The changing landscape of vancomycin-resistant Enterococcus faecium in Australia: a population-level genomic study

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Introduction
Enterococcus faecium is a leading cause of nosocomial infections worldwide.¹,² Resistance to glycopeptides, particularly vancomycin, is the most clinically relevant resistance for E. faecium;³ mortality associated with any E. faecium bloodstream infection (BSI) is >30%,³ while patients with vancomycin-resistant E. faecium (VREfm) bacteremia have ~2.5-fold higher odds of death compared with those with vancomycin-susceptible infection.⁴ Resistance to vancomycin in E. faecium can be conferred by different van gene clusters (A, B, D, E, G, L, M and N⁵–⁸), with most infection attributed to vanA and vanB genotypes.⁹ VREfm is thought to be transmitted between hospitalized patients,

Results: vanA-VREfm comprised 17.8% of isolates. ST203, ST80 and a pstS(−) clade, ST1421, predominated (30.5%, 30.5% and 37.2%, respectively). Most vanB-VREfm were ST796 (77.7%). VanA-VREfm were more closely related within hospitals versus between them [core SNPs 10 (IQR 1–357) versus 356 (179–416), respectively], suggesting discrete introductions of vanA-VREfm, with subsequent intra-hospital transmission. In contrast, vanB-VREfm had similar core SNP distributions within versus between hospitals, due to widespread dissemination of ST796. Different vanA-harbouring plasmids were found across STs. With the exception of ST78 and ST796, Tn1546 transposons also varied. Phylogenetic analysis revealed Australian strains were often interspersed with those from other countries, suggesting ongoing cross-continental transmission.

Conclusions: Emerging vanA-VREfm in Australia is polyclonal, indicating repeat introductions of vanA-VREfm into hospitals and subsequent dissemination. The close relationship to global strains reinforces the need for ongoing screening and control of VREfm in Australia and abroad.

Background: Vancomycin-resistant Enterococcus faecium (VREfm) represent a major source of nosocomial infection worldwide. In Australia, there has been a recent concerning increase in bacteraemia associated with the vanA genotype, prompting investigation into the genomic epidemiology of VREfm.

Methods: A population-level study of VREfm (10 November–9 December 2015) was conducted. A total of 321 VREfm isolates (from 286 patients) across Victoria State were collected and sequenced with Illumina NextSeq. SNPs were used to assess relatedness. STs and genes associated with resistance and virulence were identified. The vanA-harbouring plasmid from an isolate from each ST was assembled using long-read data. Illumina reads from remaining isolates were then mapped to these assemblies to identify their probable vanA-harbouring plasmid.
healthcare staff and/or via the hospital environment, with risk of colonization highest among those receiving long courses of antibiotics, the critically ill and/or immunosuppressed, and those with prolonged hospitalization or history of nursing home residence. VREfm may also arise de novo in a patient, following transfer of the vanB gene cluster from commensal gut anaerobes to Enterococcus faecium.

The prevalence of the vanA and vanB genotypes varies geographically; in North America, vanA has been shown to drive the VREfm epidemic, while in Europe, both vanA and vanB genotypes play a role. Australia has one of the highest rates of VREfm in the world, and until recently, was dominated by vanB-VREfm.

Multiple clones of vanB-VREfm have been detected in Australia, mostly from STs within the hospital-associated clonal complex 17,18-19. A survey of sentinel laboratories from across Australia in 2011 found that 97.2% (104 of 107 isolates) of vancomycin-non-susceptible isolates had the vanB genotype, over half of which were ST203. Subsequent country-wide surveys showed this strain dominated until 2014, when it was surpassed by the ST796 clone largely due to its widespread dissemination throughout Victoria and southeastern Australia.

Along with the spread of vanB-VREfm, there has been a recent, dramatic increase in the vanA genotype; since 2011, the prevalence of vanA-VREfm bacteraemia, as measured by these sentinel surveys, increased from <1% (2 of 341) to 22% (90 of 408 isolates genotyped) in 2016. The reasons for this increase remain unclear, but multiple STs appear to be involved. A retrospective study examining vanA-VREfm (screening and clinical isolates) across four Australian hospitals from 2011 to 2013 suggested this increase was not due to a single ST or vanA-harbouring plasmid; however, it was based on a convenience sample of only 18 isolates from across Australia, and was inconclusive.

