

## First Infection with VanD-Type Glycopeptide-Resistant *Enterococcus faecium* in Europe

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**We report the first strain of glycopeptide-resistant *Enterococcus faecium* from Europe that contains a *vanD* allele isolated from blood cultures of an immunocompromised patient hospitalized in a French university hospital. Based on phenotypic results, PCR sequencing, pulsed-field gel electrophoresis, and Southern blotting, the isolate was assigned to *E. faecium* with a chromosomally located *VanD* allele most closely related to the *VanD1* allele.**

Glycopeptides inhibit cell wall synthesis through the interaction of their N termini with the D-alanyl-D-alanine termini of peptidoglycan precursors. The resulting complexes prevent the transglycosylases from incorporating disaccharide pentapeptide subunits into the growing peptidoglycan chain, thereby inhibiting cell wall synthesis (15). Since 1988, many reports described the increase of vancomycin resistance in enterococci from Europe and the United States (6, 13). This resistance to glycopeptides occurs via a common mechanism involving modification of the peptidoglycan biosynthetic pathway (8). Five phenotypes of acquired glycopeptide resistance have been identified in enterococci. *VanA* and *VanB* are the most common types. They are caused by complex gene clusters that may be plasmid and/or transposon carried (8). *VanE* and *VanG* types have each been identified in single strains of *Enterococcus faecalis* (11, 15). Finally, to date, five *Enterococcus faecium* strains with *VanD* resistance have been reported in the United States, in Canada, and in Brazil (4, 7, 16, 18, 19). These isolates were recovered from stool cultures, urine, and blood cultures from immunocompromised patients. Each contained a distinct *vanD* allele, *vanD1* to *vanD5* (4, 7, 16, 18, 19).

Since November 2003, a 56-year-old man had been regularly admitted to the hematology and oncology unit of Nîmes University Hospital for the treatment of an acute myeloid leukemia. He was hospitalized on February 2004 for febrile neutropenia. Physical examination did not reveal a focal site of infection. *E. faecium* was isolated from three blood cultures. Despite an intravenous antibiotic therapy using levofloxacin and quinupristin/dalfopristin, the patient died 5 days after his admission.

*E. faecium* was determined biochemically using the Vitek 2-AST N017 identification card (Biomérieux, Marcy-l'Etoile,

France). Susceptibility to antibiotics was determined with the E-test method (AB Biodisk, Solna, Sweden) on blood agar plates, and antibiotic MICs were interpreted according to the recommendations of the Antibiotic Susceptibility Testing Committee of the French Society for Microbiology with *Enterococcus faecalis* ATCC 29212, *E. faecium* B91 (*VanA*<sup>+</sup>), and *E. faecium* B132 (*VanB*<sup>+</sup>) as reference strains (20). The three isolates from the blood cultures showed the same resistant pattern. They were resistant to penicillin G (MIC, 16 µg/ml), gentamicin (MIC, >2,000 µg/ml), streptomycin (MIC, >2,000 µg/ml), tetracycline (MIC, 16 µg/ml), linezolid (MIC, >4 µg/ml), daptomycin (MIC, 16 µg/ml), and macrolide-lincosamide-streptogramin B-type antibiotics (MIC, >8 µg/ml). They were also resistant to vancomycin (MIC, >256 µg/ml) and teicoplanin (MIC, >256 µg/ml). They were susceptible only to quinupristin/dalfopristin (MIC, 0.5 µg/ml) and intermediate to levofloxacin (MIC, 2 µg/ml). Levels of growth of these strains in the absence of and following the addition of vancomycin (8 µg/ml) were identical (data not shown), indicating that resistance was expressed constitutively. One of the three isolates was genotypically studied and named *E. faecium* NEF1.

To determine the genotype responsible for the unusual resistance of this strain, we used a multiplex PCR assay previously described by Dutka-Malen et al., which allows simultaneous detection of the glycopeptide resistance genotypes *vanA* and *vanB*, which encode D-alanine-D-lactate (D-Ala-D-Lac) ligases, and *vanC-1* and *vanC-2/3*, which encode D-Ala-D-Ser ligases (9). At the same time, multiplex PCR allows the identification to the species level of the following clinically relevant enterococci: *E. faecium*, *E. faecalis*, *E. gallinarum*, and *E. casseliflavus/E. flavescentis*. In this assay, PCR amplicons are discriminated from each other based on their molecular sizes. This protocol was modified according to Patel et al. in order to bypass the DNA extraction procedure and to process the PCR assay directly on an isolated colony from blood agar or Mueller-Hinton plates (17). Four previously characterized vancomycin-resistant *Enterococcus* strains, which carry either *vanA*,

