Prevalence and Molecular Epidemiology of Glycopeptide-Resistant Enterococci in Belgian Renal Dialysis Units

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The molecular epidemiology of glycopeptide-resistant enterococci (GRE) colonizing the intestinal tracts of Belgian renal dialysis patients was studied among 1318 patients of a population of 1800 dialysis patients from 29 dialysis centers. Of these, 185 patients (14.0%) were colonized with a VANA-positive GRE; GRE harboring the VANB gene were not detected. The majority of the VANA GRE (80.5%) were identified as Enterococcus faecium; 14.8% were identified as E. faecalis; and a limited number were identified as E. avium, E. casseliflavus, E. dispar, E. durans, or E. gallinarum. Genome analysis of 277 VANA-positive GRE by pulsed-field gel electrophoresis revealed a high genetic variability both within the different dialysis centers and within the patients’ own GRE flora. No high-level gentamicin-resistant VANA-positive GRE were detected, and most strains remained susceptible to ampicillin. These findings do not support a hospital-driven endemicity of VANA-positive enterococcal isolates in Belgium.

Although enterococci are intestinal commensals and are not highly pathogenic, they may cause a wide range of infections, including urinary tract infection, bacteremia, and endocarditis [1]. In recent years, they have become important nosocomial pathogens, especially since the acquisition of resistance to multiple antibiotics, including the glycopeptides. The recent rapid spread of glycopeptide-resistant enterococci (GRE) is of concern, not only because of the problems related to treatment but also because of the potential for vancomycin resistance genes to spread into other, more virulent organisms [2, 3].

Although the first reports of vancomycin resistance came from the United Kingdom [4] and France [5], the major problems are located in the United States. Although GRE are widespread in Europe, the incidence of infection caused by these European GRE strains is indeed very low [6, 7]—except for some reported outbreaks, particularly in the United Kingdom [8]—probably because these strains remain susceptible to ampicillin and genta, which is in contrast to the situation in the United States. In Europe, VANA-positive GRE are widespread among hospitalized and nonhospitalized patients [9, 10] and have been found in the community [11], in farm and pet animals [12–14], and on raw meat and meat products [14–16]. In contrast to the United States, animals and animal meat products contaminated with VANA GRE are considered the major reservoir of glycopeptide resistance genes in Europe [14–17]. The origin of such isolates in European hospitals is controversial. It has been hypothesized that GRE may have entered the hospital via the food chain [6] or, alternatively, were selected by the intensive use of glycopeptides and other antibiotics [12]. Clearly, all possible reservoirs of GRE, including the intestinal flora, may provide a potential risk of GRE infections for hospitalized patients.

The first hospital outbreak of GRE occurred in 1986 in a renal unit in Dulwich, United Kingdom [18]. Jordens et al. [19] showed that in 1992 in a hospital in Oxford, United Kingdom, the rate of colonization with VANA GRE was 3 times greater (15% vs. 5%) for patients in the renal unit than for patients elsewhere in the hospital. A point prevalence study conducted in 1996 in a 600-bed university hospital in Antwerp, Belgium, revealed a higher prevalence of VANA-positive GRE among renal dialysis patients (13.8%) than among hospitalized patients (4.9%) [20].

In the present study, we report on a national prevalence study of intestinal GRE carriage among Belgian renal dialysis patients, conducted between May and September 1997. All VANA-positive isolates were studied by genome analysis with pulsed-field gel electrophoresis (PFGE), by organization of the VANA gene cluster, and by susceptibility patterns.

Materials and Methods

Patient specimens. Between May and September 1997, 29 (51.8%) of 56 Belgian renal dialysis centers participating in the present study were invited to send a stool specimen from each of their patients together with a completed questionnaire. The participating centers are equally spread over the country, with 17 cen-
ters from the Flemish community, 8 centers from the French community, and 4 centers from Brussels, and serve ~1800 hemodialysis patients. A total of 1318 stool specimens were received together with completed questionnaires. Demographic and clinical data were recorded, including glycopeptide use during the year preceding the sampling and the use of antibiotics during or 1 month prior to the sampling.

