J Antimicrob Chemother 2017; **72**: 40–47
doi:10.1093/jac/dkw360 Advance Access publication 7 September 2016

Journal of Antimicrobial Chemotherapy

Genomic analysis of 495 vancomycin-resistant *Enterococcus faecium* reveals broad dissemination of a *vanA* plasmid in more than 19 clones from Copenhagen, Denmark

Mette Pinholt[1,2]*, Heidi Gumpert[1], Sion Bayliss[3], Jesper B. Nielsen[1], Veronika Vorobieva[4], Michael Pedersen[5], Edward Feil[3], Peder Worning[1] and Henrik Westh[1,2]

[1]Department of Clinical Microbiology, Hvidovre University Hospital, Hvidovre, Denmark; [2]Institute of Clinical Medicine, University of Copenhagen, Copenhagen, Denmark; [3]Department of Biology and Biochemistry, University of Bath, Bath, UK; [4]Department of Clinical Microbiology, Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark; [5]Department of Clinical Microbiology, Herlev University Hospital, Herlev, Denmark

*Corresponding author. Department of Clinical Microbiology, Hvidovre University Hospital, Hvidovre, Denmark.
Tel: +45-38623210; Fax: +45-38623357; E-mail: mette.pinholt@regionh.dk

Received 21 April 2016; returned 22 June 2016; revised 23 July 2016; accepted 2 August 2016

Objectives: From 2012 to 2014, there has been a huge increase in vancomycin-resistant (*vanA*) *Enterococcus faecium* (VREfm) in Copenhagen, Denmark, with 602 patients infected or colonized with VREfm in 2014 compared with just 22 in 2012. The objective of this study was to describe the genetic epidemiology of VREfm to assess the contribution of clonal spread and horizontal transfer of the *vanA* transposon (Tn*1546*) and plasmid in the dissemination of VREfm in hospitals.

Methods: VREfm from Copenhagen, Denmark (2012–14) were whole-genome sequenced. The clonal structure was determined and the structure of Tn*1546*-like transposons was characterized. One VREfm isolate belonging to the largest clonal group was sequenced using long-read technology to close a 37 kb *vanA* plasmid.

Results: Phylogeny revealed a polyclonal structure where 495 VREfm isolates were divided into 13 main groups and 7 small groups. The majority of the isolates were located in three groups ($n=44$, 100 and 218) and clonal spread of VREfm between wards and hospitals was identified. Five Tn*1546*-like transposon types were identified. A dominant truncated transposon (type 4, 92%) was spread across all but one VREfm group. The closed *vanA* plasmid was highly covered by reads from isolates containing the type 4 transposon.

Conclusions: This study suggests that it was the dissemination of the type 4 Tn*1546*-like transposon and plasmid via horizontal transfer to multiple populations of *E. faecium*, followed by clonal spread of new VREfm clones, that contributed to the increase in and diversity of VREfm in Danish hospitals.

Introduction

Enterococcus faecium, and MDR *E. faecium* in particular, has emerged as an increasingly important nosocomial pathogen. MLST data and genomics have shown that a distinct genetic subpopulation of *E. faecium* has adapted to the hospital environment[1,2] and several studies have shown that hospital-associated *E. faecium* is capable of surviving for long periods on hospital surfaces.[3,4]

E. faecium is inherently resistant to cephalosporins and has the capacity to acquire and disseminate mobile genetic elements containing virulence and resistance genes (e.g. gentamicin and vancomycin resistance genes). In 2012, >90% of Danish *E. faecium* blood isolates were resistant to ampicillin, 62% were high-level gentamicin resistant and only 1.8% were vancomycin resistant.[5] However, from 2012 to 2014, there has been a huge increase in vancomycin-resistant *E. faecium* (VREfm) in the Capital Region of Denmark (Copenhagen) with 602 patients infected or colonized with VREfm in 2014 compared with just 22 patients in 2012.[6] In total, 894 VREfm patients were identified.[6]

The *vanA* and *vanB* genotypes are the major vancomycin resistance genotypes regarding human infections.[7] The *vanA* gene cluster, dominant in Denmark, consists of two regulatory genes (*vanR* and *vanS*) and vancomycin resistance genes (*vanH*, *vanA*, *vanX*, *vanY* and *vanZ*).[7] Together with the *vanA* gene cluster, two transfer-related genes, a transposase (*orf1*) and a resolvase (*orf2*), form the Tn*1546*-like transposon,[8] which is often carried on a transferable plasmid.[7] Variations in the Tn*1546*-like transposon structure are well described among clinical VREfm isolates including nucleotide variations, insertion of IS elements and deletions of the left-end (*orf1*/*orf2*) and right-end (*vanZ*) genes of the transposon.[9–13]

In a recent publication, we described hospital outbreaks of *vanA* VREfm in Denmark, 2012–April 2013.[14] In the Capital Region, four clones and six singletons were identified using WGS. Considering the rapid increase and diversity of VREfm, we hypothesized that clonal spread of VREfm as well as horizontal transfer of Tn*1546*-like transposons into hospital-adapted *E. faecium* [ampicillin-resistant and vancomycin-susceptible *E. faecium* (VSEfm)] may have contributed to the spread of VREfm in hospitals. Introduction of VREfm from patients returning from foreign hospitals or a VREfm reservoir outside hospitals (e.g. farm animals or food products) may also contribute to the diversity of VREfm.

The present study uses WGS to describe the genetic epidemiology of VREfm in the Capital Region of Denmark for an extended time period, 2012–14, to assess the contribution of clonal spread as well as horizontal transfer of Tn*1546*-like transposons in the dissemination of *vanA* VREfm. All Tn*1546*-like transposons were characterized to assess their similarity. One VREfm isolate was selected for PacBio long-read sequencing to close a *vanA* plasmid and to study the dissemination of this resistance plasmid.

Methods

The project was approved by the Danish Data Protection Agency (2012-58-0004) and the Danish Health and Medicines Authority (3-3013-1168/1).

