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A *vanA* vancomycin-resistant *Enterococcus faecium* ST80 outbreak resulting from a single importation event

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Background: A marked genotype shift among vancomycin-resistant *Enterococcus faecium* (VREfm) from *vanB* to *vanA* in Australia between 2011 and 2015 is a well-known phenomenon. It is hypothesized that this was caused by multiple independent clones emerging simultaneously in different settings and/or regions.

Objectives: To gain insights into the circumstances surrounding the shift from *vanB* to *vanA* VREfm in one Australian hospital.

Methods: The genomes of 69 *vanA* VREfm isolates from St George Hospital collected between 2009 and 2018 were studied. An expansion of ST80 *vanA* VREfm was noted following a single introduction. ST80 isolates were thus further characterized using hybrid sequencing and contextualized through comparisons with other published Australian ST80 isolates. Phylogenies were constructed with plasmid sequences compared with the index isolate.

Results: The 2011 expansion of ST80 *vanA* VREfm isolates in our institution originated from the 2009 index isolate, from a patient transferred from overseas. Phylogenetic analysis with other Australian ST80 *vanA* VREfm isolates showed that the 2011 expansion event was unique, with limited spread to adjacent local health districts. Plasmid analysis showed multiple variants, which can also be traced back to the 2009 isolate, consistent with ongoing plasmid adaptation over time.

Conclusions: These findings confirm an expansion event following a VREfm introduction event leading to a sustained clonal and plasmid outbreak over several years. Moreover, it demonstrates the complexity of countrywide replacement events. This study also highlights the use of hybrid sequencing in establishing an epidemiological relationship to the index isolate that was initially inapparent.

Introduction

Infection and colonization with vancomycin-resistant *Enterococcus faecium* (VREfm) contribute to substantial healthcare problems, particularly in Australia where the prevalence of VREfm is greater than in many European countries.[1,2] Colonization with VREfm is common among inpatients; however, systemic infection experienced by haematology/oncology patients and those admitted to ICU contributes to greater mortality than sepsis from susceptible strains of enterococci.[3–5]

Following two decades of *vanB* VREfm dominance, the rapid emergence of the *vanA* genotype since 2011 has been recognized throughout Australia.[1,6,7] This was exemplified by national enterococci bloodstream surveillance data showing that *vanA* prevalence increased from 2% in 2011 to 53% in 2018.[8,9] Genomic examination of isolates around this time showed different STs across different regions of Australia and between hospitals within the same region.[6,7,10,11] One possible explanation for these observations is multiple introduction events of the *vanA* genotype, followed by local selection of fit strains.[6]

Since 2008, all ICU and haematology/oncology patients in our institution have been screened for VRE on admission, weekly during hospitalization, and on discharge for infection control purposes. A similar shift from a *vanB* to a *vanA* genotype predominance was observed over a 12 month period in early 2011. Prior to this event, only two screening *vanA* isolates had been detected. The first was

detected 15 months earlier from a patient transferred from Bangladesh, who also carried other multiply resistant organisms[12] while the second was isolated in mid-2010. To better understand this genotype-replacement event, stored *vanA* VREfm isolates from our hospital were analysed using WGS.

Materials and methods

Isolates and settings

St George Hospital is a 660 bed acute care, tertiary referral, university-affiliated facility located in south eastern Sydney. All major surgical and medical specialties are delivered as well as maternity and child health services. A large haematology/oncology unit is supported by a 45 bed ICU. The Emergency Department records 70 000 patient encounters annually and is the receiving point for the Major Trauma Service. The hospital also receives inbound travellers requiring urgent treatment due to its close proximity to Sydney International Airport.

Isolates of *E. faecium* were confirmed by a combination of phenotypic characteristics including growth on chromogenic agar (chromID® VRE agar plate, bioMérieux, France), with speciation subsequently confirmed by MALDI-TOF MS (Bruker Daltonics, Germany). The isolates were tested for the presence of the putative *vanA/B* genotypes by real-time PCR using the LightCycler VRE detection kit (Roche, Germany) performed on the LightCycler® 2.0 instrument (Roche) according to the manufacturer's instructions. If the growth on selective screening media or routine culture media appeared to be pure, without regard for its pathogenic significance, isolates were stored prospectively at −70°C in Nutrient Broth No. 2 (Oxoid, UK) with 20% glycerol (Sigma, USA). Selected isolates were resuscitated by subculture on 5% horse blood agar (Oxoid) and incubated overnight in 5% CO_2 at 35°C. Vancomycin MIC was determined using ETEST® (bioMérieux) and interpreted using CLSI guidelines.[13]

