (24). The reasons for this increase remain unclear, but multiple STs
88 appear to be involved. A retrospective study examining *vanA*-VREfm (screening and clinical
89 isolates) across four Australian hospitals from 2011-2013 (25) suggested this increase could
90 not be attributed to a single ST or *vanA*-harbouring plasmid; however, this was based on a
91 convenience sample of only 18 isolates from across the whole country, making this
92 inconclusive.

93 To further explore the potential reasons for the shift in *van* genotype, and increase our
94 understanding of the molecular epidemiology of VREfm, we conducted a population-level
95 study of VREfm in the State of Victoria – the second most inhabited State in Australia
96 (<http://www.abs.gov.au/AUSSTATS/>). All VREfm isolates from colonization and clinical
97 infection detected across the State over a month were subjected to whole genome sequencing,
98 as well as isolates from VSEfm bacteremia. In doing so, we provide an in-depth assessment
99 of the genomic diversity of *E. faecium* and novel insights into the epidemiology of VREfm in
100 this region.

101

102 **Materials and Methods**

103 *Study design and population.*

104 A cross-sectional survey of VREfm was conducted between November 10th – December 9th,
105 2015 in the State of Victoria (population: 5,931,100 for 2015, per the Australian Bureau of
106 Statistics; <http://www.abs.gov.au/AUSSTATS/>). During this period, all VREfm-positive

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isolates (including screening and clinical samples) from all laboratories across the State were sent to the Microbiological Diagnostic Unit Public Health Laboratory (MDU), as well as all vancomycin-susceptible *E. faecium* isolated from blood cultures. Final lists of isolates received by the MDU were cross-checked with primary diagnostic laboratories to ensure all eligible isolates were included.

DNA extraction and WGS.

Genomic DNA was isolated from a single colony using a JANUS Chemagic Workstation and Chemagic Viral DNA/RNA kit (PerkinElmer). Libraries were prepared with the Nextera XT DNA sample preparation kit (Illumina). Whole genome sequencing (WGS) was performed using the Illumina NextSeq 500 with 150 base-pair paired-end reads.

Bioinformatics.

Sequences were analysed using the Nullarbor pipeline (Seemann T, available at: <https://github.com/tseemann/nullarbor>).

In brief, Illumina reads were trimmed for quality using Trimmomatic (v.0.36, (26)). Contamination was assessed using Kraken (v.0.10.5, (27)). Reads were assembled into contigs using SPAdes (v.3.10.1, (28)). *In silico* identification of resistance genes was done with ABRicate (Seemann T, available at: <https://github.com/tseemann/abricate>), wherein the assembled contigs were searched for resistance genes in the ResFinder database using BLAST+ (29). Putative virulence genes were also identified in the same manner using the Virulence Factor DataBase (30), to assess whether increased virulence could be responsible for the rise in VREfm bacteremia in Australia. All positive hits were reviewed for gene completeness (i.e., gene coverage and gaps), percent identity, and depth of coverage. BLAST+ (29) was also used to search the assembled contigs for *E. faecium* alleles listed in the pubMLST database (<https://pubmlst.org>); unknown allelic combinations and allelic variants were submitted to the database for new assignment. Single nucleotide polymorphism

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(SNP)-based analyses were done by aligning reads using the Burrows-Wheeler Aligner MEM algorithm (v.0.7.16, (31)) to a novel local Victorian reference (ST796, AUSMDU00004028; see **Table A1** for details and metrics). This reference was selected because preliminary analyses indicated the majority of our samples were also ST796. A minimum mapping quality of 60 was required, and ambiguously-mapped reads were excluded. SNPs were called using Freebayes (v1.0.2, (32)) under a haploid model, with a minimum depth of coverage of 10 and allelic frequency of 0.9 required to call a SNP. Integrative Genomics Viewer (v.2.3.88) was used to review alignments and SNPs as needed (33).

Phylogenetics and population structure.

ClonalFrameML was used to identify potential recombination (v.1.11, (34)). Putative recombination sites were then masked using a custom script (available at: <https://github.com/kwongj/cfml-maskrc>), and concatenated core SNP sites were extracted using snp-sites -c (35). A maximum likelihood tree was then produced using RAxML (v.8.2.11, (36)) under a General Time Reversible model with four gamma categories. One thousand bootstrap replicates were performed to assess confidence in the tree. Figures were produced using ggtree (37) in R (v.3.3.2). Bayesian Analysis of Population Structure with hierarchical model-based clustering (hierBAPS; v.6.0, (38)) was also used to identify major clades, using the alignments of recombination-adjusted core SNPs as input.