Detection of GRE. Stool specimens were processed within 24–48 h after collection. A 10% suspension was made in sterile physiologic saline. One drop (50 μL) was plated onto Enterococcus agar (Becton Dickinson, Cockeysville, MD), and 0.1 mL was inoculated in 3 mL of Enterococcus broth (Becton Dickinson); both media were supplemented with 6 μg/mL vancomycin. Cultures were incubated at 37°C in ambient air and were examined after 48 and 72 h. Enterococcal growth on the selective Enterococcus agar is recognized by the typical large gray-black colonies with a brown halo. In general, 1 colony per plate was subcultured. If different enterococcal colony types were observed, 2–5 colonies per plate were subcultured. When no enterococci were detected on the selective agar, 1 drop of the corresponding 48-h-old Enterococcus broth was plated onto a fresh Enterococcus agar plate containing 6 μg/mL vancomycin and was further processed as described. All isolates were used for purity on Mueller-Hinton agar (Becton Dickinson) containing 5% defibrinated horse blood by incubation overnight at 37°C in 5% CO₂.

Species identification. The isolates were tested for pyrrolidonyl arylamidase activity (Rosco Diagnostica, Taestrup, Denmark), which is characteristic for Enterococcus species. Isolates positive for pyrrolidonyl arylamidase activity were identified to the species level by the recently described arbitrarily primed polymerase chain reaction (AP-PCR)–based identification method [21]. PAGE of whole-cell proteins, as described by Pot et al. [22], was done on isolates that could not be identified by AP-PCR.

Detection of the vancomycin resistance genes. The VANA, VANB, VANC1, and VANC2 genes were detected by a multiplex PCR as described elsewhere [20], using the oligonucleotide primers described by Clark et al. [23] and Dukta-Malen et al. [24].

Susceptibility testing. MICs were determined by the agar dilution method with the criteria recommended by the National Committee for Clinical Laboratory Standards [25]. The following drugs were tested: vancomycin (Eli Lilly Benelux, Brussels), teicoplanin (Hoechst Marion Roussel, Romainville, France), streptomycin (Sigma, Steinheim, Germany), gentamicin (Sigma), ampicillin (Sigma), and ciprofloxacin (Bayer, Leverkusen, Germany). Enterococcus faecalis ATCC 29212 and 2 laboratory strains were used for quality control.

High-level resistance to gentamicin and streptomycin was determined on Mueller-Hinton agar supplemented with 2000 μg/mL streptomycin and 500 μg/mL gentamicin, respectively, as described by Sahm and Torres [26].

Organization of the putative transposon Tn1546. The structure of the putative Tn1546 elements was analyzed by almost complete transposon amplification as described elsewhere [27], with the 25-bp forward primer ORF1-F1 (5'-AATCTCTATTAAGCTACCTGTCCGG-3') located at position 191 and the 25-bp reverse primer VanY-R1 (5'-TATCTCTATAACGAGATTAGTCCGGC-3') located at position 9867 of transposon Tn1546 (GenBank accession no. M97297). Briefly, amplification was done in a GeneAmp PCR system 9600 (Perkin-Elmer Cetus, Norwalk, CT) by use of 50-μL PCR reaction mixtures containing 5 μL of 10× ExTaq buffer (Takara, Gennevilliers, France), 4 μL of dNTP mixture (2.5 mM each dNTP), 1 μL of each primer (20 pmol/μL), 0.25 μL of ExTaq DNA polymerase (5 U/μL), 500 ng of extracted DNA, and sterile distilled water up to 50 μL. The amplification process consisted of 35 cycles of denaturation at 98°C for 20 s, annealing at 68°C for 1 min, and elongation at 72°C for 15 min. Five microliters of the amplification product was digested by use of the DdeI restriction enzyme and separated in a 1.5% pronase D1 gel (Sphaero Q, Burgos, Spain) for 2 h at 150 V in 0.5× Tris-borate-EDTA containing 0.05 mg of ethidium bromide/L. The obtained restriction fragment length polymorphism (RFLP) patterns were digitized by the Gel Doc 1000 documentation system (Bio-Rad Laboratories, Nazareth, Belgium). Conversion, normalization, and further analysis of the patterns were done with GelCompar software, version 4.0 (Applied Maths, Kortrijk, Belgium).

