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Published in:
Journal of Hospital Infection

DOI:
[10.1016/j.jhin.2020.12.015](https://doi.org/10.1016/j.jhin.2020.12.015)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2021

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Nijhuis, R. H. T., Chlebowicz-Fliss, M. A., Smilde, A. E., Rossen, J. W. A., Weersink, A. J. L., & Gigengack-Baars, A. C. M. (2021). Whole-genome sequencing analysis reveals the spread of a vanB-carrying transposon among different vancomycin-resistant *Enterococcus faecium* clinical isolates in a non-endemic setting. *Journal of Hospital Infection*, 110, 52-59. <https://doi.org/10.1016/j.jhin.2020.12.015>

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Whole-genome sequencing analysis reveals the spread of a *vanB*-carrying transposon among different vancomycin-resistant *Enterococcus faecium* clinical isolates in a non-endemic setting

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ARTICLE INFO

Article history:

Received 23 July 2020

Accepted 23 December 2020

Available online 5 January 2021

Keywords:

Antimicrobial resistance

WGS

cgMLST

VRE

vanB

Enterococcus faecium

SUMMARY

Background: Vancomycin-resistant enterococci (VRE), particularly *Enterococcus faecium* (VREfm), can cause serious nosocomial infections, and have been responsible for healthcare-associated outbreaks. Spreading of VREfm can occur both clonally and by the dissemination of mobile genetic elements.

Aim: To report prospective analysis of whole-genome sequencing (WGS) data, including both core-genome multi-locus sequence typing (cgMLST) and transposon analysis, during a *vanB* VREfm outbreak.

Methods: Screening for *vanB*-positive VREfm isolates was performed by real-time polymerase chain reaction (PCR) on an overnight enriched broth and, if positive, subculture was performed. *vanB*-positive VREfm isolates underwent WGS. Generated data were used for molecular typing that was performed by cgMLST using SeqSphere. For transposon characterization, sequence data were mapped against the reference sequence of transposon *Tn1549* using CLC Genomics Workbench, or de-novo assemblies were used for BLASTN comparisons.

Results: In total, 1358 real-time PCRs were performed. Two hundred and fifty-one specimens from 207 patients tested positive on PCR for *vanB*, of which 13 specimens obtained from six patients were identified as *vanB* VREfm positive on culture. These six patients harboured seven unique isolates belonging to four cluster types: CT118 (*N*=2), CT2483 (*N*=3), CT2500 (*N*=1) and CT2501 (*N*=1). Transposon analysis revealed the presence of an identical *vanB*-carrying transposon in the isolates cultured from all six patients that could be linked based on epidemiological data.

Conclusion: A *vanB* VREfm outbreak occurred in the study hospital, including six patients with isolates belonging to four cluster types. In-depth transposon analysis revealed that

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<https://doi.org/10.1016/j.jhin.2020.12.015>

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dissemination of transposon *Tn1549* rather than clonal spread was the cause of the outbreak.

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Introduction

Vancomycin-resistant enterococci (VRE), particularly *Enterococcus faecium* (VREfm), are highly resistant micro-organisms that can cause (severe) nosocomial infections and have been shown to cause outbreaks in different clinical settings. Although resistance to vancomycin by enterococci is related to a diversity of *van* genes (*vanA*, *-B*, *-C*, *-D*, *-E*, *-G*, *-L*, *-M* and *-N*) [1], the high-level resistance of VREfm is generally caused by the presence of *vanA* and/or *vanB* genes [1,2]. In *E. faecium*, *vanA* and *vanB* genes are usually acquired via horizontal gene transfer of specific mobile genetic elements (MGEs), such as transposons from other enterococci or anaerobic bacteria. Dissemination of successful VREfm clones within the healthcare setting can occur by contact of healthcare workers with colonized patients or through the contaminated environment [3]. It is also possible that transposons with vancomycin resistance spread to different lineages of *E. faecium*, linking genetically unrelated isolates in one outbreak [4]. The most predominant lineages of VREfm found in hospitals are ST17, ST18 and ST78 [5], in which vancomycin resistance is associated with the presence of transposons *Tn1546* or *Tn5382/1549* carrying *vanA* or *vanB*, respectively [5–7].

