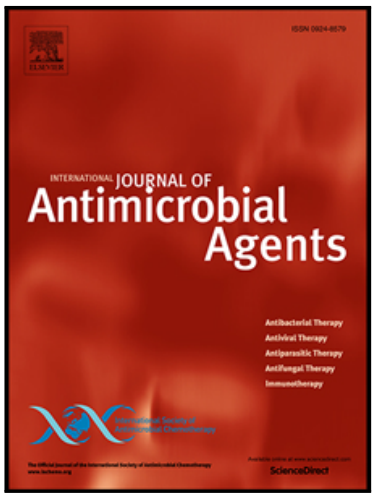

Molecular characterization and evolution of the first outbreak of vancomycin resistant Enterococcus faecium in Western Australia

Terence Lee , Stanley Pang , Sam Abraham , Geoffrey W. Coombs

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Title: Molecular characterization and evolution of the first outbreak of vancomycin resistant *Enterococcus faecium* in Western Australia.

Authors: Terence Lee[a], Stanley Pang[a], Sam Abraham[a] and Geoffrey W Coombs[a]

[a] Antimicrobial Resistance and Infectious Diseases (AMRID) Research Laboratory, Murdoch University, Western Australia

Corresponding Author: Professor Geoffrey Coombs

Email: G.coombs@murdoch.edu.au

Telephone: +6 1 8 9360 2259

Address:

Antimicrobial Resistance and Infectious Diseases (AMRID) Research Laboratory

School of Veterinary and Life Science, 90 South Street, Murdoch, Western Australia 6150

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Abstract

In 2001, Western Australia recorded its first outbreak of vancomycin resistant *Enterococcus faecium* (VREfm). A state-wide infection control effort, which oversaw patient screening and transfers, successfully terminated the outbreak within six months. However, the outbreak re-emerged two years later. Over the two outbreaks, the *vanB*-positive multilocus sequence type (ST) 173 *E. faecium* strain, was isolated from 201 patients.

Our objective was to identify differences in genetic traits leading to the successful transmission of ST173 VREfm compared to non-ST173 VREfm isolated during the same period. Additionally, we aimed to describe the changes observed in the ST173 VREfm genome collected during the two outbreaks.

Virulence factors *ecbA, fss3, psaA* and scm, identified in the non-ST173 isolates were largely absent in the ST173 isolates. The *esp* gene was not identified beyond 45% coverage for any isolate in this study. In terms of resistance genes, *tet(U)* was identified in 94.7% of ST173 VREfm isolated in the first outbreak but was largely absent in the ST173 VREfm isolated in the second outbreak and in the non-ST173 VREfm. Seven ST173 VREfm isolates (Clade A) carried *dfrG* but not *tet(M)* resistance genes. The average genome size of ST173 VREfm isolated in the first outbreak was significantly larger than the genome of the ST173 VREfm isolated in the second outbreak.

The reduced number of virulence factors in the ST173 isolates may explain the low infection and high colonization rates observed during the outbreak. In addition, isolates with larger genome sizes were found to be associated with outbreaks.

Keywords: Outbreak, *Enterococcus faecium*, Antimicrobial resistance, Genetic Evolution

1. Background

Enterococcus faecium is an opportunistic pathogen that is capable of rapid genetic mutations allowing the bacterium to quickly adapt and become resistant to antimicrobials. The rapid rise of antimicrobial resistance in *E. faecium* coupled with the slow pace of development of new novel antimicrobials is a growing threat to public health. The global issue of multi-drug resistant *E. faecium* is well reported not only as a public health issue, but also as an animal agriculture issue [1,2]. Typically, *E. faecium* only cause severe infection when the host becomes immuno-compromised. Consequently, the greatest affected group of people are hospitalized patients and the elderly [3].

Introduced in 1954, vancomycin has been a critical antimicrobial treatment option for *E. faecium* and methicillin-resistant *Staphylococcus aureus* infections. However, the development of vancomycin resistance has been increasingly identified in both species leading to the need for strict antimicrobial stewardship. In 1986, vancomycin resistant enterococci (VRE) was first isolated in the United Kingdom [4] followed by the rest of Europe [5], and then in 1989 in North America [6]. Globally, VRE are thought to account for approximately 10% of all bacteraemias [7], and are the fourth and fifth leading cause of sepsis in North America and Europe respectively [8]. In Australia, 50% of *E. faecium* bacteraemia episodes are now vancomycin resistant [1].