PFGE. All VANA gene–harboring isolates were studied by PFGE using the restriction enzyme Smal, as described by Descheemaeker et al. [21]. Briefly, 1 loopful of cells from an overnight culture on Mueller-Hinton agar was adjusted to a density of 4 × 10⁶ cfu/mL in EET buffer (100 mM EDTA, 10 mM EGTA, 10 mM Tris-HCl, at pH 8.0) and was mixed with an equal volume of 1.6% (wt/vol) low-melting-point preparative-grade agarose (Bio-Rad Laboratories). After cell wall and protein digestion, the plugs were incubated in the appropriate restriction buffer, which contained 30 U of Smal restriction enzyme (MBI Fermentas, St. Leon-Rot, Germany). The electrophoresis conditions were ramping linearly from 5 to 35 s at a constant voltage of 6 V/cm over a period of 24 h at 14°C, and separations were done in 1% pulsed-field certified agarose (Bio-Rad Laboratories).

The DNA banding profiles were stained with ethidium bromide, visualized, and digitized by the Gel Doc 1000 documentation system (Bio-Rad Laboratories). Conversion, normalization, and further analysis of the patterns were done with GelCompar software, version 4.0 (Applied Maths). The levels of similarity between the PFGE patterns were calculated by use of the Pearson product moment correlation coefficient.

Results

GRE detection and identification. In total, 601 GRE were detected in the stool specimens of 437 persons (table 1); 1 isolate was obtained from each of 313 stool specimens, 2 isolates from each of 92 stool specimens, 3 isolates from each of 27 specimens, 4 isolates from each of 2 specimens, and 5 isolates from each of 3 specimens.

Identification by the AP-PCR technique was successful for 572 isolates, whereas the PCR patterns of 29 isolates did not group within the clusters of species-specific patterns [21]. These 29 isolates were identified by SDS-PAGE.

The majority, 277 (46.1%) of the 601 isolates, carried the VANA gene; 223 (80.5%) of these were identified as E. faecium, 41 (14.8%) as E. faecalis, and a limited number as E. avium, E. casseliflavus, E. dispar, E. durans, and E. gallinarum (table
Table 1. Vancomycin resistance genotype and species distribution of 601 glycopeptide-resistant enterococcal isolates.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. (%) of patients</th>
<th>No. of isolates</th>
<th>Enterococcus species</th>
<th>No. (%) of isolates</th>
<th>No. of PFGE clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>VANA</td>
<td>185 (14.0)</td>
<td>277</td>
<td>E. faecium</td>
<td>223 (80.5)</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>E. faecalis</td>
<td>41 (14.8)</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>E. avium</td>
<td>3 (0.7)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>E. casseliflavus</td>
<td>3 (0.7)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>E. dispar</td>
<td>1 (0.4)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>E. durans</td>
<td>2 (0.7)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>E. gallinarum</td>
<td>2 (0.7)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Unidentified</td>
<td>2 (0.7)</td>
<td>2</td>
</tr>
<tr>
<td>VANC1</td>
<td>158 (12.0)</td>
<td>200</td>
<td>E. gallinarum</td>
<td>200 (100)</td>
<td>ND</td>
</tr>
<tr>
<td>VANC2</td>
<td>106 (8.0)</td>
<td>124</td>
<td>E. casseliflavus</td>
<td>124 (100)</td>
<td>ND</td>
</tr>
</tbody>
</table>

NOTE. No isolates were VANC genotype. ND, not done; PFGE, pulsed-field gel electrophoresis.