Comparative genomics of vanA-VREfm.

One strain from each of the major *vanA*-harbouring STs was sequenced using PacBio Single Molecule, Real-Time sequencing with P6-C4 chemistry (Pacific Biosciences, CA). *De novo* assembly was done using Canu (v.1.3 (39)); see **Appendix** for further detail. QUAST (v.4.5) was used for additional quality metrics (40). *vanA*-harbouring plasmids from each of these strains were identified using ABRicate, and subsequently annotated using Prokka (v. 1.11, (41)) with the *Enterococcus* database

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(<https://github.com/tseemann/prokka/blob/master/db/genus/Enterococcus>). To compare the gene content of *vanA*-harbouring plasmids, these plasmids were aligned to one another using progressiveMauve (build date 2014-12-19) and visualized in Geneious (v.9.1.7, (42)).

Tn1546 transposons were identified and genes were manually compared across plasmids.

Victorian isolates in global context.

To assess how Victorian *E. faecium* strains fit in the global context, we re-ran our analyses including 877 additional strains from Europe (872), North America (2), Africa (2) and Asia (Israel; 1) (43-46), and isolates from the Wellcome Trust Sanger BSAC Resistance Surveillance Project (from NCBI BioProject PRJEB344). A maximum likelihood tree was produced using IQ-TREE (v.1.6.1, (47)); see **Appendix** with model selection based on the lowest Bayesian Information Criterion.

Statistical analysis.

Pairwise SNP distributions were compared using the Mann-Whitney rank-sum test. Differences in proportion were compared using either the Chi-Square test or Fisher's Exact test, as appropriate. Analyses were done in Stata (v.14.2, College Station, TX: StataCorp).

Data availability.

Illumina data, and the PacBio reads with corresponding assemblies for the five isolates listed in **Table A1** are available on the National Center for Biotechnology Information's (NCBI) Sequence Read Archive under BioProject PRJNA433676.

Results

Between November 10th – December 9th 2015, 333 *E. faecium*-positive samples were detected at 19 primary diagnostic laboratories from across Victoria (two additional laboratories reported no *E. faecium* during this time). Two eligible VSEfm isolates were not

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submitted, thus 331 isolates were available for sequencing. All isolates passed WGS quality control (see the **Appendix** for details).

In total, 321 isolates (from 286 patients) were VREfm - 20 of which were from BSI (from 17 patients). Ten VSEfm isolates (from ten patients) were also collected; one of these was likely a false negative, as the patient also had a *vanA*-VREfm isolated from blood collected the same day (further detail in the **Appendix**). Thus, 27 patients had *E. faecium* bacteremia over this period; an incidence of 5.5/100,000 when extrapolated to one year.

Most patients (n=257, 87.1%) were from hospitals in major metropolitan centers, compared to rural areas (n=29, 9.8%), consistent with the geographical distribution of the population in this State. Overall, 35 hospital networks (HCNs) were represented, with isolates received from up to five affiliated hospitals from a single network. Five isolates were collected in community medical/dental clinics (3.1%). Sex and age were not available for patients from HCN1 (n=61, 20.7%); among the remaining patients, 113 were male (48.3%), and the median age was 71 (range 18-98, IQR 58-80). At sample collection, 256 persons were inpatients and 36 were outpatients. Four patients were missing location of isolation and had unknown admission status.

Twenty-seven patients had >1 sample positive for *E. faecium* (range 2-5 per patient; **Table A2**), with a maximum of 40 SNPs between repeat isolates of the same ST (**Figure A1**). Repeat samples were collected within the same hospital as the original sample for 26/27 patients (96.3%, **Table A2**).

Identification of van genotype.