Vancomycin resistance is mediated via the acquisition of a *van* operon of which three types, *vanA, vanB* and *vanM,* are clinically important due to the horizontal transfer of the operon amongst bacterium. The *vanA* type, which is predominant in North America and Europe and confers high levels of vancomycin and teicoplanin resistance, can be found on the mobile transposon genetic element Tn*1546* [9]. The *vanB* type, which is predominant in Australia and New Zealand and is increasingly being reported in Europe, confers variable levels of vancomycin resistance and is found on the mobile genetic transposable element Tn*1547* [9]. The *vanM* type, which has been reported in China and Singapore [10], confers high levels of inducible vancomycin resistance, and can be found downstream of an IS*1216*-like element akin to the IS*1216V* element found widely in *vanA.*

In July 2001, the first isolate of *vanB* multilocus sequence type (ST) 173 vancomycin resistant *E. faecium* (VREfm) at Royal Perth Hospital, a major Australian teaching hospital located in the Perth metropolitan area of Western Australia (WA), was cultured from an ICU patient [11]. Quarterly surveillance of the hospital's high-risk areas had previously only detected four patients with non-epidemiologically related cases of *vanA* VREfm. By screening patients which shared the same ward as the index patient, the spread of *vanB* ST173 VREfm was traced to 11 wards. In an attempt to prevent ST173 VREfm from becoming endemic in the hospital, and throughout WA, extensive state-wide outbreak and infection control measures were implemented. By late September, 60 patients were found to be colonized with ST173 VREfm. Consequently, a hospital executive committee was established to manage all aspects of infection control at the hospital, including patient and staff movements, microbiology diagnostics and surveillance. By the end of the outbreak in December 2001, 9,658 patient and 24,396 hospital-environmental VRE screening swabs were processed and a total of 141 patients from 23 wards across two hospitals were colonized with *vanB* ST173 VREfm. An additional two patients had infections of varying severity. As a result of the increased VRE screening, seven non-ST173 *E. faecium* strains were also identified during the outbreak. However, wide spread transmission of the strains did not occur.

An additional 14 isolates were recovered sporadically over 2002 and no ST173 isolate was recovered in 2003. In mid-2004 *vanB* ST173 VREfm re-emerged at the hospital and 40 patients admitted to the hospital's burns unit were colonized. Only an additional four ST173 VREfm have been isolated in WA since; the last in November 2005.

To determine if there were any genetic advantages of the outbreak isolate, next generation whole genome sequencing was used to compare the genomes of *vanB* ST173 VREfm isolates recovered from the two outbreaks with the non-ST173 VREfm isolates. In addition, we compared the evolutionary modifications that may have occurred in the *vanB* ST173 VREfm isolates during the two outbreaks.

2. Materials and Methods

2.1. Isolates

Overall 201 *vanB* ST173 VREfm and 20 non-ST173 VREfm (16 *vanB* and four *vanA*) isolated from patients admitted to Royal Perth Hospital from June 1998 to November 2005 were included in the study. Previous antibiogram and pulsed-field gel electrophoresis (PFGE) typing information for all isolates were sourced from a previously published study (Sup.Figure 1) [11].

2.2. Whole genome sequencing

All isolates were retrieved from -80°C storage and cultured onto blood agar. Identities of secondary subcultures were confirmed using matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) (Bruker, United States). DNA extraction was performed on overnight sub-cultures using the Invitrogen Applied Biosystems™ MagMAX™ Multi-Sample DNA extraction kit (ThermoFisher, United States). The DNA library was prepared using the Ilumina Nextera XT DNA kit (Illumina, United States) and sequenced on either the Illumina Miseq or NextSeq platforms (Illumina, United States). Only DNA sequences with greater than 40x coverage were used for analysis.

