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Development of a strain-specific PCR as a diagnostic tool for surveillance, detection, and monitoring of vancomycin-resistant *Enterococcus faecium* during outbreak

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Abstract

Introduction Vancomycin-resistant *Enterococcus faecium* (VREfm) poses a significant concern in healthcare settings, particularly during outbreaks. Traditional antibiotic susceptibility testing may fail to detect occult vancomycin resistance, and long culture times delay diagnosis. While whole genome sequencing (WGS) is the most accurate method for tracing infectious disease transmissions, its response times are not rapid enough to optimally support controlling of ongoing outbreaks. To address this limitation, we investigated the genomic diversity among outbreak isolates and developed outbreak-specific PCR tests for rapid VREfm carrier screening using strain-specific biomarkers identified through comparative genomics.

Materials and methods Total DNA from VREfm isolates was sequenced using Oxford Nanopore and Illumina platforms. Multi locus sequence types (MLST-ST) and core genome sequence type clusters (cgMLST-CT) were determined with Ridom SeqSphere + software. Comparative analysis of whole genomes was conducted using Lasergene software (DNASTAR).

Results A large VREfm outbreak involving 111 patients caused by *E. faecium* ST117-CT469 was identified in the Northern Netherlands, spanning from August 2021 to September 2024. A subset of 55 *E. faecium* ST117-CT469 isolates were evaluated by WGS and outbreak specific PCRs. Antibiotic susceptibility testing revealed occult vancomycin resistance in the outbreak strain. Comparative genomics identified unique markers specific to *E. faecium* ST117-CT469. Two PCR assays were developed for rapid outbreak detection: a traditional PCR assay distinguishing outbreak from non-outbreak strains based on amplicon size and a TaqMan real-time PCR assay. Both assays demonstrated 100% reproducibility and specificity. The TaqMan assay was able to detect as little as 5 fg of bacterial DNA in the presence of human DNA, equivalent to approximately one bacterial genomic copy. Sequence analysis of WGS data for all 55 outbreak isolates showed perfect nucleotide sequence conservation in the regions where the primers and probe

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hybridized. Sequence comparison against NCBI GenBank entries confirmed the perfect specificity of both PCR assays for detecting the ST117-CT469 outbreak strain.

Conclusions These PCR tests maintain the accuracy and discriminatory power of WGS for identifying the ST117-CT469 outbreak strain but are more cost-effective, faster, and easier to use compared to WGS. They enhance VREfm outbreak management by providing an efficient method for rapid screening. Application of strain-specific PCR based on WGS data is currently the most effective screening method during large, ongoing outbreaks.

Keywords Vancomycin-resistant *Enterococcus faecium* (VREfm), Whole-Genome sequencing (WGS), Outbreak, Biomarker, TaqMan real-time PCR

Introduction

Vancomycin-resistant *Enterococcus faecium* (VREfm) is a significant nosocomial challenge worldwide [1]. VREfm can spread rapidly among patients in healthcare settings, especially in hospitals. This rapid transmission is facilitated by several factors, including the high prevalence of antibiotic use, which selects for resistant strains, and the presence of vulnerable patient populations, such as those with compromised immune systems. Additionally, VREfm can survive on surfaces for prolonged periods, increasing the risk of environmental contamination and subsequent infection. This leads to local outbreaks resulting in increased length of hospitalization, higher mortality rates, and elevated healthcare costs [2].

The treatment of VREfm infections is often limited by the spread of strains resistant to ampicillin and frequently multidrug-resistant [3]. VREfm acquires antibiotic resistance through sporadic chromosomal mutations or exogenous gene exchange and are intrinsically resistant to many antibiotics, including cephalosporins, trimethoprim-sulfamethoxazole, and lincosamides [4]. In particular, vancomycin resistance poses a significant challenge for treating VREfm [1].

To date, at least nine operons related to vancomycin resistance have been identified for enterococci and are described as *vanA*, *vanB*, *vanC*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM*, and *vanN* [5–8]. The most common vancomycin resistance operons found in VREfm are *vanA* and *vanB*, where *vanA* confers high-level resistance to both vancomycin and teicoplanin, whereas *vanB* retains susceptibility to teicoplanin [7, 9]. Both *vanA* and vanB operons are linked to mobile genetic elements: *vanA* with Tn1546 of the Tn3 family and *vanB* with Tn1547 or Tn1549/Tn5382-like conjugative transposons, which enable vancomycin resistance spreading within and between species. The transmission of the *vanB* operon from anaerobic flora to glycopeptide-susceptible enterococcal strains in the human gut is considered a key factor driving the emergence of *vanB* VRE [10]. While the natural reservoir of the *vanA* operon remains uncertain [8], ancient environmental reservoirs have been identified. For instance, permafrost cores approximately 30,000 years old contained *vanA* operons in environmental strains highly similar to those in current VREfm strains, suggesting a deep evolutionary history of this resistance mechanism [11].

The expression of VanA- and VanB-type glycopeptide resistance in enterococci is regulated by two-component systems, $VanR_AS_A$ and $VanR_BS_B$, respectively [12]. These systems consist of a membrane-associated sensor kinase ($VanS_A$ and $VanS_B$) and a cytoplasmic response regulator ($VanR_A$ and $VanR_B$) [12]. In both the *vanA* and *vanB* operons, upon sensing vancomycin, VanS undergoes autophosphorylation on a conserved histidine residue. This phosphorylation is an essential step in signal transduction. VanS then transfers the phosphate group from its histidine residue to an aspartate residue on the response regulator VanR, activating it. The activated VanR binds to the promoter regions of the van operons (*vanA* or *vanB*), initiating the transcription of resistance genes. In the vanA operon, $VanR_A$ controls the transcription of the $vanR_AS_A$ regulatory genes and *vanHAXYZ* resistance genes by activating their respective PR and PH promoters. Similarly, in the vanB operon, $VanR_B$ regulates the $vanR_BS_B$ regulatory genes and $vanY_BWH_BBX_B$ resistance genes by activating the PR_B and PY_B promoters [12].

Recently, vancomycin-variable *Enterococcus* (VVEfm) strains have been reported. These *E. faecium* strains appear phenotypically susceptible to vancomycin but possess a *vanA* or *vanB* gene [13–15]. These isolates employ numerous mechanisms to silence vancomycin resistance, including deletions of the *vanRS* regulatory genes, as well as deletions, disruptions, or mutations of resistance genes [16–20]. During vancomycin therapy, VVEfm can revert to vancomycin-resistant phenotypes through multiple mechanisms [13, 19, 21]. Such strains pose diagnostic challenges for laboratories, evading routine antimicrobial tests like chromogenic screening media or the Vitek2 system, leading to treatment failures, silent transmission, and outbreaks [22, 23]. Therefore, additional methods are essential for accurately identifying all *vanA*- and *vanB*-positive VREfm in clinical practice.

Whole-Genome Sequencing (WGS) is the most accurate method for surveying infectious disease

transmissions. However, the current response times associated with WGS are not sufficiently rapid to optimally support controlling an ongoing outbreak. To address this limitation, we developed an outbreak-specific PCR test to rapidly screen all patients for vancomycin-resistant *E. faecium* (VREfm)-carriership. This test utilized a strain-specific biomarker identified through comparative genomics.