To explore further the potential reasons for the emerging dominance of vanA-VREfm in our region, we conducted a population-level study of VREfm. All VREfm isolates from colonization and clinical infection detected across Victoria (the second most populous state in Australia) over a month were subjected to WGS, as well as isolates from VSEfm bacteraemia. In doing so, we provide an in-depth assessment of the genomic diversity of E. faecium, and, situating these in context with global VREfm, novel insights into the genomic epidemiology of this clinically relevant pathogen.

Methods

Ethics

This research was conducted in accordance with the Declaration of Helsinki and Australian National and institutional standards. Data were collected as part of public health surveillance and de-identified for analysis. Individual patient consent was not required.

Study design and population

A cross-sectional survey of VREfm was conducted between 10 November and 9 December 2015 in the State of Victoria (population: 5 931 100 for 2015; http://www.abs.gov.au/AUSSTATS/). During this period, all VREfm-positive isolates (including screening and clinical samples) collected by laboratories across the state were sent to the Microbiological Diagnostic Unit Public Health Laboratory (MDU PHL), as well as all vancomycin-susceptible E. faecium (VSEfm) isolated from blood cultures. Final lists of isolates received were cross-checked with primary diagnostic laboratories to ensure all eligible isolates were included.

Laboratory methods

See Supplementary data (available at JAC Online) for detail. Isolates were sequenced using the Illumina NextSeq 500, with 150 bp paired-end reads.

Bioinformatics

In brief, sequences were analysed using the Nullarbor pipeline (Seemann T, available at: https://github.com/tseemann/nullarbor). Reads were assembled into contigs using SPAdes (v. 3.10.1). Resistance genes present in the ResFinder database and putative virulence genes from the Virulence Factor Database were identified in silico from these assemblies using ABRIcator (Seemann T, https://github.com/tseemann/abri cate). Assembled contigs were also searched for E. faecium alleles listed in the pubMLST database (https://pubmlst.org). For SNP-based analyses, reads were aligned using the Burrows–Wheeler Aligner MEM algorithm (v.0.7.16) to a novel local reference (ST796, AUSMDU00004028; see Table S1). SNPs were called using Freebayes (v1.0.2). Further details are given in the Supplementary data.

Phylogenetics and population structure

ClonalFrameML was used to identify potential recombination (v.1.11). This was masked using a custom script (https://github.com/kwongj/cfml-masker) and the adjusted core SNP alignment was used to produce a maximum likelihood tree using RaXML (v.8.2.11). Bayesian analysis of population structure with hierarchical model-based clustering (hierBAPS; v.6.0), was used to identify major clades.

Comparative genomics of vanA-VREfm

A bacteraemia strain from each of the major vanA-harbouring STs was sequenced using PacBio single molecule, real-time sequencing (Pacific Biosciences, CA, USA). vanA-harbouring plasmids were identified using ABRIcator, and annotated using Prokka (v. 1.11), with the Enterococcus database (https://github.com/tseemann/prokka/blob/master/db/genes/Enterococcus). To compare the gene content, these plasmids were aligned to one another using progressiveMauve (build date 19 December 2014) and visualized in Geneious (v.9.1.7). To investigate whether the same vanA-harbouring plasmids were present in the other isolates in our dataset, we mapped reads to each complete vanA-harbouring plasmid (see Methods in Supplementary data).

Australian isolates in global context

To assess how our E. faecium strains fit in the global context, we searched for publicly available short-read data; this resulted in inclusion of strains from Europe (872), North America (6), Africa (2) and Asia (Israel, 1). To investigate whether the same vanA-harbouring plasmids were present in the other isolates in our dataset, we mapped reads to each complete vanA-harbouring plasmid (see Methods in Supplementary data).

Statistical analysis

Analyses were done in Stata v.14.2 (StataCorp, College Station, TX, USA). Pairwise SNP distributions were compared using the Mann–Whitney rank-sum test. Differences in proportion were compared using the Fisher’s exact test or a two-proportion Z-test.

Data availability

Sequencing data and PacBio assemblies are under BioProject PRJNA433676 on the NCBI Sequence Read Archive.
Results

Between 10 November and 9 December 2015, 321 VREfm-positive samples were detected at 19 primary diagnostic laboratories across Victoria (two additional laboratories reported no E. faecium during this time). Twelve VSEfm BSI samples were also detected, two of which were not submitted. Thus, 331 isolates were available for sequencing. All isolates passed WGS quality control (see Supplementary data).

