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Development of a PCR assay for rapid and accurate detection of an emerging *vanB Enterococcus faecium* clone in the Capital Region of Denmark

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Objectives: To develop and validate a real-time PCR assay detecting the sequence bridging Tn*1549* and the *Enterococcus faecium* chromosome in the emerging *vanB* vancomycin-resistant *E. faecium* (VREfm) clone (ST80/CT2406).

Methods: The Tn*1549* insertion site was determined on routinely sequenced VREfm isolates. The outer boundaries of Tn*1549* and adjoining host bacterial sequences were determined using a BLAST search in the silent information regulator gene *sir2*. Next, the primers and probe were developed, targeting the sequence bridging Tn*1549* and the *E. faecium* chromosome. Finally, the PCR assay was validated on well-characterized strains and prospectively performed on rectal screening samples submitted to our laboratory.

Results and conclusions: The PCR assay proved to be accurate and provide rapid diagnosis of the emerging *vanB* VREfm in rectal screening samples.

Introduction

Vancomycin-resistant *Enterococcus faecium* (VREfm) is a major cause of nosocomial infections. The *vanA* and *vanB* genotypes are the most prevalent among clinical isolates of *E. faecium*.[1] The *vanB* gene cluster is carried by a 34 kb conjugative Tn*1549* transposon and most often integrated into the chromosome.[1] *vanA*/*vanB* PCR performed on rectal screening samples is accurate in detection of *vanA* VREfm; however, the positive predictive value for *vanB* in faecal screening samples is low, because of the presence of *vanB* in non-enterococcal gut bacteria. Therefore, *vanB* VREfm diagnosis on rectal screening samples is challenging as definite identification of *vanB* VREfm depends on culture (2–3 days) compared with PCR (6 h). *vanA* was initially dominant in the Capital Region of Denmark (2012–19); however, in 2018 a *vanB* VREfm clone [ST117, cgMLST complex type (CT) 36] was introduced to the region.[2,3] Tn*1549* was found to be inserted in an L-arabinose isomerase (*araA2*) gene.[3,4] A specific PCR was developed to detect the sequence bridging Tn*1549* and *araA2* (*araA2*/Tn*1549* PCR) allowing rapid and accurate detection of this *vanB* VREfm clone.[4] In 2020–22, a shift in the dominating *vanB* clone was observed. The emerging *vanB* clone was ST80, cgMLST CT2406, which has a different insertion site for the Tn*1549*.[3]

The aim of the present study was to develop a real-time PCR assay detecting the sequence bridging Tn*1549* and the *E. faecium* chromosome in the emerging *vanB* VREfm clone (ST80/CT2406). In addition, the PCR assay was to be validated on well-characterized strains and prospectively on rectal screening samples. VREfm are routinely whole-genome sequenced and used for validation.

Materials and methods

Determination of Tn1549 insertion site in the emerging vanB VREfm clone

VREfm is routinely sequenced at the Department of Clinical Microbiology (DCM), Hvidovre Hospital and the Tn*1549* insertion site in the emerging *vanB* VREfm cluster (ST80/CT2406) was determined in contigs encoding Tn*1549*. A BLAST search of the Tn*1549* complete sequence (AF_192329.1) was used to identify contigs. Most often, the Tn*1549* sequence was identified in one contig. The outer boundaries of Tn*1549* were identified and adjoining host bacterial sequences were determined. The adjoining host bacterial sequences were used for a BLAST search against the NCBI nr/nt database to determine the gene in which Tn*1549* was inserted. BLAST revealed this to be a silent information regulator gene (*sir2*).

Primer and probe design

The target sequence for the *sir2*/Tn*1549* PCR assay was selected with a forward primer in *sir2* (TGTCAGTTCATAATTATATCTCTCTTGATCA), a reverse primer in Tn*1549* (GGCTATACCGACATTCAAGAACTTC) and a TaqMan probe (LC640- TCCTCAGAATCGACAAAATTTTCCT-BBQ), amplifying a 121 bp fragment (Table S1, available as Supplementary data at *JAC-AMR* Online). The *sir2*/Tn*1549* PCR assay was multiplexed to the existing *araA2*/Tn*1549* TaqMan assay on the Roche FLOW System (Roche Diagnostics, Basel, Switzerland) that identified the ST117/CT36 clone.[4]

To determine the analytical specificity, a BLAST search against the NCBI nr/nt database (highly similar sequences) was performed for the 121 bp amplicon to ensure that this sequence was only found in *E. faecium*.