Setting and bacterial isolates

The study was conducted in the Capital Region of Denmark (1.7 million inhabitants) from January 2012 to December 2014. The healthcare service in the region is managed by 12 public hospitals and general practice healthcare services. In Denmark, all residents receive tax-supported healthcare including free access to primary care and public hospitals. Three departments of clinical microbiology (DCM) perform all microbiological analyses in the Capital Region. VREfm isolates from all three DCM were submitted for WGS to the DCM at Hvidovre Hospital. One DCM did not submit VREfm isolates from the last 9 months of 2014 due to financial constraints. The first submitted isolate from a patient was included in the study ($n=495$). VREfm isolates were identified in clinical samples as well as screening samples (rectal swab and gastric aspiration). Date of sampling, submitting hospital, hospital ward, sample site and sample type (clinical or screening sample) were collected from laboratory information systems. Vancomycin resistance genotype (*vanA* or *vanB*) was detected by WGS. Only *vanA* VREfm was included in the study as the VREfm epidemic was mainly caused by *vanA* VREfm.

WGS and assembly

DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Germany) and sequenced with the use of Illumina technology (Illumina, USA) as described previously.[14] The genomes were multiplexed to 24 isolates per run on an Illumina MiSeq platform producing 2×150 bp paired-end reads. The isolates were sequenced with a mean coverage depth of 62× (range 14–194×). Genomes were assembled using Velvet[15] and an in-house script has been developed to analyse *E. faecium* genomes for MLST and vancomycin resistance genotype.

Phylogenetic analysis and the pangenome

Contigs were annotated by Prokka[16] and the pangenome was estimated using Roary.[17] Core genes were defined as those present in 99% of the isolates with a minimum percentage identity of 95%. Multiple sequence alignment of concatenated core genes was used to build a neighbour-joining tree with bootstrapping (1000 replicates) in RapidNJ.[18] Tree drawing was managed with FigTree.[19]

In addition, a distance matrix with pairwise comparisons (SNP differences) of all VREfm isolates was achieved by a reference-based mapping approach. Reads were mapped to a *vanB E. faecium* reference genome (GenBank: AUS0004_NC_017022[20]) using Stampy[21] and SNPs were called using SAMtools as previously described.[14] The pipeline used is a β-version of a pipeline developed at the Oxford Centre for Gene Function.[22] SNP differences among epidemiologically related isolates identified in (i) the same hospital ward during the study period, or (ii) the same hospital ward on the same date were determined to divide the isolates into subgroups of closely related isolates.

Characterization of Tn1546-like transposon structures

Rapid Annotations using Subsystems Technology (RAST) was used to annotate all contigs from sequenced VREfm isolates.[23,24] Most VREfm isolates had transposon elements on more than one contig. To reconstruct the Tn*1546*-like transposons, a reference sequence was used as a template to order the contigs containing transposon elements. The linkage between neighbouring contigs was supported by overlapping contig ends and via paired-end reads aligning to each end of the neighbouring contigs. Briefly, paired-end reads were aligned to the contigs via Bowtie2,[25] regions of contigs sharing homology to the Tn*1546*-like transposon reference were identified via MUMmer[26] and then the discordant paired-end reads that aligned to these contigs were enumerated by executing custom-written Python scripts that used BioPython and PySam libraries.[26–28] In this way, additional insertion elements were also identified as contigs with discordant reads linking contigs with *van* genes. The insertion elements were annotated via blastn searches.[29]

Multiple sequence alignments were used to identify variant nucleotide positions and minor deletions. A reference sequence derived from a VREfm isolate (V98), which contained *orf2*, IS*1251* in the *vanSH* intergenic region and all *van* genes on one contig, was selected. Subsequently, the reference sequence was divided into minor fragments [resolvase-*vanZ* (including IS*1251*), resolvase-*vanR*, resolvase-*vanS*, *vanH-vanZ*, *vanH-vanX*, *vanY-vanZ*, *orf1*, *orf2*, *vanR*, *vanS*, *vanH*, *vanA*, *vanX*, *vanY* and *vanZ*] and all VREfm contigs were searched for DNA sequences that matched the fragments using BLAST (90% identity and 90% query cover). Matching DNA sequences were aligned via Muscle.[30]

Finally, flanking regions of the transposon, i.e. the regions upstream of *orf1* or *orf2* and downstream of *vanZ*, were determined.

Reconstruction of a vanA plasmid from Illumina and PacBio data

One VREfm isolate belonging to the largest clonal group (V24 from group 2) was selected for PacBio long-read sequencing. DNA was extracted as described for Illumina sequencing and sequenced on a Biosciences PacBio sequencing platform producing an average subread length of 5.8 kb and coverage depth of 441× (Duke University, USA). A hybrid assembly using Illumina and PacBio data was performed using SPAdes.[31] Longer contigs produced by the hybrid assembly were used to reconstruct and close the *vanA* plasmid (pHvH-V24) applying the same method as we used to reconstruct the transposon structures. The plasmid was annotated by RAST[23,24] and plasmid replicons and resistance genes were identified via PlasmidFinder and ResFinder online tools, respectively.[32,33] To determine whether the remaining VREfm isolates had a related *vanA* plasmid, reads from each VREfm isolate were mapped to the closed plasmid using Bowtie2 and the percentage of the plasmid that was covered by reads was determined.[25] Read coverage breadth and depth of the plasmid were determined using the bedtools coverage tool[34] and the percentage of the plasmid that was covered by reads was determined. For a region in the plasmid to be considered covered by reads (breadth), the read depth

had to be ≥20% of the average plasmid read depth. Finally, BLAST and the NCBI database were used to find plasmids sharing homology with the closed plasmid.

Accession numbers

Illumina sequence data for a representative isolate of each WGS group (20 isolates) have been deposited in the European Nucleotide Archive under study accession numbers PRJEB14625 and PRJEB8719. The association between WGS group number, accession number and isolate identification number is listed in Table S1 (available as Supplementary data at *JAC* Online). Also, sequences of transposon types and pHvH-V24 were deposited in NCBI with accession numbers listed in Table S1.

