Novel genomic islands and a new vanD-subtype in the first sporadic VanD-type vancomycin resistant enterococci in Norway

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Abstract

Background
Vancomycin-resistant enterococci (VRE) represent several types of transferable vancomycin resistance gene clusters. The vanD type, associated with moderate to high level vancomycin resistance, has only sporadically been described in clinical isolates. The aim of this study was to perform a genetic characterization of the first VanD-type VRE strains detected in Norway.

Methods
The VanD-type VRE-strains (n = 6) from two patient cases were examined by antimicrobial susceptibility testing and whole genome sequencing (WGS) to uncover Van-phenotype, strain phylogeny, the vanD gene clusters, and their genetic surroundings. The putative transferability of vanD was examined by circularization PCR and filter mating.

Results
The VanD-type Enterococcus faecium (n = 4) and Enterococcus casseliflavus (n = 2) strains recovered from two cases (A and B), expressed moderate to high level vancomycin resistance (MIC 64—>256 mg/L) and various levels of teicoplanin susceptibility (MIC 2—>256 mg/L). WGS analyses revealed phylogenetically different E. faecium strains (A1, A2, and A3 of case A and B1 from case B) as well as vanD gene clusters located on different novel genomic islands (GIs). The E. casseliflavus strains (B2 and B3 of case B) were not clonally related, but harbored nearly identical novel GIs. The vanD cluster of case B strains represents a novel vanD-subtype. All the vanD-GIs were integrated at the same chromosomal site and contained genes consistent with a Clostridiales origin. Circular forms of the
vanD-GIs were detected in all strains except B1. Transfer of vanD to an *E. faecium* recipient was unsuccessful.

**Conclusions**

We describe the first VanD-type *E. casseliflavus* strains, a novel vanD-subtype, and three novel vanD-GIs with a genetic content consistent with a Clostridiales order origin. Despite temporal occurrence, case A and B *E. faecium* strains were phylogenetically diverse and harbored different vanD subtypes and vanD-GIs.

**Introduction**

Vancomycin resistant enterococci (VRE) have become a global nosocomial problem three decades after the first description in the late 1980s [1]. Eight different acquired vancomycin resistance gene clusters (*vanA*, *vanB*, *vanD*, *vanG*, *vanE*, *vanL*, *vanM*, and *vanN*) have been identified [2]. The *vanC* gene cluster is intrinsic in *E. casseliflavus* and *E. gallinarum* [2]. In general, *van* gene clusters encode three groups of co-acting enzymes; 1) enzymes necessary for the synthesis of new peptidoglycan precursors, 2) enzymes that erase the inherent D-Ala-D-Ala-ending precursors, and 3) a two-component signal transduction system for inducible resistance [3]. The normal enterococcal cell wall side chain terminal residue D-Ala-D-Ala, to which vancomycin binds with high affinity, are replaced by D-Ala-D-Lac in *vanA*, *vanB*, *vanD*, and *vanM* gene clusters or D-Ala-D-Ser in the other *van* gene clusters [3]. Vancomycin binds to D-Ala-D-Ser with seven times lower affinity compared to D-Ala-D-Ala, causing low-level vancomycin resistance, while the binding affinity of vancomycin to D-Ala-D-Lac is almost 1000 times lower mediating high-level resistance [4]. The *vanA* and *vanB* clusters dominate worldwide, likely due to linkage to successful mobile genetic elements (MGEs) [5]. Although the *vanA*, *vanB*, and *vanD* clusters have a similar organization, the *vanD* gene clusters have so far only been sporadically described on chromosomal genomic islands (GIs) that have not been shown to be transferable between enterococci [6–9]. The *vanD* gene cluster has up till now been reported in five species of enterococci (*Enterococcus faecium*, *Enterococcus faecalis*, *Enterococcus gallinarum*, *Enterococcus avium*, and *Enterococcus raffinosus*) [10].

The VanD-phenotype is characterized by moderate to high level vancomycin resistance and various levels of susceptibility to teicoplanin [3, 11, 12]. The housekeeping *ddl* gene (D-Ala-D-Ala ligase) is often inactivated by mutations in *vanD* containing strains causing an impaired chromosomal peptidoglycan synthesis pathway and addiction to *vanD*-expression as the alternative peptidoglycan precursor pathway [3, 7, 13]. Based on sequence differences, there are five known subtypes of *vanD*. The sequence diversity in *vanD* gene cluster subtypes mostly is in the *vanYD*, *vanHD*, *vanD*, and *vanXD* genes and at the intergenic sequence between the two operons of the cluster [11]. VanD VRE are rare and have only been reported sporadically from the Netherlands, France, Canada, Japan, Sweden, Australia, the US, and Brazil during the last decades [7, 8, 10, 12–18].

In this study, we aim to determine the genetic relatedness between the first Norwegian VanD-type VRE strains, their Van-phenotype, and the putative MGEs harbouring the *vanD*-gene cluster.
Material and methods

Case descriptions

**Case A.** A middle-aged previously healthy female presented with acute hepatic failure. An urgent transplantation with an ABO-incompatible liver was performed. At week eight, a subphrenic abscess was diagnosed supported by the growth of *E. coli* and *E. faecium* and treated by local drainage. In week 16, a new subphrenic abscess was diagnosed and a vanD *E. faecium* in pure culture was isolated from the abscess drainage pigtail catheter. Screening for fecal VRE-carriage at week 20 after transplantation yielded vanD *E. faecium*. Several negative rectal VRE-screening samples were obtained during the subsequent 9 months, except for one vanC *E. casseliflavus* strain. Several screening samples were collected during linezolid treatment. Antibiotic treatment was successfully terminated almost a year after the transplantation.

**Case B.** An elderly female, undergoing hemodialysis for the last five years after kidney transplant failure, presented with recurrent urinary tract infections (UTIs), predominantly caused by *Klebsiella pneumoniae*, but occasionally by *E. faecium*. Due to relapsing *Clostridiodides difficile* infections (CDIs), she had received oral vancomycin prophylaxis the last three years. The urine yielded vanD *E. faecium* in pure culture. Repeated fecal VRE-screening (follow-up 2 years) revealed the presence of vanD *E. casseliflavus*, but not vanD *E. faecium*. The vanD *E. faecium* UTI was successfully treated with linezolid, while the *C. difficile* prophylaxis was changed to metronidazole.

