Whole-genome sequencing revealed independent emergence of vancomycin-resistant *Enterococcus faecium* causing sequential outbreaks over three years in a tertiary care hospital

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Abstract

Vancomycin-resistant *Enterococcus faecium* (VREfm) emerged as an important cause of nosocomial infections worldwide. Previous studies based on molecular typing revealed that VREfm outbreaks are mainly associated with a particular genetic lineage, namely clonal complex 17 (CC17), which harbours either *vanA* or *vanB* gene cluster. The University Hospital of Lausanne faced several VREfm episodes of transmissions between 2014 and 2017. In this study, we used whole-genome sequencing (WGS) to investigate the relatedness of 183 VREfm isolates collected from 156 patients. Sequence type (ST) 17, ST80 and ST117 were the most predominant clones. Based on epidemiological data, 10 outbreaks were identified, which were caused by at least 13 distinct genotypes. The majority of isolates involved in outbreaks (91%) differed by only 0 to 3 SNPs. Four outbreaks involved more than one genotype and half of the cases considered as sporadic were possibly linked to an outbreak. By sequencing all isolates, we were able to better understand our local epidemiology of VREfm. The polyclonal structure observed between the different outbreaks strains, the high level of recombination detected in isolates, the time elapsed between admission and the first VREfm detection and the negative screening at admission support the hypothesis of the emergence of new VREfm clones within the hospitalized population.

Introduction

Vancomycin-resistant *Enterococcus faecium* (VREfm) colonization and infection represent a major problem mostly in the hospital setting. Since VREfm was first reported in 1988 in the United Kingdom and in France, it emerged worldwide causing outbreaks (1, 2). Patients infected by VREfm are likely to have longer hospitalization stays and exhibit higher mortality rates compared to vancomycin-susceptible infections (3, 4). In addition, asymptomatic VREfm colonized patients can serve as potential sources for transmission and environmental contamination.

VREfm belonging to clonal complex 17 (CC17) has been successfully disseminated in hospital settings and became endemic in many countries. *E. faecium* encode resistance to vancomycin through of *van* gene clusters of either type A (*vanA*) or B (*vanB*). Previous studies have shown that VREfm with *vanA* type are widely spread in the United States, Europe, Korea, Africa and South America, while VREfm with *vanB* are predominant in Australia and Singapore (5).

Transmission is suspected when two or more VREfm-positive patients are identified in the same unit during an overlapping period. Genotyping of isolates should complement this epidemiological approach, especially when whole-genome sequencing techniques are used, providing greater discrimination between isolates (6-8).

Here, we report the findings of a prospective study of several sequential VREfm outbreaks, which occurred between 2014 and 2017 at the University Hospital of Lausanne, Switzerland. Whole genome sequencing (WGS) was used to assess the genetic relatedness among 183 VREfm isolates collected from 156 patients during this period. In addition, we investigated the evolution of VREfm within individual long-term carriers.

Materials and methods

Setting, case definition and infection control measures. The University Hospital of Lausanne is a 1100-bed tertiary care hospital with 1 to 5-bedrooms, which faced several VRE outbreaks in 2011, 2015 and 2016. A case was defined as a patient colonized/infected with VREfm during a hospitalization stay. All VRE cases were placed on contact isolation. Contact patients (i.e. patients who shared the same room or the same open unit of a new VRE case during at least 24 hours in the last month) were screened and placed on contact isolation until at least 3 rectal samples taken a week apart were found negative for VRE. When one or more contacts were found positive, all patients from the unit were screened on a weekly basis and at discharge until no new case was revealed.

Laboratory. Screening for VRE was performed by culturing a rectal swab or a stool sample in an enrichment brain–heart infusion with 3.3 mg/L of vancomycin and chromogenic selective agar plates (Biorad, Marnes la Coquette, France). Identification was confirmed with MALDI-TOF mass spectrometer (Bruker, Daltonics, Germany) and the presence of the *van* genes was assessed using the Xpert vanA/vanB rapid test (Cepheid, Sunnyvale, CA, USA). At least one isolate per patient was stored at -80 °C for further analysis.

Epidemiological definition. Epidemiological links between cases were considered i) between a new case and positive patients identified following the screening of contact patients, ii) new cases identified in the same unit during weekly screening and iii) after reviewing the hospitalisation chart of patients and the detection of links with VRE-positive cases during previous hospitalisation. An outbreak was defined as two or more cases with epidemiological links. A case with no epidemiological links was considered as a single case.

Bacterial isolates. In this study, we analysed a total of 183 consecutive VREfm isolates. They were collected during successive outbreaks occurring between January 2014 and May 2017 at the University hospital of Lausanne. For only 10 patients, the VREfm was detected in a clinical sample, for the others patients, the VRE (n=146) were detected from screening samples (Table S1).

Whole-genome sequencing. Genomic DNA of isolates was extracted using the Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The extracted DNA was quantified by the Qubit double-stranded DNA high-sensitivity (HS) assay kit (Life Technologies, Waltham, MA, USA). Sequencing library preparation was carried out using Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) with indexed adapters, following the manufacturer's guidelines, followed by sequencing using version 2 chemistry protocol on the Illumina MiSeq platform (Illumina, San Diego, CA, USA) to generate 150 bp paired-end reads.