2.3. Sequence data analysis

An *E. faecium* ST17 strain, AUS004 (NCBI Reference sequence: NC_017022), complete genome was used as the reference genome for all sequence analysis. Raw DNA sequences were cleaned by trimmomatic [12], assembled using SPAdes De novo assembly v3.10.1 [13] and annotated using Prokka v1.12 [14]. The resistome and virulence factors were identified using the ABRicate v0.4 screening tool with the ResFinder database [15] and the virulence factor database [16] respectively. Virulence factors with greater than 75% coverage and identity were considered present. Single nucleotide polymorphism (SNP) identification and core genome alignments were performed using Snippy v3.217 and the multilocus sequence type (MLST) was identified using the *E. faecium* multilocus sequence typing scheme [18]. The genome alignment was parsed through gubbins [19] which identified and remove recombination regions. A phylogenetic tree was constructed using the

resulting SNPs with recombination regions removed in Mega using the maximum parsimony algorithm. Visualization of the phylogenetic tree was performed on the Interactive tree of life (iTOL) web-service [20]. A pan-genome analysis of the ST173 isolates was performed by mapping raw sequences in CLC Genomic Workbench (CLC Bio, Denmark) to a pan genome generated by Roary [21].

2.4. Statistical analysis

All statistical analysis was performed in R [22]. Results of $P<0.005$ was considered statistically significant.

3. Results

3.1. Sequences

Sequences obtained for 198 of the 201 vanB ST173 VREfm isolates and 15 of the 20 non-ST173 VREfm isolates were above the minimum coverage threshold. Of the 198 vanB ST173 VREfm isolates, 153 were isolated from the first outbreak and isolates from 2002. The MLST and the van operon type for the 15 non-ST173 VREfm included *vanB* ST17 (9 isolates), *vanA* ST17 (2) and single isolates of *vanB* ST25, *vanA* ST154, *vanA* ST195 and *vanB* ST666.

3.2. *vanB* ST173 *E. faecium*

The average genome size for the ST173 VREfm isolates was 2.88mb with a standard deviation of 31.6kb. The average number of genes for all ST173 VREfm isolates was 2,759 with a standard deviation of 37.4. The smallest and largest genome sizes were 2.76mb and 2.98mb respectively. Of the 3,682 genes identified, 61.6% were classified as core genes (present in ≥99% of isolates).

Using an unpaired t-test, the genome size of the *vanB* ST173 VREfm collected in the first outbreak was significantly larger (average 2.89mb) than the genome size of the ST173 VREfm collected in the second outbreak (average 2.84mb) ($p<2.2x10^{-16}$). (Figure 1).

3.3. Non-ST173 vancomycin resistant *E. faecium*

The average genome size for the non-ST173VREfm isolates was 2.82mb with a standard deviation of 153kb. The average genome size for ST17 was 2.89mb with a standard deviation of 98kb while the genome sizes of ST25, ST154, ST195 and ST666 were 2.65mb, 2.80mb, 2.47mb and 2.64mb respectively. The average number of coding sequences for the non-ST173 VREfm isolates was 2,675 with a standard deviation of 158.

An unpaired t-test showed no statistical differences between the genome sizes of the ST173 and the non-ST173 VREfm isolates (Figure 2).

3.4. Phylogenetic tree

The phylogenetic tree of the 213 VREfm showed three STs (ST195, ST666 and ST25) were more distantly related to ST173 compared to STs 17 and 154. The clustering of 198 *vanB* ST173 VREfm isolates in the same tree branch suggests that the outbreak clone was introduced at the hospital only once and expanded clonally (see figure 2).

The phylogenetic tree for *vanB* ST173 VREfm was rooted at the most distant isolate based on the phylogenetic tree of all isolates. One-to-one patient spread of *vanB* ST173 VREfm across seven patients (clade A) was observed prior to the clonal expansion of the strain into clade B then further into clade C (Figure 3).

Clade B contained 13% of *vanB* ST173 VREfm isolates all of which were collected during the first outbreak period. The isolates within clade B were distributed across six smaller sub-clades of two to twelve isolates each.

Clade C derived from isolates in clade B contained 84% of *vanB* ST173 VREfm isolates and could be further sub-divided into three sub-clades; C1, C2 and C3. Clade C1 contained 9% of *vanB* ST173 VREfm isolates, with one isolate having the smallest genome amongst the *vanB* ST173 VREfm. Clade C2 contained 20% of *vanB* ST173 VREfm isolates, all of which were collected from the second

outbreak period. The largest clade, clade C3 contained 55% of *vanB* ST173 VREfm isolates. Clade C3 also housed the index isolate cultured from the first outbreak.

