Polyclonal emergence of *vanA* vancomycin-resistant *Enterococcus faecium* in Australia

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**Objectives:** To investigate the genetic context associated with the emergence of vanA VRE in Australia.

**Methods:** The whole genomes of 18 randomly selected vanA-positive Enterococcus faecium patient isolates, collected between 2011 and 2013 from hospitals in four Australian capitals, were sequenced and analysed.

**Results:** In silico typing and transposon/plasmid assembly revealed that the sequenced isolates represented (in most cases) different hospital-adapted STs and were associated with a variety of different Tn1546 variants and plasmid backbone structures.

**Conclusions:** The recent emergence of vanA VRE in Australia was polyclonal and not associated with the dissemination of a single ‘dominant’ ST or vanA-encoding plasmid. Interestingly, the factors contributing to this epidemiological change are not known and future studies may need to consider investigation of potential community sources.

**Introduction**

Enterococcus faecium became prominent as a nosocomial pathogen in the 1990s with the emergence of hospital-adapted clones (a polyclonal subpopulation grouped together and previously designated as clonal complex 17) associated with antimicrobial resistance. Although first identified in the USA, these E. faecium clones quickly spread around the world, resulting in the widespread use of vancomycin as first-line therapy for the treatment of hospital-acquired enterococcal infections. Not surprisingly, VRE emerged shortly thereafter in North America and Europe. Since then VRE have been detected worldwide, including Australia.

The epidemiology of vancomycin resistance differs between regions, with the use of antibiotics in agriculture (such as avoparcin in Europe) thought to be a contributing factor. Irrespective of these differences, vancomycin resistance is acquired (in most cases) through the acquisition of either the vanA or the vanB operon, carried by the transposons Tn1546 and Tn1549 (and structural variants
thereof), respectively. While the spread of these transposons amongst enterococcal populations is largely facilitated by MDR plasmids, integration (of the transposon) into the chromosome can occur, particularly in the context of vanB; however, this is not required for operon stability or function.

Although the first VRE reported in Australia was a vanA-positive E. faecium in 1995, vancomycin resistance in enterococci has largely been mediated by a chromosomally encoded vanB determinant,\textsuperscript{2,3} which was associated with \( \sim 36.5\% \) of all E. faecium and 98\% of all VRE bacteraemia isolates in 2011.\textsuperscript{4} Unexpectedly, in 2012 vanA was reported in 32\% of vancomycin-resistant E. faecium isolated (predominantly from rectal surveillance specimens) in Western Australia.\textsuperscript{5} The proportion of vancomycin-resistant E. faecium bacteraemia isolates in Australia harbouring vanA genes increased from <1\% in 2011\textsuperscript{4} to 9.5\% in 2014.\textsuperscript{6} Some Australian centres have even witnessed a dramatic shift, with vanA replacing vanB VRE since 2012.\textsuperscript{7} In order to investigate the genetic context associated with this phenomenon, a random selection of vanA-positive E. faecium isolates from hospitals in four Australian capital cities were collected and subjected to WGS. All isolates were obtained from patients between 2011 and 2013 and corresponded with the ‘emergence’ of vanA within each institution.

**Methods**

**DNA manipulations**

DNA was extracted from each isolate using the ISOLATE Genomic DNA Kit (Bioline, London, UK). DNA fragments were PCR-amplified using BioTaq (Bioline) and capillary sequencing was performed by Macrogen Inc. (Seoul, Korea).

**Isolates and vanA screening**

Eighteen vanA E. faecium isolates collected between 2011 and 2013 were obtained from hospitals in Brisbane \((n = 5)\), Melbourne \((n = 5)\), Perth \((n = 5)\) and Sydney \((n = 3)\) and confirmed as vanA positive by conventional PCR using primers vanA-F (5\'-atgaatagaataaaggaactg-3) and vanA-R (5\'-
tcaccttaacgtaatac-3). All isolates demonstrated similar phenotypes with resistance to ampicillin, vancomycin and teicoplanin (MICs >8, 2 and 4 mg/L, respectively).