Materials and methods

Identification of *E. faecium* and susceptibility testing

Screening samples were collected using faecal ESwab medium and then transferred into selective brain heart infusion (BHI) broth, supplemented with 16 µg/µl amoxicillin, 20 µg/µl amphotericin B, 20 µg/µl aztreonam, and 20 µg/µl colistin. Amoxicillin was included because VRE outbreaks are typically associated with amoxicillin-resistant enterococci that have acquired vancomycin resistance [24]. Additionally, it restricts the growth of amoxicillin-sensitive anaerobes, such as *Clostridium* species, which are among the primary bacteria that may harbor *vanB* genes [24]. After incubation for 17–24 h at 35 °C, the enriched broth cultures were analyzed for the presence of *vanA* and *vanB* genes using a laboratory-developed TaqMan assay. Only samples with a *vanB* ct-value of ≤30 were further cultured, while all samples with a *vanA* ct-value above 1 were cultured. The broth cultures were then subcultured onto VRE Brilliance agar plates (Oxoid) and incubated at 35 °C for 24 h up to 5 days. Enterococci were identified using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (Bruker). Confirmed *E. faecium* isolates underwent antibiotic susceptibility testing using Vitek2 (bioMérieux) or the disc diffusion method, with minimum inhibitory concentrations (MIC) breakpoints interpreted according to EUCAST recommendations.

Whole genome sequencing

The cells were homogenized with a TissueLyser II (Qiagen, Germantown, MD, USA) and total DNA was purified using the MagAttract HMW DNA Kit (Qiagen, Hilden, Germany). DNA was quantified using a Qubit 2.0 fluorometer (ThermoFisher Scientific, Waltham, Massachusetts, US) and the quality was assessed by the 2200 TapeStation software (Agilent Technologies, Santa Clara, California, US). A NanoDrop 2000c spectrophotometer (ThermoFisher Scientific) was used to measure the purity of extracted DNA. To obtain the complete genome sequences for each isolate, Illumina genomic libraries were prepared using a Nextera XT kit (Illumina, San Diego, California, US) and sequenced on a MiSeq platform (Illumina) with a 2 × 300 bp paired-end protocol. Oxford Nanopore sequencing libraries were prepared using the Ligation Sequencing Kit and sequencing was carried out on a MinION Mk1C device using flow cell type R10.4.1 (Oxford Nanopore, United Kingdom). Nanopore reads were *de novo* assembled with SeqMan NGen assembler version 17.4.0.81 (DNASTAR, Madison, Wisconsin, US). Consensus sequences were refined using the Illumina reads and the SeqMan NGen assembler with automated polishing workflow. Polished assemblies were manually corrected using SeqMan Pro (DNASTAR). Automated genome annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline (https://www.ncbi.nlm.nih.gov/genome/annotation_prok).

Data analyses

The DNA sequences were aligned using BLASTn software (https://blast.ncbi.nlm.nih.gov/Blast.cgi). MLST typing and core genome MLST typing was performed using the SeqSphere software (Ridom GmbH). The presence of acquired antimicrobial-resistance genes was assessed using Res-Finder 4.1 (https://cge.food.dtu.dk/services/ResFinder/) and CARD (The Comprehensive Antibiotic Resistance Database, https://card.mcmaster.ca/). PHASTEST (PHAge Search Tool with Enhanced Sequence Translation) was used to analyze prophages in the genome [25]. SNP analysis was performed using the CSI Phylogeny 1.4 server [26]. The phylogenetic tree produced by CSI Phylogeny 1.4 server was visualized in MEGA6 software [27].

Development of outbreak-specific PCR assays based on WGS data

To identify an outbreak-specific biomarker for the ST117-CT469 strain, we performed a comparative genomic analysis between the first outbreak isolate, NN-EF1, and a genetically closely related isolate of ST117-CT71 that was not associated with the outbreak. Whole-genome sequence data, analyzed using DNASTAR software, allowed us to systematically compare regions of genomic divergence, focusing on structural variations such as single nucleotide polymorphisms (SNPs), insertions, deletions, and the presence of mobile genetic elements like insertion sequences (IS elements), prophages, and plasmids.

Two PCR assays, named Marker3 and Marker4, were developed to specifically detect the outbreak strain ST117-CT469. Comparative genomics revealed a unique integration site of the ISL3 insertion sequence specific to the ST117-CT469 strain. This site was located in the intergenic region between the *clpB* gene encoding ATP-dependent chaperone ClpB (GenBank accession number CP172608, locus_tag ACH2TX_07985) and the gene encoding hypothetical protein (locus_tag ACH2TX_07975).

The first assay, Marker3, was designed using a primer pair (forward: 5'-CGTGGATTGCTGAAAATGC,

reverse: 5'-TGCCTTAATGGGATTGTTTG) flanking the ISL3 element. This PCR test was designed to yield a single amplicon when *E. faecium* DNA was present, with the amplicon size distinguishing the outbreak strain (1936 bp - outbreak-specific) from non-outbreak *E. faecium* strains (434 bp). As a result, this test is also species-specific. The PCR reaction was performed using Platinum SuperFi PCR Master Mix (ThermoFisher Scientific) in a 20 µl volume, containing 500 nM of each primer and 50 ng of template DNA. Amplification was carried out on a C1000 Touch Thermal Cycler (Bio-Rad Laboratories) with the following conditions: 98 °C for 30 s, followed by 40 cycles of 98 °C for 10 s, 66 °C for 10 s, and 72 °C for 60 s. Amplicon sizes were measured using the Agilent 2200 TapeStation system and D5000 ScreenTape assay. The PCR test Marker3 was validated according to the ISO 15,189 standard and approved for routine detection of *E. faecium* ST117-CT469 at the UMCG.

The second outbreak-specific PCR assay, Marker4, used a primer set (forward: 5'-TTGATGGACAACTTCATTTCA, reverse: 5'-TCTCACTCTATCAGTCCTAT) along with a TaqMan probe (6-FAM-CAGAAGAGCTAGAAGAAATCGTC-BHQ®-1), amplifying a 109 bp fragment. The forward primer targeted the gene encoding the ATP-dependent chaperone ClpB, while the reverse primer hybridized to the ISL3 insertion sequence region. Each 20 µl reaction mix contained 500 nM of each primer, 250 nM of the probe, and 50 ng of template DNA. Amplification was conducted using a CFX96 Touch Real-Time PCR detection system (Bio-Rad Laboratories) with thermal cycling conditions of 95 °C for 5 min, followed by 30 cycles of 95 °C for 5 s and 59 °C for 10 s. Negative controls (50 ng of non-outbreak control DNA) and positive controls (50 ng/µl of outbreak control DNA) were included in each run of both PCR assays. To assess the sensitivity of the Marker4 TaqMan assay, we used 50 ng of human DNA (Jurkat Genomic DNA, ThermoFisher) mixed with 10-fold serial dilutions of *E. faecium* genomic DNA from isolate NN-EF1, tested in triplicate, and the number of PCR cycles was extended to 50.

Nucleotide sequence accession numbers

The complete genome sequences of the *E. faecium* ST117-CT469 isolates have been deposited in the GenBank database under Bioproject PRJNA1178465.

Results

Description of the outbreak

An outbreak caused by VREfm ST117-CT469 was identified in the Northern Netherlands, beginning in August 2021 and ending in September 2024. In total, 111 patients were identified based on genotypic confirmation, or presumed cases based on strong epidemiological links to confirmed cases. Among these patients, three developed infections requiring treatment, while all others were cases of colonization. The 38 cases at the University Medical Center Groningen (UMCG) hospital were all genotypically confirmed, whereas a subset of isolates ($n = 17$) from the secondary hospitals were retrospectively confirmed, firstly by unique marker PCR runs on all isolates, and later evaluated by WGS (Table 1).