Sequence data analysis and core-genome phylogeny. *In silico* multi-locus sequence typing (MLST) analysis and identification of STs were performed using SRST2 pipeline (9). Sequence reads of the 183 VREfm were mapped to the VREfm reference genome AUS004 (GenBank accession number: NC-017022) using Snippy version 3.1 pipeline (https://github.com/tseemann/snippy). Furthermore, Snippy used BWA-MEM version 0.7.15 (10) for aligning reads against the reference genome, while Freebayes version 1.0.2 (11) was used for variant calling. Single nucleotide polymorphisms (SNPs) were identified based on the following parameters: first, a minimum read coverage of 10 x, a minimum base quality score of 30, and a minimum proportion for variant evidence of 0.9. SNPs found within the core-genome of the 183 VREfm isolates (1,755,155 bp in size) were included, while SNPs located within mobile

genetic elements and repetitive regions were excluded. Briefly, mobile genetic elements such as insertion sequences, transposons, and conjugative plasmids were identified from the reference genome annotations AUS004 (GenBank accession numbers: NC-017022) and the repeat-match algorithm that is implemented in MUMmer package version 3.23 (12), while putative prophages and repetitive regions were identified using PHAST (phast.wishartlab.com). Gubbins version 2.2.2 (13) with default settings was used to identify and exclude regions of high SNPs densities and suspected to undergo recombination events based on the isolates' phylogeny. Subsequently, the final alignment of non-recombinant core genome SNPs was generated and used to construct a maximum likelihood phylogenetic tree using PhyML, which was visualized with the Seaview software version 4.6 (14).

A cluster was defined as a group of isolates showing a high degree of similarity based on SNPs analysis, suggesting they belong to the same chain of transmission.

Detection of antibiotic resistance and virulence genes. The determination of acquired resistance and putative virulence genes were performed through mapping and from de novo assembled using both SRST2 and ARIBA (rapid antimicrobial resistance genotyping directly from sequencing reads) (http://www.biorxiv.org/content/early/2017/03/18/118000) with default settings, respectively. Furthermore, the public databases ARG- ANNOT (http://en.mediterranee-infection.com/article.php?laref=283&titre=arg-annot) and CARD (15) were used as a reference for detecting the antimicrobial resistance determinants, while the VFDB database (16) was used for identifying the virulence factor genes. To study the genetic variation within *vanA* and *vanB* transposons among the investigated isolates, sequence reads were mapped against *Tn1549* (ENA

accession number M97297) and Aus0004 (2835430-2869240 bp) reference genomes, respectively, using the Snippy pipeline version 3.1.

Results

From January 2014 to May 2017, 156 patients were found infected/colonized with VREfm; the majority being hospitalized in the visceral surgery ward (n=76), the medical ward (n=33), the septic surgical ward (n=25) and the intensive care units (n=10). A total of 183 VREfm isolates were sequenced (one per patient for 146 patients, 2-17 isolates for 10 patients). Outbreaks were defined on epidemiological links as described in Material and Methods. During the study period, a total of ten outbreaks were recorded (Figure 1 and Table S1). The median age of VREfm positive patients was 66 years (range 1 to 96). The median number of days between patients' admission and first detection of VREfm was 25 days (range 0 to 280). Moreover, temporary universal VRE screening of patients at admission in the surgical unit during six weeks revealed only one VREfm case out of 187, suggesting intra-hospital emergence of vancomycin-resistance in *E. faecium* instead of importation.

Population structure of VREfm within the hospital setting

In silico MLST analysis revealed eleven different STs among the 156 patients: ST80 (n = 77), ST117 (n = 36) and ST17 (n = 30) were the most prevalent STs, while few isolates were assigned to ST203 (n = 3), ST18 (n = 3), and ST192 (n = 2). In addition, ST82, ST182, ST412 and ST721 were represented each by a single isolate, and one remaining isolate had a novel ST (not assigned to any of the previously published STs) (Table S1).

Recombination

Using Gubbins, a high rate of recombination events was detected, especially among VREfm isolates that belongs to ST80 (216 recombination events) and ST117 (95 recombination events) (Figure S1). These recombination events were distributed over the entire genomes of both ST80 and ST117 and varied broadly in size between 19 bp to 109,812 bp and between 31 bp to 93,428 bp, respectively. The majority of these recombination events were due to the acquisition of insertion sequences that encode for antimicrobial resistance and phosphotransferase system (PTS). Consequently, the entire SNPs detected within the identified recombinant sequences were excluded from the final core-genome SNPs alignment that was used to build the phylogeny of the investigated VREfm isolates.

Phylogenetic analysis

The phylogenetic analysis based on core genome SNPs of the 183 VREfm revealed that isolate H32990 (ST82) was distantly related to the remaining 182 isolates and, therefore, was used as an outgroup to root the phylogenetic tree. Three major phylogenetic clades were identified, namely, clade ST117, clade ST17 and clade ST80-ST18 (Figure 2), which were correlated to the different successive VREfm outbreaks, suggesting clonal disseminations of different VREfm clones within the hospital. The monophyletic clade ST17 corresponded to the first and second outbreaks and consisted of 51 ST17 VREfm isolates with a median SNP difference of zero (range 0-2), suggesting direct transmission between patients.

In contrast, clade ST80-ST18 was subdivided into six distinct clusters (ST80-A, ST80-B, ST80-C, ST80-D, ST80-E and ST18), which represented different lineages introduced into the hospital on different occasions (Figure 2).