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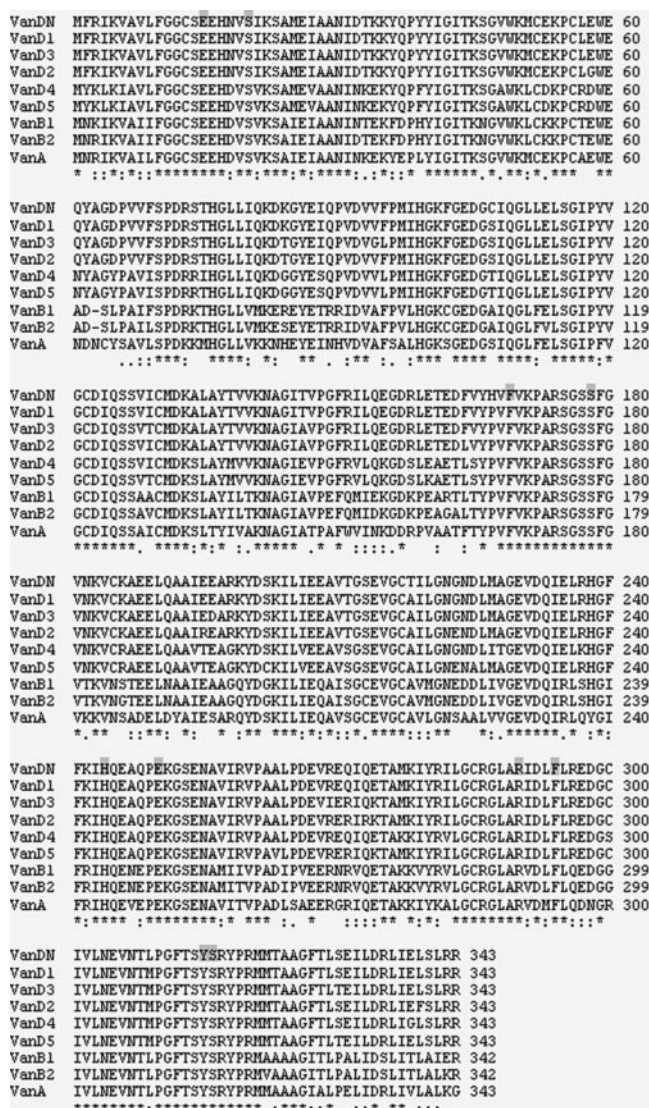


FIG. 1. Comparison of the amino acid sequences of *E. faecium* NEF1 (VanDN) and other D-Ala-D-Lac ligases from glycopeptide-resistant enterococci. The conserved residues that form the active site and those important for ligand binding in the VanA ligase are shaded. Data for comparison are taken from GenBank accession no. AF130997 (VanD1), AF153050 (VanD2), AF175293 (VanD3), AF277571 (VanD4), AAR37060 (VanD5), M97297 (VanA), U35369 (VanB1), and AY145441 (VanB2). Deduced amino acid sequences were aligned using ClustalW.

*vanB*, *vanC-1*, or *vanC-2/3* genes, were used as controls. Multiplex PCR performed on the enterococcal isolate resistant to vancomycin and teicoplanin yielded a 550-bp amplified fragment specific for an internal fragment of the gene encoding D-Ala-D-Ala ligase of *E. faecium*. However, despite the high level of resistance to glycopeptides of this *E. faecium* isolate, none of the *vanA*, *vanB*, *vanCI*, or *vanC2/3* resistance determinants could be amplified. Using degenerate V3 (5'-GAR GAT GGI TSC ATM CAR GGW-3') and V4 (5'-MGT RAA ICC IGG CAK RGT RTT-3') primers, which allow amplification of fragments internal to genes encoding related ligases (12), we obtained a 630-bp fragment. This fragment was cloned

into pGEMT (Promega, Madison, WI) to yield the pGEMT-Van plasmid and introduced into *Escherichia coli* via transformation. The insert was sequenced. The deduced 223-amino-acid partial peptide showed approximately 90% identity with five VanD ligases listed in the GenBank database. Primer walk sequencing was carried out to amplify the entire D-Ala-D-Lac ligase (MWG-Biotech, Ebersberg, Germany). Deduced amino acid sequences were aligned using ClustalW (<http://www.ebi.ac.uk/clustalw/>). The complete region detected was predicted to encode a ligase of 343 amino acids (Fig. 1). This VanD peptide showed 85 to 95% amino acid identity to the VanD2 (AF153050 [16]), VanD3 (AF175293 [3]), VanD4 (AF277571 [7]), and VanD5 (AAR37060 [2]) ligases and 68% and 67% identity with, respectively, the VanA and VanB (B1 and B2) ligases (M97297 [1], U35369 [10], and AY145441 [14]) (Table 1). It shared a maximum of 98% amino acid identity with the VanD1 ligase (AF130997 [5]).