3.5. Virulence factors

Ten virulence factors were identified amongst the 213 isolates.

All 198 ST173 VREfm carried *bopD* which putatively encodes a sugar-binding transcriptional regulator critical for the process of biofilm formation and *bsh*, a bile salt hydrolase. 98.9% of ST173 VREfm isolates also carried the *sgrA* gene encoding an LPxTG surface adhesin that binds to fibrinogen and nidogen and is commonly implicated in biofilm formation. Another common gene found in 98.5% of ST173 VREfm was *acm*, a collagen- binding microbial surface components recognizing adhesive matrix molecules (MSCRAMM). Additionally, two isolates harboured *fss3*, another fibrinogen-binding MSCRAMM and *psaA* encoding pneumococcal surface adhesin A. There were no notable differences in the compliment of virulence factors between the ST173 VREfm isolated in the first and second outbreaks.

The 15 non-ST173 VREfm harboured the previously described *acm*, *bopD* and *bsh* genes. All except one isolate harboured additional virulence genes. The additional genes include *fss3*, *psaA, scm*, *sgrA and ecba*, a collagen type-V binding MSCRAMM, which were found in, 73%, 73%, 33%, 67% and 73% of non-ST173 VREfm (Table 1).

The *esp* gene, encoding the enterococcal surface protein, commonly found in clinical isolates of clonal complex (CC) 17 *E. faecium* was partially found in eleven non-ST173 isolates and two ST173 isolates. In both sets of isolates, the *esp* gene only had a maximum of 45% coverage and 89% identity.

3.6. Resistome

In addition to the *vanB* (209 isolates) and *vanA* (4 isolates) operons, antimicrobial resistance genes to aminoglycosides, lincosamides, macrolides, streptogramins, tetracyclines and trimethoprim were identified (table 2).

Aminoglycoside resistance genes were identified in 98% of all isolates in this study. Overall 97%, 93% and 92% of the ST173 VREfm carried the *ant(6)-Ia*, *aph(3')-III* and *aac(6')-aph(2")*, aminoglycoside resistance genes respectively. For the non-ST173 VREFm, 67%, 67% ,33% and 20% carried the *ant(6)-la*, *aph(3')-III*, *aac(6')-aph(2")* and *aadE*, aminoglycoside resistance genes respectively.

The *erm(B)* erythromycin resistance gene, was identified in 97% of ST173 VREfm and 67% of non-ST173 VREfm. A second erythromycin resistance gene, *erm(T)*, was only identified in ST173 VREfm isolates in Clade A (Figure 3).

Four types of tetracycline resistance genes were identified. Overall 91% of isolates carried *tet(M)*, 93% *tet(L)* and 72% *tet(U).* One isolate (ST17) carried *tet(32)*, a novel tetracycline resistance gene with a 76% amino acid similarity to *tet(O)* and 71% similarity to *tet(M)*. For ST173 VREfm isolates, all except one isolate carried the *tet(L)* gene. The *tet(M)* and *tet(U)* genes were however generally absent in isolates of particular clades; clades A and C2 respectively. For non-ST173 VREfm isolates, 20% carried *tet(M)*, 20% *tet(U)* and only 13% *tet(L)*.

The *msrC* gene, conferring enhanced erythromycin resistance, was identified in all but one isolate (ST195).

The *dfrG* gene, which encodes trimethoprim resistance, was identified in 60% of non-ST173 VREfm isolates and 4% of ST173 VREfm, all except one isolate belonged to clade A. The *cat* gene which encodes chloramphenicol resistance, commonly found on plasmid pC233, and the *lnu(B)* gene which encodes lincosamide resistance were not detected in ST173 VREfm isolates but were identified in 53% and 7% of non-ST173 VREfm respectively.

4. Discussion

Using whole genome sequencing we have compared non-ST173 VREfm with *vanB* ST173 VREfm isolated from two distinct *vanB* ST173 VREfm outbreaks to understand the genetic factors leading to

the success of the outbreaks. Additionally, we have compared isolates recovered from the two outbreaks to understand the genetic factors leading to the reduced success of the second outbreak.