Forty-six isolates were stored from 109 patients shown to have *vanA* VREfm in 2011 (Figure S1, available as Supplementary data at *JAC* Online) and all were selected for WGS. Of these, 2/15 isolates from ICU, 0/20 from haematology/oncology wards and 8/11 from other wards in the hospital were from clinical samples; the remainder were from screening samples. The two screening isolates detected prior to 2011 were retrieved and included. In later years, fewer isolates were stored. The remaining 21 isolates included 15 screening isolates and 6 clinical isolates (obtained from bloodstream infections, urine and sputum samples) originating from locations including the Emergency Department, ICU and surgical and haematology/oncology wards between 2012 and 2018. A total of 69 isolates of *vanA* VREfm were selected for WGS.

WGS

DNA was extracted from an overnight pure culture on the QIAGEN EZ1 Advanced XL using the DSP virus card (QIAGEN, Germany) as per the manufacturer's instructions and quantified using the Qubit Fluorometer (Thermo Fisher, USA). Illumina libraries were generated using the Illumina DNA Prep Kit (Illumina, USA) and WGS was performed using 150 bp paired-end chemistry on the Illumina MiSeq platform (Illumina). The ST80 subset of our isolates also underwent long-read sequencing. Libraries were generated using the Rapid Barcoding Sequencing Kit (SQK-RBK004) and sequenced on a Flongle flow cell using a MinION device (Oxford Nanopore Technologies, UK).

Comparison with external ST80 isolates

To place our observed events related to ST80 in an Australian-wide context, short-read sequences from the NCBI Sequence Read Archive (SRA) were downloaded following literature searches. Australian isolates of unknown ST metadata ($n = 2496$) were downloaded and screened for ST80 manually using stringMLST[14] on the FASTQ files. Overall, 220 external ST80 genomes (Table S1) were included and represented, but are not limited to, short-read sequences from the Australian Group on Antimicrobial Resistance (AGAR) Australian Enterococcus Sepsis Outcome Programme (AESOP) for the 2015–17 period (NCBI PRJNA562395, PRJNA562414, PRJNA562407),[11] screening and clinical samples from Victoria, Australia (PRJNA433676)[7] and a 2016 New South Wales study of VREfm in ICUs (PRJNA415172)[15] that included strains from our hospital.

Genome analysis

Short-read sequences in this study were initially mapped against the reference Aus0004 (GenBank CP003351.1) using the Burrows–Wheeler Aligner (BWA-MEM).[16] The resulting variants were filtered for read depth 20, base quality 30 and mapping quality 30 using FreeBayes.[17] Consensus sequences were based on location of SNP ignoring indels. *De novo* assemblies were generated using SPAdes[18] with *in silico* MLST performed using the mlst package (https://github.com/tseemann/mlst).

The ST80 subset of isolates was subjected to long-read sequencing and each underwent hybrid assembly using Unicycler[19] under normal mode, with the results manually inspected using Bandage[20] to ensure high-fidelity assemblies. The short-read analysis was then repeated, as above, inclusive of the 220 downloaded sequences using the long-read RP01 (2009-ST80 isolate) scaffolds as the reference.

Plasmids harbouring the Tn*1546* gene cluster were identified and extracted from the assemblies and annotated using RAST[21] and the ISFinder service.[22] Manual curation of Tn*1546*-harbouring plasmid assemblies were undertaken to confirm their structure and to categorize plasmid variants. Additional comparative analysis was performed on completed hybrid assemblies using the BLAST Ring Image Generator[23] and progressiveMauve[24] under default settings to interrogate homology of core genome sequences.

Phylogenetic trees were subsequently generated from the SNP alignments using RAxML-NG[25] with 100 bootstrap replicates following masking of recombination events identified using Gubbins.[26] Visualizations of genomic features were created using *R* software[27] with *ggplot2*,[28] *ggtree*[29] and *genoPlotR*[30] packages.

Ethics approval

The study was approved by the SESLHD Human Research Ethics Committee (reference no 17/175 LNR/17/POWH/519), NSW Health, Australia.

Sequence data

Nucleotide sequences of this study (Table S2) have been deposited in NCBI BioProject accession number PRJNA690640.