In The Netherlands, the prevalence of VREfm is low [2,8,9], but outbreaks in clinical settings are reported occasionally [4,10]. In the case of a suspected outbreak, it is recommended that VREfm isolates should be compared by molecular typing to reveal transmission and to help control such an outbreak [10,11]. Nowadays, whole-genome sequencing (WGS) is often applied as it has the highest discriminatory power to distinguish clinical isolates [12]. Generated data can be used for core-genome (cg) or whole-genome multi-locus sequence typing (MLST) analysis using a specific *E. faecium* scheme containing 1423 or 2547 genes, respectively [12]. However, cgMLST is only helpful when investigating possible clonal transmission. Recently, Zhou *et al.* reported a retrospective analysis of cgMLST data, complemented by the characterization of *vanB*-carrying transposons, and concluded that horizontal transfer of these transposons occurred rather than clonal spread of *E. faecium* isolates in some outbreaks. The authors advised the use of WGS data for both cgMLST analysis and transposon analysis to create a complete overview of the (suspected) outbreak [4].

This article reports the (prospective) analysis of WGS data, including both cgMLST and transposon analysis, during a *vanB*-harbouring VREfm outbreak in a clinical setting.

Methods

Setting and study population

A VREfm outbreak occurred at Meander Medical Centre (MeanderMC), a tertiary hospital in central Netherlands. The hospital is housed in a new building (2014) with single-person

rooms in all wards except for haemodialysis and day treatment. The haematology department (HEM), vascular surgery unit (VSU) and intensive care unit (ICU), as well as the haemodialysis department, house patients at high risk of carrying VRE. Apart from the VSU, point-prevalence screening is performed in these departments four times each year. In the case of unexpected VRE, screening of contacts is performed to assess possible spread within the hospital. This screening includes both hospitalized and discharged patients that had been nursed on the same ward and in the same period as the VRE-carrying patients. The outbreak described in this study involved two different wards at MeanderMC hospital: HEM and VSU.

VRE diagnostics

Screening for VRE was on rectal swabs. One swab was obtained for inpatient point-prevalence screening, and five swabs, obtained on consecutive days, were obtained for contact screening. For hospitalized patients, swabs were sent to the medical microbiology laboratory at MeanderMC on the day of collection. Outpatient swabs were collected daily and sent to the laboratory together on the fifth day. In the laboratory, swabs were inoculated into brain heart infusion (BHI) enrichment broth with amoxicillin (MP Media, Groningen, The Netherlands). The five swabs from outpatients were pooled. After overnight incubation, real-time polymerase chain reaction (PCR) for detection of the *vanB* gene was performed on a lysate of BHI, including PhHV as an internal control [13]. Detection of *vanB* was performed in a 25- μ L duplex reaction, together with PhHV that acted as a control for inhibition and correct PCR amplification. The reaction mixture consisted of 12.5 μ L Fast Universal Mastermix, 0.5 μ L uracil-N-glycosylase, 5 μ L of lysate, 300 nM of primers and 100 nM of probe, described previously [10,13]. The *vanB* assay was tested with a cycling profile that included initial incubation at 50°C for 5 min, followed by denaturation at 95°C for 20 s, and 50 cycles at 95°C for 3 s and 60°C for 30 s. A cycle threshold (Ct) value ≤ 40 indicated suspected VRE in a sample.