In total, 321 isolates (from 286 patients) were VREfm, 20 of which were from BSI (from 17 patients). Ten VSEfm isolates (from 10 patients) were also received; one of these was likely a false negative, as the patient also had a vanA-VREfm isolated from blood collected the same day (see Supplementary data). Thus, 27 patients had E. faecium bacteraemia over this period. Extrapolated to 1 year, this gives an incidence of 5.5/100,000 for all E. faecium, and 0.6/100,000 and 3.2/100,000 for vanA-VREfm and vanB-VREfm, respectively (excluding one case of vanA + vanB-VREfm bacteraemia).

Most patients (257 of 295, 87.1%) were from hospitals in major metropolitan centres, compared with rural areas (29 of 295, 9.8%), consistent with the geographical distribution of the population in this state. Overall, 35 hospital networks (HCNs; groups of ≥1 affiliated public or private hospitals that provide services to distinct geographical areas in Victoria) were represented, with isolates received from up to five 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 one HCN (HCN1, 61 of 295, 20.7%); among the remaining patients, 113 of 295 were male (38.3%), and the median age was 71 (range 18–98, IQR 58–80). At sample collection, 256 persons were inpatients and 36 were outpatients. The location of sample collection was missing for four patients, who also had unknown admission status.

Twenty-seven patients had more than one sample positive for E. faecium (range 2–5 per patient; Table S2), with a maximum of 40 SNPs between repeat isolates of the same ST (Figure S1). Repeat samples were collected within the same hospital as the original sample for 26 of 27 patients (96.3%, Table S2).

Identification of van genotype

In silico genotyping revealed that 260 (78.5%) isolates were exclusively vanB, and 59 (17.8%) were exclusively vanA. There was no association between vanA 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 a minimum of zero pairwise core SNPs compared with vanB-VREfm isolates (versus 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/L24). Neither vanA nor vanB was found in isolates classified as VSEfm (3.0%).

Overall, vanB-VREfm was more widely dispersed across hospitals and HCNs compared with vanA-VREfm; excluding the two vanA + vanB-positive samples, vanB-VREfm isolates were identified in 34 of 35 HCNs, while vanA-VREfm was only identified in 10 of 35 during this period (P < 0.00005). VSEfm isolates were from six different HCNs.

MLST

Eighteen previously known STs 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 S3).

Excluding vanA + B-positive isolates, vanA was found in fewer STs than vanB (4 versus 15, respectively, P < 0.00005). vanA was almost exclusively present in ST203, ST80 and ST1421 isolates (Table 1). Most vanB-positive isolates were ST796. Among the nine probable VSEfm isolates, eight different STs were represented; only ST80 (n = 2) was also present among VREfm.

Phylogenetics and population structure

Adjusting for recombination reduced the median core SNPs from 20 (IQR 14–2653) to 12 (IQR 9–238). The adjusted maximum likelihood tree is shown in Figure 2 (recombination blocks are shown in Figure S2). Many branches had very low bootstrap support; therefore, BAPS was used to validate clusters.

Five major BAPS groups were identified, which were largely consistent with the phylogeny (Figure 2). While all BAPS groups had at least one VSEfm, one was closely related to any VREfm within the same cluster (Table S4). Overall, BAPS-1 was predominantly ST796 (203 of 214, 94.9%), and was highly clonal, with a median of 10 core SNPs separating isolates (IQR 7–15). In contrast, BAPS-2 comprised multiple STs. The highest proportions were from ST203 (36 of 82, 43.9%), ST1421 (22 of 82, 26.8%) and ST17 (12 of 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 S3). BAPS-3 comprised a single VSEfm isolate from ST54, which was over 3800 SNPs from other isolates (not shown in Figure 1). BAPS-4 isolates were predominantly from ST80 (25 of 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 VREfm from ST80; excluding repeat isolates, 8 of 15 of these pairs were collected at the same hospitals as one another (H1, H2 and H4). BAPS-5 comprised 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 S2), the median pairwise core SNP distances among VREfm were similar within and between hospitals [196 (IQR 13–297) versus 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 analysed separately. vanA-VREfm were significantly less diverse within hospitals compared with 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 versus between them (Figure 3c), P = 0.0001, consistent with widespread dissemination and long-term establishment of these strains across institutions within the Victorian healthcare system.