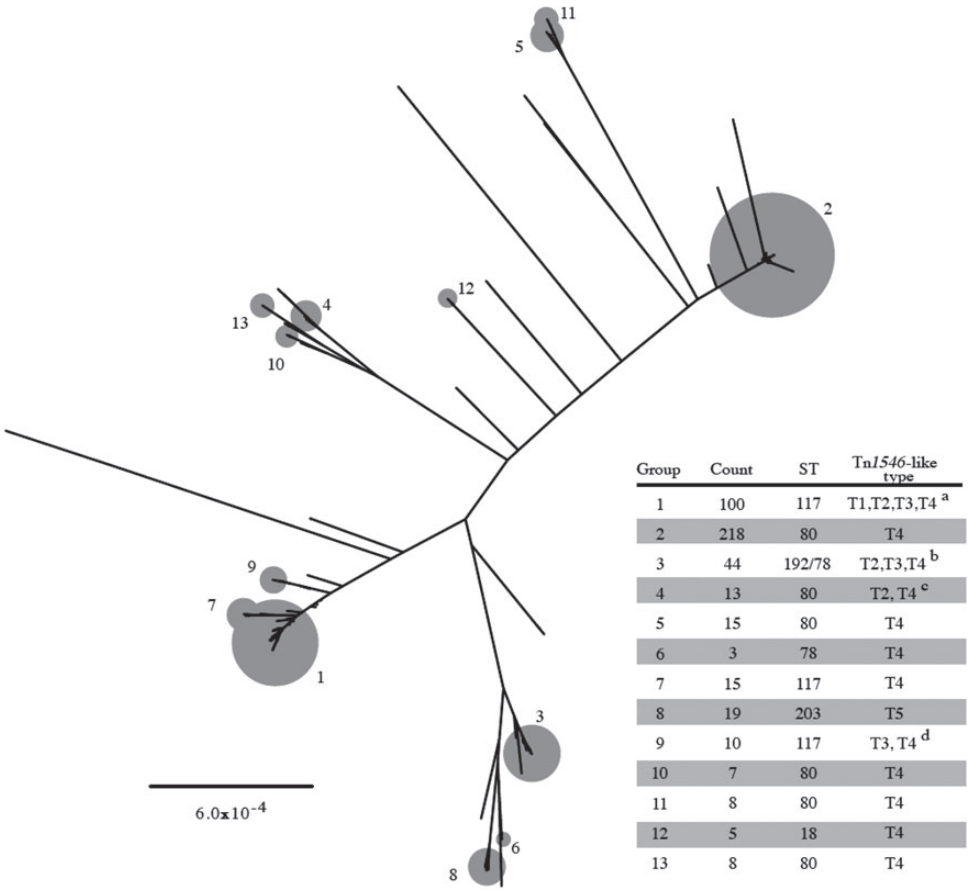
Results

Descriptive data

Four hundred and ninety-five VREfm isolates from the Capital Region of Denmark, 2012–14, were sequenced, which represent an isolate from 55% of all VREfm patients. The mean age of the patients was 70 years (range 4–100 years) and 53% were men. VREfm isolates were identified in 10 hospitals in the Capital Region although the vast majority (90%) were identified in three acute care hospitals and one national referral hospital. The predominant departments with VREfm were departments of gastroenterology/surgery (27%), orthopaedic surgery (21%) and ICU (15%). Half of the isolates (55%) were from screening samples (Table S2). Isolates from the urinary tract and bloodstream accounted for 27% and 6%, respectively.

Genomic epidemiology of VREfm

The phylogenetic tree, built from sequence alignment of 1119 concatenated core genes, revealed a picture of genetic diversity of VREfm with isolates divided into 13 distinct main groups (at least three isolates), 7 small groups (two isolates) and 16 singletons (Figure 1). Three major groups consisting of 218, 100 and 44 isolates, respectively, were identified. Four VREfm main groups were observed in 2012 and new groups were observed throughout the study period demonstrating an increasing diversity among the VREfm isolates (Figure 2). A few groups caused a local outbreak and subsequently disappeared, while most of the groups were observed throughout the study in several locations (Figure 2).



Group	Count	ST	Tn*1546*-like type
1	100	117	T1,T2,T3,T4 [a]
2	218	80	T4
3	44	192/78	T2,T3,T4 [b]
4	13	80	T2, T4 [c]
5	15	80	T4
6	3	78	T4
7	15	117	T4
8	19	203	T5
9	10	117	T3, T4 [d]
10	7	80	T4
11	8	80	T4
12	5	18	T4
13	8	80	T4

Figure 1. Phylogeny based on 1119 core genes showing the genetic relationship among *vanA E. faecium* in the Capital Region of Denmark from 2012 to 2014. Three outliers have been removed from the phylogenetic tree. Each circle represents a group and was scaled in relation to the number of isolates in the group. Circles are located around the centre of the group on the phylogenetic tree. All main groups had ≥80% bootstrap support (1000 replicates). The group numbers are assigned according to when the group was first identified. The group identification number, the number of VREfm isolates, STs and transposon types (T1–T5) are given for each main group. [a]*n* (T1)=1, *n* (T2)=2, *n* (T3)=7. [b]*n* (T2)=3, *n* (T3)=1. [c]*n* (T2)=1. [d]*n* (T3)=2.