Suspected VRE samples on real-time PCR were subcultured on to Brilliance VRE agar (Oxoid, Basingstoke, UK) and incubated for 48 h. Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (Bruker, Billerica, MA, USA) was used for species identification of suspected VRE colonies. Antibiotic susceptibility of the species was tested by Vitek (bioMérieux, Marcy-l'Étoile, France), and MIC test strips (Liofilchem, Roseto degli Abruzzi, Italy) were used in cases of uncertain vancomycin minimum inhibitory concentrations by Vitek (i.e. <32). Regardless of vancomycin susceptibility, all *E. faecium* isolates were tested by real-time PCR to confirm the presence of *vanB*.

In the case of VRE isolates detected in contact patients, genotyping of the isolates was performed as recommended in the Dutch guidelines [11].

WGS and typing methods

Genomic DNA was extracted using the Ultraclean Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. DNA libraries were prepared using the Nextera XT v2 kit (Illumina, San Diego, CA, USA) and run on a MiSeq sequencer (Illumina) for generating paired-end 250-bp reads. De-novo assemblies were performed by CLC Genomics Workbench v12.0 (QIAGEN, Hilden, Germany) after quality trimming ($Q_s \geq 20$) with optimal word sizes. All procedures were performed as described previously [14].

MLST sequence types (STs) and cgMLST cluster types (CTs) were extracted from the draft genomic sequences using SeqSphere+ Version 5.1.0 (Ridom GmbH, Münster, Germany). For cgMLST analysis, SeqSphere+ used the *E. faecium* scheme published previously [12] considering a cluster alert distance of 20 different alleles. *vanB*-carrying transposons were identified by BLAST comparisons of assemblies with the reference sequence of Tn1549 (GenBank AF192329.1) using BLASTN under default settings. Detailed analysis of each transposon, as well as the integration points, was performed using the Artemis Comparison Tool [15]. For identification of single nucleotide polymorphisms (SNPs), trimmed reads were mapped to the Tn1549 reference sequence using CLC Genomics Workbench v12.0.

Nucleotide sequence accession numbers

The sequence data obtained in this study have been deposited at the European Nucleotide Archive under BioProject No. PRJEB36167.

Results

Study population

vanB VREfm was first identified on 15th March 2019 in a patient (Patient 1) during 3-monthly point-prevalence VRE screening in the HEM. The second and third cases of *vanB* VREfm were found in the following weeks through contact screening in the VSU (Patient 2) and 3-monthly point-prevalence screening in the haemodialysis department (Patient 3). Investigation revealed that all three patients had been nursed in the VSU before transfer to either the HEM or the haemodialysis department. Due to possible dissemination of *vanB* VREfm, screening of contacts was initiated, and sampling was performed in two rounds. The first round included 174 patients that had been in contact with Patient 1, and involved four different departments of the hospital. For the second round, 120 contacts, all hospitalized in a single department, were screened. For Patient 3, no contact screening was performed as this patient was nursed in isolation for >1 month. In addition, weekly VRE point-prevalence screening was started in the HEM and VSU; this ended 2 weeks after the last VRE-positive patient nursed on the respective wards was discharged from the hospital.

Three new patients colonized with *vanB* VREfm were found. One additional case was detected in both the first and second contact screening rounds; these cases resulted in further rounds of screening of 192 and 92 contacts (all negative). The third new case was identified through weekly VRE point-prevalence screening in the HEM. Thus, the final tally was six patients colonized with *vanB* VREfm.

Figure 1. Minimum spanning tree based on core-genome multi-locus sequence typing (cgMLST) using the Ridom SeqSphere+ *Enterococcus faecium* cgMLST scheme based on 1423 columns. The numbers next to the lines correspond to allele differences between the isolates. Sequence types, blue circles; clone types, black squares. Isolates labelled with a patient number (e.g. Patient 2) belong to the outbreak, whereas isolates labelled with a year (e.g. 2018-1) are historical isolates.