Relevant case characteristics are summarized in **Table 1**. Antibiotic treatment and microbiological findings for case A are presented in S1 Fig.

Ethical approval

Since this study contain only limited anonymized patient data, the study was approved by the Data Protection Officer at Oslo University Hospital and the Chief of Department of Microbiology at St Olavs Hospital. The written consents of the patients were obtained to use anonymized data from their patient journal in publication of this work.

VRE strains and data collection

The first two cases of VanD-type VRE were identified in Norway in 2017. The Norwegian National Advisory Unit on Detection of Antimicrobial Resistance received the strains for further characterization (**Table 2**). Three VanD-positive *E. faecium* (VanD-type VREfm) (A1, A2, and A3) strains were isolated from case A. The strains of case A were recovered from a subphrenic abscess (A1 and A2) and through rectal screening (A3). A month later, a VanD-type

| Case | Underlying condition | Indication antimicrobial treatment | Antimicrobial treatment | Time to isolation of vanD *E. faecium* | Infection focus | Rectal carriage | Hospital |
|------|----------------------|----------------------------------|------------------------|----------------------------------------|-----------------|----------------|----------|
| A    | Acute liver Tx—otherwise healthy | Postoperative subphrenic abscesses | Broad spectrum beta-lactams, vancomycin, trimethoprim/sulfamethoxazole (PJP prophylaxis) | 19 weeks post liver tx | Subphrenic abscess | vanD *E. faecium, E. casseliflavus* | 1 and 2 |
| B    | Tx kidney failure, hemodialysis, recurrent UTIs and CDIs | Recurrent CDI | Vancomycin p.o. (CDI prophylaxis) | 3 years from start of vancomycin prophylaxis | Urinary tract infection | vanD *E. casseliflavus* | 3 |

Tx: transplantation, UTI: Urinary tract infection, CDI: *C. difficile* infection, PJP: Pneumocystis jiroveci pneumonia, p.o.: postoperative.

Fecal screening with CHROMagar™ VRE.

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VREfm (B1) strain was isolated from the urinary tract in a hemodialysis patient (case B). Further, two vanD-positive E. casseliflavus strains were recovered from case B by rectal screening, three weeks (B2) and two years (B3) later. Both patients had received vancomycin treatment before the isolation of the VanD-type VRE.

### Antimicrobial Susceptibility Testing (AST) and van genotype determinations

AST was performed by broth microdilution using the GPALL1F or EUENCF Sensititre plates (Thermo Fisher Scientific, Waltham, Massachusetts, USA), ComASP™ Vancomycin, and Teicoplanin MIC Test Strip (Liofilchem, Roseto Degli Abruzzi, Italy). The results (MICs) were interpreted according to EUCAST clinical breakpoints v. 10.0 2020 [19]. The van genotype was initially determined by a vanDEG multiplex PCR as described previously [20, 21] and JumpStart REDTaq ReadyMix (Merck KGaA, Darmstadt, Germany). DNA extractions for PCRs were performed using the NucliSens EasyMAG instrument and reagents (BioMérieux, Marcy-l’Étoile, France) according to the manufacturer’s instructions.

### Species identification and Whole Genome Sequencing (WGS)

Strains were subcultured on blood agar to ensure pure culture. Species identification was performed by MALDI-TOF (Bruker, Billerica, USA) according to the manufacturer’s instructions. Genomic DNA was extracted using DNeasy Blood and tissue kit (Qiagen, Hilden, Germany). The total DNA concentration was quantified by Qubit fluorometer (Invitrogen, Thermo Fisher Scientific). Libraries were prepared by the Nextera XT DNA library preparation kit (Illumina, San Diego, USA) and sequenced using Illumina NextSeq500 and the Mid Output 300 cycles cell.

### Genomic analyses

Adapter removal and quality trimming of the raw reads were performed by trimmomatic v0.39 [22]. Later, genome assembly was done using SPAdes v3.13.0 [23] and the quality of assembled genomes was assessed using QUAST v5.0.2 [24]. The annotation of the transposons was carried out using the National Center for Biotechnology Information (NCBI) prokaryotic genome annotation pipeline (PGAP) [25]. Antimicrobial resistance (AMR) genes were

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Table 2. Relevant strain characteristics.

| Strain ID | Strain name | Species | MLST | VAN* | TEC | AMP | LIN | GEN | Ddl ligase changes compared to E. faecium | Source | Isolation day |
|-----------|-------------|---------|------|------|-----|-----|-----|-----|-----------------------------------------|--------|--------------|
| A1        | VRE1736     | E. faecium | 1486 | 64  | 4   | >8  | <1  | >500| S185 changed to F185                     | Abcess drainage Day 1
| A2        | VRE1737     | E. faecium | 1486 | >128| 4   | >8  | <1  | >500| S185 changed to F185 | Abcess drainage Day 1
| A3        | KresVRE0001  | E. faecium | 117  | 64  | 2   | >8  | 2   | <32 | S319 changed to G319 # | Screening Day 10
| B1        | KresVRE0002  | E. faecium | 203  | >256| >256| >8  | 2   | >500| Truncated protein of 110 aa # | Urine Day 42
| B2        | KresVRE0003  | E. casseliflavus | - | >256| >256| 1   | 2   | <2 | | Rectal screening Day 65
| B3        | KresVRE0012  | E. casseliflavus | - | >256| >8  | <0.25| 2   | <32 | | Rectal screening Day 665

* MICs in mg/L for VAN (vancomycin), TEC (teicoplanin), AMP (ampicillin), LIN (linezolid), and GEN (gentamicin).

# These changes are not within the part of the ddd gene used for sequence typing.

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identified \textit{in silico} from the assemblies using NCBI bacterial AMR reference gene database (PRJNA313047) \cite{26} in ABRicate tool v0.8.7 \cite{27}. Identification of Type IV secretion systems genes was carried out by BLASTp \cite{28} searches against the SecReT4 database \cite{29}.