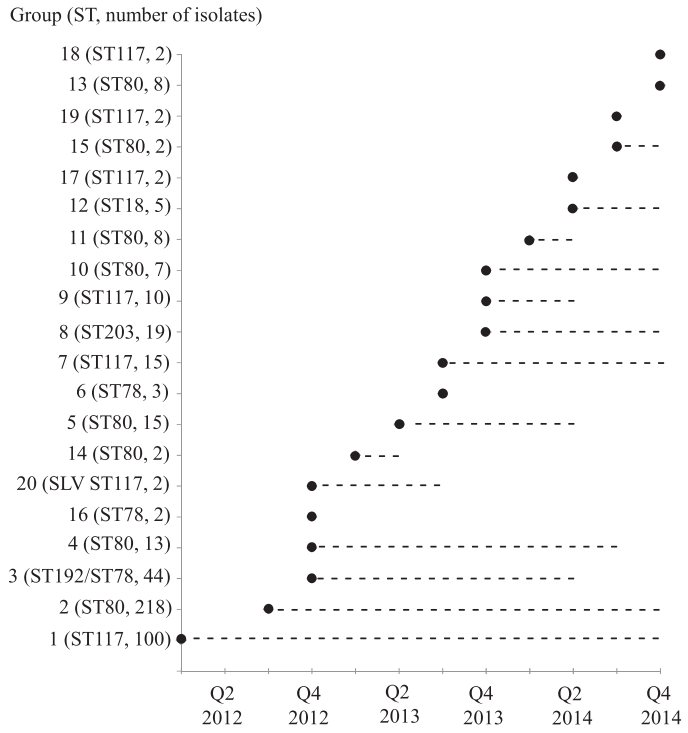


Figure 2. Occurrence of new *vanA E. faecium* groups during the study period, 2012–14. The filled circles indicate the first quarters the VREfm groups were identified and the broken lines indicate the time periods the VREfm isolates were identified. Due to missing data from the last 9 months of 2014, we could not decide whether an outbreak had been resolved in this period.

The pangenome contained 10544 genes of which 1119 were conserved in 99% of the VREfm isolates (core genes). Of the remaining 9425 genes (accessory genome), 4412 were present in ≤1% of the isolates.

The SNP distance matrix supported the grouping of all but one VREfm isolate and isolates within each group differed from each other by ≤400 SNPs. To divide the groups into subgroups of closely related isolates, the diversity among epidemiologically related isolates was determined. Pairwise comparisons of isolates from three hospital wards (∼3801 pairwise comparisons) showed that 98% of related isolate pairs (0–400 SNPs) had between 0 and 27 SNP differences. Using 27 SNPs as a threshold, the VREfm isolates were divided into 29 subgroups (at least two isolates). Isolates belonging to a particular subgroup were identified in several locations and the subgroups were on average identified in three hospitals (range from one to nine hospitals), suggesting cross-hospital spread of related VREfm (Table S2). Furthermore, in each of the four hospitals with the highest burden of VREfm, 13–17 subgroups were circulating.

Another SNP threshold of ≤3 SNPs was set based on the fact that 98% of the screening isolates collected on the same date and same hospital ward differed from each other by ≤3 SNPs (∼300 pairwise comparisons). Data were derived from isolates collected during general screening of six hospital wards. A total of 297 (60%) VREfm isolates differed by ≤3 SNPs from at least five of the other VREfm isolates. These findings supported the presumption of direct or indirect transmission of VREfm between hospitalized patients.

Characterization of Tn1546-like transposons

The structure of the transposons was characterized in all but two isolates. The majority (70%) of the transposons were assembled in more than one contig, which hampered the characterization of the structure. Assembly problems were located around IS elements present in multiple copies in the genome.

Five Tn*1546*-like transposon types were detected (Figure 3). Three IS elements (IS*1251*, IS*1216* and IS*Ef1*) were observed within the transposons and all IS elements were well-known transposases. A truncated transposon was frequent (96%) with a total deletion of *orf1* combined with a partial or total deletion of *orf2*.

Transposon type 1, identical to the Tn*1546* prototype characterized by Arthur *et al.*[8] (Figure 3), was only identified in two (0.4%) non-related isolates. In contrast, a dominant truncated transposon (type 4) with an insertion of a transposase IS*1251* in the *vanSH* intergenic region was detected in 455 isolates (92%). Type 4 consisted of four subtypes. Type 4a, identified in one isolate from group 2, was probably a predecessor to the remaining subtypes as it contained all the Tn*1546* elements and IS*1251*. Types 4b and 4c had a deletion of the left end of the transposon including *orf1* and the left end (156 bp) of *orf2*. Type 4b was the most dominant type (74%). In addition to the left-end deletion, type 4c had an IS*Ef1* element inserted between *vanR* and *vanS*. Along with the IS*Ef1*, RAST annotation of the type 4c transposon showed an extension of *vanR* (18 bp) and *vanS* (33 bp) resulting in two genes that are likely to be functional as the isolates are resistant to vancomycin. In the Tn*1546* prototype, *vanR* and *vanS* overlap.[8] Subtype 4c was present in group 3 (ST192) and group 4 (ST80) (Table S2) and all isolates containing type 4c were collected in the same geriatric ward. The remaining isolates in groups 3 and 4 contained type 4b, suggesting that the insertion of IS*Ef1* occurred during the outbreak in the geriatric ward. VREfm containing type 4c were only identified in the second and third quarter of 2013 and disappeared when the outbreak in the geriatric ward was resolved. Type 4d had a larger left-end deletion including *orf1* and the left end (516 bp) of *orf2*. Figure 1 presents the clonal structure of the VREfm isolates and their corresponding Tn*1546*-like transposons. More detailed information regarding the association between VREfm groups/subgroups and transposon types/subtypes is shown in Table S2. Notably, type 4 was the dominant transposon in all but one VREfm group (group 8). Isolates belonging to group 8 contained only the type 5 transposon, which is a truncated transposon with an IS*1216* element in the *vanXY* intergenic region. Group 8 was recognized from the end of 2013 and represents a new clone in our region. Transposon types 1–3 were mainly identified in a subset of isolates belonging to groups 1, 3, 4 and 9 (Figure 1).

Multiple sequence alignments of transposon fragments demonstrated that the transposon elements were highly conserved. Compared with the prototype from 1993,[8] only five positions in the type 4 transposon were affected by nucleotide variations (Figure 3). All nucleotide variations were non-synonymous and only affected a single codon.