Antibiotic susceptibility testing - occult vancomycin resistance

The majority of isolates were susceptible to key treatment antibiotics, vancomycin ($n = 45$, MIC ≤ 0.5) and teicoplanin ($n = 54$, MIC ≤ 1) (Table 1). Nine isolates exhibited low-level resistance to vancomycin (MIC = 8), while one isolate showed high-level resistance to both glycopeptide antibiotics (MIC ≥ 32.0) (Table 1). Most isolates were resistant to imipenem ($n = 54$, MIC ≥ 16.0) (Table 1). All isolates were resistant to amoxicillin (MIC ≥ 32.0) and levofloxacin (MIC ≥ 8.0) but remained susceptible to linezolid (MIC ≤ 4), nitrofurantoin (MIC ≤ 64), quinupristin-dalfopristin (MIC ≤ 1) and tigecycline (MIC ≤ 0.12).

SNP-based phylogeny

FastQ files containing Illumina reads were obtained from the genome sequencing of 55 outbreak isolates and five non-outbreak (outgroup) isolates of ST117-CT469, ST117-CT6605, and ST117-CT71. These outgroup isolates were selected as the most genetically related to the outbreak ST117-CT469 isolates. The files were analyzed using the CSI Phylogeny 1.4 server to call SNPs and infer phylogeny. Reads were mapped to the reference chromosome NN-EF1, the first isolate identified during the outbreak, and SNPs were called (Fig. 1). The outbreak isolates displayed low genetic diversity, with NN-EF1 differing from other outbreak isolates by 0 to 10 SNPs, while differences among the outbreak isolates ranged up to 15 SNPs. The exceptional outbreak isolate, NN-EF38, differed from other outbreak isolates by 22–34 SNPs. The most closely related outgroup isolate, Outgroup5 of ST117-CT71, differed from the outbreak isolates (excluding NN-EF38) by 23 to 32 SNPs.

Genetic diversity of chromosomes among the VREfm ST117-CT469 outbreak isolates

To explore genetic variation in the ST117-CT469 outbreak population, 18 isolates were selected for further DNA sequencing using the Oxford Nanopore platform to obtain complete genome sequences. The isolates were chosen based on the SNP-based phylogenetic tree and phenotypic antibiotic resistance, aiming to include both indistinguishable isolates and those with significant differences.

The complete chromosome sequences of the outbreak isolates ranged from 2,928,838 to 3,015,443 bp. A key

Table 1 Summary of *E. faecium* isolates evaluated by WGS and outbreak specific PCRs, including the isolate name, source, date of isolation, and their antibiotic susceptibility or resistance. Antibiotics to which all isolates were either resistant or susceptible have been excluded from the table

Isolate name	Source[a]	Date of isolation	Imipenem	Vancomycin	Teicoplanin	Nitrofurantoin
NN-EF1	UMCG	27-8-2021	>= 16.0	<= 0.5	<= 0.5	64
NN-EF2	UMCG	25-9-2021	>= 16.0	<= 0.5	<= 0.5	64
NN-EF3	UMCG	1-10-2021	>= 16.0	<= 0.5	<= 0.5	32
NN-EF4	UMCG	5-10-2021	>= 16.0	<= 0.5	<= 0.5	64
NN-EF5	UMCG	27-10-2021	>= 16.0	<= 0.5	<= 0.5	32
NN-EF6	UMCG	13-11-2021	>= 16.0	<= 0.5	<= 0.5	64
NN-EF7	UMCG	31-1-2022	>= 16.0	<= 0.5	<= 0.5	64
NN-EF8	UMCG	22-3-2022	>= 16.0	<= 0.5	<= 0.5	64
NN-EF9	UMCG	6-5-2022	>= 16.0	<= 0.5	<= 0.5	64
NN-EF10	UMCG	1-6-2022	>= 16.0	8	<= 0.5	32
NN-EF11	UMCG	13-6-2022	>= 16.0	8	<= 0.5	64
NN-EF12	UMCG	12-8-2022	>= 16.0	8	<= 0.5	32
NN-EF13	UMCG	18-8-2022	>= 16.0	<= 0.5	<= 0.5	32
NN-EF14	UMCG	27-9-2022	>= 16.0	<= 0.5	<= 0.5	32
NN-EF15	UMCG	14-11-2022	>= 16.0	<= 0.5	<= 0.5	<= 16.0
NN-EF16	UMCG	21-11-2022	>= 16.0	<= 0.5	<= 0.5	64
NN-EF17	UMCG	26-11-2022	>= 16.0	<= 0.5	<= 0.5	64
NN-EF18	UMCG	28-11-2022	>= 16.0	<= 0.5	<= 0.5	64
NN-EF19	UMCG	5-12-2022	>= 16.0	<= 0.5	<= 0.5	64
NN-EF20	UMCG	6-12-2022	>= 16.0	<= 0.5	1	64
NN-EF21	UMCG	11-12-2022	>= 16.0	<= 0.5	<= 0.5	64
NN-EF22	UMCG	12-12-2022	>= 16.0	8	<= 0.5	32
NN-EF23	CERTE	9-1-2023	>= 16.0	<= 0.5	<= 0.5	<= 16.0
NN-EF24	UMCG	10-1-2023	>= 16.0	<= 0.5	<= 0.5	64
NN-EF25	UMCG	24-1-2023	<= 1.0	>= 32.0	>= 32.0	<= 16.0
NN-EF26	CERTE	4-2-2023	>= 16.0	<= 0.5	<= 0.5	64
NN-EF27	UMCG	9-2-2023	>= 16.0	<= 0.5	<= 0.5	64
NN-EF28	UMCG	14-2-2023	>= 16.0	<= 0.5	<= 0.5	64
NN-EF29	CERTE	17-2-2023	>= 16.0	<= 0.5	<= 0.5	64
NN-EF30	UMCG	24-2-2023	>= 16.0	<= 0.5	<= 0.5	32
NN-EF31	UMCG	28-2-2023	>= 16.0	<= 0.5	<= 0.5	<= 16.0
NN-EF32	UMCG	14-3-2023	>= 16.0	<= 0.5	<= 0.5	32
NN-EF33	UMCG	21-3-2023	>= 16.0	<= 0.5	<= 0.5	32
NN-EF34	UMCG	24-3-2023	>= 16.0	<= 0.5	<= 0.5	64
NN-EF35	CERTE	31-3-2023	>= 16.0	<= 0.5	<= 0.5	64
NN-EF36	CERTE	9-4-2023	>= 16.0	8	<= 0.5	32
NN-EF37	UMCG	17-4-2023	>= 16.0	8	<= 0.5	32
NN-EF38	CERTE	14-5-2023	>= 16.0	<= 0.5	<= 0.5	64
NN-EF39	CERTE	15-5-2023	>= 16.0	<= 0.5	<= 0.5	32
NN-EF40	UMCG	16-5-2023	>= 16.0	<= 0.5	<= 0.5	64
NN-EF41	CERTE	4-6-2023	>= 16.0	<= 0.5	<= 0.5	64
NN-EF42	CERTE	5-6-2023	>= 16.0	<= 0.5	<= 0.5	32
NN-EF43	CERTE	6-6-2023	>= 16.0	<= 0.5	<= 0.5	64
NN-EF44	UMCG	6-6-2023	>= 16.0	8	<= 0.5	64
NN-EF45	CERTE	8-6-2023	>= 16.0	<= 0.5	<= 0.5	64
NN-EF46	CERTE	7-6-2023	>= 16.0	<= 0.5	<= 0.5	64
NN-EF47	CERTE	12-6-2023	>= 16.0	<= 0.5	<= 0.5	32
NN-EF48	UMCG	13-6-2023	>= 16.0	<= 0.5	<= 0.5	64
NN-EF49	CERTE	14-6-2023	>= 16.0	<= 0.5	<= 0.5	64
NN-EF50	CERTE	29-6-2023	>= 16.0	<= 0.5	<= 0.5	64
NN-EF51	CERTE	23-5-2023	>= 16.0	<= 0.5	<= 0.5	64