To search for a possible chromosomal location of the *vanD* allele, we used pulsed-field gel electrophoresis of I-CeuI-restricted DNA as previously described (2). A transfer of DNA from electrophoresis gel to a nylon membrane was accomplished by capillary transfer. For DNA probing of the Southern blot, we used an amplification product obtained by PCR of *E. faecium* NEF1 using the *vanD1* primers previously described (18). This product was labeled with digoxigenin-dUTP following the manufacturer's instructions (Boehringer Mannheim, Indianapolis, Ind.) and hybridized on I-CeuI-restricted patterns (Fig. 2). The *vanD1* probe hybridized with *E. faecium* NEF1 DNA, confirming the chromosomal localization of this resistance gene as previously described for the *vanD1* gene (18). All these results point to the fact that the resistance gene in *E. faecium* NEF1 is most closely related to the *vanD1* allele.

In this study, we reported to the best of our knowledge the first strain of vancomycin-resistant *E. faecium* from Europe that contains a *vanD* allele. Since this episode of bacteremia, no further strains belonging to this genotype have been isolated in our hospital. Similar to previous patients reported with VanD-type glycopeptide-resistant *Enterococcus*, this patient

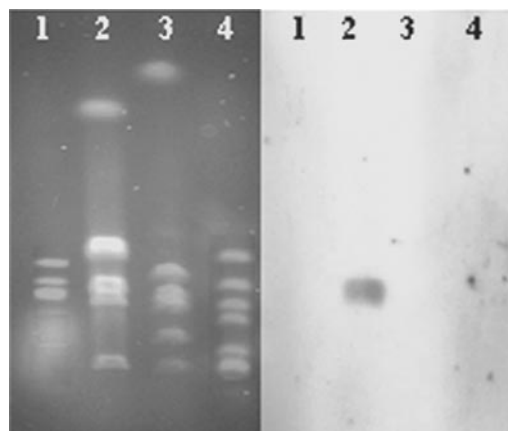


FIG. 2. Analysis of I-CeuI-digested genomic DNA by pulsed-field gel electrophoresis (left) and hybridization of the restricted patterns (right) with a *vanD*-specific probe. Lane 1, *E. faecium* ATCC 29212; lane 2, *E. faecium* NEF1 (VanD1); lane 3, *E. faecium* B91 (VanA); lane 4, *E. faecium* B132 (VanB).



TABLE 1. Percent amino acid identities between *E. faecium* NEF1 (VanDNEF1) and selected D-Ala-D-Lac ligases

Ligase	% Amino acid identity to:								
	VanDNEF1	VanD1	VanD2	VanD3	VanD4	VanD5	VanA	VanB1	VanB2
VanDNEF1	100	98	95	95	84	84	68	67	67
VanD1		100	96	97	85	85	68	67	67
VanD2			100	96	84	86	67	67	67
VanD3				100	83	86	68	67	67
VanD4					100	95	68	69	68
VanD5						100	67	68	67
VanA							100	75	76
VanB1								100	95
VanB2									100

had multiple medical problems, i.e., underlying medical illness (diabetes mellitus), immunocompromised status (neutropenic leukemia or lymphoma), prolonged hospital stays, multiple courses of antibiotics (vancomycin, expanded-spectrum cephalosporins), and instrumentation (central venous catheter) (16). For a few months, our patient had been treated several times with vancomycin, but, during this period, the patient had no enterococcal infections. He received also  $\beta$ -lactams (amoxicillin plus clavulanic acid, imipenem, and cephalosporins), fluoroquinolones, aminoglycosides, and pristinamycin. However, he did not have any links with the United States, Canada, or Brazil, the countries where the five VanD-type glycopeptide-resistant *E. faecium* strains have been previously described. Interestingly, the five *E. faecium* strains with VanD-type resistance reported before were isolated in patients with multiple medical problems: diabetes mellitus, aplastic anemia, and, above all, three cases of orthoptic liver transplantation.

Phenotypically, this *E. faecium* presents a very high level of resistance against teicoplanin compared with the other described *vanD* phenotypes. It may represent constitutive high-level expression of the *vanD* gene cluster. The resistance gene in *E. faecium* NEF1 is most closely related to the VanD1 allele. The *E. faecium* NEF1 peptide showed 98% amino acid identity to the VanD1 ligases and a *vanD1* probe hybridized with the *E. faecium* NEF1 DNA. The residues believed to comprise the active site of VanA are conserved in all enterococcal D-Ala-D-Ala ligases, including our isolate (7) (Fig. 1). It will be interesting to characterize the entire *vanD* operon in *E. faecium* NEF1 to detect potential variations in its organization compared to *vanD1* to *vanD5* operons.

We showed that the *vanD* gene of our isolate was chromosomally located as previously described for the other VanD isolates (8). This distribution may contribute to the paucity of VanD-type resistant strains in contrast to the widespread and increasing prevalence of vancomycin-resistant enterococci carrying *vanA* or *vanB* genes in France.

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