The flanking region upstream of the transposon was not assessable in most isolates as the contig started just before the first transposon element. A cadmium efflux system protein was

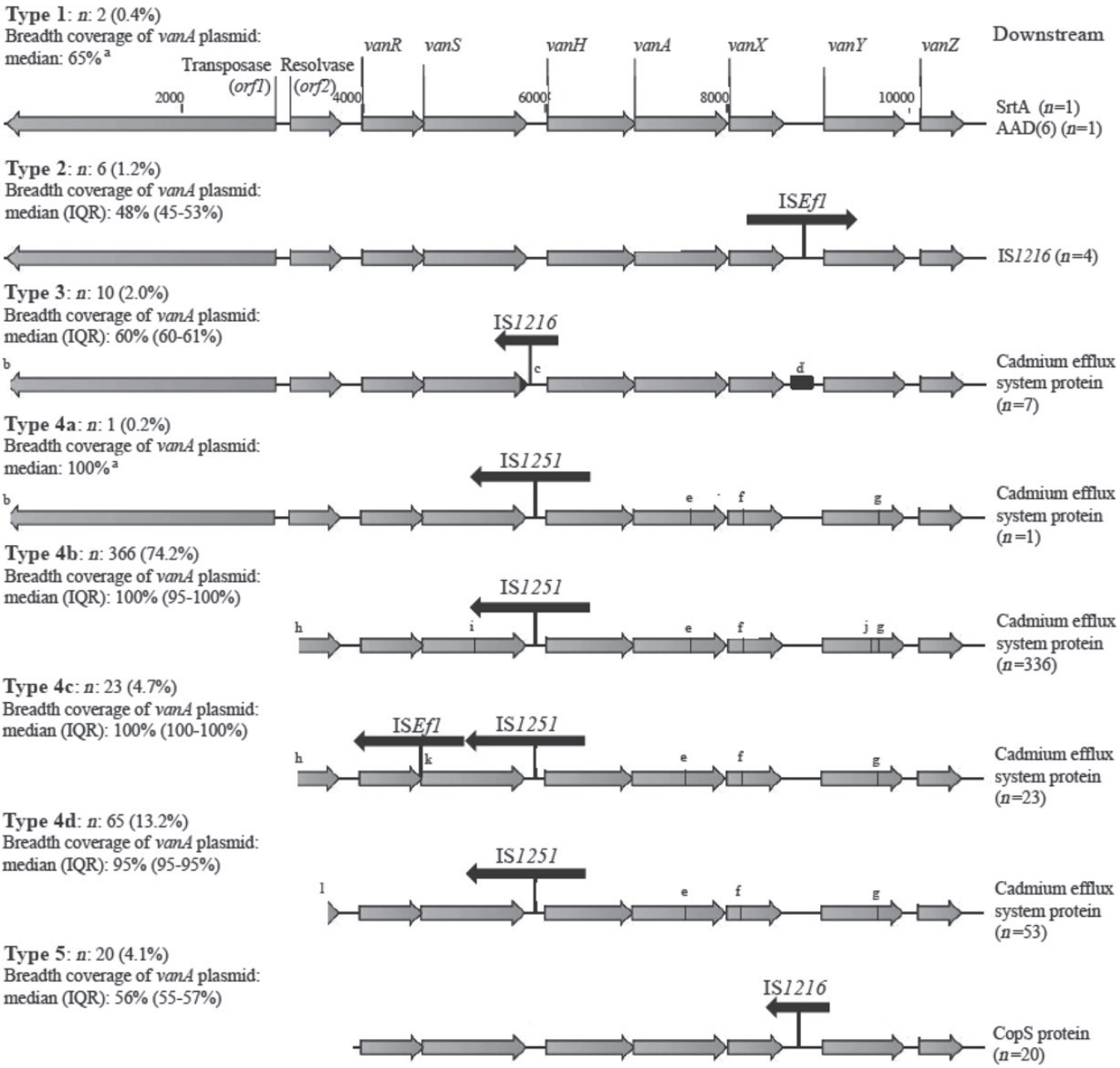


Figure 3. Tn*1546*-like transposon types among 493 *vanA E. faecium* in the Capital Region of Denmark from 2012 to 2014. The number and percentage of isolates containing the transposon type and the percentage of the closed *vanA* plasmid (pHvH-V24) that was covered by mapped reads (breadth coverage) from isolates containing the same transposon type [median (IQR)] are given. Type 1 was identical to the Tn*1546* prototype characterized in 1993.[8] The positions of the transposase (*orf1*), resolvase (*orf2*) and *van* genes and their direction of transcription are depicted with arrows. Black arrows represent IS elements and their direction of transcription. Filled black boxes represent sequences in the transposon that are not identical to the prototype. A vertical line represents a nucleotide variant in relation to the prototype. [a]IQR could not be determined due to the limited number of isolates. [b]Deletion of the left end (42 bp) of the transposase. [c]IS*1216* was inserted after *vanS* changing the last 11 amino acids in *vanS* to 10 different amino acids compared with the prototype. [d]A region of 216 bp has been replaced by a region of 245 bp (this region does not align to the prototype). [e]Nucleotide variant in *vanA* (position 680 T>C). [f]Nucleotide variant in *vanX* (position 219 G>T). [g]Nucleotide variant in *vanY* (position 647 C>T) present in 38%, 100% and 83% of types 4b, 4c and 4d, respectively. [h]Deletion of the transposase and the left end (156 bp) of the resolvase. [i]Nucleotide variant in *vanS* (position 589 T>A) present in 20% of type 4b. [j]Nucleotide variant in *vanY* (position 549 C>T) present in 3% of type 4b. [k]Insertion of IS*Ef1* between *vanR* and *vanS*. *vanR* and *vanS* were extended with 18 and 33 bp, respectively, according to RAST annotation. [l]Deletion of the transposase and the left end (516 bp) of the resolvase. The first element downstream of *vanZ* is given together with the number of isolates that contained the element. SrtA, sortase A; AAD(6), putative aminoglycoside 6-adenylyltansferase.

identified downstream of *vanZ* in types 3 and 4, while type 5 differed with a CopS protein downstream of *vanZ* (Figure 3). Also, types 1 and 2 had different flanking regions downstream of *vanZ*.