Table 1 (continued)

Isolate name	Source[a]	Date of isolation	Imipenem	Vancomycin	Teicoplanin	Nitrofurantoin
NN-EF52	CERTE	26-5-2023	>= 16.0	<= 0.5	<= 0.5	32
NN-EF53	UMCG	24-10-2023	>= 16.0	<= 0.5	<= 0.5	64
NN-EF54	UMCG	22-11-2023	>= 16.0	8	<= 0.5	64
NN-EF55	UMCG	4-1-2024	>= 16.0	8	<= 0.5	32

[a] Isolates from secondary hospitals were submitted to CERTE for analysis, a medical center in the Northern Netherlands that provides specialized medical diagnostics and advice

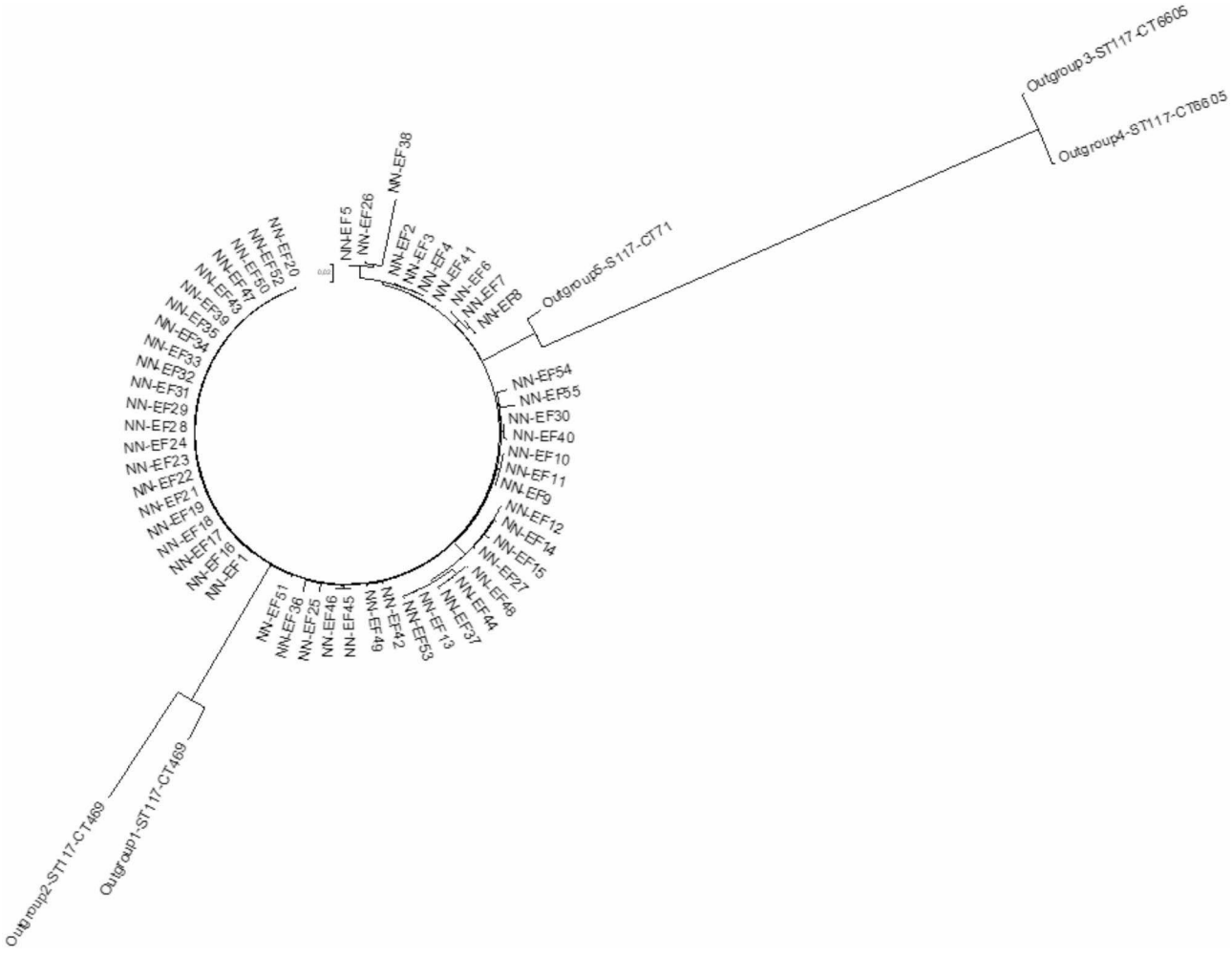

Fig. 1 Phylogenetic relationships among outbreak VREfm ST117-CT469 isolates from Northern Netherlands. A phylogenetic tree was constructed based on SNPs using CSI phylogeny 1.4 (https://cge.food.dtu.dk/services/CSIPhylogeny/). The scale bar represents evolutionary distance between the sequences measured as 0.02 substitutions per nucleotide at the variable positions

feature of the chromosomal variation was the presence of phages. Among the 18 complete outbreak genomes, twelve distinct phages were integrated into the chromosomes as prophages. Most isolates carried five phages; however, isolates NN-EF9 and NN-EF11 had four, while NN-EF26 and NN-EF38 carried six prophages. Two phages, phiEFM-UMCG-4 and phiEFM-UMCG-5, were present in all isolates, while the other phages were distributed heterogeneously. The genomic positions and isolate distribution are detailed in Tables S1-S12.

We also identified a novel composite transposon, designated TnEFM-UMCG-1, which was 19,000 bp in size and flanked by IS110 family transposases (Fig. 2). Among the 18 analyzed genomes, only four isolates (NN-EF43, NN-EF50, NN-EF51, and NN-EF52) lacked TnEFM-UMCG-1. The genes within the composite transposon are involved in various metabolic pathways, including carbohydrate metabolism, arsenic detoxification, and potentially xenobiotic degradation. These findings suggest a functional role in the adaptation of *E. faecium* to its