In silico genotyping revealed that 260 (78.6%) of isolates were exclusively *vanB*, and 59 (17.8%) were exclusively *vanA*. There was no association between *van* genotype and BSI (**Figure 1**, Fisher's Exact p=0.52). Two isolates were positive for *vanA* and *vanB* (<1%). These were most closely related to *vanB*-VREfm isolates, with as few as 0 pairwise core

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SNPs compared to *vanB*-VREfm isolates (vs. 195 for *vanA*-VREfm), suggesting they already had *vanB* when *vanA* was acquired. As expected, all isolates with *vanA* and/or *vanB* were phenotypically resistant to vancomycin (MIC > 4 mg/L (48)). Neither *vanA* or *vanB* were found in isolates classified as VSEfm (3.0%).

Overall, *vanB*-VREfm was more widely dispersed across Victorian hospitals and HCNs compared to *vanA*-VREfm; excluding the two *vanA*+*vanB*-positive samples, *vanB*-VREfm isolates were identified in 34/35 HCNs, while *vanA*-VREfm was only identified in 10/35 during this time period ($p < 0.00005$). VSEfm isolates were from six different networks.

Multi-locus sequence typing.

Eighteen previously-known MLSTs and four novel STs were identified. Most isolates were ST796 (61.3%, **Table 1**). The *pstS* allele was missing for 22 (6.6%) isolates; these were classified as ST1421 based on remaining alleles (**Table A3**).

vanA was found in fewer STs than *vanB* (3 vs. 11, respectively, excluding *vanA*+*B* positive isolates), though this trend was not statistically significant (Fisher's Exact test, $p=0.17$). *vanA* was almost exclusively present in ST203 and ST80, and ST1421 isolates (**Table 1**). Most *vanB*-positive isolates were ST796.

Phylogenetics and population structure.

Prior to adjusting for recombination, there were a median of 20 core SNPs between study isolates and the reference (IQR 14-2,653); adjusting for recombination reduced this to a median of 12 core SNPs (IQR 9-238). An alignment of these adjusted core SNPs was used to produce a maximum likelihood tree (**Figure 2**; recombination blocks are shown in **Figure A2**). Many branches had very low bootstrap support, therefore BAPS was used to validate clusters.

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Five major BAPS groups were identified, which were largely consistent with the phylogeny (**Figure 2**). BAPS-1 was comprised predominantly of ST796 isolates (203/214, 94.9%), and was highly clonal, with a median of 10 core SNPs separating isolates (IQR 7-15). In contrast, BAPS-2 was comprised of multiple STs. The highest proportions were from ST203 (36/82, 43.9%), ST1421 (22/82, 26.8%) and ST17 (12/82, 14.6%). Isolates in BAPS-2 were separated by a median of 257 core SNPs (IQR 167-312). Excluding repeat samples from the same patient, 81 pairs of isolates were within one SNP. Sixty-six of these pairs (81%) were isolated at the same hospitals as one another (H1, H3, H5), while the remainder were predominantly from different HCNs (see **Figure A3**). BAPS-3 was comprised of a single VSEfm isolate from ST54, which was over 3,900 SNPs from other isolates (not shown in **Figure 1**). BAPS-4 isolates were predominantly from ST80 (25/31, 80.6%), but also included isolates from ST17, ST78 and ST262. Isolates were separated by a median of 252 core SNPs (IQR 6-346). All pairs within one core SNP were from ST80; excluding repeat isolates, 8/15 of these pairs were collected at the same hospitals as one another (H1, H2 and H4). BAPS-5 was comprised of three VSEfm isolates (ST21, ST22, and ST32), with a minimum of 369 core pairwise SNPs.

Genetic diversity across hospitals and van genotypes.

Excluding comparisons within the same patient (shown in **Figure A2**), the median pairwise core SNP distances among VREfm were similar within and between hospitals, at 196 (IQR 13-297) and 193 (IQR 13-207), respectively (**Figure 3A**).

As we hypothesized there were differences in diversity between isolates with different VREfm genotypes, *vanA*-VREfm and *vanB*-VREfm were analyzed separately. *vanA*-VREfm were significantly less diverse within hospitals compared to between them (**Figure 3B**, $p=0.0001$), suggesting multiple, independent introduction events with subsequent intra-hospital transmission. In contrast, *vanB*-VREfm was more diverse within hospitals vs.

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between them (**Figure 3C**), $p=0.0001$), consistent with wide-spread dissemination and long-term establishment of these strains across institutions within the Victorian healthcare system.