Reconstruction and dissemination of a vanA plasmid

Incorporating PacBio long reads into the assembly of a VREfm isolate reduced the number of contigs from 203 to 34 and increased the N50 by nearly 10-fold from 45 000 to 425 000, when compared with assembly using Illumina data alone. The *vanA* plasmid was assembled in three contigs, but was possible to close resulting in a 37 372 bp plasmid, termed pHvH-V24. This plasmid contained a *rep-17* replicon. In addition to the type 4b Tn*1546*-like transposon, the plasmid contained genes involved in aminoglycoside (*aph-3*, *sat4* and *ant-6*) and macrolide [*erm*(B)] resistance. Furthermore, the plasmid contained a toxin–antitoxin (TA) system (*axe-txe*), which enhances plasmid stability. This plasmid was highly similar to the non-conjugative, MDR pS177 plasmid of 39 kb,[35] sharing 95% of pS177 at 99% identity. Relative to pS177, pHvH-V24 contained a 3191 bp deletion in the Tn*1546* transposon corresponding to the truncated transposase (*orf1*) and the first 156 bp of the resolvase (*orf2*). The truncated transposon represents ∼20% of the plasmid.

Mapping of reads from all the VREfm isolates to the plasmid sequence (pHvH-V24) showed that 45%–100% of the plasmid was covered by reads and in the majority (74%) of the isolates ≥95% of the plasmid was covered by reads. This suggests the existence of a *vanA* plasmid that has spread through distinct populations of *E. faecium*. In isolates containing the type 4 transposon, a high percentage of the plasmid was covered by reads (median ≥ 95%) compared with isolates containing the other transposon types (median = 48%–65%) (Figure 3), showing a correlation between transposon type 4 and the closed plasmid (pHvH-V24).

Discussion

The increase in hospital-acquired VREfm in the Capital Region of Denmark was recognized towards the end of 2012. In total, 894 VREfm patients were identified from 2012 to 2014, whereas <20 VREfm patients were detected in previous years.[6] Part of the increase is due to screening of VREfm contact patients and general screening of hospital wards; however, almost half of the VREfm isolates in this study were from clinical samples. WGS of 495 isolates collected from the start of the VREfm epidemic revealed a polyclonal structure with clonal spread of several new VREfm groups (Figure 1). Previous typing of VREfm by methods such as PFGE and MLST has shown clonal dissemination of VREfm in hospitals.[36,37] Applying greater discrimination provided by WGS, the present study suggests transmission of VREfm between hospitalized patients via direct or indirect transmission. In line with a recent study of VREfm in Denmark,[14] we confirmed spread within wards, between wards and between hospitals, which is probably due to frequent transfer of patients between hospitals and specialties. Transfer of patients between hospitals and specialties may explain why it has been so difficult to control the VREfm spread in our region despite improved infection control and enhanced cleaning.

Although polyclonal structures of *vanA* VREfm in hospital outbreaks have been reported in other studies,[9,11,38] the diversity was surprising given that the VREfm frequency was very low before 2012. However, the vast majority of the transposons were similar and belonged to transposon type 4 (92%) with type 4b (74%) as the dominant subtype. Transposon type 4 had spread across several distinct WGS groups, suggesting horizontal transfer of the Tn*1546*-like transposon (Figure 1). Adding PacBio sequencing of one VREfm isolate enabled us to close a *vanA* plasmid carrying the type 4b transposon variant and the vehicle for the transposon was detected. Most VREfm isolates with a type 4 transposon contained a highly related *vanA* plasmid determined by the percentage of the plasmid (pHvH-V24) that was covered by reads (Figure 3). This indicates the existence of a successful plasmid that has spread through different populations of pre-existing VSEfm. The genetic relationship between VREfm and endemic VSEfm in our hospitals needs to be elucidated. WGS of temporally related VSEfm from our region has not been done yet due to financial constraints. However, recent studies from Sweden, the UK and Australia have shown that VREfm and VSEfm belong to the same circulating population in hospital settings.[39–41]

The origin and reason for the success of this type 4 *vanA* plasmid remain to be elucidated. Recently, a Tn*1546*-like structure similar to type 4 has been shown to be prevalent in clinical isolates from Taiwan, Brazil and Paraguay.[9,10,42] Furthermore, the pS177 plasmid, highly similar to our plasmid, was identified in a VREfm clinical isolate sampled in a hospital in Illinois, USA.[35,43] The complete sequence and gene content of pS177 was reported in 2011 and it was the first report of a completely sequenced plasmid carrying both vancomycin resistance genes and a TA system (*axe-txe*).[35] In addition to the selective advantage of resistance mechanisms against aminoglycosides, vancomycin and macrolides, the TA system enhances plasmid stability, which might explain the success of this plasmid.[35,43] Despite the ability of pHvH-V24 to disseminate to a wide range of hospital-associated *E. faecium* populations, genes involved in mobilization could not be identified. In addition, pHvH-V24 is highly similar to pS177, which is a non-conjugative plasmid.[35] The plasmid belongs to the narrow host range *rep-17* (pRUM) replicon group, which is common in hospital-associated *E. faecium* populations with reported prevalences from 39% to 58%.[44,45] This group of plasmids is frequent and seems to be highly adapted to hospital-associated *E. faecium*, which might explain the rapid spread of the *vanA* plasmid after its appearance in the Capital Region. Another explanation is the fact that the VREfm epidemic occurred shortly after a major outbreak of *Clostridium difficile* in our region resulting in a 2-fold increase in the consumption of vancomycin from 2008 to 2013,[46,47] which probably favoured vancomycin-resistant bacteria in the gut.

Very recently, it has been reported that an IS element in the *vanSH* region can influence the vancomycin phenotype.[48] However, the IS elements described in the present study did not affect the vancomycin phenotype as all clinical isolates and a subset of screening isolates had vancomycin MICs >32 mg/L (data not shown).