To examine the genomic context of vanA-positive isolates relative to vanB, the dataset was supplemented by a single isolate from each city (collected during the same time period). During this period three STs (ST203, ST341 and ST796) accounted for 90% of all vanB-positive isolates.8,9 Of these, only a single ST (ST203) was disseminated across Australia, accounting for 54% of all vanB VRE.

WGS analysis

All isolates underwent WGS following library preparation using either the Ion Plus Fragment Library kit (Life Technologies, Carlsbad, USA) or the NEBNext Fast DNA Fragmenon & Library Prep Set (New England Biolabs, Ipswich, USA). Sequencing was performed on the Ion Torrent PGM (Life Technologies) according to the manufacturer’s instructions. De novo assemblies were constructed using SPAdes software v3.5,10 with in silico MLST performed against the generated contigs. For isolates where no match was obtained, for one or more alleles, a mapped approach was taken using the closed genome of the E. faecium ST203 isolate AUS0085 (GenBank accession number CP006620). Identified ST groups were assigned to clonal complex 17 using eBURST 3 (efaecium.mlst.net), which defines groupings if ≥5/7 allele loci are shared between isolates. A maximum-likelihood phylogenetic tree was generated from the core single-nucleotide variants using RAxML v8.2.811 with tree visualization and manipulation performed within Figtree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree).

Transposon and plasmid assembly

Transposon assembly for all isolates was accomplished via mapping reads to the Tn1546 structure of the E. faecium isolate BM4147 (GenBank accession number M97297), in conjunction with manual curation using CLC Genomics Workbench v5.5 (CLCbio, Aarhus, Denmark). Based on BLASTn matches of de novo assembled contigs, a similar approach was taken for the assembly and closure of pJEG40 and pJEG43, using the previously characterized plasmids pS177 (GenBank accession number
NC_014959) and pRE25 (GenBank accession number NC_008445), respectively. Gaps in plasmid read mappings were closed (and assemblages confirmed) via PCR amplification and capillary sequencing. In silico plasmid replicon typing for all isolates was performed using BLASTn and the Gram-positive database available via the PlasmidFinder 1.3 website (https://cge.cbs.dtu.dk/services/PlasmidFinder/).

The complete sequences of pJEG40 and pJEG43 have been deposited in GenBank and assigned accession numbers KX810025 and KX810026, respectively.

**Results and discussion**

Eighteen isolates from four capital cities representing nine different hospitals were sequenced. These isolates represented both clinical [bloodstream and urine infection (n = 5)] and screening (n = 13) isolates. De novo assembly resulted in an average of 215 contigs with a mean N50 of >30 kb per isolate. From the 43 300 SNPs detected relative to the reference (Aus0085), 39 167 mutations were present in all isolates, leaving 4133 to construct the phylogeny. In silico MLST identified 12 different STs, with multiple STs observed in each capital city except in the case of Sydney, which only had ST80 isolates. Identical MLST isolates (ST80, ST203 and ST18) differed on a core genome level confirming non-identical isolates between Australian cities and institutions (Figure 1). Overall, 90% of isolates belonged to CC17, suggesting vanA has largely emerged from within the hospital-adapted clones (data not shown). Furthermore, the data suggest that vanA acquisition has occurred within the circulating clones, with all vanB ST203 isolates sharing the same ancestry.