Virulence.

Isolates were interrogated for the presence of putative virulence factors; as shown in **Figure 2**, only two isolates (both ST17) had the *cylA* gene which is required for expression of cytolysin (49). No genes encoding hyaluronidase (*hyl*_{Efm} (50)), agglutination substance (*agg/asaI* (51) or gelatinase (*gelE* (52)) were detected; the latter two genes are thought to be more prevalent in *E. faecalis* than *E. faecium* (53).

Microbial surface components recognizing adhesive matrix molecules including *acm* (54), which has been found predominantly in clinical isolates, as well as *ecbA* (55) and *sgrA* (55) were also investigated. *acm* was present in 98.5% of study samples, across nearly all STs. *ecbA* was present in 62 isolates (30.5% of *vanA*-VREfm; 15.8% of *vanB*-VREfm), including all ST192, ST400 and ST893 isolates, and most ST17 and ST203 isolates (71% and 97%, respectively). *sgrA* was present in 294 isolates (91.9%, of *vanA*-VREfm; 89.2% of *vanB*-VREfm) from nearly all STs. No isolates had *pilA* and *pilB*, which are also potentially involved in adhesion (56).

Finally, the gene *esp*, which has previously been associated with hospital outbreaks (57) and encodes enterococcal surface protein (58), was investigated. While repeat regions made it impossible to completely assemble *esp* using short-read data (see **Appendix, Figure A4**), we found that 266 isolates had at least 80% coverage (81.6% of *vanA*-VREfm; 86.2% of *vanB*-VREfm), suggesting that these isolates were likely *esp*-positive. In this context, *esp* was more prevalent in bacteremia due to VREfm than VSEfm (17/20 vs. 1/9, $p=0.0001$, excluding the VSEfm that had likely lost the *vanA* operon).

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The *vanA*-harbouring plasmid from one isolate per ST was completely assembled using long-read data; no putative virulence genes were found to co-localize with *vanA* on the same plasmid (**Figure A5**).

Comparative genomics of vanA-VREfm.

The *vanA*-harbouring plasmids from ST78 and ST796 had the greatest homology (**Figure 4**), with similar plasmid backbones and 100% amino acid identity of the Tn1546 transposon, which carries the *vanA* gene cluster (**Figure A5**). The main difference between these plasmids was a large insertion in ST796 compared to ST78, carrying *repB*, *IS1216* and two hypothetical proteins (**Figure A5**). The *vanA*-harbouring plasmid from ST80 was similar to that from ST796 (**Figure A6**), but had lost *ISEfa7-birA* from the plasmid backbone (**Figure A5**) and had a 48-bp deletion in *vanS* compared to the ST796 plasmid. Both *vanA*-harbouring plasmids from the ST203 and ST1421 isolates had unique plasmid backbones and Tn1546 transposons (**Figure A5**). Together, these findings suggest that horizontal transfer of a single *vanA*-harbouring plasmid and/or Tn1546 carrying the *vanA* gene cluster is not responsible for the increase in *vanA*-VREfm in Victoria.

Victorian isolates in global context.

Compared to published sequences, the *vanA*-harbouring plasmids from ST78, ST796 and ST80 shared closest homology to a *vanA*-harbouring plasmid from VREfm in Denmark (46) (**Table A4**). The *vanA*-harbouring plasmid from ST203 was most closely related to plasmids from several STs of VREfm isolated in Western Australia and Queensland (25), while the plasmid from ST1421 was closely-related to that from another ST1421 *E. faecium*, isolated in New South Wales in 2014 (59) (**Table A4**).

Phylogenetic analysis based on core SNPs from Victorian isolates and global *E. faecium* strains showed that, with the exception of those from ST796, Victorian isolates were

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widely distributed across the tree (**Figure A7**). This suggests ongoing, inter-continental transmission of *E. faecium*.

Discussion

For the past two decades, the majority of VREfm in Australia have been *vanB* genotype (13, 18, 19, 21-23, 60-62). Recently, however, *vanA*-VREfm has been increasing. In this population-based study, we looked at factors potentially contributing to this change in epidemiology.