VRE diagnostics

Between 16th March and 20th May 2019 (i.e. from initial finding until end of the outbreak), a total of 1358 real-time PCRs were performed on specimens obtained from 727 patients. Two hundred and fifty-one real-time PCRs (from 207 patients) tested positive for *vanB*, with Ct values ranging from 18.32 to 39.98. *vanB*-positive VREfm isolates were identified by culture in 13 of the PCR-positive specimens (5.2%) obtained from six patients (including Patient 1 patient). In two of the patients, follow-up samples were collected that remained positive for VREfm. Mean Ct values for culture-positive and culture-negative specimens were 24.38 (range 18.32–36.19) and 36.26 (range 23.32–39.98), respectively. Interestingly, the initial sample from Patient 4 contained two *vanB* VREfm isolates that differed both phenotypically and by susceptibility pattern (Table S1, see [online supplementary material](#)).

WGS typing and transposon analysis

Once it was clear that the first three patients carrying *vanB* VREfm had been nursed in the same department, the decision was made to send the isolates for WGS and typing analysis. All subsequently identified VRE isolates were sent prospectively, as soon as *vanB* VREfm was confirmed on PCR. In total, seven isolates obtained from six patients suspected to belong to the outbreak were sent for WGS and typing (Table S1, see [online supplementary material](#)). WGS analysis confirmed the presence of the *vanB* gene in all isolates. Three different STs were identified by MLST: ST17 (*N*=3), ST18 (*N*=1) and ST117 (*N*=3). cgMLST typing identified four different clusters: CT118 (*N*=2), CT2483 (*N*=3), CT2500 (*N*=1) and CT2501 (*N*=1). Figure 1 shows the minimum spanning tree of the MLST and cgMLST typing results of the seven isolates in this outbreak. Based on the cgMLST data alone, it was concluded that there was no spread of a single clone of *vanB* VREfm between the patients, but rather two clusters of two and three patients (ST117-CT118 and ST17-CT2483, respectively), and two single cases (ST117-CT2500 and ST18-CT2501).

Detailed analysis of the sequences of the *vanB* gene showed 100% similarity between all seven isolates (Figure 2). An in-depth investigation of the transposons revealed that all seven isolates contained transposon *Tn1549*. Six isolates had identical sequences (transposon 1, *Tn1549*-1), and one of the two isolates obtained from the same patient (Patient 4-isolate 1) carried a transposon (*Tn1549*-2) that had a minor difference compared with the other six isolates (Table S2, see [online supplementary material](#)).

All *vanB* VREfm isolates identified previously in the authors' laboratory were analysed retrospectively in the same way as the outbreak strains to determine whether the cause of this outbreak was the spread of *Tn1549*-1, and to investigate its presence in historical VREfm isolates. In total, four isolates identified between 2016 and 2018 underwent WGS and were confirmed as *vanB* positive. MLST and cgMLST analysis identified three isolates with ST80-CT16 (all identified within the study

hospital) and a single case with ST80-CT2325 (identified in a neighbouring hospital) that were distinct from the isolates in the current outbreak. DNA sequences of *vanB* of the retrospective isolates were different from the *vanB* sequences found in the outbreak isolates (Figure 2). The historical isolates also contained *Tn1549*, but this differed from *Tn1549*-1 and *Tn1549*-2. Interestingly, all three of the ST80-CT16 isolates contained an identical *vanB* gene and *Tn1549* (Table S2, see [online supplementary material](#)). In addition, in-silico analysis of the *vanB* protein of *Tn1549*-1 using BLASTP identified a total of six sequences with 100% agreement. Two sequences belonged to *E. faecium* isolates: one from Poland (AMJ43235.1) and one from Sweden (KWW11699.1). The remaining four sequences belonged to *Clostridioides* sp. (AAV58816.1, AAV74564.1), *Ruminococcus* sp. (AAV74565.1) and unknown species (WP_060763488.1). Furthermore, 477 amino acid sequences were downloaded from GenBank and included in an alignment to compare the *vanB* proteins. The alignment showed a wide variety of sequences, of which only 22 sequences, identified by institutes from Sweden, Poland or the USA, clustered with the *vanB* protein identified in the outbreak isolates (Figure 2). Finally, seven proteins of *vanB* operons of the outbreak isolates were compared with corresponding proteins from previously identified *vanB* VREfm isolates and the reference transposon *Tn1549* (Table S3, see [online supplementary material](#)). No amino acid substitutions were identified amongst the proteins of the outbreak isolates; however, when compared with the previously identified isolates, multiple substitutions were identified in proteins.