Results

Initial analysis of the 69 isolates identified a predominance of ST80 (45/69; 65%) with the majority of these observed in 2011 (42/45; 93%). *In silico* MLST of the 2009 and 2010 isolates (prior to clonal expansion) revealed ST80 and ST117, respectively. The remaining 2011 *vanA* isolates included: ST17 ($n = 2$), ST203 ($n = 1$) and ST341 ($n = 1$). Although ST80 was still present after 2011 ($n = 2$), *pstS*-null strains of ST1421 ($n = 10$), ST1424 ($n = 8$) and ST1478 ($n = 1$) were increasingly detected between 2015 and 2018 following similar patterns seen in Australian surveillance data.[9,31] The phylogeny, inclusive of all 69 isolates, generally grouped isolates by ST with ST80 forming a single group inclusive of the earliest *vanA E. faecium* isolate from 2009 (Figure S2).

ST80 subset analysis

All ST80 isolates ($n = 45$) underwent nanopore long-read sequencing. Nine complete chromosomes with an average size of 2.8 Mb were generated by hybrid assembly, including the 2009-ST80 index strain (RP01). Comparative genomics of these chromosomes showed three locally collinear blocks that were highly conserved and syntenic to each other (Figure S3). A 116 kb cryptic inversion region incorporating the *ddl* housekeeping gene was observed in the middle block of RP01, flanked by opposing IS*Ef1* ISs. The GC skew of this region showed an asymmetrical pattern from neighbouring regions, indicative of recombination (Figure S3). Upon reanalysis of genome alignment, excluding RP01, one collinear block was observed.

Placing our ST80 isolates into a wider context, the dataset was supplemented with 220 identified ST80 sequences obtained following screening of available Australian *E. faecium* short-read sequences. The phylogeny of the ST80 datasets (using RP01 as the reference and masking of recombination sites) was unbalanced, with all of the 2011 *vanA* VREfm isolates sequenced in this study clustering on a separate branch consistent with a local hospital expansion event (Figure 1a). Moreover, RP01 is ancestral to the clonal expansion event and therefore likely represents the index isolate.

Twenty-four strains from external sources clustered among our isolates (Figure 1b). Of these, 10 were isolates from our hospital sequenced as part of a previous study.[15] The remaining 14 isolates originated from hospitals adjacent to our local healthcare district ($n = 11$) and from other Australian sources ($n = 3$).[11,15,32,33] The remaining Australian ST80 isolates ($n = 196$) formed distinct lineages. Two previously sequenced isolates from our hospital were found to reside remotely in one of these clusters.

In total, 2691 core-genome SNP sites were identified in all mapped ST80 isolates. The median pairwise difference of RP01