**Phylogenetic analyses**

To explore the phylogenetic relationship between the \textit{vanD} strains and publically available genome sequences on NCBI, the global phylogenetic trees were generated based on the core genome. All closed genomes of \textit{E. faecium} (n = 135) and \textit{E. casseliflavus} (n = 3) from NCBI as of 04.04.2020 were retrieved and phylogenetic trees were constructed using Parsnp v1.2 \cite{30}. Another core genome SNP tree was built for the publicly available VanD-type VRE\textit{fm} genome sequences together with the Norwegian vanD-type VRE\textit{fm}. Also, a SNP tree was generated for \textit{vanD} gene cluster sequences using parsnp. Multilocus Sequence Typing (MLST) was performed using MLST tool version 2.11 \cite{31}. For high-resolution typing, Minimum Spanning Tree was generated based on the 1423 core genes of \textit{E. faecium} scheme of SeqSphere+ software V6.0.2 (Ridom GmbH, Münster, Germany [http://www.ridom.de/seqsphere/]). We used the default ≤ 20 allelic differences as a threshold for cluster calculation and clonal relatedness \cite{32}.

**Comparative genomics**

The closest non-VRE strains to each of the Norwegian VanD-type VRE were selected from the global phylogenetic tree. We used Mauve \cite{33} to sort the contigs according to the reference genomes (E1 (NZ_CP018065.1) for A1-3 strains, E4402 (NZ_LR135174) for B1 strain, and EC20 (CP004856.1) for B2-3 strains) followed by Easyfig v2.2.2 \cite{34} for comparison. The Artemis comparison tool \cite{35} was used to visualize the BLASTn v2.6.0 search result and to locate the mobile genetic structures containing \textit{vanD} gene clusters and their insertion site in the genome. Sequences of the GIs harboring the \textit{vanD} gene clusters were BLASTed against the NCBI \textit{nr} database to find the homologous sequences. Pyani v0.2.7 was used to determine the average nucleotide identity (ANI) between genomes, GIs and \textit{vanD} gene clusters \cite{36}. For the novel GIs, transposon numbers were registered at the Transposon Registry \cite{37}.

**Excision of putative GIs**

The ability of the GIs to circularize was examined by PCR using the following pair of primers which directed outwards from the GIs ends: 5´–GCGTGAGAAGCTGACAACAA–3´ and 5´–GTTTCAGCCGCCAACTATTC–3´. Subsequent Sanger sequencing of PCR products using BigDye 3.1 technology (Applied Biosystems, CA, USA) was performed to confirm the expected sequence.

**Transferability of putative GIs**

Transferability of \textit{vanD} gene clusters was examined as described previously \cite{38} using \textit{E. faecium} BM4105-RF \cite{39} as a recipient. To determine transfer frequency, colony forming units were counted on Brain heart infusion agar with rifampicin (30 mg/L) and fusidic acid (20 mg/L), and/or vancomycin (8 mg/L).

**Results and discussion**

Most of the reported VanD-type VRE have been sporadic clinical isolates \cite{7, 10, 12, 13, 15}. Despite an increasing prevalence of VRE in Norway since 2010, only \textit{vanA} and \textit{vanB} have been reported until now \cite{40}. The detection of VanD-type VRE from two different patients within two months in 2017, therefore raised a concern of facing a VanD-type VRE outbreak in
Norway, although no obvious epidemiological link between the patients was identified. Thus, the pheno- and genotype of the six VanD VRE strains were examined (Table 2). All three VRE from case A were *E. faecium*, while in case B, one *E. faecium* and two *E. casseliflavus* were isolated. To our knowledge, B2 and B3 are the first VanD-type vancomycin resistant *E. casseliflavus* strains reported.

AST results

The AST-results are summarized in Table 2. Briefly, all strains expressed high-level vancomycin resistance (MIC ≥ 64 mg/L), various levels of susceptibility to teicoplanin (MIC 2 mg/L to >256 mg/L), and susceptibility to linezolid. All four *E. faecium* strains were ampicillin resistant and three also demonstrated high-level gentamicin resistance.

*In silico* analysis showed that all strains contained the *vanD* gene cluster integrated into their chromosome. The *E. casseliflavus* genomes (B2 and B3) also contained the intrinsic *vanC* gene cluster [2]. In the *E. faecium* strain B1, alignment of the housekeeping D-Ala-D-Ala ligase deduced from the *ddl* gene sequence showed a truncated protein of only 110 amino acids caused by a deletion resulting in a frameshift and a premature stop codon (Table 2 and S2 Fig). All the other VanD-type VREfm strains showed point mutations in essential positions that presumably could lead to a non-functional Ddl ligase. In the literature, most VanD-type VRE strains described have had an impaired Ddl ligase and are thus dependent on the constitutively expressed *vanD* cluster to synthesise peptidoglycan [10].

The VanD *E. faecium* strains from the two cases were not closely related

The VanD VREfm strains from cases A and B had different MLST profiles (Table 2). A1 and A2 genomes had an identical MLST profile which was registered as the novel ST1486, a single locus (*ddl* allele) variant of ST117 (strain A3) belonging to the hospital associated ST78 lineage. The *E. faecium* strain from case B belonged to ST203 which is part of the ST17 hospital associated lineage. Population genetic modeling based on the seven MLST genes using the Bayesian Analysis of Population Structure (BAPS) software have shown that 80% of the *E. faecium* nosocomial strains cluster in two different groups (2–1 and 3–3) [41]. *E. faecium* A and B strains belonged to lineages within these different main BAPS groups (lineage ST78 to 2–1 and lineage ST17 to 3–3) [41], confirming a large phylogenetic distance. This was further shown by cgMLST analysis which revealed that A1-3 strains belonged to the same novel cluster type (CT) 3198 (Fig 1). The B1 strain belonged to another novel CT3199 and showed at least 354 allelic differences to A1-3 strains. The two ST1486 strains had only one allelic difference, while the maximum allelic differences (eight) within CT3198 were between A1 and A3. One of these allelic differences was in the *ddl* allele which is one of the seven MLST scheme genes. Our results show that even strains with different MLST profiles could be clonally closely related and have the same CT.