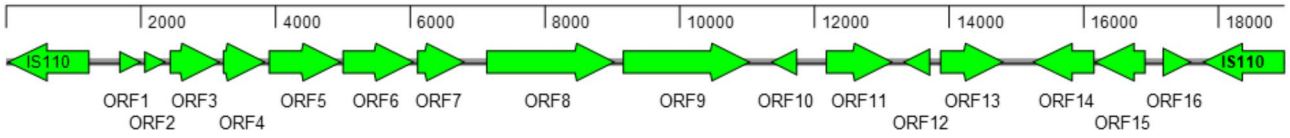

Fig. 2 Structure of composite transposon TnEFM-UMCG-1 (GenBank accession number: CP101129, bases 1092882.1111881). The arrows indicate open reading frames and their orientations: IS110 - IS110 family transposase, ORF1 - glycine-rich SFCGS family protein, ORF2 - DUF4312 family protein, ORF3 - DUF4311 domain-containing protein, ORF4 - DUF4310 family protein, ORF5 - amidohydrolase/deacetylase family metallohydrolase, ORF6 - DgaE family pyridoxal phosphate-dependent ammonia lyase, ORF7 - KDGP aldolase family protein, ORF8 - fructose-specific PTS transporter subunit EIIC, ORF9 - BglG family transcription antiterminator, ORF10 - arsenate reductase (thioredoxin), ORF11 - LLM class flavin-dependent oxidoreductase, ORF12 - MerR family transcriptional regulator, ORF13 - alpha/beta hydrolase, ORF14–1-phosphofructokinase family hexose kinase, ORF15 - DeoR/GlpR family DNA-binding transcription regulator, ORF16 - PTS sugar transporter subunit IIA

Fig. 3 Comparison of isolate genomes with and without the 878,888 bp chromosomal inversion

environment, contributing to nutrient utilization, resistance to toxic compounds, and the metabolism of diverse substrates.

A large chromosomal inversion

A single isolate, NN-EF38, exhibited a large chromosomal inversion of 878,888 bp (Fig. 3A). The phenotypic impact of this inversion is currently unclear. This inversion was located between two phages: phiEFM-UMCG-11 (61.8 kb) and phiEFM-UMCG-12 (82.3 kb). In all other outbreak isolates, phage phiEFM-UMCG-2 (56.4 kb) occupied the position corresponding to phiEFM-UMCG-11, while phages phiEFM-UMCG-3 (75.6 kb) and phiEFM-UMCG-7 (79.5 kb) were found at the position corresponding to phiEFM-UMCG-12 in 11 and 6 isolates, respectively.

Phage phiEFM-UMCG-12 was entirely composed of segments from phages phiEFM-UMCG-2 and phiEFM-UMCG-3, covering lengths of 15.3 kb and 67.0 kb, respectively, and displaying an almost identical nucleotide identity of 99.9% (Fig. 3B). Moreover, the phiEFM-UMCG-2 segment within phiEFM-UMCG-12 was inverted. Notably, phages phiEFM-UMCG-2 and phiEFM-UMCG-3 did not exhibit substantial identity with each other. A larger portion of phage phiEFM-UMCG-11 (77.5%) comprised sequences from phage phiEFM-UMCG-2 and the inverted sequence of phage phiEFM-UMCG-3, covering lengths of 5.5 kb and 42.4 kb, respectively. Additionally, phages phiEFM-UMCG-11 and phiEFM-UMCG-12 shared an inverted 33.3 kb fragment derived from phage phiEFM-UMCG-3 (Fig. XB). Phage phiEFM-UMCG-7 exhibited shorter lengths of identical sequences to phiEFM-UMCG-3,

phiEFM-UMCG-12, and phiEFM-UMCG-11, measuring 41.3 kb, 32.8 kb, and 13.0 kb, respectively, with no substantial similarity to phiEFM-UMCG-2.

These observations suggest that the hybrid phages phiEFM-UMCG-11 and phiEFM-UMCG-12 likely originated through recombination events involving phages phiEFM-UMCG-2 and phiEFM-UMCG-3. This analysis is supported by multiple attL and attR sequences surrounding and within the phages (Tables S2, S3, S11, and S12). Recombination events created 33.3 kb inverted sequences with identical nucleotide sequences within phiEFM-UMCG-11 and phiEFM-UMCG-12. When homologous recombination occurred between these inverted sequences, the chromosomal region between them inverted, ultimately resulting in the observed large 878,888 bp inversion. Importantly, phiEFM-UMCG-7 did not share the 33.3 kb inverted sequences with phiEFM-UMCG-11 and phiEFM-UMCG-12 that were necessary for the recombination events leading to the 878,888 bp chromosomal inversion (Fig. 3B). Therefore, phiEFM-UMCG-7 was not involved in this inversion.

Structure of Tn1549-type transposon

To identify the insertion site and structural composition of a transposon carrying genes conferring resistance to glycopeptide antibiotics in the ST117-CT469 genomes, we used the nucleotide sequence of the prototype Tn1549 transposon, which carries the *vanB*$_2$ operon responsible for vancomycin resistance (GenBank accession no. AF192329), as a reference for BLAST searches (Fig. 4). Our analysis revealed that all ST117-CT469 isolates shared the same Tn1549 insertion site within the chromosome, with the transposon integrated into a gene encoding a silent information regulator 2 (SIR2) family protein. Among the 18 complete ST117-CT469 genomes analyzed, we identified two novel variants of the *vanB*$_2$ operon in the Tn1549 transposon (Fig. 4). These variants were used as templates for transposon assembly in the remaining isolates based on available Illumina reads.

Variant 1, found in 53 out of 55 outbreak isolates, had two differences from that of the prototype *E. faecalis* Tn1549 (GenBank accession no. AF192329). This variant included a 0.6 kb deletion resulting in a 3'-terminally truncated *vanY* (426 bp deletion) and a 5'-terminally truncated *vanW* (192 bp deletion). Additionally, it contained an insertion of an IS*L3* family transposase between *vanS* and truncated *vanY* (Fig. 4).

Variant 2 of the Tn1549-type transposon, found in two isolates (NN-EF10 and NN-EF11), was similar to Variant 1 but carried an additional copy of an IS*110* family transposase inserted between truncated *vanW* and *vanH* genes (Fig. 4). Insertions of the IS*L3* and IS*110* family transposases did not alter the regulatory sequences located upstream of the *vanY* and *vanH* genes, respectively, within the *vanB* operon.

The entire Tn1549 nucleotide sequences among the outbreak isolates were identical, with minor exceptions. Single Nucleotide Polymorphisms (SNPs) were identified in the *vanS* gene, resulting in amino acid changes in the histidine kinase VanS protein: L189F in isolate NN-EF25 and A191V in isolate NN-EF37. Additionally, a non-synonymous mutation, V130M, was found in the *vanH* gene of isolate NN-EF37.

Extrachromosomal genetic elements

A total of seven plasmids, ranging in size from 1.6 kb to 150 kb, were identified among the 18 complete outbreak genomes analyzed. None of the isolates contained