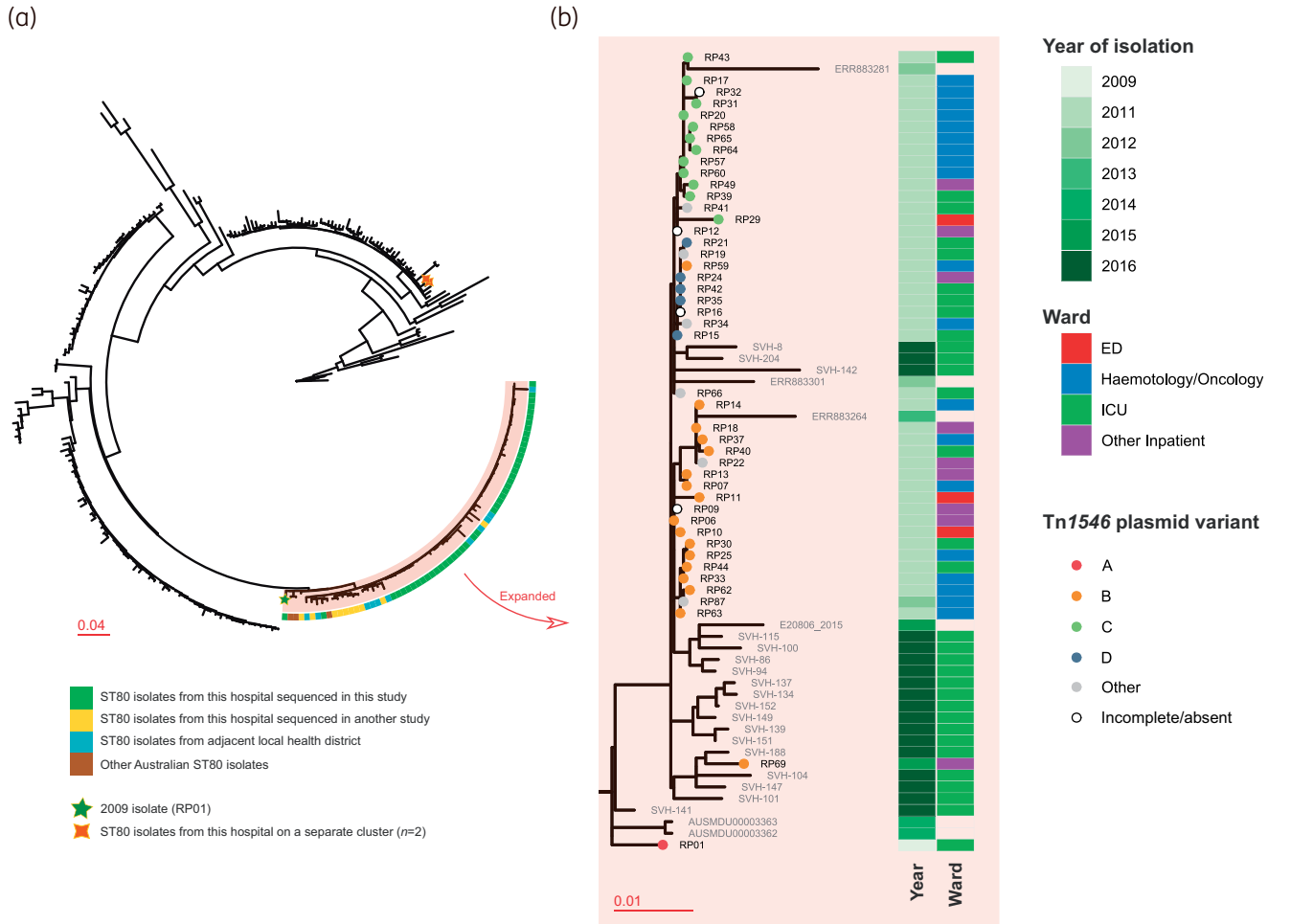

Figure 1. Maximum-likelihood phylogeny of selected Australian ST80 VREfm isolates following masking of recombination and 100 bootstrap replicates. (a) The circular phylogeny constructed from 265 ST80 genomes. Of the 69 genomes within the highlighted clade, 55 originated from our institution. Of these, 10 and 45 were sequenced as part of a previous (indicated by yellow squares) or current study (indicated by green squares), respectively. (b) This clade is expanded, with coloured tree tips corresponding to Tn*1546*-harbouring plasmid variants (additional details can be found in Figure 2). Additional metadata, including year of isolation and location of the isolate, are shown in columns to the right of the tree. Scale bar represents the average number of base substitutions per branch site. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Figure 2. (a) Alignment of four major plasmid variants corresponding to homologue variants A–D in Figure 1 and (b) plasmid structures of six remaining plasmids corresponding to the 'other' variant in Figure 1. The Tn*1546* gene cluster is highlighted by orange shading, ISs are represented by teal shading and other antibiotic resistance genes are in pink. Regions with >99% shared identity are shaded in grey. Partial sequences are denoted with an asterisk, *. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

and the cluster associated with our 2011 hospital expansion (Figure 1a, highlighted) was 39 SNPs (range 22–80). Of these, a median of 37 SNPs (range 33–64) were detected in the 2011-ST80 isolates. A higher diversity was demonstrated among the remaining ST80 isolates, with a median of 256 SNPs (range 86–1046).

Plasmid analysis

Seven complete circular plasmids were detected in RP01, including a rep_{17} pRUM-like plasmid encoding the Tn*1546* cluster and a rep_2 pRE25-like plasmid. Overall, a total of 41 Tn*1546*-harbouring plasmids were successfully constructed by hybrid assembly. The *vanA* context was similar in the majority of these Tn*1546* plasmids, characterized by an IS*1251* element between the *vanS and vanH* genes, absence of the *orf1* transposase gene and partial deletion of the *orf2* resolvase gene (Figure 2).

Thirty-five closed plasmids, ranging from 37 638 to 41 654 bp in length, were categorized into four distinct variants (A–D) based on genomic content (Figure 2a). Plasmid variant A from RP01 closely matched an Indian plasmid (pA15023_P2; GenBank: CP059825.1) and a Danish plasmid (pV24-5; GenBank: CP036156.1),[34] both with 99% cover and 100% identity. Variant B matched a previously closed Australian *E. faecium* plasmid (pJEG040; GenBank: KX810025.1)[6] with 100% coverage and >99.9% identity. This plasmid varied from plasmid A across a 4016 bp region flanked by IS elements (IS*1216E*), containing four hypothetical genes and a *repB*-encoding sequence. No published exact matches could be found for plasmid variants C and D. Consistent with ongoing plasmid adaptation, these variants were related to variant B with differences mediated by insertion of either one or two IS elements (IS*1251*), respectively.