Firstly, we examined the genetic diversity of *vanA*-VREfm across our State, to assess for potential transmission. Consistent with the hypothesis of van Hal et al. (25), we found that a single clone is not driving this increase; instead, our findings suggest that multiple introductions of *vanA*-VREfm have occurred, with subsequent spread within Victorian hospitals. This pattern is similar to that of early *vanB*-VREfm in Australia (63), though the latter has become dominated by a single ST in Victoria (ST796) (23).

Another possibility was that a single *vanA*-harbouring plasmid and/or Tn1546 transposon was being transmitted within and across different STs, as has been shown in Denmark (46). As a unique *vanA*-harbouring plasmid was present in representatives from each ST, and nearly all had different Tn1546 transposons, this suggests that horizontal gene transfer is not the sole driver of *vanA*-VREfm in Victoria.

Finally, we assessed whether there were differences in putative virulence factors between our isolates, and those from other studies/settings. Overall, few putative virulence factors were identified, and their prevalence was largely consistent with previous data from *vanB*-VREfm in Australia (64), and similar to or lower than those found in *vanA*-VREfm from settings (e.g., in China (65) or Brazil (66)). While much is still unknown about virulence factors in VREfm (compared to *Enterococcus faecalis*), this suggests the rise in

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vanA-VREfm in Australia, and the continued success of *vanB*-VREfm, is not due to changes in virulence. Consistent with this, the rate of *E. faecium* BSI in our study was similar to that of another population-based study in Denmark (3). This has important implications for hospital infection control; as the patient population affected by VREfm has also remained consistent over time, we propose that environmental factors may be mediating the observed changes in VREfm epidemiology. Further studies are needed to investigate this hypothesis.

In addition to gaining new insights in *vanA* epidemiology in this context, we have also provided valuable information on *vanB*-VREfm; given that we found genetic diversity was slightly lower across hospitals compared to within, this suggests a potential community reservoir for these strains. Transmission of VREfm has previously been shown in nursing homes (67), with carriage strains closely related to those causing infection; investigation is warranted to determine if this is also the case for Victoria. The seemingly widespread diversity of *vanB* VREfm could also be due to a “diffusion” effect, wherein colonization and infection have slowly spread across hospitals through many years of patient exchanges. Such a pattern would be reminiscent of that observed in Denmark (68); however, while in Denmark the “diffusion” process was marked by a hub-and-spoke pattern, with patients mainly being exchanged between regional hospitals and central hospitals in Copenhagen, in Victoria, the process was likely more random. If such a “diffusion” scenario is correct, we hypothesize that the pattern of genetic diversity of *vanA*-VREfm will eventually mirror that of the *vanB*.

This work has a number of key strengths. Firstly, by including >99% of VREfm collected in Victoria over this one-month period, we have provided a comprehensive assessment of local strain diversity and the prevalence of clinically-relevant *van* genotypes, as well as a baseline to monitor for changes in VREfm population in Victoria. Secondly, we used local reference genomes for all SNP-based analyses. As using more genetically-distant

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reference genomes can result in false positive SNPs, and/or loss of information due to differences in genes present in the study sample, but not in the reference, this increased the accuracy of our short-read analyses. Since the first complete *E. faecium* genome was published by our group in 2012 (69), few other genomes have been completed; by sharing these complete VREfm genomes (from five different STs), we are also providing an invaluable resource for public health as well as future studies on *E. faecium*. Finally, the use of long-read data has also allowed us to completely assemble and characterize the *vanA*-harbouring plasmids in each of the different STs – a task that is not always feasible with short-read data alone.

There are several limitations of this work. Firstly, screening protocols for VREfm colonization are not standardized across Victoria; each hospital adhered to its own infection control guidelines, and as such, there may be incomplete capture of colonizing strains in this dataset. This may bias our dataset somewhat towards strains causing clinical infection, and cause us to miss potential transmission. Another limitation is that we did not have any data on patient contact or intra-hospital transfer (except where isolates were collected at different hospitals) – preventing us from confirming direct person-to-person transmission events. However, we do not think this affects our overall population-level inferences, given the dramatically lower genetic diversity of *vanA* within hospitals compared to *vanB*. Finally, as this was predominantly a lab-based study, we also did not have data on clinical presentation at time of sample collection and were therefore unable to discriminate clinical infection aside from bacteremia. Thus, we may have underestimated the overall prevalence of infection (though the most clinically important cases - associated with bacteremia - have been included).