Our study has limitations. A complete collection of VREfm isolates has not been included in the study, which means diversity could be even higher. Also, double VREfm infections might have been missed by sequencing a single colony subculture. As we were only able to determine closed plasmid sequences for one isolate due to the inclusion of long reads, variations in the structure of the *vanA* plasmid could not be identified for all isolates.

The main strengths of the study were an almost complete collection of VREfm isolates at the beginning of the VREfm epidemic

and a collection of isolates throughout the study period from ≥8 out of 12 hospitals. All submitted isolates have been sequenced, which resulted in a large collection of WGS data. Furthermore, we managed to close all Tn*1546*-like transposon types and the inclusion of long reads via PacBio sequencing of one isolate made it possible to close the predominant *vanA* plasmid.

In summary, applying high discrimination provided by WGS, the present study identified clonal spread of VREfm within wards, between wards and between hospitals in the Capital Region of Denmark. The majority of the VREfm isolates contained the type 4b Tn*1546*-like transposon and its variants in a conserved plasmid (pS177-like). This suggests that it was the spread of the type 4 Tn*1546*-like transposon and plasmid by horizontal transfer to pre-existing VSEfm, followed by clonal spread of new VREfm, that contributed to the increase and diversity of VREfm in hospitals. Specifically, this study supports the existence of a successful *vanA* plasmid (pHvH-V24) that has spread across several populations of *E. faecium*. This is cause for concern because the plasmid has spread with relative ease and the spread of the plasmid has proven difficult to control.

Acknowledgements

Data have been presented at the Twenty-fifth European Congress of Clinical Microbiology and Infectious Diseases, Copenhagen, Denmark, 2015 (Oral Presentation O168) and at the Eleventh International Meeting on Microbial Epidemiological Markers, Estoril, Portugal, 2016 (Oral Presentation OP43). Sequence data from 2012 to April 2013 were presented in: *J Antimicrob Chemother* 2015; **70**: 2474–82.

We thank Louise Christensen, Susanne Rhode, Marianne S. Studstrup and Maria Kristin Bjornsdóttir for excellent technical assistance. We thank the Oxford Centre for Gene Function for the use of the pipeline that handles the WGS data, the Scandinavian Society for Antimicrobial Chemotherapy (SSAC) Foundation and the Danish Ministry of Health. We also thank Toyotafonden, Denmark, for a grant to purchase the Illumina MiSeq instrument.

Funding

This work was supported by the Scandinavian Society for Antimicrobial Chemotherapy (SSAC) Foundation, the Danish Ministry of Health and internal funding.

Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

References

1 Willems RJ, Top J, van Schaik W *et al*. Restricted gene flow among hospital subpopulations of *Enterococcus faecium*. *MBio* 2012; **3**: e00151–12.

2 Galloway-Pena J, Roh JH, Latorre M *et al*. Genomic and SNP analyses demonstrate a distant separation of the hospital and community-associated clades of *Enterococcus faecium*. *PLoS One* 2012; **7**: e30187.

3 Kramer A, Schwebke I, Kampf G. How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. *BMC Infect Dis* 2006; **6**: 130.

4 Wagenvoort JH, De Brauwer EI, Penders RJ *et al*. Environmental survival of vancomycin-sensitive ampicillin-resistant *Enterococcus faecium* (AREfm). *Eur J Clin Microbiol Infect Dis* 2015; **34**: 1901–3.

5 *DANMAP 2012—Use of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Bacteria from Food Animals, Food and Humans in Denmark*. http://www.danmap.org/~/media/Projekt%20sites/Danmap/DANMAP%20reports/DANMAP%202012/Danmap_2012.ashx.

6 *Task Force: Prevention of Hospital-Acquired Infections in the Capital Region of Denmark*. https://www.regionh.dk/hospitalsinfektioner/data/Sider/vancomycinresistente-enterokokker-vre.aspx.

7 Werner G, Coque TM, Hammerum AM *et al*. Emergence and spread of vancomycin resistance among enterococci in Europe. *Euro Surveill* 2008; **13**: pii=19046.

8 Arthur M, Molinas C, Depardieu F *et al*. Characterization of Tn*1546*, a Tn*3*-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. *J Bacteriol* 1993; **175**: 117–27.

9 Kuo AJ, Su LH, Shu JC *et al*. National surveillance on vancomycin-resistant *Enterococcus faecium* in Taiwan: emergence and widespread of ST414 and a Tn*1546*-like element with simultaneous insertion of IS*1251*-like and IS*1678*. *PLoS One* 2014; **9**: e115555.

10 da Silva LP, Pitondo-Silva A, Martinez R *et al*. Genetic features and molecular epidemiology of *Enterococcus faecium* isolated in two university hospitals in Brazil. *Diagn Microbiol Infect Dis* 2012; **74**: 267–71.

11 Wardal E, Markowska K, Zabicka D *et al*. Molecular analysis of VanA outbreak of *Enterococcus faecium* in two Warsaw hospitals: the importance of mobile genetic elements. *Biomed Res Int* 2014; **2014**: 575367.

12 Willems RJ, Top J, van den Braak N *et al*. Molecular diversity and evolutionary relationships of Tn*1546*-like elements in enterococci from humans and animals. *Antimicrob Agents Chemother* 1999; **43**: 483–91.

13 Novais C, Freitas AR, Sousa JC *et al*. Diversity of Tn*1546* and its role in the dissemination of vancomycin-resistant enterococci in Portugal. *Antimicrob Agents Chemother* 2008; **52**: 1001–8.

14 Pinholt M, Larner-Svensson H, Littauer P *et al*. Multiple hospital outbreaks of *vanA Enterococcus faecium* in Denmark, 2012–13, investigated by WGS, MLST and PFGE. *J Antimicrob Chemother* 2015; **70**: 2474–82.