The remaining six plasmids (range: 29 426 to 68 560 bp) differed in length and content from the aforementioned variants (Figure 2b). These were scattered among the isolates, suggestive of adaptation or introduction of new plasmids into the ST80 backbone (Figure 1). Two large plasmids (>60 kb) were observed harbouring a region from a concurrent rep_2 plasmid, suggesting possible hybrid plasmid recombination (pRP66, pRP34; Figure 2b). One strain with a plasmid containing a truncated Tn*1546* transposon between the *orf1* and the *vanR-S* regions (pRP87; Figure 2b) was found to be phenotypically susceptible to vancomycin, with an MIC of 1.0 mg/L. The remaining plasmids differed by apparently random insertion or deletion of elements that can be ancestrally traced back to the variants above.

Discussion

The combination of short- and long-read sequencing has allowed us to trace the origin of our institution's initial *vanA* VRE problem. The 2009 isolate originating from a patient following a medical transfer from Bangladesh is the likely index case, with similar isolates found at a chromosomal level.[12] The pairwise SNP differences of the 2009 case versus 2011 ST80 isolates revealed a median of 37 SNPs, consistent with molecular clock rates of 5–58 SNPs per year reported previously.[15,35,36] Analysis of plasmids harbouring the Tn*1546* from our ST80 isolates revealed a consistent backbone structure, suggesting a single plasmid lineage. This plasmid structure has been observed in multiple STs of *E. faecium*, including our 2009 isolate in different regions of Australia and overseas.[6,7] Although plasmids vary, these differences are relatively minor given the 2 year time frame. Overall, we demonstrate a complex evolution of both the plasmid and the core genome stemming from an initial introduction in 2009.

The index isolate is likely to have become established and disseminated undetected within the general hospital environment for 15 months, as only ICU and haematology/oncology patients were routinely screened for VRE. A study by Lee *et al.*[37] supports this theory by showing persistence of *vanA* VRE on surfaces as a reservoir for continuing indirect transmission.

By 2011, ST80 was the predominant ST, with ongoing transmission in subsequent years in our hospital. Moreover, the presence of closely related strains in adjacent hospitals is consistent with the regular transfer of patients between local healthcare facilities. Although ST80 has been widely reported in many countries previously (i.e. Denmark,[38] Canada[39] and India[40]), this transmission pattern seems to be limited to our institution as the Australian surveillance data found only a single *vanA* ST80 isolate among 107 bloodstream VREfm isolates in 2011 from 29 participating Australian hospital laboratories.[8] Concomitant with the 2011-ST80 expansion, four local *vanA* VREfm isolates were typed as either ST17 ($n = 2$), ST341 or ST203. Nationally, these alternative STs were prevalent in 2011,[8] consistent with heterogeneous introduction and expansion events in other institutions driving the emergence of *vanA* in Australia.[6] This pattern seems to have occurred once more with the dominance of *pstS*-null strains ST1421 and ST1424 as early as 2015, and coincides with the increasing prevalence of these strains across Australia.[15]

Our comparative analysis with external sequences is limited by virtue of an incomplete collection of isolates following the initial 2011 *vanA* VRE expansion. As this retrospective study relied partly on randomly stored screening isolates, there may have been additional introductions that were undetected. However, this seems unlikely as ST80 isolates were preferentially found in our institution by another study drawing on ICU isolates in 2016.[6] Our screening isolates originated from haematology/oncology and ICU patients, as these patients tend to be at highest risk of VRE and transfer between these hospital wards. Thus, our findings may underrepresent the total genomic ST80 diversity within our institution.

In summary, we have shown an ST80 introduction event and clonal expansion that differs from other contemporaneous Australian isolates. This work confirms the hypothesis of multiple entry and expansion events of *vanA* VREfm to an Australian setting.[6] Rapid clonal expansion in one location was followed by apparent spread to neighbouring hospitals with minor genomic changes over time. The study also highlights the value of both short- and long-read WGS technology in understanding the introduction and subsequent transmission of VRE in healthcare facilities. Hybrid sequencing enables us to pair an ancestrally related genome to obtain a more accurate SNP profile, instead of using an unrelated index reference that can be more divergent.[41] Additionally, the ability to close plasmids and compare their genes gives us another perspective on the dynamic nature of nosocomial VRE transmission. These are fundamental to infection prevention and control strategies as regular molecular techniques or short-read WGS alone do not give enough resolution to infer relatedness, especially for plasmid-transmissible antibiotic resistance genes. To our knowledge, this is the

first publication showing an introduction event leading to a sustained plasmid outbreak over several years.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 and Figures S1 to S3 are available as Supplementary data at *JAC* Online.

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