Herein, we have provided a comprehensive snapshot of VREfm strain diversity across Victoria. We highlight the complexity of *E. faecium* genomic epidemiology, revealing key

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differences in transmission dynamics of clinically-relevant *van* genotypes in this setting. Our findings are applicable to not only to Victoria, but other regions with VREfm - particularly those where the prevalence of *vanB* is high. While *vanB*-VRE continues to predominate in Victoria, repeated introductions and dissemination of *vanA*-VREfm within our hospitals suggest this is changing. To better understand the clinical significance of this shift, prospective studies with detailed corresponding clinical data are needed. As plasmids carrying *vanA* or their Tn1546 transposons may be may be readily transferred to VSEfm and/or rarely to other species (e.g., (70)), preventing an increase in *vanA*-VREfm is critical. This reinforces the overall importance of ongoing prevention and control of VREfm.

Transparency. Authors have no conflicts of interest to declare.

Funding. RSL is supported by a Fellowship from the Canadian Institutes of Health Research (Funding Reference Number 152448). SLB holds an Australian Government Research Training Program Scholarship. JCK is supported by an early career fellowship from the NHMRC (GNT1142613). BPH has a Practitioner Fellowship from the National Health and Medical Research Council (NHMRC), Australia (GNT1105905), and is supported by the Centre of Research Excellence on Emerging Infectious Diseases (NHMRC GNT1102962). The Microbiological Diagnostic Unit Public Health Laboratory is funded by the Victorian Government, Australia.

Acknowledgements. We would like to thank the participating laboratories for their provision of samples and the clerical and technical assistance of staff at MDU PHL. We would also like to thank Dr. Sharon Peacock (Department of Medicine, University of Cambridge, Cambridge, United Kingdom; Wellcome Trust Sanger Institute, Hixton, United Kingdom; London School

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of Hygiene and Tropical Medicine, London, United Kingdom) and Dr. Kathy Raven (Department of Medicine, University of Cambridge, Cambridge, United Kingdom) for helpful discussions about global VREfm.

Contributions. RSL designed and ran the primary analyses, interpreted results, made the tables and figures, and wrote the first draft of the manuscript. AG designed analyses, advised on bioinformatics approaches, helped interpret results, and contributed to writing the manuscript. SLB assembled the PacBio genomes, provided input on analyses, and helped interpret results. JS recruited all labs, coordinated submission of isolates and data from primary diagnostic laboratories, and did the initial processing of samples at MDU PHL. SB designed sample collection and advised on the initial interpretation of the results. GPC did the laboratory work to generate the PacBio sequences. JCK, MS, DB and TS provided bioinformatics tools, and advised on bioinformatics analyses. TPS and BPH conceived the cross-sectional study, helped decide on analyses, and edited the manuscript. All authors critically reviewed the manuscript for content.

Figure Legends.

Figure 1. Specimen type by *van* gene for Victorian *E. faecium* isolates

Only 2 isolates were *vanA+vanB*; only one was from a blood-stream infection (BSI). As all 10 VSEfm isolates were from BSI by design, these have not been shown. Fisher Exact test comparing the proportion of BSI among isolates with *vanA* vs *vanB*: $p = 0.52$.

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6,497 recombination-adjusted core SNPs from all 331 isolates (321 VREfm and 10 VSEfm) were concatenated, and used to produce a maximum likelihood tree in RAxML (36) under a General Time Reversible model with gamma rate heterogeneity. One thousand bootstrap replicates were performed to assess confidence in the phylogeny. BAPS-3 is not shown; AUSMDU00004157 (an ST54 VSEfm) was the only isolate in this clade, and was >3,800 SNPs from all other isolates, so it was excluded from this figure for easier visualization. Branches with bootstrap support >90 are shown. Many branches had very low bootstrap support, thus hierarchical Bayesian Analysis of Population Structure (hierBAPS) was used to further validate the clusters identified. Main BAPS groups are shown. The reference is identified with an arrow. No isolates had *ebpA*, *hyl*, *gelE*, *agg*, *pilA* or *pilB*, therefore these genes have not been shown.