For *E. casseliflavus* strains, a core genome SNP tree was constructed together with publically available closed genomes. Interestingly, the two VanD strains (B2 and B3) clustered in two separate branches, showing that they were not clonally related (S3 Fig).

The vancomycin susceptible *E. faecium* strain E1 (GCF_001886635.1) isolated from Spain in 2010, was identified as the closest genome to A1-3 strains using a core genome SNP tree of all closed *E. faecium* genomes in NCBI and the Norwegian VanD-type VREfm genomes (S4 Fig). Strain E1 was therefore used as a reference genome for sorting contigs and further comparative genomic analyses. Genomic comparison using Easyfig confirmed that the A1-3 genomes were very similar. The ANI between A1 and A2 was the highest (99.99%).
Comparison of case B VREfm (B1) to case A VREfm genomes, confirmed observed genomic differences (S5 Fig).

The significant phylogenetic difference between the vanD E. faecium strains from case A and B is consistent with the observed sporadic occurrence of vanD-type VRE strains in contrast to the epidemic vanA/B-type VRE [7, 12, 13, 15]. Our patient characteristics with underlying diseases and long-term antibiotic exposure including vancomycin are also consistent with previous observations in vanD VRE cases [12, 17].

A novel vanD-subtype was found in strains from case B
Sequence comparison and phylogenetic analysis of complete vanD gene clusters from this study and reference sequences representing the five known vanD subtypes (vanD1-D5) [8, 11, 42, 43], showed that the Norwegian vanD gene clusters belonged to two different vanD-subtypes. In case A, the vanD gene clusters of strains A1 and A2 were 100% identical and showed 99.96% ANI to the cluster in A3. The vanD genes of case A clustered with the vanD5 reference sequence (E. faecium strain N03-0072) (Fig 2). ANIs between the vanD5 reference sequence and A1-3 strains were >99.9%. In case B strains, B2 and B3 vanD gene clusters were 99.98% identical and the B1 vanD gene cluster showed > 99.96% ANI with them. The ANI between case A and B vanD gene clusters was around 91%. B1-3 vanD gene clusters are significantly different from the known vanD-subtypes (maximum 93.7% identity to the known subtypes) (S1 Table). Thus, we propose that the B vanD gene cluster is a new subtype termed vanD6. Identification of the novel vanD6 gene cluster in two different species of enterococci suggests interspecies genetic exchange.

Three novel vanD-containing GIs identified
Comparison alignments with non-VRE reference genomes using Artemis comparison tool showed that all vanD gene clusters in the Norwegian vanD-type VRE were part of GIs ranging...
between 112–126 kb (Table 3). The GC content of the GIs was higher (44.1–44.3%) than the average GC content range of 38% of *E. faecium* strains [44–46]. For B2 and B3 *E. casseliflavus* strains, the genomic GC content was 42.4% and 42.3%, in contrast to 44.6 and 44.7% for their GIs, respectively. The GI Tn\(^{6711}\) of A1-3 strains showed identical size and had an ANI above 99.99% suggesting a common origin. The GI Tn\(^{6713}\) of the *E. casseliflavus* strains (B2 and B3) was identical in size and showed only 0.001% difference (S2 Table). The GI Tn\(^{6712}\) in *E. faecium* strain B1 was 7230 bp larger than that of *E. casseliflavus* GI (Tn\(^{6713}\)), while it was 6134 bp shorter and showed more rearrangements compared to Tn\(^{6711}\) of strains A1-3 (Table 3 and Fig 3). ANIs were lowest (below 98%) between case A and B *E. faecium* GIs (S2 Table). Thus, the overall genetic differences between the GIs of A1-3 and B1-3, do not support a direct

![Fig 2. Phylogenetic SNP tree of the vanD gene clusters of the Norwegian and vanD1—vanD5 subtype reference clusters retrieved from NCBI. Flags represent the countries that vanD-types were discovered in first. Case A strains clustered with vanD5 reference N03-0072 while case B strains clustered separately.](https://doi.org/10.1371/journal.pone.0255187.g002)

**Table 3. Characteristics of the GIs of the Norwegian VanD-type VRE.**

| Strain (case) | Genomic island | Repeats in the insertion site (5'-3' strand) |
|---------------|----------------|---------------------------------------------|
|               | Name           | GC content (%) | Size in bp | Number of CDSs | lysS side | 16S rRNA side* |
| A1 (A)        | Tn\(^{6711}\)  | 44.1           | 125858     | 157            | TTCCCACAAATGA | TTCCCACAAATGA |
| A2 (A)        | Tn\(^{6711}\)  | 44.1           | 125858     | 157            | TTCCCACAAATGA | TTCCCACAAATGA |
| A3 (A)        | Tn\(^{6711}\)  | 44.1           | 125858     | 157            | TTCCCACAAATGA | TTCCCACAAATGA |
| B1 (B)        | Tn\(^{6712}\)  | 44.3           | 119724     | 149            | TTCCCACAAATGA | TTCCCACAAATGA |
| B2 (B)        | Tn\(^{6713}\)  | 44.6           | 112494     | 143            | TTCCCACAAATGA | TTCCCACAAATGA |
| B3 (B)        | Tn\(^{6713}\)  | 44.7           | 112494     | 143            | TTCCCACAAATGA | TTCCCACAAATGA |

*, difference compared to repeat on the lysS side is indicated by underlined nucleotide

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spread between the two cases. However, in case B strains, we suggest one genetic event has evolved Tn6713 of *E. casseliflavus* to the longer Tn6712 in *E. faecium* or vice versa (Fig 3).