Validation of the PCR assay

The PCR assay was validated on 75 *E. faecium* isolates. Five isolates were vancomycin-susceptible *E. faecium* from clinical samples, and 70 isolates were VREfm well characterized by WGS. They either had Tn*1549* integrated into *sir2* or an intact *sir2*, as predicted by genome analysis. The validation of the *sir2*/Tn*1549* PCR is summarized in Table 1. We found no false-positive and no false-negative isolates.

In addition, the PCR assay was performed on 1393 rectal screening samples submitted for VREfm screening to the DCM, Hvidovre Hospital, from 10 March 2022 to 24 October 2022, from the Southern part of the Capital Region of Denmark (approximately 1.0 million inhabitants). When receiving VREfm rectal screening samples, PCR assays for *vanA*/*vanB* in addition to *sir2*/Tn*1549* and *araA2*/Tn*1549* were performed. All positive samples (*vanA*, *vanB*, *araA2*/Tn*1549* or *sir2*/Tn*1549*) were cultured and compared with WGS data. Routinely, a minimum of one VREfm isolate per patient per year was whole-genome sequenced. *In silico* PCR for *vanB*, *araA2*/Tn*1549* and *sir2*/Tn*1549* was performed on WGS data for the validation.

Table 1. WGS results and PCR results for all isolates used in the validation of the multiplex PCR

WGS	*sir2*/Tn*1549* PCR *n* detected/ *n* not detected
Vancomycin susceptible	0/5
vanA	0/33
vanB (non-*sir2*)	0/4
vanA + *vanB*	0/1
vanB + *araA2*/Tn*1549*	0/6
vanB + *sir2*/Tn*1549*	26/0

In silico PCRs were performed for the following targets: *vanA*, *vanB*, *araA2*/Tn*1549* and *sir2*/Tn*1549*; *in silico* PCR detected targets are listed in the first column.

Culture and identification of VREfm

First, the sample was inoculated into a selective brain heart infusion (BHI) broth, containing 4 mg/L vancomycin and 60 mg/L aztreonam. On the next day, 10 µL of the enrichment broth was inoculated on a selective chromogenic agar (bioMérieux, Marcy-L'Étoile, France) for 48 h. If *E. faecium* grew on the agar (purple colonies) the species identity was confirmed using MALDI-TOF.

WGS and in silico PCR

WGS was performed locally at the DCM, Hvidovre Hospital. DNA was purified using the Nucleic Acid Extraction Kit For Bacteria Genomic DNA Extraction (#738, PentaBase, Denmark). DNA libraries were prepared on a Biomek 4000 (Bechman Coulter, IN, USA) with the Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA, USA). Shotgun sequencing was performed on a MiSeq instrument (Illumina Inc.) using MiSeq® Reagent Kit v2 (300 cycle) (Illumina, cat. no. 15033412), generating 2 × 150 bp paired-end reads. [5,6] Sequencing reads were *de novo* assembled using SKESA v. 2.2 with default settings except inclusion of the parameter –allow_snps.[7] MLST and cgMLST were performed in SeqSphere v.8.2.0 (Ridom GmbH, Münster, Germany).[8] All sequences were visualized on a minimum spanning tree (MST) with the setting of a maximum of 20 alleles between clusters.

In silico PCR was performed to detect the PCR amplicon in WGS data. A script performed a BLAST search of the genome with the sequences of the primers and probe with a perfect (100%) match. The script identified all matches where the forward and reverse primers were on the same contig with the direction pointing towards each other, and where the probe matched a sequence between the two primers. The script only identified amplicons with a perfect match (100%) to both primers and probe. Finally, the size of the amplicon was determined.

Ethics

This project was approved by the Danish Data Protection Agency (*P*-2022-653) and the Danish Health and Medicines Authority (3-3013-1118/1).