* Harboring both VANA and VANC2 genes.

† Harboring both VANA and VANC1 genes.

1). Two VANA isolates remained unidentified. Eighty-eight (31.8%) of the VANA GRE were isolated after enrichment only.

No VANC-harboring enterococci were detected. Two hundred GRE, identified as E. gallinarum, carried the VANC1 gene, and 124 isolates, identified as E. casseliflavus, carried the VANC2 gene.

GRE colonization. Of the 1318 renal dialysis patients, 437 (33.2%) were colonized with GRE (table 2). The prevalence of GRE carriers in the different dialysis centers varied between 14.3% (center 24) and 61.1% (center 8). One hundred eighty-five patients (14.0%) were colonized with a VANA-positive GRE; the carriage rate of VANA-positive GRE varied from none among the patients in centers 11 and 16 to 23.4% among the patients in center 26. Without prior enrichment cultures, 20.0% (37) of the patients would not have been identified as carriers of a VANA-positive GRE. Isolates harboring VANC1 or VANC2 genes were detected in 12.0% and 8.0% of the patients, respectively, and the carriage rates varied considerably among the different centers (table 2).

Mixed colonizations were detected in 23 patients: 12 patients were colonized with a VANA and a VANC1 isolate, 5 patients with a VANA and a VANC2 isolate, and 5 patients with a VANC1 and a VANC2 isolate. One patient carried a VANA isolate and an isolate harboring both a VANA and a VANC1 gene. The presence of mixed colonizations may be underestimated, because only 1 isolate was characterized in each of 313 stool specimens.

Clonal distribution of VANA strains. All VANA GRE isolates were typed by PFGE with the restriction enzyme Smal. The inter- and intragel reproducibility of different restriction digests and electrophoretic runs was >92%, as determined by the Pearson product moment correlation coefficient.

On the basis of visual and computerized analysis, a difference of ≥3 bands in the PFGE pattern was used as a criterion to define a separate clone. Isolates characterized by 2-band differences or indistinguishable PFGE patterns were regarded as belonging to the same clone. By use of these criteria, 157 PFGE clones were distinguished, of which 108 were represented by a single isolate. Because we evaluated no epidemiologically related strains in the present study, the criteria we used are more strict than the criteria of Tenover et al. [28] to ensure the equality of isolates represented by indistinguishable PFGE patterns.

In 5 dialysis centers (centers 7, 8, 13, 18, 24), only 1 clone was detected, but these had only 1–3 VANA GRE–colonized patients (table 2). Up to 25 clones were detected in the other centers. In 15 centers, 2 or 3 patients per center were colonized by GRE indistinguishable by PFGE. Indistinguishable isolates were present among patients from 2 centers in 18 instances, among patients from 3 centers in 5 instances, among patients from 4 centers in 3 instances, and among patients from 6 centers in 3 instances; 1 clone, characterized by 1 unique PFGE pattern, was distributed over 9 different centers geographically dispersed over Belgium.

From 124 stool specimens from which ≥2 VANA isolates were recovered, 31 carried 2 different GRE clones, 5 carried 3 different clones, and 1 carried 4 different clones.

RFLP analysis of the Tn1546-like elements. Transposon typing was done on a selection of 120 VANA GRE isolates (all isolates of the 29 most frequently detected clones) originating from 83 stool specimens. Two transposon types were detected. Transposon type 1, identical to the prototype transposon Tn1546 [29], was present in 19 (15.8%) of the strains studied. The second transposon type, differing from Tn1546 in 1 base substitution in the vanX gene [27], was present in 63 (52.5%) of the strains studied. No RFLP pattern could be obtained from the remaining strains; these probably represent additional transposon types.