Combining epidemiological data and WGS analysis

Based on the epicurve of the initial three *vanB* VREfm findings (Figure 3), VSU was thought to be the department where this *vanB* VREfm circulated. MLST and cgMLST showed different clones, suggesting that *vanB* VREfm of Patient 1 was not transferred to or from Patients 2 and 3. Transposon analysis showed identical *Tn1549*-1 in all three isolates, raising awareness of the possible spread of this mobile element. Regarding the fourth finding (Patient 5), the only connection to this outbreak was initially based on hospitalization in the HEM. This was confirmed by WGS as Patients 1 and 5 appeared to carry the same isolate (both ST17-CT2483). Hence, it was supposed that there were two separate clusters of *vanB* VREfm: Patients 1 and 5 (HEM) and Patients 2 and 3 (VSU).

Patient 4 had only been nursed in the VSU and was found to carry two phenotypically diverse isolates. Initially, only one isolate was sent for WGS, together with *vanB* VREfm identified from Patient 6. Typing analysis revealed two distinct isolates that could not be clustered with the isolates found previously. However, transposon analysis and comparison with all isolates revealed that *Tn1549*-1 was found in all patients, except for Patient 4 (*Tn1549*-2). Accordingly, potential spread of *Tn1549*-1 was suspected, rather than clonal spread between two patients (Patients 1 and 5, and Patients 2 and 3, respectively) and two additional coincidental findings (Patients 4 and 6).

Figure 2. Phylogenetic tree showing relatedness of the *vanB* protein of the isolates found in this study compared with 477 *vanB* protein sequences of *Enterococcus faecium* obtained from GenBank. For each cluster, a maximum of six sequences from GenBank are included. For each box, the number of additional sequences is mentioned. Blue rectangles indicate clustering of sequences from isolates of the outbreak, and orange rectangles indicate clustering of sequences from historical isolates from the study hospital.

numbers are presented, with each column representing a single day. The first day of each round of contact screening is highlighted. Blue bars, green bars and grey bars indicate hospitalization in the vascular surgery unit, haematology department and any other department, respectively. Department and room numbers are noted at the beginning of each bar. C, collection date of first specimen containing *vanB*-positive VREfm for each patient (Patients 4 and 6 were outpatients and collected swabs on 5 consecutive days); P, positive on polymerase chain reaction (only the first finding is included in this schema); S, date sent for typing; W, date when whole-genome sequencing results were available.

Discussion

Typing of VRE isolates is recommended in cases of suspected spread. WGS, in combination with cgMLST, is the preferred method because of its higher discriminatory power in comparison with, for instance, pulsed-field gel electrophoresis and MLST [4, 17]. However, although WGS and core genome analysis showed excellent identification of clonal spread, possible transmission of MGEs amongst different clones is not examined using this method. Previous studies have demonstrated the importance of investigating the presence of MGEs, as combining this with the investigation of clonal spread can lead to different conclusions in an outbreak setting [4, 18]. For that reason, Zhou *et al.* advised that transposon analysis should be added to the diagnostic algorithm to better understand transmission routes during VREfm outbreaks [4].