Results

Primer and probe design

A sequence of 151 bp bridging Tn*1549* and the *E. faecium* chromosome in isolates belonging to the dominant *vanB* VREfm cluster was identified for primer and probe design. The PCR design resulted in a 121 bp amplicon (Figure S1). The analytical specificity of the *sir2*/Tn*1549* PCR assay was examined. The 121 bp amplicon was identified in 10 *E. faecium* isolates (100% query cover and 100% identity). The remaining sequences identified by BLAST had a query cover of ≤66%, showing that the *sir2*/Tn*1549* PCR assay did not have any significant hits in gut microbes.

Validation and application of the specific sir2/Tn1549 PCR assay

Twenty-six isolates with Tn*1549* integrated into the *sir2* gene were correctly identified. In addition, 49 isolates carrying an intact *sir2*

Cluster	MLST	cgMLST	March – October 2022
	ST	CT (n)	n
1	ST80	CT2406(18), CT2946(2), CT2949(2), CT3234(7), CT5120(1), CT5165(6), CT5211(25), CT5999(1), CT6610(2), CT6692(3), CT6739(1)	68
2	ST80	CT6117(11)	11
3	ST80	CT1065(4)	4
4	ST80	CT3239(2)	2
5	ST203	CT5121(2)	2
No group assigned	ST80 ST117	CT6507(1), CT6606(1), CT6668(1), CT6998(1)	4
Total	-	-	91

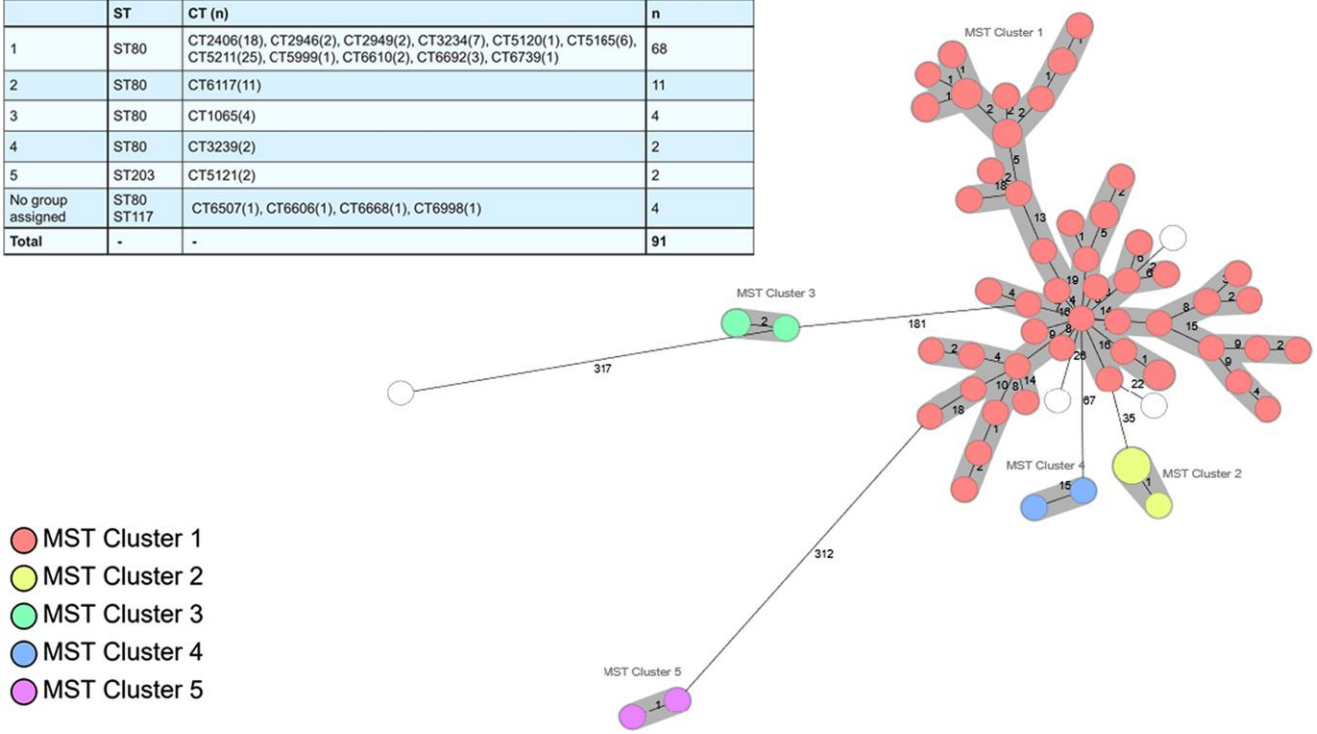

Figure 1. MST of 91 isolates made with SeqSphere v.8.2.0 (Ridom GmbH, Münster, Germany). The table shows the MLST and the cgMLST for each cluster.