Two strains with an indistinguishable PFGE pattern, isolated from different patients, harbored different transposon types. In general, identical transposon types were found in GRE isolates originating from the same stool specimen, whereas in 3 stool specimens, GRE isolates harboring transposon type 1 or type 2 were detected.

Susceptibility testing. The susceptibility patterns of all VANA-positive GRE were determined. All strains were resistant to both vancomycin (MIC ≥32 µg/mL) and teicoplanin (MIC ≥32 µg/mL). High-level gentamicin resistance was not detected, but 23.3% of strains had high-level streptomycin resistance. Resistance to ampicillin (MIC ≥16 µg/mL) was detected among 10.9% of strains; the MIC90 was 8 µg/mL, and the MIC50 was 16 µg/mL. For ciprofloxacin, 31.9% of the strains were resistant (MIC ≥4 µg/mL), with a MIC90 of 1 µg/mL and a MIC50 of 8 µg/mL.

Discussion

The VANA-positive isolates were identified as predominantly E. faecium (80.5%), whereas E. faecalis was less prevalent (14.8%). A limited number of other enterococcal isolates, be-
longing to the species *E. avium*, *E. casseliflavus*, *E. dispar*, *E. durans*, and *E. gallinarum*, also harbored the *VANA* gene. The higher prevalence of *E. faecalis*, compared with the prevalence of *E. faecium* and the other enterococcal species harboring the *VANA* gene, corroborates the findings in other studies [30, 31]. This species distribution may be explained by the emergence of glycopeptide resistance predominantly in *E. faecium* [6, 23], possibly as a result of its inherent higher resistance to the frequently used β-lactam antibiotics [32]. However, glycopeptide-resistant *E. faecium* may also be more prevalent in humans because of its predominance in food animals [33].

Enterococcal isolates harboring the *VANB* gene were not detected. This probably results from their rarity rather than from technical problems. The medium used for screening GRE contained 6 μg/mL vancomycin, which is in the MIC range for *VANB* GRE [6]. In Europe, *VANB* isolates have been detected most frequently in the United Kingdom, where they have caused distinct episodes of infections in a renal unit [34] and in leukemia and bone marrow transplantation units [3, 35]. Still, *VANA* isolates predominate in the United Kingdom [36]. In Belgium and The Netherlands, *VANB* isolates were infrequently found in some studies [11, 37, 38], whereas others [39–42] did not detect them. In contrast, *VANB*-associated outbreaks are frequently reported in the United States [43–45].

The species identity of the isolates harboring the *VANC1* and *VANC2* genes, characteristic for the low-level glycopeptide-resistant *E. gallinarum* and *E. casseliflavus*, was confirmed by the identification techniques used. Because the intrinsic low-level-resistant *VANC1*- and *VANC2*-carrying isolates may be part of the normal human intestinal flora, and no risk factors for colonization or infection with these isolates could be identified [46], further discussion will concern only the *VANA* GRE isolates.

The *VANA* gene cluster, conferring high-level glycopeptide resistance, was detected in 277 enterococcal isolates originating from 185 (14.0%) of the 1318 participating patients. This figure is in line with the previously reported 13.8% prevalence of GRE among the renal dialysis patients in a 600-bed university hospital in Belgium [20]. This prevalence was significantly higher than the 4.9% carriage rate in patients located on other wards [20]. In the United Kingdom, the stool carriage rate among renal patients was estimated at 6%, but was only 1% for patients on other wards [19]. These reports confirm the significant association found by Tornieporth et al. [47] between hemodialysis treatment and acquisition of GRE. However, among the different Belgian renal dialysis centers, the prevalence of *VANA* GRE differed significantly and ranged between zero and 23.4% (table 2).