With exception of three isolates, all VREfm ST117 isolates were grouped into the same clade, which is subdivided into four distinct clusters (ST117-A, ST117-B, ST117-C and

ST117-D). Pairwise SNPs distance analysis revealed that isolates within each cluster of clades ST80-ST18 and clade ST117 were closely related with a median SNP difference of 0-2 SNPs (range 0-5), with exception of cluster ST117-D that had a range of 0 to 51 SNPs. This wide range of SNPs was due to isolate H32231, which is single locus variant from ST117 and differed by 49 to 51 SNPs compared to the remaining isolates within this cluster. Noteworthy, the inter-clades/clusters median SNPs differences were considerably high with up to 50 SNPs (range 2-82).

Seventeen patients carried an isolate that was not closely related to another isolate (unique genotypes).

Comparison between epidemiological and genotyping data

The relation between outbreaks (documented based on epidemiological data) and genetic clusters (defined based on genomic data) is shown in Figure 3. This figure is highly informative. First, among the 129 cases involved in outbreaks, 109 (91%) belonged to the same genetic clusters. The remaining 20 cases (16%) could be excluded from the transmission chains because they carried an isolate genetically different. In four of the ten outbreaks, patients with a unique genotype or belonging to another cluster were observed, excluding these cases from the transmission chain. More interesting, on one occasion, a small outbreak (#1, Figures 1 and 3) was followed two months later by a larger outbreak (#2), suggesting the resurgence of transmission that was considered under control. On the other hand, among the 27 cases with no epidemiological link with other cases (singles), only 12 carried a unique VRE genotype, the other 15 carried isolates genetically highly similar to isolates from other cases, strongly suggesting they were associated to some putative chains of transmission. We also observed patients hospitalized at different times, with no obvious epidemiological link, but carrying isolates belonging to the same cluster and therefore putative

transmission (ST80-A, ST117-C, ST117-D and ST117-E). The most striking example is cluster ST117-D which was first observed in a single case in March 2016, followed by 5 cases between October 2016 and January 2017 that were detected during weekly screenings performed to control outbreak #8 (cluster ST80-D), and a last case in May 2017. These observations strongly suggest the existence of undetected carriers.

Genetic characterisation of VRE isolates

In this study, the antimicrobial resistance genes detected in the 183 VREfm isolates genomes are summarized in Table S2. The majority harboured the *vanA* gene (91.3%), whereas the remaining 8.7% carried the *vanB* gene. In addition, the presence or absence of antimicrobial resistance genes was consistent within each cluster (Table S2).

To estimate the number of different origins of the *van* genes in our isolates' collection, the genetic contents of *van* transposon were investigated. Sixteen isolates carried the *vanB* genes and constituted clusters ST18, ST117-A and ST117-B. Mapping the *vanB* transposon of these isolates against the *vanB* transposon of the reference genome AUS004 (GenBank accession number: NC-017022) grouped the isolates into three distinct clusters with 8 to 11 SNPs differences among them. The tree topology agreed with the core-genome SNPs phylogeny (Figure S2). Similar analysis was performed with the *vanA* transposon, showing a limited diversification (zero to five SNPs) and no correlation with clusters (data not shown).

Several genes conferring resistance to various aminoglycosides were detected among the isolates collection. Most of these genes were associated with particular phylogenetic clusters. For example, all 33 isolates belonging to cluster ST80-D lacked the high-level gentamicin resistance *aac(6´)-aph(2")* gene, which was detected in 47% (86/183) of the isolates. Similarly, isolates from clusters ST80-C and ST80-D lacked

both *sat4* and *aph(3')-IIIa* genes, while ST17 isolates lacked the *ant(6')-Ia* gene that mediate only high-level streptomycin resistance; these genes were detected in 47.5% (87/183), 53% (97/183) and 67% (128/183) of the investigated isolates, respectively. The *msrC*, *ermA*, *ermB*, and *ermC* genes that confer resistance to macrolides were detected in 100% (183/183), 0.5 % (1/183), 98% (180/183) and 4.9% (9/183) of the investigated VREfm isolates, respectively. The *dfr* gene, that encodes resistance to trimethoprim, was found in 46.5% (85/183) of the isolates. Of note, all isolates belonging to clade ST17 and cluster ST117-D lacked this gene.

Interestingly, mutations in *liaS* gene and/or *liaR* conferring resistance to daptomycin were detected in 20% (37/183) of isolates, which all but one belonged to ST80 and were mainly located in clusters ST80-B and ST80-D.

Similarly, screening all the 183 VREfm for putative virulence factors genes using the Virulence Factor Database (VFDB) (17) revealed that all isolates but two harboured the *acm* gene. In addition, clusters ST80-B and ST80-D isolates lacked pilin gene cluster, while the *ecbA* gene was present in > 50% of isolates but was absent from ST17 isolates (Table S2).

In-host evolution

To investigate the in-host diversity and the evolution of VREfm within host, we analysed 36 isolates that were sampled between 0 to 391 days apart from nine patients (P003 [n = 2], P008 [n = 2], P009 [n = 17], P020 [n = 2], P025 [n = 2], P033 [n = 5], P037 [n = 2], P089 [n = 2] and P159 [n = 2]). For Patient P009, on two occasions, different colonies from the same sample were isolated for WGS (P009-02 to P009-05; and P009-08 to P009-16, Figure 2). For six patients, phylogenetic analysis revealed evidence of restricted numbers of in-host variations: isolates sampled up to 391 days apart differed by only 0 to 3 SNPs. Evidence for multiple colonization was found in

patients P033 and P089. Over a period of 370 days, P033 was simultaneously colonized by two different genotypes ST80-A and ST80-B which differed by a mean of 20 SNPs. The two isolates from P089 were obtained 182 days apart and belonged to two different genotypes (unique and ST117-C; 42 SNP differences), suggesting successive colonization by different genotypes.