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A. Pairwise SNP comparisons are shown regardless of *van* gene. There were 54,567 total pairwise comparisons, after excluding those involving isolates with missing data on hospital/HCN, those from unknown clinics, and pairs within the same patients. Between hospitals, the median pairwise SNPs was 193 (IQR 11-298), while within hospitals, the median was 196 (IQR 13-297). For easier visualization, as one VSEfm isolate

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Figure 4. Homology between *vanA*-harbouring plasmids

PacBio Single Molecule Real-Time sequencing was performed on a *vanA*-positive isolate from each of the main *vanA*-associated STs (ST 78, ST80, 203, 796, and ST1421). Plasmids harbouring *vanA* were initially identified using ABRicate (<https://github.com/tseemann/abricate>), and subsequently annotated using the *Enterococcus* database (<https://github.com/tseemann/prokka/blob/master/db/genus/Enterococcus>). This figure was produced using EasyFig (v. 2.2.2, version); BLASTn was used to compare sequence homology. Only hits with a minimum length of 200 bp and at least 99% identity are shown for clarity. *van* genes are indicated in red, all other genes are indicated in blue.

Table 1. Multi-locus sequence types (MLSTs) identified

MLST	Number of isolates, n (%)	<i>vanA</i> only, n (%)	<i>vanB</i> only, n (%)	<i>vanA+B</i> , n (%)	Neither <i>vanA</i> nor <i>vanB</i> , n (%)
796	203 (61.3)		202 (77.7)	1 (50.0)	
203	37 (11.2)	18 (30.5)	19 (7.3)		
80	26 (7.9)	18 (30.5)	5 (1.9)		3 (30.0) ^d
17	17 (5.1)		15 (5.8)	1 (50.0)	1 (10.0)
1421 (<i>pstS</i> (-))	22 (6.6)	22 (37.2)			
1429 ^a	5 (1.5)		5 (1.9)		
?new ^b	1 (0.3)		1 (0.4)		
Other ^c	20 (6.0)	1 (1.7)	13 (5.0)		6 (60.0)
Total, n (%)	331	59 (17.8)	260 (78.5)	2 (1.0)	10 (3.0)

N=331 isolates. ^a A novel single-locus variant of ST796. ^b The *ddl* gene might be interrupted in this isolate, but we were unable to resolve it using short-read data, and were therefore not able to type it. It clusters with ST17 isolates in the recombination-adjusted phylogenetic tree. ^c Sixteen additional STs were identified, each represented by three or fewer isolates. The single *vanA* isolate here corresponds to ST78. ^d This includes the one isolate that likely lost the *vanA* -harbouring plasmid during passage.

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Legend. 6,497 recombination-adjusted core SNPs from all 331 isolates (321 VREfm and 10 VSEfm) were concatenated, and used to produce a maximum likelihood tree in RAxML (36) under a General Time Reversible model with gamma rate heterogeneity. One thousand bootstrap replicates were performed to assess confidence in the phylogeny. BAPS-3 is not shown; AUSMDU00004157 (an ST54 VSEfm) was the only isolate in this clade, and was >3,800 SNPs from all other isolates, so it was excluded from this figure for easier visualization. Branches with bootstrap support >90 are shown. Many branches had very low bootstrap support, thus hierarchical Bayesian Analysis of Population Structure (hierBAPS) was used to further validate the clusters identified. Main BAPS groups are shown. The reference is identified with an arrow. No isolates had *ebpA*, *hyl*, *gelE*, *agg*, *pilA* or *pilB*, therefore these genes have not been shown.

Figure 3. Recombination-adjusted pairwise core SNPs separating Victorian isolates

Panel A. Overall pairwise comparisons, including isolates with both *vanA* and *vanB* and VSEfm

Panel B. Pairwise comparisons between *vanA*-only isolates

Panel C. Pairwise comparisons between *vanB*-only isolates

Legend. A. Pairwise SNP comparisons are shown regardless of *van* gene. There were 54,567 total pairwise comparisons, after excluding those involving isolates with missing data on hospital/HCN, those from unknown clinics, and pairs within the same patients. Between hospitals, the median pairwise SNPs was 193 (IQR 11-298), while within hospitals, the median was 196 (IQR 13-297). For easier visualization, as one VSEfm isolate (AUSMDU00004157) was a minimum of 3,893 SNPs from all others, only comparisons between the 330 other isolates are shown in the figure.

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