The use of an enrichment technique for the detection of GRE may have influenced the measured prevalence of *VANA* carriage, since this method results in much higher recovery rates [20]. The need for enrichment suggests that *VANA* GRE occur in very low numbers in the intestinal tract. Previously, we showed that, among these patients, <10^4 cfu/g of feces was present [20]. Indeed, 37 (20%) of the patients colonized with a *VANA* GRE would not have been identified if only the direct plating method had been used to screen for GRE. Ieven et al. [20] described an increase of 46.5% with broth enrichment. Renal dialysis patients receiving antibiotics may, as a consequence, harbor higher numbers of *VANA* GRE than do other hospitalized patients, resulting in a better recovery rate by the direct plating method. The altered pharmacokinetics of glycopeptides in patients with renal failure results in prolonged low concentrations of the drug, which could contribute to the selection of GRE [48].

A high genetic variability, as was detected by PFGE, points to a nonepidemic spread of GRE. Indeed, in 1 center, up to 25 genetically unrelated clones were detected among 20 different patients (table 2). In contrast, indistinguishable clones were found in patients from the same centers and in patients from different centers. Although this might indicate a possible inter- and intrahospital spread, a phenomenon reported frequently in the United States [49, 50], no epidemiologic links between these centers could be found in Belgium. Alternatively, the omnipresence of particular clones (1 clone was found in 9 different
centers geographically distributed over the country) may indicate a common reservoir outside the hospital [40]. This was additionally supported by the detection of identical clones in dialysis patients and in a healthy volunteer without a previous hospital stay (present authors, unpublished data).

The high genetic variability among GRE found in this and other studies [10, 11, 51] does not favor the interpretation of a clonal spread of GRE. It has been suggested that the GRE in hospitalized patients may originate from unknown sources in the community [11]. Alternatively, the glycopeptide resistance genes, located on the Tn546 transposon or related elements [52, 53], may spread horizontally among intestinal enterococcal strains in the patients, resulting in the genetic heterogeneity observed. The presence of several genetically unrelated GRE strains in 1 patient, all characterized by the same transposon, underscores this possibility. The presence of numerous PFGE types among the GRE flora of the same patient was also reported by Schoonmaker et al. [54] and Bonten et al. [55]. In the present study, different isolates belonging to particular E. faecium and E. faecalis clones were shown to harbor different transposon types, possibly the result of their horizontal spread. However, the stability of the transposons must also be questioned, because the transposon types most frequently detected differ only in a nucleotide change in the VanA gene and a deletion at the 5' end of the transposon [27, 56–58].

This polyclonality may also be correlated with the susceptibility patterns. Our study and others [31] show that the European VanA GRE strains are not resistant to gentamicin at a high level. This is in contrast to the situation in the United States, where most VanA GRE are multiresistant [44, 59–61]. Gentamicin antibiotic therapy in Europe may eliminate VanA GRE from, or at least suppress its growth in, the gut, maintaining polyclonality, whereas in the United States, GRE are selected during therapy, resulting in a clonal expansion. Indeed, in the United States, identical multiresistant VanA clones have been identified in outbreaks in different hospitals, spread over different states [49]. Except for sporadic outbreaks, clinical problems due to GRE in Europe remain relatively low, probably because of the susceptibility of these GRE to gentamicin and, in general, to ampicillin.

In conclusion, this study showed a high rate of intestinal carriage (14%) of genetically diverse VanA GRE isolates among Belgian renal dialysis patients. Polyclonality within the dialysis centers and within the patients' own GRE flora points to a community origin of these GRE. Alternatively, a horizontal spread of the glycopeptide resistance genes and the susceptibility of these GRE to gentamicin may be additional factors maintaining polyclonality. The epidemiology of GRE is still not well understood, and effective control measures are needed to control transmission. Precautions against resistant organisms, adapted antibiotic policies, and elimination of patient carriage may be useful measures for controlling the spread of GRE. In addition, there is a need for surveillance of the susceptibility of GRE isolated from all sources, including clinical, environmental, and food, for early detection of multiresistance.

Acknowledgments


References