Evidence of in-host recombination was found in patients P037 and P089. After excluding the SNPs located within recombinant genomic regions, four (P037) and 42 (P089) SNPs differences were detected among the repetitive isolates from these patients.

Infection control

These outbreaks led to reinforcement of infection control measures in units with VRE cases, including information, training and observations of health care workers. Procedures for disinfection of the patient's environment were reinforced, and additional staff was dedicated to this task. Weekly screenings by culture of all patients were performed until two weeks passed without any new case. In addition, patients in units with recurrent VRE outbreaks were screened on a weekly basis for several months. We also transformed some of the 5-bed rooms into 2-bed rooms with sanitation. Since the introduction of these infection control measures, a marked decrease in new cases has been observed (N=16, including only three small outbreaks of 2, 2 and 3 cases) during the year following this study.

Discussion:

Using WGS analysis, we prospectively investigated the epidemiology of VREfm at the University hospital of Lausanne over a period of three years. Among the 156 studied patients, thirteen clusters (genotypes) of genetically highly related isolates were

involved in the sequential outbreaks, suggesting several independent introductions of VRE isolates into the hospital followed by direct or indirect transmission. These findings are supported by previous studies, which revealed the emergence of several VREfm clones within hospitals through multiple independent introductions followed by intra-hospital transmissions (6, 7, 18, 19).

Our results showed that 91% of the cases involved in outbreaks were confirmed by WGS results. Moreover, isolates of the same outbreak differed by only 0 to 3 SNPs, which is in agreement with the estimated molecular clock rate of ~10 SNPs per genome per year (19). Conversely, based on WGS results, we were able to completely exclude patients from a chain of transmission when they were harbouring a different genotype. Moreover, WGS highlighted the co-circulation of several genotypes in some wards. For example, during outbreaks #3 and #9 (four patients each), only one transmission was confirmed, whereas during outbreak#7, two genotypes (ST80D and ST117-D) were spreading concomitantly in the ward.

Another added value of WGS analysis lies in its very high discriminatory power, which enables to cluster patients for whom no apparent epidemiological link was recorded. For example, WGS linked the first large outbreak in January 2015 to two epidemiologically unsuspected cases in November 2014, which highly suggests the presence of undetected cases between both episodes. This is corroborated in our study by the presence of several patients hospitalized at different times, with no obvious epidemiological link, but carrying isolates belonging to the same cluster. The hypothesis of a persistent contamination of the environment was considered. Several other factors contributing to the spread of VREfm were also identified: i) multiple movements of patients within the hospital, ii) five-bed rooms and open units, iii) lack of individual toilet facility, iii) suboptimal disinfection of the environment. Interestingly,

VREfm cases transferred from a foreign hospital, for whom contact precautions were taken since admission, had a unique genotype, suggesting they were not the source of transmission to other patients.

Investigating the in-host evolution of VREfm revealed that most patients carried isolates with only 0 to 3 SNP differences despite long period of carriage (up to 391 days). Analysing various repetitive isolates from the same sample showed a small number of SNP difference emphasising that patients harbour a collection of VREfm isolates that have evolved independently following the first colonization/infection event. Conversely, different genotypes were observed for two patients, suggesting colonization on different occasions from various sources. These findings are consistent with previous studies that suggested the carriage of several VREfm lineages by patients (7, 20). Hence, a larger number of sequenced isolates per sample and per patient are required to better characterize the in-host population dynamic of VREfm. Of note, recombination between patient's isolates was observed, highlighting the necessity to consider this diversification process when analysing SNP similarity between isolates.

The diversity of genotypes within our population of patients raised the question of their emergence. Van Hal *et al*. (21) hypothesised that the emergence of new clone is a result of continuous recombination. Our study supports this hypothesis since a high recombination rate was observed among the investigated isolates. Moreover, recent studies showed that resistance to vancomycin is repeatedly introduced in the *E. faecium* population (7, 19). A limitation of these studies was that only blood culture isolates were included and carriage isolates were missing. Our study includes carriage isolates and we identified various resistance patterns that were lineage specific, emphasizing the role of horizontal gene transfer in the emergence of new genotypes.

However, the limitation of our study to investigate this hypothesis resides in the lack of sequencing isolates of *E. faecium* susceptible to vancomycin. Together, these findings suggest the common *de novo* emergence of VREfm. The time elapsed between patients' admission and first VREfm detection (median 26 days) and the fact that only one patient out of 187 was positive for VREfm at admission in a unit with recurrent outbreaks suggest that the emergence occurred within the hospitalized patients. Therefore, control measures should focus on (i) the prevention of VREfm emergence with an effective antibiotic stewardship program and (ii) a bundle of infection control actions to prevent cross transmission, particularly with early identification of cases by repeated screening throughout the hospitalization as well as prompt implementation of contact precautions.

In conclusion, WGS of all VREfm isolates enable us to better understand our local epidemiology. Of interest, sporadic cases were often found to be related to a past or future outbreak. The polyclonal structure observed between the different outbreaks, the high level of recombination detected in studied isolates, the time elapsed between admission and the first VREfm detection and the negative screening at admission support the hypothesis of the emergence of new VREfm clones within hospitalized patients.

Note

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Compliance with Ethical Standards

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Conflict of Interest

The authors declare no conflict of interest.