All GIs lacked conjugative apparatus genes and the *vanD* gene cluster was the only AMR gene within the islands (GenBank Acc. No. MT951615-7). The nucleotide sequence of integrase genes in Tn6712 and Tn6713 was identical and had only one SNP compared to Tn6711. Despite the existence of the same GIs in *E. casseliflavus* strains (B1 and B3) while the *E. faecium* island Tn6712 of B1 is about 7.2 kb larger. The Dutch E8429 and E9354 showed the highest identity with case B GIs. *vanD* gene cluster and the integrase gene are marked in green and turquoise, respectively.

Comparisons of the Norwegian *vanD*-GIs to those of the newly isolated VanD-type VREfm from the Netherlands and Japan with publically available WGS data revealed a high rate of identity. Two VanD-type Dutch VRE strains (E8429 and E9354) [7] contained *vanD*-GIs with 99.99% sequence identity to Tn6712 of B1. Moreover, the *vanD5*-containing GI from the Japanese *E. faecium* SMVRE20 [17] (AP019408.1) showed 99.98% sequence identity to Tn6711 of case A. Another Japanese *vanD*-GI (157 kb) from *E. faecium* strain AA620 (LC467712.1) showed 96% identity covering 81% of Tn6711. Although the *vanD*-GIs are similar between the Norwegian, Dutch, and Japanese VREfm strains, phylogenetic analyses based on SNPs suggest that the strains are not closely related (S6 Fig). The GI of the Japanese SMVRE20 has an additional gene compared to Tn6711. Likewise, Tn6712 and the Dutch GIs show only one gene in

**Fig 3. Comparison of the Norwegian, Dutch, and Japanese *vanD*-GIs built using Easyfig.** A1-3 GIs have similar gene organization and showed high similarity with the Japanese SMVRE20 GI differing only in one hypothetical protein coding gene which contains transposase DDE domain. In case B, a high similarity exists between *E. casseliflavus* islands (B2 and B3) while the *E. faecium* island Tn6712 of B1 is about 7.2 kb larger. The Dutch E8429 and E9354 showed the highest identity with case B GIs. *vanD* gene cluster and the integrase gene are marked in green and turquoise, respectively.

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difference. Both these genes encode hypothetical proteins (Fig 3). The high identity between Tn6711 and the GIs of the Japanese VanD-type VREfm and between Tn6712 and two Dutch VanD-type VREfm GIs indicate a global spread of similar MGEs.

Due to the intrinsic vanC gene cluster of E. casseliflavus clinical strains, they already express low level resistance to vancomycin. Thus, E. casseliflavus strains often are not investigated further to see if they contain additional van clusters. In this study, we show that E. casseliflavus may be the intermediate source of the vanD type cluster containing GI (Tn6713) that spread to E. faecium (Tn6712) in case B. Based on this finding, MIC investigation of clinically important strains of E. casseliflavus should be considered to reveal possible acquired van gene clusters.

The GIs show site specific integration in E. faecium and E. casseliflavus
The insertion sites of the vanD GIs were identical for all six strains and located in the 3′ end of the lysS gene which is positioned upstream of a 16S ribosomal rRNA gene. The integration resulted in a 13 bp direct repeat located 17 bp from the 3′ end of the lysS gene. The left and right repeats in the different vanD-containing strains showed maximum one SNP difference. For case A GIs the imperfect direct repeats were identical. In strain B1 of case B, the repeat is identical to case A GIs but localised on opposite sides. The perfect direct repeat in B3 differed by one nucleotide compared to the other strains (Table 3). The same integration site was also found in the recently isolated Dutch and Japanese VanD-type VREfm [7, 17]. Thus, this insertion site may be a hotspot in some enterococcal species including E. faecium and E. casseliflavus.

Putative origin of vanD-containing GIs
BLAST searches revealed 89% identity with several regions of Blautia producta SCSK genome covering only 59% of the Tn6711 length. Another hit of Tn6711 BLAST showed 89% identity to Blautia coccoides YL58 with 59% coverage, spanning some small fragments that were not covered by B. producta SCSK. An even higher identity (93%) was seen between the shorter Tn6712 and Tn6713 with fragments from B. coccoides YL58 covering 59% of these GIs. Previous reports have shown that vanD-type vancomycin resistance gene clusters can be found in non-enterococcal species like Ruminococcus gauvreauii, Lachnospiraceae bacterium, and Ruthenibacterium lactatiformans [7]. The above mentioned species and Blautia genus belong to the same taxonomic order of Clostridiales and are found in both the human and animal gut microbiome [47–49]. Thus, anaerobic Blautia genus or other members of the Clostridiales order are possible sources for vanD GIs.

Activity and transferability of putative GIs
Mobile chromosomal genetic elements, excise and circularize before transfer [50]. Circularization PCR and amplicon sequencing confirmed that Tn6711 and Tn6713 were able to circularize supporting that they are active MGEs. Agarose gel electrophoresis of PCR products repeatedly showed stronger bands for Tn6713 in E. casseliflavus which could be due to higher activity compared to Tn6711 in E. faecium (S7 Fig). However, we were not able to transfer vanD to an E. faecium recipient in this study (detection limit 10⁻¹⁰ to 10⁻⁹ transconjugants/donor cell) which is not surprising since a conjugation apparatus was not found in any of the GIs carrying the vanD gene clusters nor in other sites of the VanD-type VRE genomes. Type IV secretion systems play an important role in conjugation and can mediate the transfer of the conjugative plasmids and transposons. They have an impact on the spread of antimicrobial resistance among bacteria [29]. Non-conjugative MGEs can use the conjugative apparatus of other MGEs to mobilize. Thus, a mobility test can be conducted to confirm mobilization of the
However, the strains in this study already had several acquired resistance determinants that are used as markers in mobilization tests. Thus, we did not attempt to mobilize the islands.