Whole genome sequencing classified the *vanB* ST173 VREfm into three main clades. The uneven and increasing distribution of isolates from clade A to clade B to clade C suggests that the isolates were becoming increasingly more successful as they evolved compared to the clades before them.

In the original study [11], Christiansen *et al*. raised the possibility undetected *vanB* ST173 VREfm colonized patients could have been present in the hospital prior to the index isolate of the first outbreak. In our study, the index isolate collected was identified further down the evolutionary path of the phylogenetic tree which supports the hypothesis that the index isolate was not the founder.

From the phylogenetic tree, we were able to track isolates from the second outbreak to isolates derived from clade B suggesting the second outbreak was not a result of an introduction of an independently evolving ST173 strain but a remnant from the first outbreak.

Comparing the genome size of the ST173 and non-ST173 VREfm, we observed the *E. faecium* STs often associated with outbreaks such as ST17 (AUS0004: 3.0Mb [23], this study: 2.89Mb) , ST203 (AUS085: 3.2Mb) [24] and ST173 (this study: 2.88Mb) possess larger genomes compared to STs associated with sporadic transmission such as STs 666 (this study: 2.64Mb), ST25 (this study: 2.65Mb) and ST195 (this study:2.47Mb). In addition, the genome size of isolates in the first outbreak was larger than the genome size of isolates from the second outbreak which affected less than one third the number of patients and was able to be controlled in a shorter time period. Although genome size alone cannot be used to definitively determine outbreak strains of VREfm, it does suggest it may be advantageous for isolates to carry additional genes.

Of the non-ST173 sequence types, ST25, ST195 and ST666 were notably more distant on the phylogenetic tree. Additionally, the remaining STs, ST154 and ST17 that were more closely related to ST173 have subsequently been reported as epidemic strains of VREfm [25].

We investigated the presence and absence of virulence factors and identified fewer virulence factors in the *vanB* ST173 VREfm isolates compared to the non-ST173 VREfm isolates. In particular, the *esp* gene, often found in CC17 *E. faecium* isolates was not identified beyond 45% coverage in any isolate in this study. The lack of notable differences in the distribution of virulence factors between isolates from the first and second outbreaks suggests the success of an outbreak is not associated with virulence factors. In addition, the *ecbA, fss3 and psaA* gene, which were present in most of the ST17 isolates were largely absent in ST173 isolates, however ST173 was the more successful clone, representing an inverse relationship. The reduced number of virulence factors in the *vanB* ST173 VREfm isolates may also explain the low rate of infection (0.5%) and high rates of colonization observed during the outbreak.

A key factor in enterococcal survival in hospitals stems from the organism's ability to rapidly adapt to antimicrobials. As expected most of the isolates possessed genes encoding for aminoglycoside, erythromycin and tetracycline resistance. The phylogeny of *vanB* ST173 VREfm presented above suggests the acquisition of *tet(M)* and possibly other associated genes through horizontal transfer triggered the clonal expansion and the outbreak of *vanB* ST173 VREfm. In addition, the reduced scale of the second outbreak was associated with the absence of *tet(U)* in *vanB* ST173 VREfm isolates. Although it has been suggested the *tet(U)* gene is possibly a misannotated 3' end of a replication initiation (*rep*) protein and does not contribute to tetracycline resistance [26], it is possible that *tet(M), tet(U)* and/or genes associated during their transfer could provide an advantage to *E. faecium*. Other resistance genes encoding chloramphenicol, trimethoprim and lincosamide resistance were largely absent in the *vanB* ST173 VREfm isolates which suggests the genes were not critical for survival in ST173 isolates.

4.1. Conclusion

The objective of our study was to use next generation whole genome sequencing to determine the molecular characteristics of the first *vanB* positive VREfm hospital-outbreak in WA and to determine the molecular attributes that led to the success of *vanB* ST173 VREfm. We have characterized and

compared the genome of the outbreak strain, *vanB* ST173 VREfm, to sporadic non-ST173 VREfm background isolates collected at the same hospital during the outbreak. We have identified a correlation between isolates with large genome sizes and those from STs associated with outbreaks. We have also been able to identify a decrease in the presence of certain virulence factors with isolates from the ST173 outbreak. In this study, the gain and loss of antimicrobial resistance genes in ST173 appear to contribute most to the rise and decline of the outbreak.