Ethical approval

All procedures performed in this study involving human participants were in accordance with the ethical standards of the regional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

For this type of study, informed consent is not required.

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Figure legends

Figure 1. Incidence of VREfm cases and outbreaks identified with epidemiological data.

Figure 2. Maximum likelihood tree based on core-genome SNPs in the 182 vancomycin-resistant *Enterococcus faecium* isolates collected in 156 patients from January 2014 to May 2017. MLST sequence type, isolate number and patient number are indicated. Isolates carrying the *vanA* gene are in black and *vanB* in red. A "$" indicates isolates retrieved at admission of patients transferred from a foreign hospital.

Figure 3. Distribution of the different genetic clusters within outbreaks defined by epidemiological data

Supplementary materials

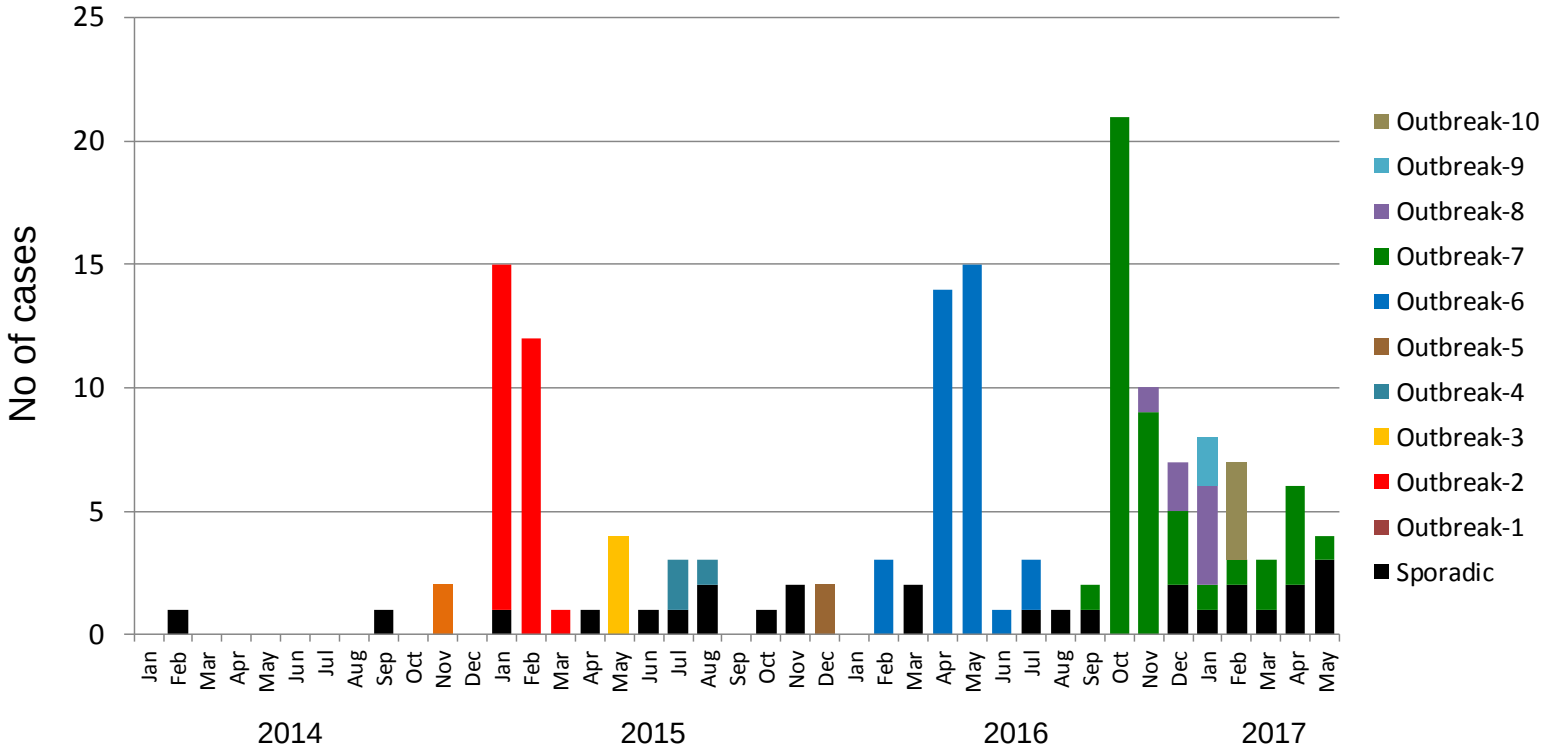
Figure S1. Phylogenetic tree of the 183 vancomycin-resistant *Enterococcus faecium* isolates inferring recombination events using Gubbins. Red blocks represent regions of high SNPs density detected in multiple isolates, while blue blocks represent high SNPs density only found in a single isolate.

Figure S2. Phylogenetic tree reconstruction of 16 VREfm isolates based on core SNPs in *vanB Tn1549*.

Table S1. Patient's epidemiological data.

Table S2. Entire resistance and virulence factors detected in the 183 VREfm isolates.