Conclusions

We have performed a genetic characterization of the first VanD-type VRE strains recovered from two patients treated with broadspectrum antibiotics including vancomycin before VRE detection. All VanD-type VRE strains of case A were *E. faecium* while both *vanD E. casseliflavus* and *E. faecium* were recovered from case B. To our knowledge, this is the first two *vanD E. casseliflavus* strains reported. Based on our finding, we recommend MIC investigation of clinically important *E. casseliflavus* strains to reveal possible additional *van* gene clusters. In the VREfm strains of case A, we identified a unique novel ST1486, an SLV of ST117, which were phylogenetically distant from case B VREfm (ST203). Sequence analyses revealed a novel *vanD*-type cluster termed *vanD6* subtype in case B strains. The large phylogenetic distance between the VREfm strain of the two cases, as well as differences in *vanD*-cluster subtypes and *vanD*-GIs, rejected the hypothesis of a clonal outbreak. We identified three novel similar *vanD*-GIs of putative *Clostridiales* order origin integrated at the same chromosomal site in both *E. faecium* and *E. casseliflavus*.

Supporting information

S1 Fig. Antibiotic treatment and microbiological findings for case A. Tx: Transplantation, BAL: Bronchoalveolar lavage.

S2 Fig. Amino acid sequences alignment of the products deduced from the *ddl* genes of the *vanD*-containing *E. faecium* strains using Clustal omega online tool compared to the reference sequence (E1). Cov and pid represent the coverage and percent identity. The *ddl* gene of B1 showed a stop codon which resulted in a 110 amino acid protein. A1 and A2 showed a point mutation in a position involved in binding of D-Ala1 (S185 changed to F185) of the D-Ala:D-Ala ligase while A3 showed a point mutation in a position involved in binding of ATP (S319 changed to G319) [Depardieu F, Foucault M, Bell J, Dubouix A, Guibert M, Lavigne J, et al. New combinations of mutations in VanD-type vancomycin-resistant *Enterococcus faecium*, *Enterococcus faecalis*, and *Enterococcus avium* strains. Antimicrob Agents Chemother. 2009;53(5):1952–63]. The point mutations are highlighted by red boxes.

S3 Fig. Core genome SNP tree for the Norwegian *E. casseliflavus* strains and the available closed genomes of the species in the NCBI database on 04.04.2020.

S4 Fig. Extended core genome SNP tree for all *E. faecium* closed genomes retrieved from the NCBI database on 04.04.2020 and VREfm of this study. The Norwegian samples are colored red and the closest genomes to them are in green.

S5 Fig. Genomic comparison between all Norwegian VanD-type VREfm strains and *E. faecium* E1 reference genome using Easyfig tool. The red and blue gradient bars represent percent sequence matches. Red shows the direct and blue the inverted sequence matches. Arrows show the coding sequences and their direction. *vanD* gene cluster is marked in green. The similarities between case A strains (A1, A2 and A3) and their differences with case B VREfm (B1)
is reflected in their matching patterns.

S6 Fig. Parsnp tree for the Norwegian, Dutch and Japanese VanD-type VREfm genomes. Case A strains and the Japanese SMVRE20 which have the most identical GIs clustered separately. Likewise the Dutch E8429 and E9354 and B1 strain of case B also clustered separately.

S7 Fig. Agarose gel electrophoresis of PCR products using pairs of primers directed outwards from the GI ends to confirm the presence of the active form of the GIs (circular form). All but B1 contain the active form.

S1 Table. Average nucleotide identity between vanD gene cluster references (vanD1–vanD5) and the novel vanD6 gene clusters from case B strains.

S2 Table. Average nucleotide identity between GIs of the Norwegian VanD-type VRE samples.

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References

1. Patel R. Clinical impact of vancomycin-resistant enterococci. J Antimicrob Chemother. 2003; 51:13–22. https://doi.org/10.1093/jac/dkg272 PMID: 12801938

2. García-Solache M, Rice LB. The Enterococcus: a model of adaptability to its environment. Clin Microbiol Rev. 2019; 32:1–28.

3. Courvalin P. Vancomycin resistance in Gram-positive cocci. Clin Infect Dis. 2006; 42:25–34. https://doi.org/10.1086/491711 PMID: 16323116

4. Cattoir V, Leclercq R. Twenty-five years of shared life with vancomycin-resistant enterococci: is it time to divorce? J Antimicrob Chemother. 2013; 68:731–42. https://doi.org/10.1093/jac/dks469 PMID: 23208830

5. Pinholt M, Gumpert H, Bayliss S, Nielsen JB, Vorobieva V, Pedersen M, et al. Genomic analysis of 495 vancomycin-resistant Enterococcus faecium reveals broad dissemination of a vanA plasmid in more than 19 clones from Copenhagen, Denmark. J Antimicrob Chemother. 2017; 72:40–7. https://doi.org/10.1093/jac/dkw360 PMID: 27605596

6. Tomita H. Identification of VanD-type resistance plasmid pMG1-like conjugative plasmids in E. faecium clinical isolate in Japan. 5th Int Conf Enterococci. Chamonix, France; 2018.

7. Top J, Sinnige JC, Brouwer EC, Werner G, Corander J, Severin JA, et al. Identification of a novel genomic island associated with VanD-type vancomycin resistance in six Dutch vancomycin-resistant Enterococcus faecium isolates. Antimicrob Agents Chemother. 2018; 62:1–7. https://doi.org/10.1128/AAC.01793-17 PMID: 29311068

8. Boyd DA, Lalancette C, Lévesque S, Gelding GR. Characterization of a genomic island harbouring a new vanD allele from Enterococcus faecium N15-508 isolated in Canada. J Antimicrob Chemother. 2016; 71:2052–4. https://doi.org/10.1093/jac/dkw063 PMID: 27084917

9. Depardieu F, Kolbert M, Pruul H, Bell J, Courvalin P. VanD-type vancomycin-resistant Enterococcus faecium and Enterococcus faecalis. Antimicrob Agents Chemother. 2004; 48:3892–904. https://doi.org/10.1128/AAC.48.10.3892-3904.2004 PMID: 15388450

10. Depardieu F, Foucault M, Bell J, Duboux A, Guibert M, Lavigne J, et al. New combinations of mutations in VanD-type vancomycin-resistant Enterococcus faecium, Enterococcus faecalis, and Enterococcus avium strains. Antimicrob Agents Chemother. 2009; 53:1952–63. https://doi.org/10.1128/AAC.01348-08 PMID: 19258279