Declarations

Funding: No funding

Competing Interests: None

Ethical Approval: Not required

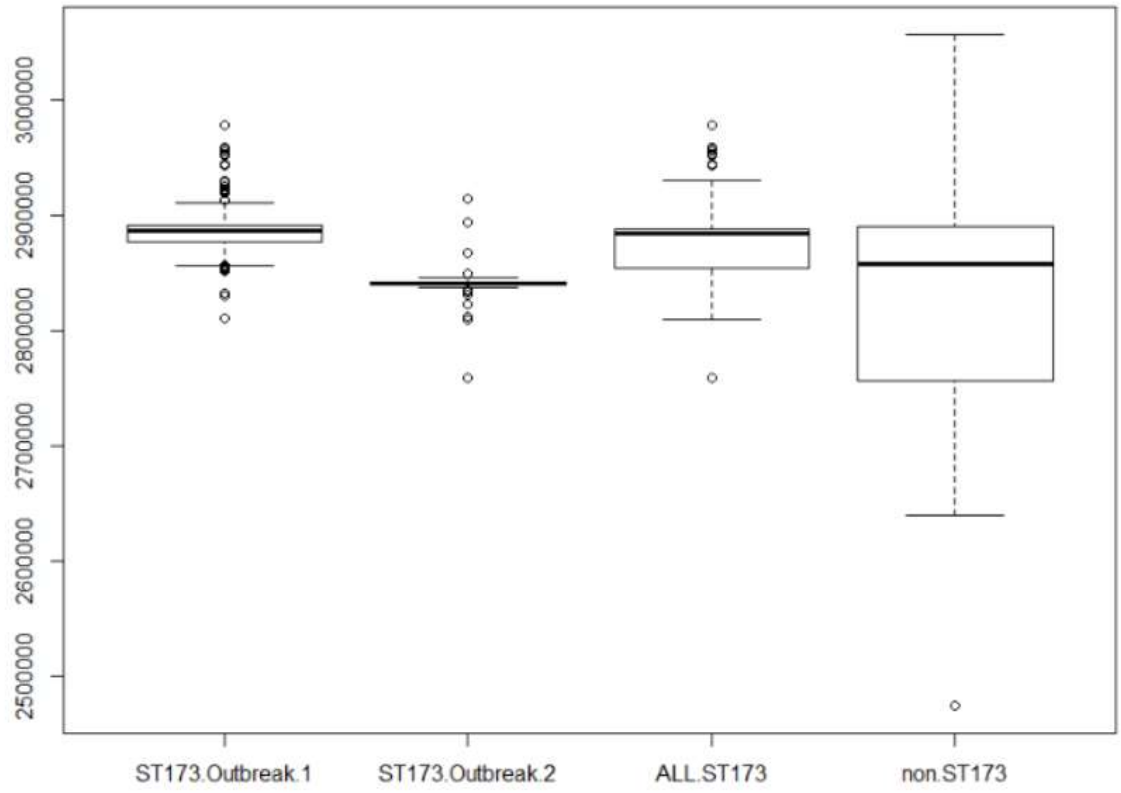

Figure 1 A boxplot of *E. faecium* genome sizes: From left: ST173 vancomycin resistant *E. faecium* (VREfm) isolated in the first outbreak (2001), ST173 VREfm isolated in the second outbreak (2004), all ST173 VREfm isolates from both outbreaks and non-ST173 VREfm isolates.