Figure 1


No of cases
25
20
15
10
5
0
Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec Jan Feb Mar Apr May
2014
2015
2016
2017
Outbreak-10
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Outbreak-4
Outbreak-3
Outbreak-2
Outbreak-1
Sporadic

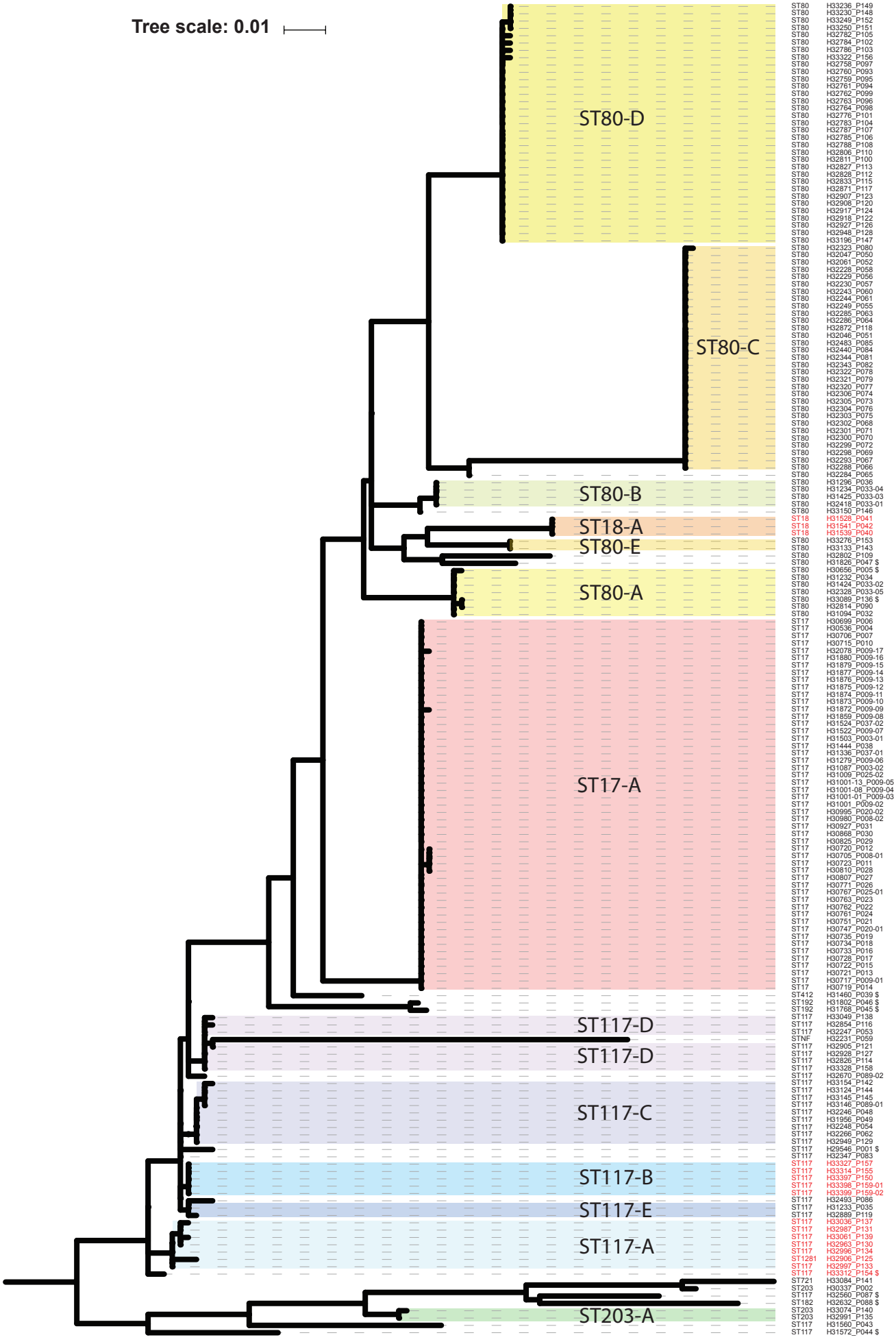
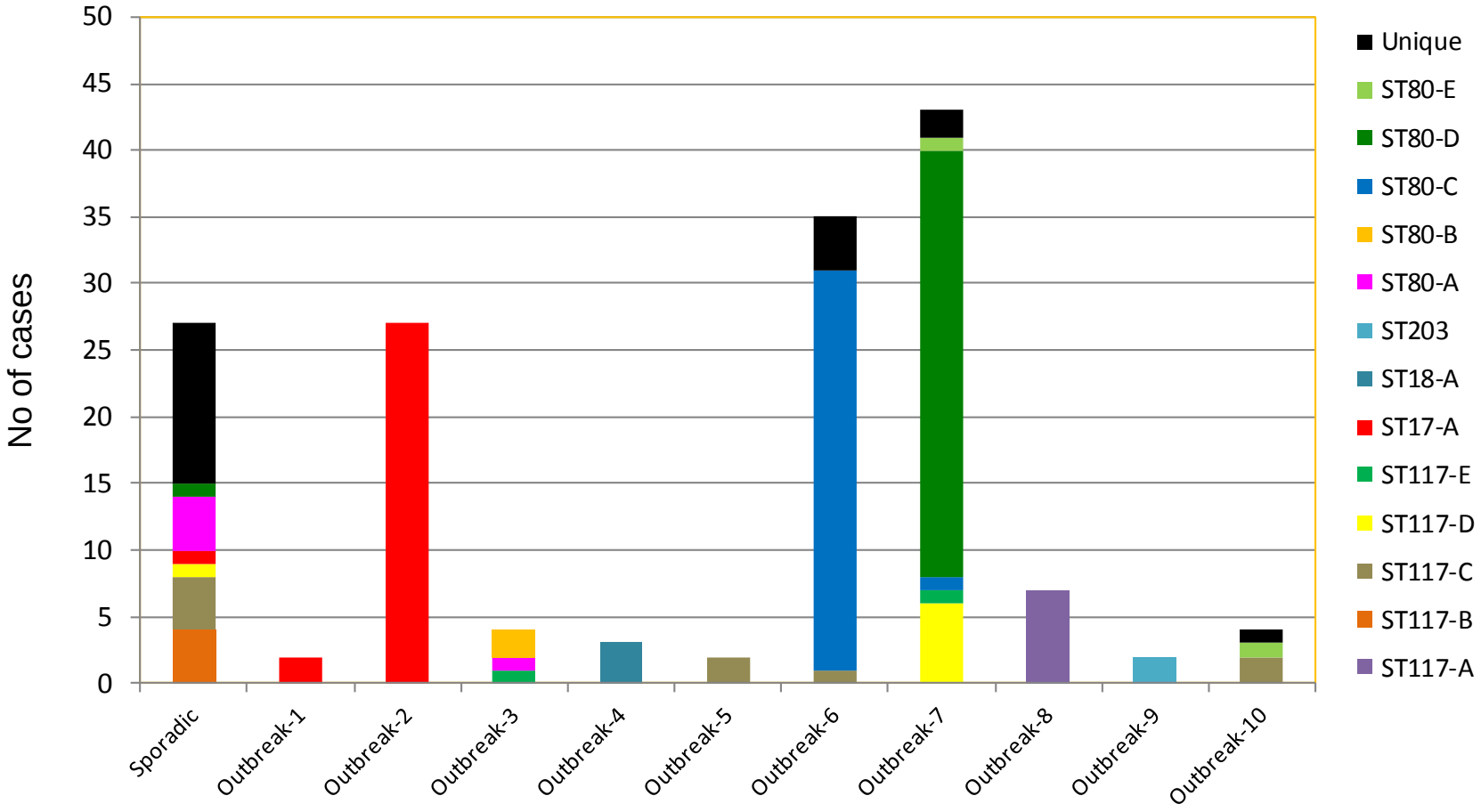