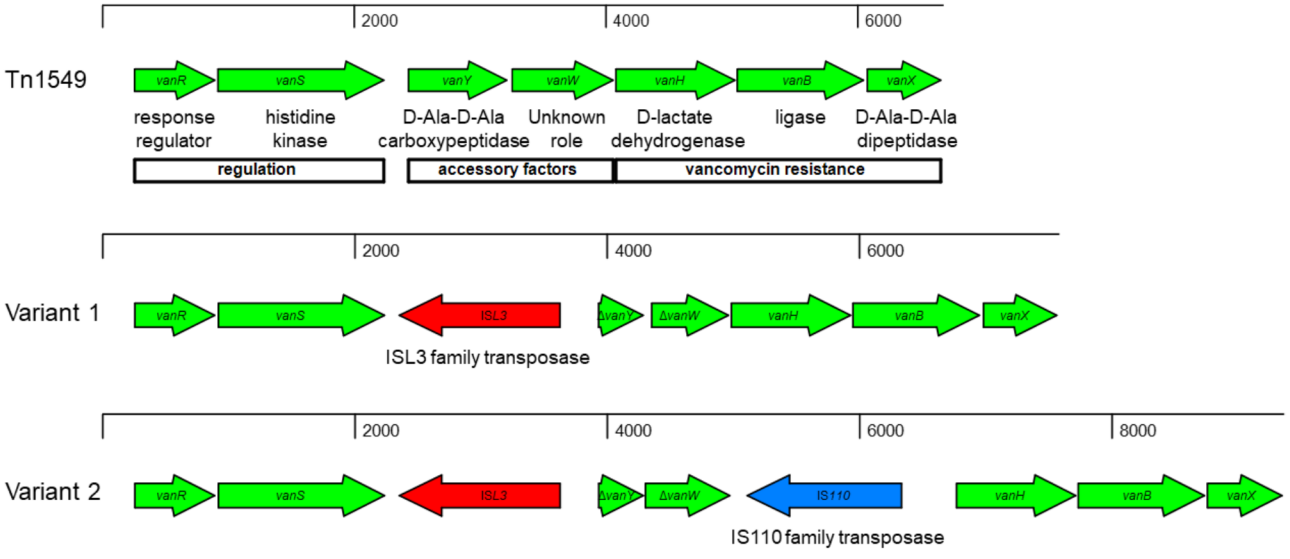

Fig. 4 Structural variants of the *vanB*$_2$ operon. The arrows indicate the genes

all seven plasmids. Three plasmids, pNN-1 (including its pNN-1B variant), pNN-2, and pNN-4, were found in every isolate. Additionally, plasmids pNN-5 and pNN-6 were present in nearly all isolates ($N = 17$). In contrast, plasmids pNN-3 and pNN-7 were detected in only one and two isolates, respectively. Notably, only two plasmids, pNN-2 and pNN-3, carried antibiotic resistance genes, conferring resistance to streptomycin, aminoglycosides, lincosamides, macrolides, and streptogramins. Detailed information regarding the plasmid characteristics and their closest counterparts in the GenBank database is provided in Table 2.

In two isolates, NN-EF15 and NN-EF37, an 18.3 kb episomal phage named phiUMCG-EPI-Ef1 was identified. This phage existed as an independent molecule in the cytoplasm, separate from the chromosome. A BLASTN search in the NCBI GenBank database returned only three hits: *E. faecium* isolate 2014-VREF-63 plasmid p63-3 (18.1 kb; 95.07% identity; 98% query coverage), *E. faecium* strain BMT-1-1-11 plasmid pIN-1-11_03 (18.0 kb; 98.06% identity; 99% query coverage), and the

Table 2 Plasmids characteristics and their closest counterparts

Plasmid	Size	Number of isolates harboring plasmid	Closest counterpart in the GenBank database	Characteristics
pNN-1	155,772 bp	16	Enterococcus faecium isolate E8440 genome assembly, plasmid: 2 (query cover: 99%; Identity: 99.97%)	Antibiotic Tolerance: Encodes two-component response regulator VncR for vancomycin tolerance and several toxin-antitoxin systems for survival. Metabolic Functions: Includes PTS systems and ABC transporters for carbohydrate uptake, enhancing adaptability to nutrient availability. Stress Response: Encodes DNA topoisomerase and glyoxalase proteins for DNA integrity and oxidative stress protection.
pNN-1B	129,445 bp	2	Enterococcus faecium isolate E8440 genome assembly, plasmid: 2 (query cover: 98%; Identity: 99.97%)	All the main characteristics of pNN-1 are retained in pNN-1B (21-kb deletion variant).
pNN-2	28,557 bp	18	Enterococcus faecium isolate E7356 genome assembly, plasmid: 4 (query cover: 100%; Identity: 100%)	Antibiotic Resistance: Contains genes for 23 S rRNA dimethyltransferase (ermB), aminoglycoside 3′-phosphotransferase (aph(3′)-III), aminoglycoside 6-nucleotidyltransferase (ant(6)-Ia), and acetyltransferase (sat-4), conferring resistance against macrolide, aminoglycoside, streptomycin, and streptothricin antibiotics, respectively. Enzymatic Functions: Encodes DNA polymerase (beta-like region), DNA topoisomerase III, and DNA-directed RNA polymerase beta subunit, all involved in nucleic acid synthesis and processing. Toxin-Antitoxin Systems: Features the Epsilon antitoxin and Zeta toxin, which may contribute to stability and survival under stress conditions.
pNN-3	102,041 bp	1	Enterococcus faecium strain VVEswe-R plasmid pVVEswe-R2 (query cover: 87%; Identity: 99.99%)	Antibiotic Resistance: Contains gene aac(6′)-aph(2″) that provide resistance against aminoglycosides. Enzymatic Functions: Encodes several enzymes, including alpha-N-arabinofuranosidase and ketopantoate reductase, involved in carbohydrate metabolism and essential biochemical pathways. Stability Mechanisms: Features the RelE/StbE toxin for plasmid stabilization and a variety of hypothetical proteins that may contribute to unknown functions.
pNN-4	4,462 bp	18	Enterococcus faecium strain 2016C07-254 plasmid p2016C07-254-6 (query cover: 100%; Identity: 99.98%)	Contains 6 CDS, including one plasmid replication initiation protein and five hypothetical proteins.
pNN-5	4,372 bp	17	Enterococcus faecium JARB-OU2352 plasmid pJAOU2352_5 (query cover: 100%; Identity: 100%)	Contains 7 CDS, including one plasmid replication initiation protein and six hypothetical proteins.
pNN-6	1,979 bp	17	Enterococcus faecium strain VRE-WC072 plasmid unnamed4 (query cover: 100%; Identity: 99.60%)	Contains 2 CDS, including one plasmid replication initiation protein and one hypothetical protein.
pNN-7	6,173 bp	2	Enterococcus faecium strain A15023 plasmid pA15023_P4 (query cover: 100%; Identity: 100%)	Bacteriocin Production: Prebacteriocin and predicted prebacteriocin proteins suggest involvement in bacteriocin production, providing competitive advantages to bacterial species or strains in its environment.

Table 3 Gene list of the phiUMCG-EPI-Ef1 phage (GenBank accession number PQ560998)

#	CDS Position	Product
1	322.510	hypothetical protein
2	520.816	hypothetical protein
3	826.1179	hypothetical protein
4	1185.1496	hypothetical protein
5	1493.1666	hypothetical protein
6	complement(1669.2700)	DNA encapsidation protein
7	complement(2712.3590)	endolysin type Endo-N-acetylmuramidase
8	complement(3587.3979)	holin
9	complement(3996.5033)	tail lysin
10	complement(5045.6379)	hypothetical protein
11	complement(6390.6812)	hypothetical protein
12	complement(6824.7228)	tail fiber protein
13	complement(7239.7346)	hypothetical protein
14	complement(7348.7644)	hypothetical protein
15	complement(7644.8195)	pre-neck appendage protein
16	complement(8201.9037)	lower collar protein
17	complement(9027.10007)	p10
18	complement(10022.11836)	tail protein
19	complement(11848.13176)	major capsid protein
20	complement(13190.13480)	head morphogensis protein
21	complement(13748.13918)	hypothetical protein
22	complement(13931.15652)	DNA polymerase
23	complement(15649.16494)	hypothetical protein
24	complement(16579.16950)	single stranded DNA-binding protein
25	complement(16990.17256)	dsDNA binding protein
26	complement(17352.17651)	hypothetical protein
27	complement(17651.17932)	hypothetical protein
28	complement(17929.18312)	hypothetical protein

complete genome of *Enterococcus* phage Athos (18.3 kb; 92.00% identity; 98% query coverage). PHASTEST analysis indicated that phage phiUMCG-EPI-Ef1 contained 28 genes, with 14 encoding proteins essential to its lifecycle (Table 3) and 14 encoding proteins of unknown function.