gene were negative in the PCR assay. Next, the PCR was performed prospectively on 1393 rectal screening samples. Of these samples, 141 (10.1%) were *sir2*/Tn*1549* positive. All positive samples were cultured, and 129 (91.5%) were culturable. Of the non-culturable samples ($n=12$), five patients had a culturable *sir2*/Tn*1549* VREfm sample within 1 week, and two patients had a culturable *sir2*/Tn*1549* VREfm sample 2 months prior to the present sample. Three samples had a high Cq value (the number of PCR cycles until threshold) of 37–39, and two isolates had Cq values of 30 for both *vanB* and sir2/Tn*1549*. Next, we looked at the WGS results and identified 91 of the 129 cultured *sir2*/Tn*1549* isolates (70.5%). In all these sequences, *in silico* PCR identified the 121 bp amplicon of the *sir2*/Tn*1549* PCR assay. The results from PCR and *in silico* PCR for each WGS isolate are shown in Table S2.

Finally, we searched through our collection of WGS isolates to identify *vanB* VREfm from rectal screening samples during the study period that were negative in the *sir2*/Tn*1549* PCR assay. Sixteen isolates were identified, of which 15 had an intact *sir2* gene (true negatives). One isolate had Tn*1549* integrated in *sir2* (6.3% of negatives). This isolate had a high Cq value of 40 for *vanB*, indicating a low number of VREfm in the sample.

As shown on the MST, most of the isolates belonged to the ST80/CT2406 cluster (74.7%) (Figure 1). Four additional clusters and four singletons (ST80, ST203 and ST117) were identified, indicating that the PCR assay also identified *vanB* clusters other than the dominant one.

Discussion

The *sir2*/Tn*1549* PCR assay was developed, validated and implemented in a multiplex PCR assay combined with the *araA2*/Tn*1549* PCR assay in the routine laboratory at the DCM, Hvidovre Hospital.[4] Compared with WGS of all *vanB* VREfm identified during the study period, all *sir2*/Tn*1549* isolates were identified by the PCR assay with the exception of one isolate. This isolate had the Tn*1549* in the *sir2* gene on WGS results but had a high Cq value of 40 for the *vanB* gene, which can explain the negative outcome of the *sir2*/Tn*1549* PCR. Twelve patients had a *sir2*/Tn*1549*-positive sample that could not be cultured; however, the majority ($n=7$; 58%) of these patients had a culturable VREfm sample prior to this sample. The remaining five samples showed high Cq values. Most likely, this represents higher sensitivity of the PCR assay compared with culture rather than false-positive results of the *sir2*/Tn*1549* PCR assay.

The PCR assay provides rapid and accurate *vanB* VREfm diagnosis with direct patient-related consequences. Isolation precautions can be implemented or withdrawn in less than 12 hours after admission to hospital with our multiplex PCR. If conventional culture-based methods are used instead, the diagnosis takes 2–3 days. In the Capital Region of Denmark, VREfm clonal shifts have been observed multiple times.[2–6] It was also demonstrated by Zhou and colleagues in 2018[9] that Tn*1549* can be located in different insertion sites. Therefore, it is important to perform

and survey VREfm through WGS data to ensure that new emerging *vanB* clones do not escape the very specific PCR assays.

In conclusion, we here report a *vanB sir2*/Tn*1549* PCR assay that has been thoroughly validated and is currently part of our routine VREfm diagnostic pathway to ensure rapid and accurate VREfm diagnostic on rectal screening samples. We continuously survey our VREfm WGS data for new emergent VREfm and are ready to develop new specific *vanB* PCR assays.

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Transparency declarations

None to declare.

Data availability

Data are uploaded in BioProject PRJNA1023182.

Supplementary data

Figure S1 and Tables S1 and S2 are available as Supplementary data at *JAC-AMR* Online.

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