Tree scale: 0.01
ST80-D
ST80-C
ST80-B
ST18-A
ST80-E
ST80-A
ST17-A
ST117-D
ST117-D
ST117-C
ST117-B
ST117-E
ST117-A
ST203-A

Figure 3


No of cases
50
45
40
35
30
25
20
15
10
5
0
Sporadic
Outbreak-1
Outbreak-2
Outbreak-3
Outbreak-4
Outbreak-5
Outbreak-6
Outbreak-7
Outbreak-8
Outbreak-9
Outbreak-10
Unique
ST80-E
ST80-D
ST80-C
ST80-B
ST80-A
ST203
ST18-A
ST17-A
ST117-E
ST117-D
ST117-C
ST117-B
ST117-A

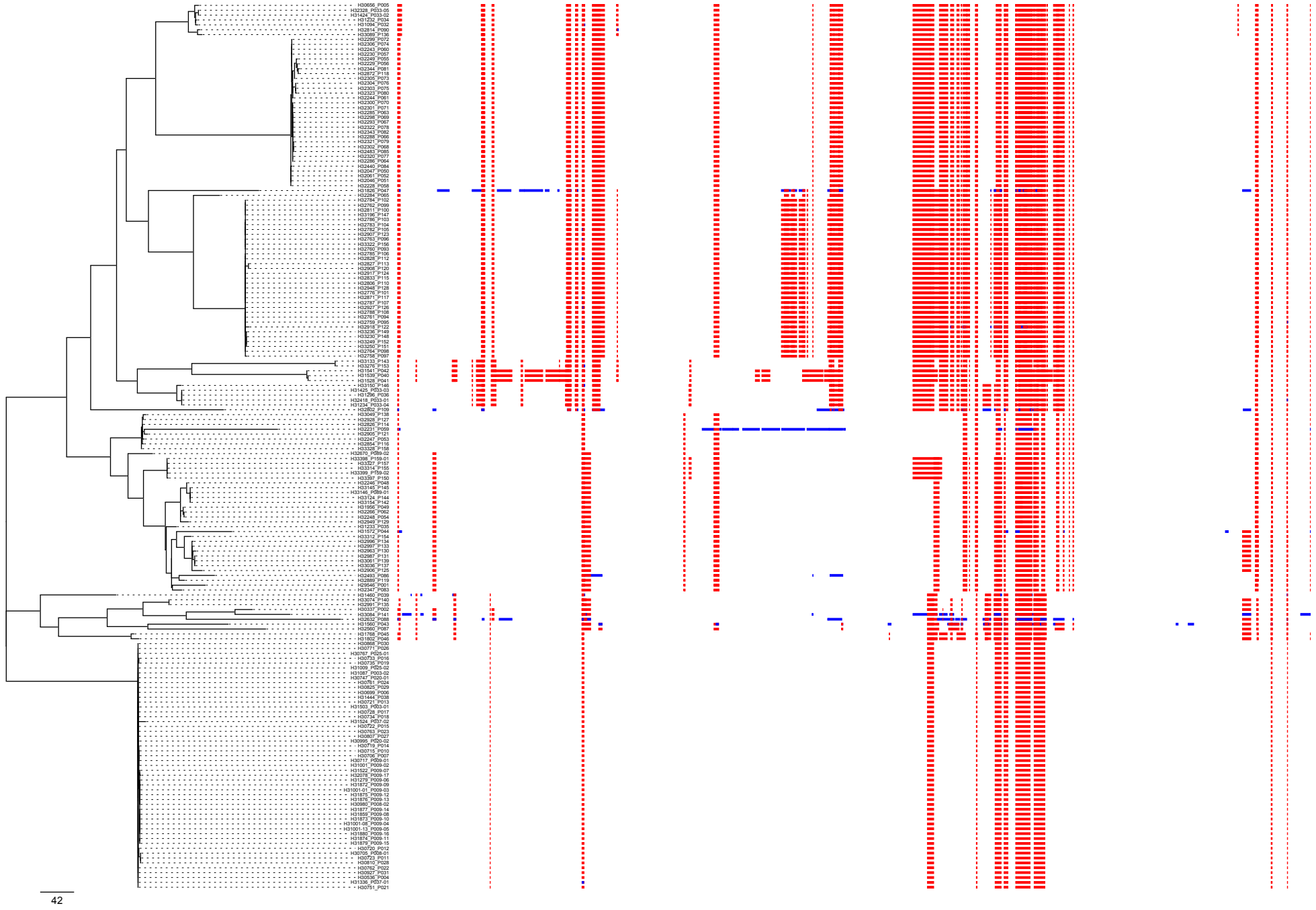