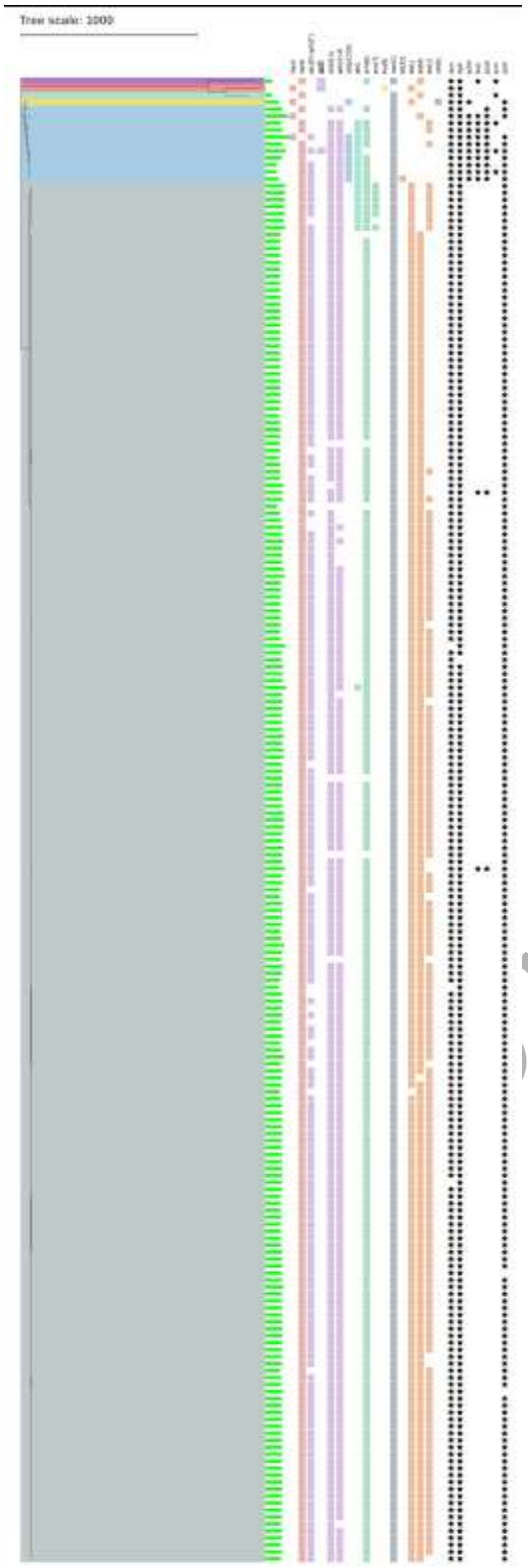

Figure 2 Phylogenetic tree of all isolates. Branch lengths reflect the number of informative single nucleotide polymorphisms. From left to right, top to bottom: Sequence types of isolates highlighted in purple are ST25, peach ST195, teal ST666, yellow ST154, blue ST17 and grey ST173. Green bar represents the genome size relative to the smallest genome. Coloured boxes represents the resistome: *vanA, vanB, aac(6')-aph(2''), aadE, ant(6)-la, aph(3')-III, cat(pC233), dfrG. erm(B), erm(T), lnu(B), msr(C), tet(32), tet(L), tet(M), tet(U)* and *vat(E).* Black stars represent virulence factors: *acm, clpP, ecbA, fss3, psaA, scm* and *sgrA*.