15 Zerbino DR, Birney E. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. *Genome Res* 2008; **18**: 821–9.

16 Seemann T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 2014; **30**: 2068–9.

17 Page AJ, Cummins CA, Hunt M *et al*. Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* 2015; **31**: 3691–3.

18 Simonsen M, Mailund T, Pedersen CNS. Rapid Neighbour-Joining. In: *Algorithms in Bioinformatics*. LNBI 5251. Springer Verlag, 2008; 113–22. doi:10.1007/978-3-540-87361-7_10.

19 *FigTree: A Graphic Viewer of Phylogenetic Trees*. v1.4.2. http://tree.bio.ed.ac.uk/software/figtree.

20 Lam MM, Seemann T, Bulach DM *et al*. Comparative analysis of the first complete *Enterococcus faecium* genome. *J Bacteriol* 2012; **194**: 2334–41.

21 Lunter G, Goodson M. Stampy: a statistical algorithm for sensitive and fast mapping of Illumina sequence reads. *Genome Res* 2011; **21**: 936–9.

22 Didelot X, Eyre DW, Cule M *et al*. Microevolutionary analysis of *Clostridium difficile* genomes to investigate transmission. *Genome Biol* 2012; **13**: R118.

23 Aziz RK, Bartels D, Best AA *et al*. The RAST Server: Rapid Annotations using Subsystems Technology. *BMC Genomics* 2008; **9**: 75.

24 Overbeek R, Olson R, Pusch GD *et al*. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Res* 2014; **42**: D206–14.

25 Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Methods* 2012; **9**: 357–9.

26 Kurtz S, Phillippy A, Delcher AL *et al*. Versatile and open software for comparing large genomes. *Genome Biol* 2004; **5**: R12.

27 Li H, Handsaker B, Wysoker A *et al*. The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 2009; **25**: 2078–9.

28 Pysam. https://github.com/pysam-developers/pysam.

29 Camacho C, Coulouris G, Avagyan V *et al*. BLAST+: architecture and applications. *BMC Bioinformatics* 2009; **10**: 421.

30 Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 2004; **32**: 1792–7.

31 Bankevich A, Nurk S, Antipov D *et al*. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol* 2012; **19**: 455–77.

32 Carattoli A, Zankari E, Garcia-Fernandez A *et al*. In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. *Antimicrob Agents Chemother* 2014; **58**: 3895–903.

33 Zankari E, Hasman H, Cosentino S *et al*. Identification of acquired antimicrobial resistance genes. *J Antimicrob Chemother* 2012; **67**: 2640–4.

34 Quinlan AR, Hall IM. BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* 2010; **26**: 841–2.

35 Halvorsen EM, Williams JJ, Bhimani AJ *et al*. Txe, an endoribonuclease of the enterococcal Axe-Txe toxin-antitoxin system, cleaves mRNA and inhibits protein synthesis. *Microbiology* 2011; **157**: 387–97.

36 Soderblom T, Aspevall O, Erntell M *et al*. Alarming spread of vancomycin resistant enterococci in Sweden since 2007. *Euro Surveill* 2010; **15**: pii=19620.

37 Suppola JP, Kolho E, Salmenlinna S *et al*. *vanA* and *vanB* incorporate into an endemic ampicillin-resistant vancomycin-sensitive *Enterococcus faecium* strain: effect on interpretation of clonality. *J Clin Microbiol* 1999; **37**: 3934–9.

38 Ryan L, O'Mahony E, Wrenn C *et al*. Epidemiology and molecular typing of VRE bloodstream isolates in an Irish tertiary care hospital. *J Antimicrob Chemother* 2015; **70**: 2718–24.

39 Howden BP, Holt KE, Lam MM *et al*. Genomic insights to control the emergence of vancomycin-resistant enterococci. *MBio* 2013; **4**: e00412–13.

40 Sivertsen A, Billstrom H, Melefors O *et al*. A multicentre hospital outbreak in Sweden caused by introduction of a *vanB2* transposon into a stably maintained pRUM-plasmid in an *Enterococcus faecium* ST192 clone. *PLoS One* 2014; **9**: e103274.

41 Brodrick HJ, Raven KE, Harrison EM *et al*. Whole-genome sequencing reveals transmission of vancomycin-resistant *Enterococcus faecium* in a healthcare network. *Genome Med* 2016; **8**: 4.

42 Khan MA, Northwood JB, Loor RG *et al*. High prevalence of ST-78 infection-associated vancomycin-resistant *Enterococcus faecium* from hospitals in Asuncion, Paraguay. *Clin Microbiol Infect* 2010; **16**: 624–7.

43 Moritz EM, Hergenrother PJ. Toxin-antitoxin systems are ubiquitous and plasmid-encoded in vancomycin-resistant enterococci. *Proc Natl Acad Sci USA* 2007; **104**: 311–6.

44 Rosvoll TC, Pedersen T, Sletvold H *et al*. PCR-based plasmid typing in *Enterococcus faecium* strains reveals widely distributed pRE25-, pRUM-, pIP501- and pHTβ-related replicons associated with glycopeptide resistance and stabilizing toxin–antitoxin systems. *FEMS Immunol Med Microbiol* 2010; **58**: 254–68.

45 Qu TT, Yang Q, Shen P *et al*. Novel vancomycin-resistance transposon, plasmid replicon types, and virulence factors of vancomycin-resistant enterococci in Zhejiang, China. *Microb Drug Resist* 2012; **18**: 183–8.

46 *The Weekly Newsletter on Surveillance and Outbreaks of Infectious Diseases*. http://www.ssi.dk/English/News/EPI-NEWS/2012/No%207-8%20-%202012.aspx.

47 *DANMAP 2013—Use of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Bacteria From Food Animals, Food and Humans in Denmark*. http://www.danmap.org/~/media/Projekt%20sites/Danmap/DANMAP%20reports/DANMAP%202013/DANMAP%202013.ashx.

48 Sivertsen A, Pedersen T, Larssen KW *et al*. A silenced *vanA* gene cluster on a transferable plasmid caused an outbreak of vancomycin-variable enterococci. *Antimicrob Agents Chemother* 2016; **60**: 4119–27.