Figure S1. Phylogenetic tree of the 183 vancomycin-resistant *Enterococcus faecium* isolates inferring recombination events using Gubbins. Red blocks represent regions of high SNPs density detected in multiple isolates, while blue blocks represent high SNPs density only found in a single isolate.

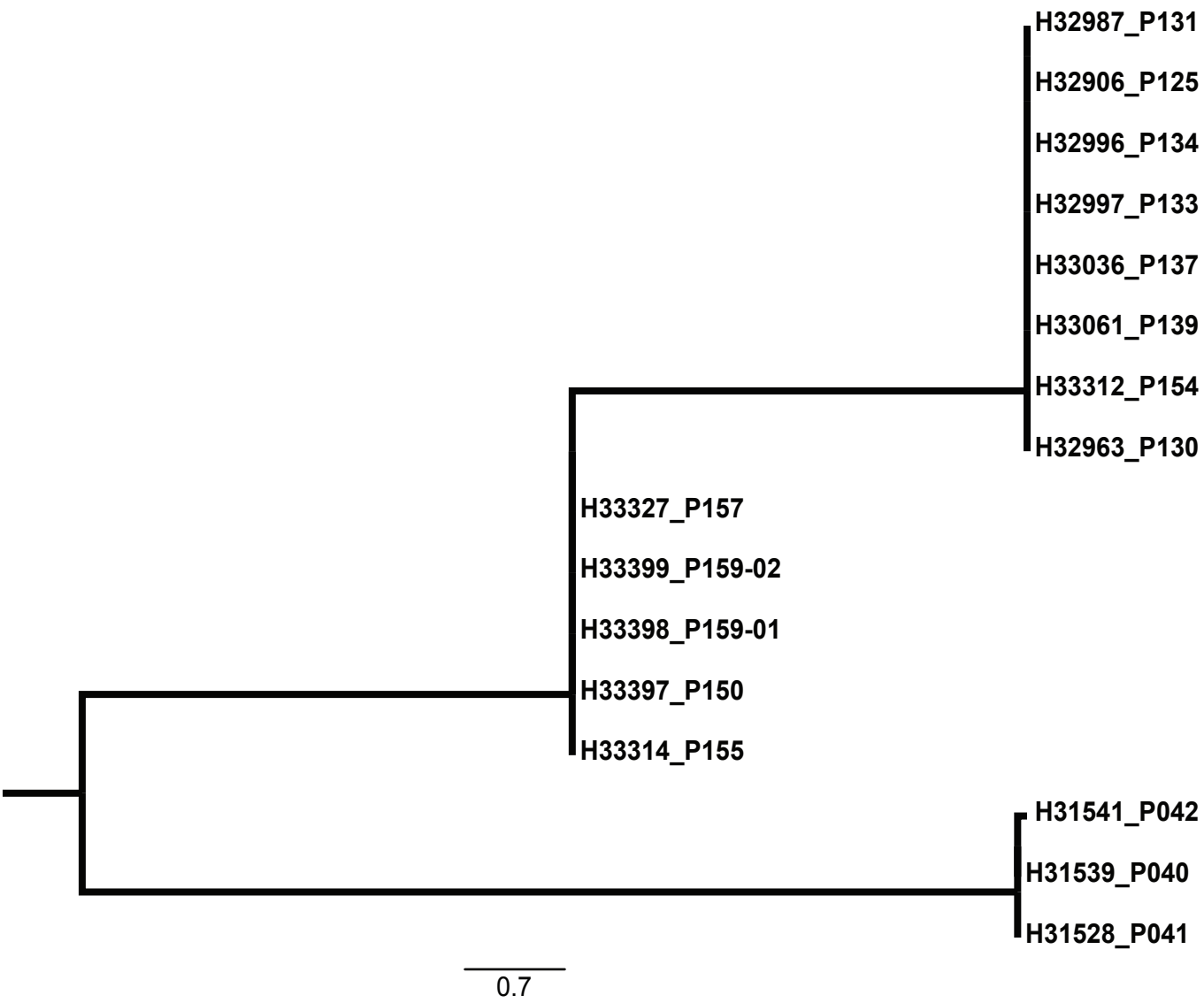


Figure S2. Phylogenetic tree reconstruction of 16 VREfm isolates based on core SNPs in vanB Tn1549.