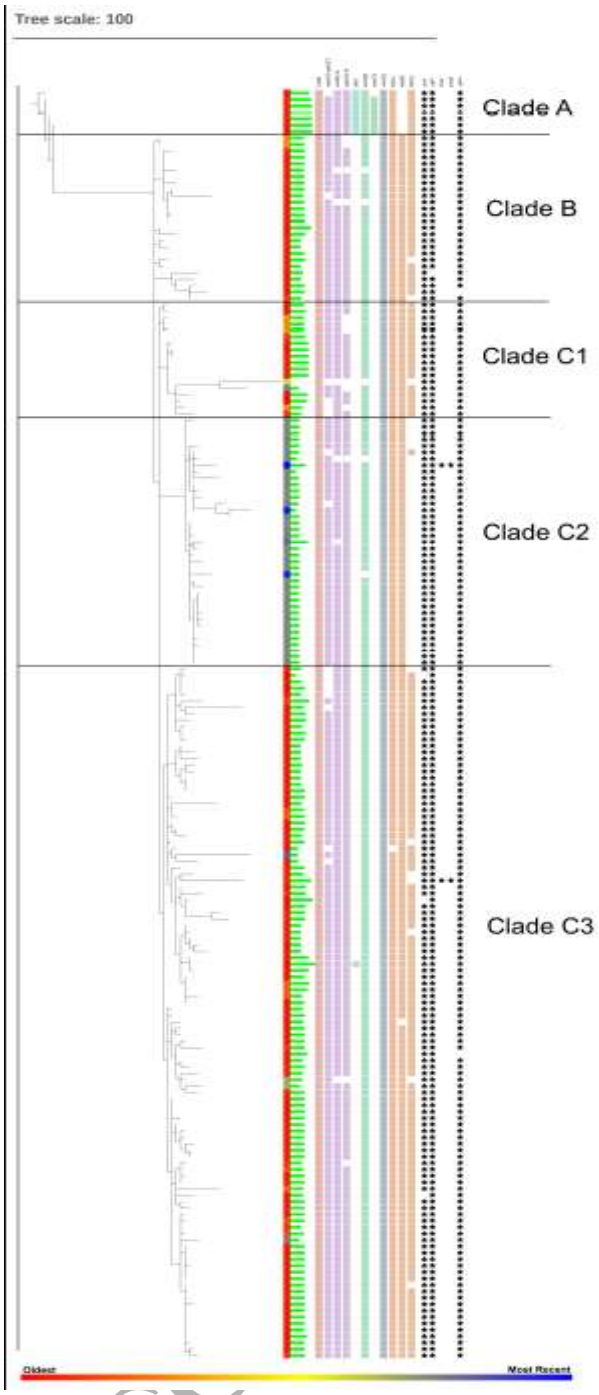

Figure 3 Phylogenetic tree of ST173 isolates. Branch lengths reflect the number of informative single nucleotide polymorphisms. From left to right: The coloured gradient represents date of isolate collection from oldest (red) to most recent (blue). The green bar represents the difference in genome size relative to the smallest genome. Coloured boxes represents the resistome with vancomycin in red, aminoglycoside in pink, chloramphenicol in navy blue, erythromycin in teal, tetracycline in green, streptogramin in yellow trimethoprim in orange and lincosamide in purple. Black stars represent virulence factors: *acm*, *clpP*, *fss3*, *psaA a*nd *sgrA*.

Supplementary Figure 1 Dendrogram, digitalized PFGE and isolate distribution of *vanB* outbreak in 2001 [11].

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Table 1. Virulence profile of all sequenced isolates.

Sequence Type	Number of isolates	*acm* Collagen-binding MSCRAMM	*bopD* Biofilm formation	*Bsh* Bile salt hydrolase	*clpP* ATP-dependent Clp protease	*scm* Collagen-binding MSCRAMM	*sgrA* Nidogen-binding LPXTG surface adhesin	*fss3* Fribrinogen-binding MSCRAMM	*psaA* Pneumococcal surface adhesin A	*ecbA* Collagen-binding MSCRAMM
ST173	190	●	●	●	●		●			
	1	●	●	●			●			
	3		●	●	●		●			
	2	●	●	●	●					
	2	●	●	●	●		●	●	●	
ST195	1	●	●	●	●					
ST154	1	●	●	●	●		●			●
ST666	1	●	●	●	●	●				
ST25	1	●	●	●	●	●		●		
ST17	6	●	●	●	●		●	●	●	●
	2	●	●	●	●	●	●	●	●	●
	1	●	●	●	●		●		●	
	1	●	●	●	●	●		●	●	●
	1	●	●	●	●			●	●	●

Table 2. Resistome of ST173 vancomycin resistant Enterococcus faecium (VREFm) from the first and second outbreaks, and background non-ST173 VREfm in percentages.

		Aac(6')-aph(2'')	*ant(6)-Ia*	*aph(3')-III*	*dfrG*	*erm(B)*	*erm(T)*	*msrC*	*tet(L)*	*tet(M)*	*tet(U)*	*aadE*	*Cat(pC233)*	*lnu(B)*	*tet(32)*	*vat(E)*
ST173	First outbreak	91.5	98.7	94.1	5.2	98.7	3.9	100	99.3	94.7	94.7	0	0	0	0	0
	Second outbreak	93.3	91.1	91.1	0	93.3	0	100	100	100	11.1	0	0	0	0	0
Non-ST173	Background	33.3	66.7	66.7	60.0	66.7	0	93.3	13.3	20.0	20.0	20.0	53.3	6.7	6.7	6.7