11. Boyd DA, Kibsey P, Roscoe D, Mulvey MR. Enterococcus faecium N03-0072 carries a new VanD-type vancomycin resistance determinant: characterization of the vanD5 operon. J Antimicrob Chemother. 2004; 54:680–3. https://doi.org/10.1093/jac/dkh391 PMID: 15308604

12. Flippe J, von Wintersdorf CJH, van Niekerk JM, Jamin C, van Tiel FH, Hasman H, et al. Appearance of vanD-positive Enterococcus faecium in a tertiary hospital in the Netherlands: prevalence of vanC and vanD in hospitalized patients. Sci Rep. 2019; 9:6949. https://doi.org/10.1038/s41598-019-42824-4 PMID: 31061446

13. Terada S, Harada T, Yokota M, Tsuchiya T, Adachi K, Asaka T, et al. First isolation and characterization of vancomycin-resistant Enterococcus faecium harboring vanD5 gene cluster recovered from a 79-year-old female inpatient in Japan. Diagnostic Microbiol Infect Dis. 2019; 95:1–6. https://doi.org/10.1016/j.diagmicrobio.2019.114883 PMID: 31495527

14. Starlander G, Tellgren-Roth C, Melhus Å. Fatal acquisition of vanD gene during vancomycin treatment of septicaemia caused by Enterococcus faecium considerations and difficulties of stethoscope disinfection for a motivated healthcare worker. J Hosp Infect. 2016; 92:409–10. https://doi.org/10.1016/j.jhin.2016.01.002 PMID: 26876745

15. Lam PW, Kozak RA, Eshaghi A, Avaness M, Salt N, Patel SN, et al. Nosocomial outbreak of vanD-carrying vancomycin-resistant Enterococcus faecium. Infect Control Hosp Epidemiol. 2018; 39:1266–8. https://doi.org/10.1017/ice.2018.174 PMID: 30111383
16. Ostrowsky BE, Clark NC, Thauvin-Eliopoulos C, Venkataraman L, Samore MH, Tenover FC, et al. A cluster of VanD vancomycin-resistant Enterococcus faecium: molecular characterization and clinical epidemiology. J Infect Dis. 1999; 180:1177–85. https://doi.org/10.1086/315030 PMID: 10479146

17. Sato T, Wada T, Shinagawa M, Fukushima Y, Nakajima C, Suzuki Y. Emergence of vancomycin- and teicoplanin-resistant Enterococcus faecium via vanDS-harbouring large genomic island. J Antimicrob Chemother. 2020; 75:2411–5. https://doi.org/10.1093/jac/dkaa220 PMID: 32585683

18. Camargo ILBC, Dalla Costa LM, Woodford N, Gilmore MS, Darini ALC. Sequence analysis of Enterococcus faecium strain 10/96A (VanD4), the original vancomycin-resistant E. faecium strain in Brazil. J Clin Microbiol. 2004; 44:2635–7. https://doi.org/10.1128/JCM.00509-06 PMID: 16825401

19. Clinical breakpoints—breakpoints and guidance [Internet]. Eur. Comm. Antimicrob. Susceptibility Test. —EUCAST. 2020. https://euca.st.org/clinical_breakpoints/.

20. Depeudieu F, Perichon B, Courvalin P. Detection of the van alphabet and identification of Enterococci and Staphylococci at the species level by multiplex PCR. J Clin Microbiol. 2004; 42:5857–60. https://doi.org/10.1128/JCM.42.12.5857-5860.2004 PMID: 15583325

21. Fines M, Perichon B, Reynolds P, Sahm DF, Courvalin P. VanE, a new type of acquired glycopeptide resistance in Enterococcus faecalis BM4405. Antimicrob Agents Chemother. 1999; 43:2161–4. https://doi.org/10.1128/AAC.43.9.2161 PMID: 10471558

22. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014; 30:2114–20. https://doi.org/10.1093/bioinformatics/btu170 PMID: 24695404

23. Nurk S, Bankevich A, Antipov D, Gurevich A, Korobeynikov A, Lapidus A, et al. Assembling genomes and mini-metagenomes from highly chimeric reads. In: Deng M, Jiang R, Sun F, Zhang X, editors. Research. 2014; 30:2114–20. https://doi.org/10.1093/bioinformatics/btu170 PMID: 24695404

24. Fines M, Perichon B, Reynolds P, Sahm DF, Courvalin P. VanE, a new type of acquired glycopeptide resistance in Enterococcus faecalis BM4405. Antimicrob Agents Chemother. 1999; 43:2161–4. https://doi.org/10.1128/AAC.43.9.2161 PMID: 10471558

25. Tatusova T, Dicuccio M, Badretdin A, Chetvernin V, Nawrocki P, Zaslavsky L, et al. NCBI prokaryotic genome annotation pipeline. Nucleic Acids Res. 2016; 44:6614–24. https://doi.org/10.1093/nar/gkw569 PMID: 27342282

26. Feldgarden M, Brover V, Haft DH, Prasad AB, Stotta DJ, Tolstoy I, et al. Validating the AMRFinder tool and resistance gene database by using antimicrobial resistance genotype-phenotype correlations in a collection of isolates. Antimicrob Agents Chemother. 2019; 63:e00483–19. https://doi.org/10.1128/AAC.00483-19 PMID: 31427293

27. Seemann T. Abricate [Internet]. Github. https://github.com/tseemann/abricate.

28. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990; 215:403–10. https://doi.org/10.1016/S0022-2836(05)80360-2 PMID: 2231712

29. Han N, Yu W, Qiang Y, Zhang W. T4SP database 2.0: an improved database for type IV secretion systems in bacterial genomes with new online analysis tools. Comput Math Methods Med. 2016; 2016:9415459. https://doi.org/10.1155/2016/9415459 PMID: 27738451

30. Treangen TJ, Ondov BD, Koren S, Phillippy AM. The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. Genome Biol. 2014; 15:524. https://doi.org/10.1186/s13059-014-0524-x PMID: 25410596