The outbreak of *vanB* VREfm reported in this study involved two different departments at MeanderMC. As recommended, typing of the identified *vanB* VREfm isolates was performed and included cgMLST analysis and (prospective) transposon analysis. Amongst the 13 *vanB* VREfm isolates, seven unique isolates

numbers are presented, with each column representing a single day. The first day of each round of contact screening is highlighted. Blue bars, green bars and grey bars indicate hospitalization in the vascular surgery unit, haematology department and any other department, respectively. Department and room numbers are noted at the beginning of each bar. C, collection date of first specimen containing *vanB*-positive VREfm for each patient (Patients 4 and 6 were outpatients and collected swabs on 5 consecutive days); P, positive on polymerase chain reaction (only the first finding is included in this schema); S, date sent for typing; W, date when whole-genome sequencing results were available.

were identified from specimens obtained from six patients. Four clones were identified, whereas transposon analysis found identical *Tn1549-1* in all six patients, implying possible dissemination of this transposon between the clones. To exclude a regional transposon found in all *vanB* VREfm isolates, historical isolates were also tested and showed different clones and transposons. This is in line with the results of Bender *et al.*, which showed a diverse phylogeny of transposons amongst 38 German VREfm isolates comprising 10 different STs identified from 2004 to 2014 [7]. In addition, extensive in-silico analysis showed the uniqueness of the transposon. Moreover, the authors believe that a coincidental finding of one transposon in four clones is not realistic, especially as the six patients were identified within 1 month and a plausible routing can be established based on the epicurve. The last case of *vanB* VREfm at MeanderMC before this outbreak was identified 2 years previously, highlighting the sporadic finding of *vanB* VREfm in general.

Interestingly, two different *vanB* VREfm isolates were found in a single specimen obtained from Patient 4. Transposons *Tn1549-1* and *Tn1549-2* were identified and differed by two nucleotides (one insertion, one deletion; Table S2, see online supplementary material). It is well known that a transposon can transfer from one isolate to another, resulting in acquired resistance. However, to the best of the authors' knowledge, it is not known how the transposon changes during this transfer. Considering three or more SNP variations as a different transposon, as used by Howden *et al.* [18], *Tn1549-2* might be considered the same as *Tn1549-1*. With that, it can potentially be concluded that all seven isolates identified in this study carried the same transposon.

Although the authors believe that dissemination of the 100% identical *Tn1549-1* is the main reason for the spread of this *vanB* VREfm, this could well be in combination with clonal spread. Clonal spread could have occurred with two clones found in three and two patients, respectively: ST17-CT2483 and ST117-CT118. This is particularly suspected for ST17-CT2483, as this clone was found in two patients from the HEM, whereas all other patients had only been nursed in the VSU.

Based on the epidemiological data available, it is impossible to identify the index patient. However, as Patient 2 was hospitalized for a long time in the VSU, overlapping with four of the other patients, it is plausible that Patient 2 was the index patient, or at least played an important role in this outbreak. Results of previous VRE diagnostics were only available for Patient 3. Since September 2016, Patient 3 had been screened regularly as part of the point-prevalence screening in the haemodialysis department, but no VRE had been found before the finding included in this study.

In conclusion, a *vanB* VREfm outbreak was identified at MeanderMC including six patients and two departments. WGS analysis, including cgMLST and in-depth transposon analysis, suggested the dissemination of *Tn1549-1* as the cause of this outbreak. By inclusion of transposon analysis in the diagnostic algorithm, the authors were able to identify the spread of the transposon as the cause of this outbreak, rather than two clusters and two unique findings. For future purposes, analysis of MGEs will be included in the case of (potential) outbreaks for rapid understanding of transmission routes.

Conflict of interest statement
None declared.

Funding sources

This work was supported, in part, by the INTERREG VA (202085)-funded project EurHealth-1Health, part of a Dutch–German cross-border network supported by the European Commission; the Dutch Ministry of Health, Welfare and Sport; the Ministry of Economy, Innovation, Digitalisation and Energy of the German Federal State of North Rhine-Westphalia; and the German Federal State of Lower Saxony.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhin.2020.12.015>.

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