Additionally, analysis of WGS reads revealed that nine Tn1546 structural variants were associated with the sequenced isolates (Figure 2a). These variants differed by the type and position of IS elements located within the transposon and in most cases the transposase genes orf1-orf2 were partially or completely deleted, preventing independent transposition (i.e. observed Tn1546 variants were likely anchored to their host plasmid backbone). The plasmid backbone structures associated with the most common Tn1546 variants, ii and iv, were completely assembled for two isolates and
designated pJEG040 (Efm008) and pJEG043 (Efm0038), respectively (Figure 2b). Subsequent read mapping using these plasmids as a reference revealed identical transposon variants were mostly associated with the same plasmid backbone (Figure 2a). Note that, with the exception of Tn1546 variant vi, which is also associated with a pJEG40-like backbone structure, sequence reads from the other isolates did not map to (or support assembly of Tn1546 variants with) pJEG040- or pJEG043-like plasmids. In this respect, *in silico* plasmid replicon typing indicated the presence of multiple plasmid backbone structures (in these isolates) that could contain the Tn1546 variant (Figure 2a).

It is also interesting to note that while pJEG040 and pJEG043 lack obvious conjugation/mobilization genes (Figure 2b), they were detected in multiple STs from across Australia. This indicates these plasmids are able to spread between strains, most likely with the help of co-resident transmissible plasmids; analysis of unassembled sequence contigs revealed the presence of conjugation-related genes. In this regard, pJEG40 is very similar to the pRUM-like plasmid pS177 (data not shown), which has mediated transfer of high-level vancomycin resistance to MRSA clinical isolates, most likely via co-integration with a conjugative plasmid present in the VRE donor.12

The presence of multiple Tn1546 variants in association with different STs and plasmid backbone structures suggests the emergence of *vanA* VRE in Australia during the 2011–13 period was not driven by the dissemination of a single ‘dominant’ ST or *vanA*-encoding plasmid. Furthermore, based on the sequences of pJEG040 and pJEG043, there are no obvious co-selectable plasmid-encoded phenotypes that might confer a selective advantage.

In context of the hospital setting, it seems unlikely that *vanA* VRE could have emerged in multiple different clones across Australia simultaneously, especially as the human gut does not have a *vanA* reservoir; contrarily, studies have shown that the *vanB* determinant is commonly carried by anaerobic gut commensals.13,14 Additionally, given the diversity within Australian hospital practice regarding *vanB* VRE treatment (e.g. use of teicoplanin), it is also unlikely that hospital antimicrobial VRE therapy is responsible for this genotypic shift. Therefore, possible explanations for the Australia-wide polyclonal emergence of *vanA* VRE include multiple previous introductions (of *vanA* VRE)
coupled with an unknown selection pressure. Alternatively, the vanA operon has been recently introduced via a community source (e.g. the food chain) and acquired by already circulating \( E. faecium \) clones, which are then enriched for via antimicrobial use in hospitals.

Ultimately, the factors contributing to this epidemiological change are not known and future studies may need to consider investigation of potential community sources. Regardless, despite the initial diversity observed, it is predicted (based on our \( vanB \) experience) that certain clones are likely to become dominant over time.

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**Transparency declarations**

None to declare.

**References**


Figure 1. Unrooted maximum-likelihood phylogenetic tree. Branch tips indicate isolates’ state of origin (New South Wales, orange; Queensland, green; Victoria, turquoise; Western Australia, red). The smallest number of single nucleotide variants (SNVs) occurs between Efm0038 and Efm0064 (18 SNVs). Grey circles indicate ST groupings. Asterisks denote ST203 vanB VRE (single isolate included from each city; collected during the same time period). This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
Figure 2. Structural features of Tn1546 variants (a) and plasmids pJEG040 and pJEG043 (b). The orf1-orf2 and van operon regions of the Tn1546 variants are represented by orange and blue lines, respectively (i–ix). Selected genes (arrows) of the plasmid maps are annotated: plasmid replication, red; transposon-related, orange; non-glycopeptide resistance, green; glycopeptide resistance, blue; all others [including antitoxin/toxin (AT/T) systems], white. Other features represented are: plasmid backbones, grey lines; ISs, orange pentagons (IS number indicated below or above; direction is with respect to the transposase gene); inverted repeats, vertical orange lines. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.