31. Jolley KA, Maiden MCJ. BIGSdb: Scalable analysis of bacterial genome variation at the population level. BMC Bioinformatics. 2010; 11:595. https://doi.org/10.1186/1471-2105-11-595 PMID: 21143983

32. de Been M, Pinholt M, Top J, Bletz S, Meilmann A, van Schaik W, et al. Core genome multilocus sequence typing scheme for high-resolution typing of Enterococcus faecium. J Clin Microbiol. 2015; 53:3788–97. https://doi.org/10.1128/JCM.01946-15 PMID: 26400782

33. Darling ACE, Mau B, Blattner FR, Perna NT. Mauve: multiple alignment of conserved genomic sequence with rearrangements, Genome Res. 2004; 14:1394–403. https://doi.org/10.1101/gr.2289704 PMID: 15217575

34. Sullivan MJ, Petty NK, Beaton SA. Easyfig: a genome comparison visualizer. Bioinformatics. 2011; 27:1009–10. https://doi.org/10.1093/bioinformatics/btr039 PMID: 21278367

35. Carver TJ, Rutherford KM, Berriman M, Rajandream M, Barrell BG, Parkhill J. ACT: the Artemis comparison tool. Bioinformatics. 2005; 21:3422–3. https://doi.org/10.1093/bioinformatics/bti553 PMID: 15976072

36. Pritchard L, Glover RH, Humphris S, Elphinstone JG, Toth IK. Genomics and taxonomy in diagnostics for food security: soft-rotting enterobacterial plant pathogens. Anal Methods. 2016; 12–24.

37. Tansirichaiay S, Rahman A, Roberts AP. The Transposon registry. Mob DNA. Mobile DNA; 2019; 10:1–6.
38. Sivertsen A, Janice J, Pedersen T, Wagner TM, Hegstad J, Hegstad K. The Enterococcus cassette chromosome, a genomic variation enabler in enterococci. mSphere. 2018; 3:1–13. https://doi.org/10.1128/mSphere.00402-18 PMID: 30404935

39. Poyart C, Trieu-Cuot P. Heterogeneous conjugal transfer of the pheromone-responsive plasmid pIP964 (IncHlyI) of Enterococcus faecalis in the apparent absence of pheromone induction. FEMS Microbiol Lett. 1994; 122:173–80. https://doi.org/10.1111/j.1574-6968.1994.tb07161.x PMID: 7958769

40. Elstrøm P, Astrup E, Hegstad K, Samuelsen Ø, Enger H, Kacelnik O. The fight to keep resistance at bay, epidemiology of carbapenemase producing organisms (CPOs), vancomycin resistant enterococci (VRE) and methicillin resistant Staphylococcus aureus (MRSA) in Norway, 2006–2017. PLoS One. 2019; 14:e0211741. https://doi.org/10.1371/journal.pone.0211741 PMID: 30716133

41. Willems RJL, Top J, van Schaik W, Bonten M, Siren J, et al. Restricted gene flow among hospital subpopulations of Enterococcus faecium. MBio. 2012; 3:e00151–12. https://doi.org/10.1128/mBio.00151-12 PMID: 22807567

42. Depardieu F, Reynolds PE, Courvalin P. VanD-type vancomycin-resistant Enterococcus faecium 10/96A. Antimicrob Agents Chemother. 2003; 47:7–18. https://doi.org/10.1128/AAC.47.1.7-18.2003 PMID: 12499162

43. Casadewall B, Courvalin P. Characterization of the vanD glycopeptide resistance gene cluster from Enterococcus faecium BM4339. J Bacteriol. 1999; 181:3644–8. https://doi.org/10.1128/JB.181.12.3644-3648.1999 PMID: 10368136

44. Tedim AP, Lanza VF, Manrique M, Pareja E, Ruiz-Gabajas P, Cantón R, et al. Complete genome sequences of isolates of Enterococcus faecium sequence type 117, a globally disseminated multidrug-resistant clone. Genome Announc. 2017; 5:e01553–16. https://doi.org/10.1128/genomeA.01553-16 PMID: 28360174

45. Qin X, Galloway-peña JR, Sillanpaa J, Roh JH, Nallapareddy SR, Chowdhury S, et al. Complete genome sequence of Enterococcus faecium strain TX16 and comparative genomic analysis of Enterococcus faecium genomes. BMC Biolog. 2012; 12:1–20.

46. Gan YQI, Zhang TAO, Gan YQ, Zhao Z, Zhu BIN. Complete genome sequences of two Enterococcus faecium strains and comparative genomic analysis. Exp Ther Med. 2020; 19:2019–28. https://doi.org/10.3892/etm.2020.8447 PMID: 32104261

47. Shkoporov AN, Chaplin AV, Shcherbakova VA, Suzina NE, Kafarskaia LI, Bozhenko VK, et al. Ruthenibacterium lactatiformans gen. nov., sp. nov., an anaerobic, lactate-producing member of the family Ruminococcaceae isolated from human faeces. Int J Syst Evol Microbiol. 2016; 66:3041–9. https://doi.org/10.1099/ijs.0.001143 PMID: 27154556

48. Kameyama K, Itoh K. Intestinal colonization by a Lachnospiraceae bacterium contributes to the development of diabetes in obese Mice. Microbes Env. 2014; 29:427–30. https://doi.org/10.1264/jsme2.ME14054 PMID: 25283478

49. Domingo M, Huletsky A, Boissinet M, Bernard KA, Picard FJ, Bergeron MG. Ruminococcus gauvreauii sp. nov., a glycopeptide-resistant species isolated from a human faecal specimen. Int J Syst Evol Microbiol. 2008; 58:1393–7. https://doi.org/10.1099/ijs.0.001150-0 PMID: 18523184

50. Wozniak RAF, Waldor MK. Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. Nat Rev Microbiol. 2010; 8:552–563. https://doi.org/10.1038/nrmicro2382 PMID: 20601965

51. Ramsay JP, Firth N. Diverse mobilization strategies facilitate transfer of non-conjugative mobile genetic elements. Curr Opin Microbiol. 2017; 38:1–9. https://doi.org/10.1016/j.mib.2017.03.003 PMID: 28391142