**Virulence**

To determine whether increased virulence played a role in the rise of vanA-VREfm, isolates were interrogated for the presence of putative virulence factors. As shown in Figure 2, only two isolates (both ST17) had the cylA gene, required for the expression of cytolysin. No isolates had the ptsD gene, which has been associated with clinical infection, or bepA, which has been implicated in biofilm formation. Genes encoding agglutination substance (agg/asa1) or gelatinase (gelE) were also not detected; these are thought to be more prevalent in *Enterococcus faecalis* than *E. faecium.*

Microbial surface components recognizing adhesive matrix molecules including acm, ecbA and sgrA 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 pIIA and pIIB, which are also potentially involved in adhesion.

Finally, the gene esp, which has previously been associated with hospital outbreaks and encodes enterococcal surface protein, was investigated. While repeat regions made it impossible to completely assemble the esp gene using short-read data (see Figure S4), 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. esp was more prevalent in bacteraemia due to VREfm than VSEfm (17 of 20 versus 1 of 9, P = 0.0001, excluding the VSEfm that had potentially lost the vanA operon).

The vanA-harbouring plasmid from one isolate per ST was completely assembled using long-read data; no putative virulence genes co-localized with vanA on the same plasmid (Figure S5).

**Comparative genomics of vanA-VREfm**

To assess whether a single vanA-harbouring plasmid or Tn1546 transposon was associated with the rise in vanA, we compared the vanA-harbouring plasmids from the long-read assemblies. 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 S5). The main difference between these plasmids was a large insertion in ST796 compared with ST78, carrying repB, IS1216 and two hypothetical proteins (Figure S5). The vanA-harbouring plasmid from ST80 was similar to that from ST796 (Figure S6), but had lost IS467-birA from the plasmid backbone (Figure S5) and had a 48 bp deletion in vanS compared with the ST796 plasmid. Both vanA-harbouring plasmids from the ST203
and ST1421 isolates had unique plasmid backbones and Tn1546 transposons (Figure S5).

Aligning reads from each isolate to the above plasmids revealed that the majority of isolates likely shared the same plasmid as the representative isolate from their ST (see Excel file on alignments in Supplementary data). The exception to this was ST80, where nearly all isolates appeared to have the same vanA-harbouring plasmid as the one vanA+ (and vanB+) isolate from ST796 (Table S5).

Figure 2. Maximum likelihood tree of E. faecium in Australia. Recombination-adjusted core SNPs (6497) from all 331 isolates (321 VREfm and 10 VSEfm) were concatenated and used to produce a maximum likelihood tree in RAxML 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 >3800 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 validate further the clusters identified. Main BAPS groups are shown. Reference is identified with an arrow. No isolates had ebpA, hyl, gelE, agg, pilA or pilB; therefore, these genes have not been shown.
**Australian isolates in global context**

Compared with published sequences, the vanA-harbouring plasmids from ST78, ST796 and ST80 shared closest homology to a vanA-harbouring plasmid from VREfm in Denmark (Table S6). The vanA-harbouring plasmid from ST203 was most closely related to plasmids from several STs of VREfm isolated in Western Australia and Queensland, while the plasmid from ST1421 was closely-related to that from another ST1421 E. faecium, isolated in New South Wales in 2014 (Table S6). Phylogenetic analysis based on core SNPs from our isolates and global E. faecium strains showed that, with the exception of those from ST796, Australian isolates were widely distributed across the tree (Figure S7), which is suggestive of ongoing, intercontinental transmission of E. faecium, with subsequent clonal expansion.

**Discussion**

For the past two decades, the majority of VREfm in Australia have been vanB genotype. Recently, however, vanA-VREfm increased dramatically. In this population-based study, we looked at factors potentially contributing to this change.

First, 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., 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 hospitals. This pattern is similar to that of early vanB-VREfm in Australia, though the latter has become dominated by a single ST in Victoria (ST796).