Validation and application of the strain ST117-CT71 specific PCR assays

The first assay, Marker3, was developed during the ongoing outbreak and validated using 21 VREfm isolates, including the first 16 outbreak isolates, 5 outbreak-unrelated isolates, and 13 enrichment cultures from rectal swabs. Electrophoresis results are shown in Fig. 5, and the raw data generated by the TapeStation analysis software are available in Supplementary File 1 (sample M585 serves as a negative control, sample M600 as a positive control, and BHI1 to BHI13 as enrichment cultures). The full uncropped gel image is provided as Supplementary File 2. The assay demonstrated 100% specificity and reproducibility. Following validation, the PCR correctly identified 13 patient isolates and 4 environmental isolates as part of the outbreak during the verification procedure in the diagnostics setting. Applied prospectively to enrichment cultures from 66 samples (33 patients), the assay identified 8 additional patients carrying the outbreak strain. WGS, routinely performed for all VREfm isolates at the UMCG, confirmed these PCR results as true positives. However, for two patients, Marker3 produced false-negative results, as revealed by WGS. This was attributed to the co-culture of outbreak and non-outbreak *E. faecium* strains from a rectal swab, where the non-outbreak strain predominated. PCR preferentially amplified the shorter 434 bp product of the non-outbreak

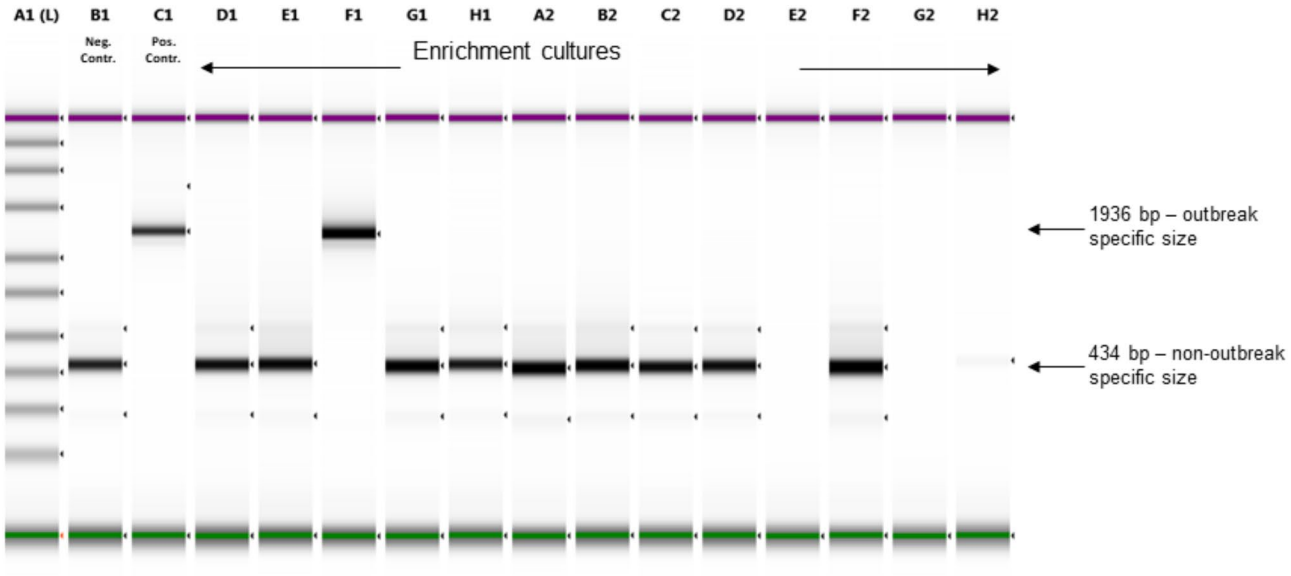

Fig. 5 Electrophoresis results of the outbreak-specific PCR test Marker3, analyzed using the Agilent TapeStation 2200 system

Table 4 Sensitivity of the Marker4 TaqMan assay in detecting *E. faecium* ST117-CT469 DNA in the presence of human DNA. This table presents the cycle quantification (Ct) values obtained from the Marker4 TaqMan assay for various concentrations of *E. faecium* DNA with 50 Ng of human DNA included where indicated. Each concentration was tested in triplicate and the mean value was used to assess the assay sensitivity

Human DNA amount in PCR reaction	E. faecium ST117-CT469 DNA amount in PCR reaction	Replicate 1 Ct value	Replicate 2 Ct value	Replicate 3 Ct value	Mean
0 ng	50 ng	12.61	12.25	12.46	12.44
50 ng	50 ng	13.07	12.7	13.03	12.93
50 ng	5 ng	16.29	16.45	16.14	16.29
50 ng	500 pg	19.86	20.12	19.86	19.94
50 ng	50 pg	23.45	23.33	23.27	23.35
50 ng	5 pg	27.1	27.01	27.02	27.04
50 ng	500 fg	30.25	30.65	30.37	30.42
50 ng	50 fg	35.41	35.3	35.2	35.31
50 ng	5 fg	39.02	40.12	Fluorescence signal not detected	39.57

strain, which was present in significantly greater abundance, effectively masking the 1936 bp outbreak-specific product.

To improve specificity for identifying the ST117-CT71 strain, a second outbreak-specific assay, the Marker4 TaqMan PCR, was developed after the outbreak ended. Marker4 was validated using 30 VREfm isolates, including the first 12 and last 12 outbreak isolates, isolate NN-EF38 (with a large chromosomal inversion), and 5 outbreak-unrelated isolates. Marker4 demonstrated 100% reproducibility and specificity. Additionally, we assessed the sensitivity of the Marker4 TaqMan assay in the presence of human DNA. The assay was able to detect as little as 5 fg of bacterial DNA, equivalent to approximately one bacterial genomic copy (Table 4).

Sequence analysis of WGS data for all 55 outbreak isolates revealed perfect nucleotide sequence conservation in the regions where the primers and probe hybridize, confirming the presence of the 1936 bp target sequence for Marker3 and the 109 bp target sequence for Marker4. These target sequences were then compared against all nucleotide sequences deposited in the NCBI GenBank database, and no contiguous sequences matching either target were found. This findings confirmed the perfect specificity of Marker3 and Marker4 for the ST117-CT469 outbreak strain.

Discussion

Screening for VREfm primarily depends on culturing on selective agars, often supplemented by PCR targeting the vancomycin-resistance genes *vanA* and *vanB*, providing a high negative predictive value. PCR-negative patients can be cleared within a day; however, PCR-positive samples still require culturing, as the presence of *vanA* or *vanB* is not specific to VREfm, leading to potential false positives. Culturing requires at least 48 h for a definitive negative or up to 72 h if *E. faecium* is isolated. The ST117-CT469 strain, however, grows poorly on VRE chromagars, delaying detection by up to five days in some cases and occasionally leading to false negatives. This delay can result in prolonged isolation of VREfm-suspected patients, increasing pressure on hospital departments and occasionally necessitating the closure of units to new admissions. To address these challenges, individual microbiology laboratories have developed outbreak-specific PCR assays tailored to their local VREfm strains, although these assays typically take several weeks to implement. At the UMCG, the ST117-CT469 outbreak PCR assay, Marker3, was developed and validated for clinical use. This assay significantly reduced the time to results, facilitating quicker patient management and easing the demand on hospital resources during outbreaks.