Another possibility was that differences in putative virulence factors might be mediating the rise in vanA-VREfm. However, few putative virulence factors were identified, and their prevalence was largely consistent with previous data from vanB-VREfm in Australia, and similar to or lower than those found in vanA-VREfm from settings (e.g. in China or Brazil). While much is still unknown about virulence factors in VREfm (compared with E. faecalis), this suggests that the rise in 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...
Figure 3. Continued
This has important implications for hospital infection control, as the patient population affected by VREfm has also remained consistent over time, environmental factors may be mediating the observed changes in VREfm epidemiology. Further studies are needed to investigate this hypothesis.

Finally, we investigated whether a single vanA-harbouring plasmid and/or Tn1546 transposon was being transmitted within and across STs, as has been shown in Denmark. A unique vanA-harbouring plasmid was identified using representatives from each ST, nearly all of which had different Tn1546 transposons. Mapping reads to these assembled plasmids also suggested that each major ST was likely dominated by a different plasmid. This further suggests that horizontal gene transfer is not the sole driver of vanA-VREfm in this context.

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 with within them, this suggests a potential community reservoir for these strains. Transmission of VREfm has previously been shown in nursing homes, with carriage strains closely related to those causing infection; investigation is warranted to determine if this is also the case in Australia. The overall low diversity of vanB-VREfm could also be due to a ‘diffusion’ effect, wherein vanB-VREfm has slowly spread across hospitals through years of patient exchanges. Such a pattern would be reminiscent of that observed in Denmark; however, while in Denmark the ‘diffusion’ process was marked by a hub-and-spoke pattern, with patients mainly being transferred 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 vanB.

This work has a number of key strengths. First, by including all VREfm collected across an entire state, 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. Second, we used local
reference genomes for all SNP-based analyses. As using more genetically distant 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. By sharing our novel, complete VREfm genomes (from five different STs), we have also provided 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 assemble and completely characterize the vanA-harboursing plasmids in each of the major STs—a task not always feasible with short-read data alone. In doing so, we illustrate that horizontal gene transfer is unlikely to be the main driver of vanA-VREfm in this context, suggesting the conventional short-read analysis may be adequate for routine public health surveillance.

This work has several limitations. First, screening protocols for VREfm colonization are not standardized across Victoria; each hospital adhered to its own infection control guidelines, thus we may have an incomplete capture of colonizing strains. This could introduce bias 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 with vanB. Finally, as this was predominantly a laboratory-based study, we also did not have data on clinical presentation at the time of sample collection and were therefore unable to discriminate clinical infection aside from bacteraemia. Thus, we may underestimate the overall prevalence of infection (though the most clinically important strains, those causing bacteraemia, have been included).

Herein, we have provided a comprehensive snapshot of VREfm strain diversity across Victoria, representing the first population-level study of VREfm from Australia. In doing so, we have shown that Australian vanA-VREfm are highly similar to those from other geographical settings, suggesting transmission of VREfm between continents is ongoing. This highlights the critical importance of continued VREfm prevention and control on a global scale. We also highlight the complexity of *E. faecium* genomic epidemiology in this setting, revealing key differences in transmission dynamics of clinically relevant van genotypes; while vanB-VRE continues to predominate in Australia, repeated introductions and dissemination of vanA-VREfm within Victorian hospitals suggest this is changing. To understand better the clinical significance of this shift, prospective studies with detailed corresponding clinical data are needed.

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Transparency declarations

None to declare.

Author contributions

R. S. L. designed and ran the primary analyses, interpreted results, made the tables and figures, and wrote the first draft of the manuscript. A. G. designed analyses, advised on bioinformatics approaches, helped interpret results, and contributed to writing the manuscript. S. L. B. assembled the PacBio genomes, provided input on analyses, and helped interpret results. J. S. recruited all labs, coordinated submission of isolates and data from primary diagnostic laboratories, and did the initial processing of samples at MDU PHL. S. B. designed sample collection and advised on the initial interpretation of the results. G. P. C. did the laboratory work to generate the PacBio sequences. J. C. K., M. B. S., D. M. B. and T. S. provided bioinformatics tools, and advised on bioinformatics analyses. T. P. S. and B. P. H. conceived the cross-sectional study, helped decide on analyses, and edited the manuscript. All authors critically reviewed the manuscript for content.

Supplementary data

Tables S1 to S6, Figures S1 to S7 and an Excel file with vanA-harboursing plasmid alignment statistics appear as Supplementary data at JAC Online.

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