At the end of the outbreak, a second PCR approach called Marker4, a TaqMan-based assay targeting ST117-CT469, was designed and validated. TaqMan PCR assays, superior to conventional PCR followed by electrophoresis, offer greater sensitivity, specificity, and faster turnaround. By using fluorescence-labeled probes that bind exclusively to target DNA, TaqMan reduces false positives from non-specific amplification and, as real-time PCR, quantifies target DNA, informing treatment decisions. With no post-amplification steps, TaqMan streamlines workflows and integrates easily into high-throughput systems, reducing contamination risks and allowing multiplexing to detect multiple targets in one reaction. For these reasons, the TaqMan assay is better suited to high-volume diagnostic laboratories, where speed, accuracy, and the ability to process large numbers of samples efficiently are essential.

As a PCR tool for outbreak identification, Marker3 and Marker4 are superior to NGS-based methods in cost and speed. The total costs of all reagents and consumables for Marker3 and Marker4 (not including labor) amount to ~8 € per sample with turnaround times of 4 h and 2.5 h, respectively. In comparison, whole-genome sequencing

typically costs above 200 € per sample and can take days to complete.

Looking ahead, our goal is to develop a VREfm-specific TaqMan PCR toolbox that detects known VREfm outbreak strains without the need for culturing. This toolbox will target loci unique to outbreak-specific clones and be made available to Dutch hospitals for rapid carrier screening and containment of these clones. Continuously updated, this toolbox will support Dutch hospitals in their efforts to control VREfm outbreaks, enabling faster insight into transmission, flagging and deflagging of patients, lifting of isolation for non-carriers, and cutting transmission rates. The project team includes members of the Dutch Society for Medical Microbiology (NVMM) from hospitals throughout the country, with expertise in diagnostics and infection control, ensuring the toolbox is highly practical and effective for use in local outbreaks.

The outbreak isolates exhibited low genetic diversity at the SNP level. The first isolate identified during the VREfm ST117-CT469 outbreak, NN-EF1, differed from other outbreak isolates by only 0 to 10 SNPs. Notably, a gradual increase in single-SNP differences was observed between NN-EF1 and the least genetically related isolate, NN-EF48 (excluding NN-EF38, which contained a large chromosomal inversion), suggesting potential transmission events between patients. McHugh and colleagues recently demonstrated that within-patient diversity typically ranges from 0 to 3 SNPs when genomes of the same sequence type are analyzed over a month [28]. In our analysis of pairwise SNP differences between NN-EF1 and the 54 other ST117-CT469 outbreak isolates, 38 isolates showed differences of 0 to 3 SNPs. Twenty-seven isolates had indistinguishable counterparts based on SNP analysis, while 24 isolates exhibited up to 3 SNPs of difference with at least one other isolate. Only four isolates had more than 3 SNPs of difference from all other outbreak isolates, including NN-EF38, which carried a large chromosomal inversion. Moreover, mutation rate estimates for *E. faecium* based on single-colony sampling suggest approximately seven mutations per year [29]. However, studies examining within-patient diversification have reported higher mutation rates, ranging from 12.6 to 128 mutations per year [30–32]. Given that the ST117-CT469 outbreak lasted for three years, our findings are consistent with these mutation rate estimates. Over this period, the ST117-CT469 strain became predominant in the Northern Netherlands, diversifying clonally and through the acquisition or loss of mobile genetic elements, such as phages, transposons, and plasmids.

ResFinder and CARD antimicrobial resistance databases are effective for detecting antibiotic resistance genes but can also yield conflicting results [33]. These discrepancies arise from variations in database content, detection algorithms, thresholds, curation practices, and update frequencies. To resolve conflicts in our workflow for antibiotic resistance gene identification, if discrepancies arise between the databases, results from ResFinder and CARD are first reanalyzed by lowering detection thresholds for sequence identity and minimum length. If discrepancies persist, the conflicting sequence is extracted from the genome and manually analyzed using BLAST against nucleotide and amino acid sequences in the NCBI database, as well as an in-house database containing antibiotic resistance determinants from various reference resources. Whenever possible, genomic findings are correlated with phenotypic data. In this study, the ResFinder and CARD databases produced consistent results. However, we observed discrepancies between genotypic and phenotypic resistance profiles. Specifically, the presence of the *vanB* gene cluster did not confer vancomycin resistance, as most outbreak isolates remained vancomycin-susceptible. The situation was further complicated by the fact that the *vanB* gene cluster confers resistance to vancomycin but not teicoplanin, yet one isolate (NN-EF25) exhibited high resistance to both antibiotics. The only nucleotide difference in the *vanB* gene cluster between NN-EF25 and all vancomycin-susceptible isolates was a novel non-synonymous SNP (L189F) in the histidine kinase VanS protein, exclusively identified in NN-EF25. Expression of the *vanB* gene is strongly influenced by transcriptional regulation via *vanR/vanS*, meaning that detection of the *vanB* gene cluster alone does not always predict glycopeptide resistance under standard testing conditions. Our findings highlight the need that antimicrobial resistance marker databases for genetic phenotype prediction need further expansion for regulatory genes along with analysis of their nucleotide sequences and more precise marker annotations per antibiotic rather than per antibiotic class.

Conclusions

The development and validation of the outbreak-specific TaqMan PCR assay for ST117-CT469 has proven to be a critical advancement in the rapid detection of VREfm during a large ongoing outbreak with a difficult-to-culture VVEfm. The nucelotides of the PCR targets were conserved perfectly over the time of the outbreak, whereas the outbreak clone evolveded into multiple variants in genotype and phenotypic vancomycin resistance. This assay has significantly reduced the time required to accurately diagnose VREfm carriers, thereby enabling faster patient management and reducing the strain on hospital resources. The TaqMan assay's higher sensitivity, specificity, and suitability for high-throughput systems make it ideal for addressing the limitations of traditional culture-based methods, especially in high-pressure outbreak settings. As part of a Dutch initiative, the creation of a comprehensive PCR toolbox targeting various

outbreak-specific VREfm clones will further strengthen the country's diagnostic capacity. By enabling hospitals to rapidly screen, identify, and isolate VREfm carriers, this toolbox promises to reduce the duration of patient isolation and minimize the risk of VREfm transmission, ultimately enhancing infection control practices across the Netherlands.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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None.

Author contributions
AV, EB, LG and MAF designed the study. AV supervised the study. EB and RFJB provided isolates. LG, MAF, AS and VA performed DNA sequencing. RFJB and ML provided epidemiological data. AS designed the strain-specific PCRs. VA performed initial validation of the PCR assays. LG validated the PCR assay for clinical use. All authors analyzed and interpreted the data. AS wrote the draft manuscript. All authors added comments and corrections and approved a final version of the manuscript.

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Data availability
All data are provided within the manuscript or supplementary information file. The complete genome sequences of the E. faecium ST117-CT469 isolates have been deposited in the GenBank database under Bioproject PRJNA1178465.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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